

EACR 2022 Congress Abstracts

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Bioinformatics and Computational Biology

EACR22-0272

microRNAome-wide detection and annotation of mutations in microRNAs and their processing motifs in lung adenocarcinoma

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Introduction

MicroRNAs (miRNAs) are short (~22 nt) non-coding RNAs that modulate gene expression at a post-transcriptional level. Expression of miRNAs is often altered in cancer, which may lead to the dysregulation of cancer-related gene expression networks. Somatic mutations in miRNAs could, in theory, also lead to major alterations in their function and promote oncogenesis. However, to date, somatic mutations in miRNAs have rarely been thoroughly analyzed or experimentally characterized. In this work, we aimed to identify somatic mutations in the miRNome in lung adenocarcinoma (LUAD) primary tumors and experimentally validate their functional impact.

Material and Methods

We performed targeted DNA sequencing of all miRBase 21 miRNAs in an in-house cohort of 70 LUAD patients. For validation, we reanalyzed LUAD cohorts from The Cancer Genome Atlas. We implemented a novel pipeline to identify high-confidence somatic mutations and to annotate the mutations in a miRNA-centric manner. In addition, we implemented a novel method for predicting sequence motifs involved in the cleavage of miRNA primary transcripts by Microprocessor and we used it to identify mutations that disrupt these motifs. Finally, we experimentally validated the phenotypical effects of a candidate mutation in LUAD cell lines.

Results and Discussions

We found ~1 mutation in miRNA genes per tumor sample, and most of them did not affect the mature miRNAs. Indeed, we found 1 mutation in a mature miRNA for every ~2.5 samples and 1 mutation in a miRNA seed for every ~9 tumor samples. In a tumor sample, we identified a single nucleotide variant in the seed of a highly conserved, well-expressed miRNA that we selected for further experimental validations. We confirmed that the mutant miRNA had an oncogenic effect in various LUAD cell lines, in which it altered cell proliferation and clonogenicity. Experiments are underway to identify the changes in targets and in mRNA expression induced by the mutant vs. wild type candidate miRNA in LUAD cell lines.

Conclusion

Mutations in miRNAs are rare, but not absent, in LUAD, and some may be functionally relevant. In particular, our candidate mutation caused major alterations in the targets of the affected miRNA, leading to phenotypical changes in cell line models.

EACR22-0306

Functional landscape in soft tissue sarcoma

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Introduction

Soft tissue sarcomas (STSs) are a group of rare cancers that are difficult to treat. The most frequent procedure is surgery, which in most cases does not prevent a relapse, leading to poor outcomes. An alternative would be Neoadjuvant Chemotherapy (NCT), which has the potential to downstage current disease and target micrometastases, thus reducing the risk of relapse. Current status of NCT use reflects the lack of consensus for treatment of STS, finding a huge variability on the treatments used. In our group, we are interested in finding new targets that could serve as biomarkers to improve the cure rates.

Material and Methods

We used RNA-Seq data from The Cancer Genome Atlas (TCGA) Program and the Genotype-Tissue Expression (GTEx) project preprocessed with the same pipeline and available through the project ReCount. We conducted three major types of analyses: analysis of signaling pathways that are differentially activated in STS with respect to putative sarcomagenic GTEx normal tissues, using the method HiPathia to convert gene expression to KEGG circuit activity; analysis of activation of transcription factors (TFs) through the enrichment of their targets across a list of genes ranked by logFC of gene expression; and *in silico* drug perturbation analysis by knocking-down the gene expression of therapeutic targets and looking at modifications at the circuit activity level.

Results and Discussions

Preliminary data visualization showed that the data from both projects do not form completely separate clusters, suggesting that both datasets are comparable and do not present a very strong batch effect. Differential activity analysis highlighted enrichment in the immune response in upregulated circuits and alteration of cell metabolism in downregulated circuits, some of them affecting patient survival. TF analysis revealed that each STS deregulates their own set of TFs, which are complex to interpret due to their often antagonistic roles. On TCGA, STS patients are treated with a plethora of drugs, not allowing for proper stratification. However, our method to simulate the effect of the drug sunitinib is concordant with the activity changes seen in patients with better performance.

Conclusion

Despite appearing close in clusters, STS subtypes possess unique characteristics that make it difficult to establish a common background of either circuit or TF activation in order to help with treatment. Nevertheless, manual perturbation demonstrates to be a promising tool for generating hypotheses on new putative targets.

EACR22-0668

Identification of undefined off-targets of novel drugs and drug combinations with network-based in silico modeling and in vitro validation in hepatocellular carcinoma

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common and deadliest cancer worldwide. It is highly resistant to conventional chemotherapies due to the hyperactivation of many critical survival signaling pathways, and targeted agents can extend the patient's survival for a few months. Furthermore, HCC patients have limited chemotherapeutics due to impaired liver functions. Thus, it is crucial to identify drugs and drug combinations for repurposing in HCC treatment.

Material and Methods

The pathways involved in hepatocarcinogenesis were used for the generation of a directed network, in which small molecule inhibitor drugs having at least one target protein were also integrated. The target proteins and their interactions were calculated; the functionality of the network was identified. Top nine ranked drugs were selected to validate their bioactivities on HCC cell lines *in vitro* by NCI-SRB assay. Transcriptomic analysis was done by Nanostring nCounter system using the PanCancer Pathways panel. Synergistic drug combinations were determined by analyzing NCI-SRB results using SynergyFinder. The molecular mechanisms induced by single drugs and drug combinations were studied by cell cycle assay, cell death assay, and western blotting.

Results and Discussions

Amrinone, Thalidomide, Chloroquine, Sunitinib, Pranlukast, Pseudoephedrine, Brigatinib, Lenvatinib, and Regorafenib were ranked as the first nine potent drugs. Except for Thalidomide, Pranlukast, Amrinone, Pseudoephedrine, the selected drugs, especially Brigatinib and Sunitinib, were remarkably bioactive on the HCC cells. Several cancer-related genes and pathways were altered in the presence of drugs, with Brigatinib having the highest number of differentially expressed genes (DEGs). The most enriched signaling pathways were Wnt-beta catenin, cell cycle, and MAPK. Brigatinib differed from the rest of the drugs in the pathway scoring matrix. Apart from the previously reported genes, HDAC1, HDAC2, HDAC4, FOS, DAXX, LEF1, RELA, and RUNX, etc., that were not associated with liver cancer therapeutics before were significantly enriched in the network. The bioactive drugs induced apoptosis involving Akt pathway inactivation due to cell cycle arrest in G0/G1 phase. Furthermore, combinations of Brigatinib with Amrinone, Sunitinib or Regorafenib, and Sunitinib with Pseudoephedrine or Chloroquine had synergistic effects on HCC cells.

Conclusion

Our results have shown that our network-based in silico model can be exploited for drug repurposing and novel target identification.

EACR22-0874

In silico Screening of Anti-breast Cancer Agent from *Lablab purpureus*

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Introduction

Lablab purpureus (LP), originating from Africa, has been a major source of nourishment. It is considered one of the most diverse domesticated legume species popular for its versatile and multifunctional utilities. LP has been reported to be comprised of significant biomedical properties, including anticancer efficacy. Additionally, the crude extracts of this plant are reported with selective cytotoxicity, while inhibiting tumorigenic cell growth without affecting normal cell growth *in vitro*.

Material and Methods

A library of reported bioactive phytochemicals from *Lablab purpureus* was created, to be used for the *in silico* evaluations against five breast cancer proteins EGFR (3W2S), ER α (3ERT), HER (3PP0, 2IOK) and PR (IE3K). Molecular docking was carried out to find the binding affinity of ligands with the help of Autodock and PyRx. The best docking conformations were selected based on the binding affinity (≥ 8.0), and RMSD values (< 2.0 Å). The interaction between protein and ligand were visualized (in 2D and 3D) with the use of Biovia Discovery Studio visualizer.

Results and Discussions

Cosmosiin, a major phytochemical found in LP, showed good binding affinity with all selected breast cancer proteins. Cosmosiin (-9.3), Trigonelline (-9.1), Dolichin A (-8.8) showed good binding affinity with EGFR protein, While cosmosiin showed good binding affinities with both HER and HER2, 2-Hydroxygenistein (-8.5), showed better affinity with HER, while Genistein (-8.9), showed better binding with HER2. Cosmosiin (-8.8), Lablaboside B (-9.6), with PR (IE3K), Brassinolide (-8.9), Genistein (-9.2), Cosmosiin (-8.5) with ER α .

Conclusion

The LP phytochemicals were successful in binding potentially with various breast cancer proteins, theoretically confirming their anticancer activity. Further validation with *in vitro* and *in vivo* evaluations could lead to the development of novel, plant-based, minimally toxic therapeutic agents against breast cancer.

EACR22-0976

Lung metastases from colorectal cancer exhibits a distinctive immune and mutational landscape

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Introduction

The most common site of metastasis in colorectal cancer (CRC) is liver followed by lung. The molecular biology underlying metastatic spread is still unclear and could influence patients' prognosis. We hypothesize that both genetic alterations in the cancer cell and tumor microenvironment may play an important role in this process. Our aim is to characterize the immune microenvironment and the mutational landscape of metastases from liver and lung in order to elucidate mechanisms determining the metastatic homing process.

Material and Methods

Gene expression data of lung metastases (n=35) and liver metastases (n=153) from CRC were downloaded from open repository GEO. Expression data was used to make a functional analysis and to perform an immune characterization of the samples. Resultant scores were analyzed for statistical differences with Wilcoxon test and used for clustering. Mutational data from 624 liver metastases and 146 lung metastases were downloaded from cBioPortal.

Results and Discussions

Lung metastases showed higher immunogenic scores than liver metastases: i) high immunophenoscore ($p=2e-10$); ii) Antigen presentation score ($p=1.7e-10$); iii) immune cells infiltrates, especially from the lymphoid lineage (Cytotoxic T cells, $p=1.9e-7$; Regulatory T cells, $p=9.2e-6$; B cells, $p=0.001$; Dendritic cells, $p=1.5e-5$; NK cells, $p=5.3e-8$). Gene set enrichment analysis between lung and liver metastases showed IL6-JAK/STAT signaling, Epithelial Mesenchymal Transition, Interferon Alpha, and Signaling by Interleukins gene sets enriched in lung metastases. At genomic level, no differences were found in total number of mutations between liver and lung metastases neither in altered genome frequency (AGF). KRAS and FBXW7 mutations were more frequent in lung metastases whereas SMAD4 and SOX9 were more frequent in liver metastases.

Conclusion

CRC lung metastases show a tendency towards a higher immunogenic environment and an inflammatory phenotype that might be susceptible to be treated with immunotherapy. A crosstalk between MAPK pathway activation and microenvironment could be involved in lung metastatic homing.

EACR22-0998

A transcriptome-wide gene expression outlier analysis pinpoints therapeutic vulnerabilities in colorectal cancer

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Introduction

Novel therapeutic strategies are urgently needed to increase survival of patients with metastatic colorectal cancer (CRC). Interestingly, previous studies have shown that pinpointing gene expression outliers enables discovering actionable oncogenic dependencies in cancer cells, but they have been mainly limited to the overexpression of kinase genes. Thus, we reasoned that novel therapeutic opportunities may be uncovered by extending the gene expression outlier analysis to other gene categories and profiling gene

downregulation events (negative outliers) along with gene overexpression events (positive outliers).

Material and Methods

Taking advantage of RNA-seq data from 246 CRC cell lines that closely recapitulates the molecular heterogeneity observed in CRC patients, we devised a computational pipeline for identifying transcriptome-wide positive and negative gene expression outliers and prioritizing those with higher likelihood of being functionally relevant in cancer cells. Then, RNA-seq, WES and DNA methylation microarray data were analysed to uncover the (epi)genetic alterations underlying gene expression outlier values. Furthermore, cell lines in which the gene expression outlier analysis suggested potential drug targets were treated with increasing doses of the corresponding inhibitors and cell viability was measured using the CTG proliferation assay after 5-7 days.

Results and Discussions

Positive and negative gene expression outliers were identified for 3,879 and 1,035 genes, respectively, and several gene expression perturbations were attributed to gene deletions and amplifications, gene fusions and differential DNA methylation in promoter regions. Intriguingly, we defined many CRC cell lines as positive and negative outliers for kinase and enzyme genes, including gene expression alterations with known therapeutic or diagnostic value and overexpressed genes for which drug inhibitors are already available and which have not been previously targeted in CRC. On the other hand, negative outliers may unveil synthetic lethal vulnerabilities that are still therapeutically unexplored in CRC. We identified 7 CRC cell lines as negative outliers for the metabolic enzyme MTAP and we observed that they are selectively sensitive to MAT2A inhibition, in accordance with previous findings.

Conclusion

We present an atlas of transcriptome-wide positive and negative gene expression outliers in a large collection of CRC cell lines and we show that it is a worthwhile resource for discovering novel therapeutic vulnerabilities.

EACR22-1020

Single-cell transcriptomics in unravelling the therapeutic potential of myeloid cells in breast cancer

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Introduction

Myeloid cells compose a high fraction of breast tumour-infiltrating immune populations, and are potentially valuable therapeutic targets in cancer immunotherapy. However, the remarkable heterogeneity of myeloid cells may conceal the real extent of anti-tumour effectors and the molecular determinants that control their functions. Thus, our project proposes to overcome this limitation by using single-cell transcriptomics to characterize individual myeloid cell types in the tumour microenvironment (TME). Particularly, we aim at:

- evaluating the cellular diversity and molecular signatures of myeloid cells with pro- and anti-tumour functions, focusing on macrophages;
- contributing to the creation of a single-cell expression atlas of myeloid lineage-specific anti-tumour molecular effectors.

Material and Methods

We generated a single-cell RNA sequencing (scRNA-seq) dataset from a mouse syngeneic triple-negative breast cancer cell line model. We FACS (fluorescence-activated cell sorting) sorted CD45⁺CD3⁻CD19⁻NK1.1⁻ cells, coupled with 10X Genomics microdroplet technology, from tumours with experimentally-induced macrophages (EIM) inducing tumour regression and pro-tumour macrophages (promoting tumour growth).

R programming language, with several packages from Bioconductor and The Comprehensive R Archive Network (CRAN), was used to perform the analysis of this dataset.

Results and Discussions

After quality control, we obtained RNA-seq data for 31,808 tumour-infiltrating immune cells, including 9,209 EIM, 11,520 macrophages from untreated tumours and 11,079 macrophages from untreated tumours in an advance stage of progression. Clustering analysis of these cells at low resolution revealed six main clusters, one of which was enriched for EIM.

Our preliminary analyses of these transcriptomes suggest the paramount importance of the TME in determining the phenotype of macrophages beyond their polarization.

Conclusion

The creation of a single-cell expression atlas of myeloid lineage-specific anti-tumour molecular effectors will contribute to the definition of good prognosis biomarkers associated to the myeloid compartment, as well as to the design of novel therapies capable of stimulating the anti-tumour potential of myeloid cells in the context of breast cancer.

EACR22-1042

A HIGH DEPTH SINGLE CELL APPROACH TO REVEAL MOLECULAR DIALOGS SUSTAINING NEUROBLASTOMA METASTATIC PROGRESSION IN THE EMBRYONIC MICROENVIRONMENT

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Introduction

Neuroblastoma (NB) is the most common extracranial solid pediatric cancer and has among the lowest survival rates. NB is suspected to arise from a highly motile transient population of embryonic cells, the neural crest (NC), giving rise to a plethora of cell lineages, which complexifies the precise identification of NB cell(s) of origin. Half of patients display a high metastatic profile detected as of the diagnostic, reflecting early and broad dissemination, hardly decipherable from clinical evaluation. Thus, whether NB metastatic properties are reminiscent of their cell(s) of origin or constitute acquired malignant features is fully unknown. Moreover, whether NB cells take advantage of signals provided by the successive microenvironments they encounter during the metastatic process remains to be determined.

Material and Methods

We previously reported the setting of an avian model in which human NB cells, grafted back within the NC, form sympatho-adrenal primary tumors and disseminate into the embryonic tissues, thus mimicking key steps of NB progression. We exploited this model to conduct a high depth single cell transcriptomic (SMART-Seq2) analysis of NB cells harvested at

distinct stages of NB progression: from the primary tumor sites (before and after metastatic onset), the routes of dissemination (embryonic nerves and aorta), and finally from the secondary metastatic site (bone marrow).

Results and Discussions

We observed the progressive *in vivo* emergence of three NB phenotypic identities, which was also observed in single cell data of neuroblastoma patient samples. We could relate these features to distinct entities of the physiologic sympatho-adrenal (SA) lineage, revealing NB cell ability to replay the NC-derived SA progenitor program. The coexistence of these transcriptional states illustrates NB plasticity that drives dynamic adaptations to the embryonic microenvironment.

Next, we developed a genomic strategy to track the metastatic journey of NB cells from each primary tumor site to the bone marrow. We succeeded to map the different dissemination paths taken by NB cells. By transcriptomic comparisons, we identified key transcriptional adaptations to the successive microenvironments along their spreading routes.

Conclusion

Our approach provides an unprecedented view of transcriptional events underlying the NB metastatic process and paves the way for novel therapies targeting embryonic signals supporting NB cell progression.

EACR22-1106

DNA Methylation-Based Profiling for Improving Sex Cord-Stromal Tumors of the Ovary Subtype Classification

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Introduction

Sex cord-stromal tumors (SCSTs) are a group of rare ovarian neoplasms comprising entities with clinical behavior ranging from benign to highly malignant. Some are associated with a distinct genetic alteration and may be familial, while the genetic driver of other tumors currently remains unknown. Pathological diagnosis can be difficult due to the relative rarity

and overlapping histopathological and immunohistochemical features of these tumors, therefore leading to potential misclassification. Recently, molecular analysis in combination with histological review, has been shown to increase diagnostic precision. Here, we report the potential value of DNA methylation profiling in a series of ovarian SCSTs.

Material and Methods

67 formalin-fixed paraffin-embedded (FFPE) tissue samples were selected from a multicenter cohort of SCSTs. Additional FFPE samples from non-SCSTs and normal ovarian tissue samples were included for comparison. Illumina Infinium HumanMethylation450 or EPIC [850k] BeadChip was used for methylation profiling. Unsupervised hierarchical clustering with t-SNE analysis was performed.

Results and Discussions

Initial pathology diagnosis classified the study cohort into 26 steroid cell tumors, 4 Leydig cell tumors, 3 signet-ring stromal tumors, 4 microcystic stromal tumors, 3 adults granulosa cell tumors, 3 juvenile granulosa cell tumors and 24 Sertoli-Leydig cell tumors. For profile comparison, 5 normal ovary samples, 27 ovarian small-cell carcinomas, hypercalcemic type (SCCOHT), and 10 high grade serous carcinomas (HGSC) were included. Analysis of methylation data showed defined segregated clusters, which matched with the diagnostic categories of ovarian neoplasms. We observed notable clustering differentiating HGSC from SCCOHT and SCSTs. Next, we plan to interrogate whether SCSTs subcluster and integrate the results with central pathology review.

Conclusion

DNA methylation analysis may be useful in understanding the pathogenesis of non-epithelial ovarian tumors and could provide an aid to diagnosis in histologically problematic cases.

EACR22-1144

Single-cell and T cell receptor sequencing analysis in pleural effusion as a tool for monitoring the evolution of the immune system during immunotherapy treatment in lung cancer

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Introduction

Patients with **advanced lung cancer stages** often suffer **malignant pleural effusions (PE)**. This pleural effusion is used as a diagnostic tool and can be considered malignant PE when a cytology or pleural biopsy culture shows malignant tumoral cells. In these patients, **immune checkpoint inhibitors** have become the main therapeutic strategy. In this study, we aim to analyze the **tumor immune microenvironment (TIME)** as well as the **T cell receptor (TCR repertoires** from paired **PE, malignant pleural biopsies, and peripheral blood samples** in a longitudinal study of probands following immune checkpoint therapy with anti-PD-L1 (ongoing).

Material and Methods

We have analyzed a pilot cohort of **four malignant PE patients** (induced by lung cancer) using **single-cell RNA sequencing techniques as well as single-cell TCR**

sequencing (over 18000 total cells) and we characterized the populations in their TIMEs (both immune and non-immune compartments). Furthermore, we applied computational methods for the joint analysis of TCR sequences, clonal expansion and gene expression, which make possible to understand not only the size of the clonal expansion, but also how the expansion profiles differ across patients and how they might relate to prognosis.

Results and Discussions

We have identified **seven major cell populations** present, in different proportions, in all patients: monocytes, macrophages, dendritic cells, T cells, B cells, fibroblasts and tumor cells; as well as **46 total minor subpopulations** (i.e. classical monocytes, M1 macrophages, naive T cells, T regs or mesothelial cells) not all of them shared between all patients; and all of this annotated by their top marker genes.

Furthermore, we identified five clusters of **patient-specific expanded TCRs**, which greatly differ in their phenotypes: exhausted, effector, cytotoxic, naive and intermediate phenotypes, which could clearly be linked to treatment response and prognosis.

Conclusion

With this pilot cohort, and thanks to single-cell RNA sequencing techniques, we showed that we can use **pleural effusions to recapitulate the composition of the TIME**. We can identify both differences and commonalities across patients with great detail. We were also able to find striking **differences in T cell expanding populations**, which can be a promising **target and predictor of treatment outcome and patient response**.

The goal is to apply this knowledge, together with the results of the ongoing clinical study, to **train a predictive model of tumor-specific TCR sequences**, that we can use for precision oncology.

EACR22-0291

Multimic analysis in neuroendocrine neoplasias displays diverse molecular subtypes with clinical significance regardless tumor origin.

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Introduction

Neuroendocrine neoplasms (NENs) are a group of tumors with a wide anatomical distribution that share common “neuro” and “endocrine” properties. Although some studies have assessed the NET molecular landscape of certain primary sites, no cross-sites molecular assessment has been performed to date. We aimed to perform a comprehensive transcriptomic and methylomic characterization of large series of NENs with different anatomical origins in order to identify subgroups and explore their potential clinical relevance.

Material and Methods

Transcriptomic and methylation data were obtained from 181 and 99 tumor samples from different primary sites. Clariom S Human microarrays and EPIC methylation arrays were used, respectively. Clustering was performed independently in each database and

differential expression with limma analysis and gene set enrichment analysis (GSEA) was applied among the resulting clusters.

Results and Discussions

NENs were classified into three different transcriptomic clusters (tC1, tC2 and tC3), while methylome differentiated two groups (mC1 and mC2). tC1 and tC3 showed an inverse transcriptomic profile, and tC2 presented mixed profile. We identified that tC3 had the highest expression levels of neuroendocrine-related genes (somatostatin receptor SSTR5: LFC: 0.77; FDR=1.52x10e-25 ; serotonin receptor HTR1A: LFC: 0.79; FDR= 1.20X10e-20 ; VIP: LFC: 0.64; FDR=1.41x10e-10 etc), in lung primary tumor samples (50%) and mC1 (82.14%). On the other hand, tC1 presented an enrichment of the some important cancer-related pathways (MYC targets NES -6.37 FDR < 10-3; MTORC1 NES -3.78 FDR < 10-3) and oxidative phosphorylation (NES -4.87 FDR < 10-3) ; and showed a higher proportion of small intestine primary tumors (46.34%), Grade 2 tumors (73.17%) and mC2 (57.58%).

Conclusion

NENs of different primary sites can be stratified into two major transcriptomic clusters, with a third cluster having a mixed expression profile. The groups with extreme transcriptomic signatures were enriched in neuroendocrine-related genes or principal cancer pathways. These results suggest a dedifferentiation from a more indolent neuroendocrine phenotype to more aggressive proliferative tumor types that could be driven partially with the methylation profile.

EACR22-0313

A potential gene mutation signature predicting immunotherapy benefits for pan-cancer

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Introduction

Immune checkpoint blockade (ICB) is a promising treatment for cancer, but predictive biomarkers are always in need. We aimed to develop a signature with low cost-effectiveness and predictive value for the immunotherapy benefits of pan-cancer.

Material and Methods

We proposed a study framework to construct a signature for predicting the survival time after ICB treatment. Specifically, we built a multivariate Cox proportional hazards regression model with the least absolute shrinkage and selection operator (LASSO) by using 80% samples of an ICB-treated cohort with 1661 patients from MSKCC. The risk score of the model was the desired signature named SIGP and was validated in the remaining 20% of patients and an external ICB-treated cohort with 249 patients from DFCI.

Results and Discussions

SIGP was based on 18 candidate genes (NOTCH3, CREBBP, RNF43, PTPRD, FAM46C, SETD2, PTPRT, TERT, TET1, ROS1, NTRK3, PAK7, BRAF, LATS1, IL7R, VHL, TP53, and STK11), and according to the SIGP score, we classified patients into SIGP high (SIGP-H), SIGP low (SIGP-L) and SIGP wild type (SIGP-WT) groups. A multi-cohort validation demonstrated that patients in SIGP-L had significantly longer OS than those in SIGP-WT and SIGP-H (44.00 months versus 13.00 months and 14.00 months, p<0.001 in the test set) for ICB therapy, while the survivals of SIGP in non-ICB-treated cohorts were different where

SIGP-WT performed best than others. Besides, SIGP-L+TMB-L (~15% of patients) had similar survivals to TMB-H, and patients with both SIGP-L and TMB-H had better survival. Further analysis on tumor-infiltrating lymphocytes demonstrated that the SIGP-L group had significantly higher abundances of CD8+ T cells.

Conclusion

Our proposed signature SIGP based on 18-gene mutations has good predictive value for the clinical benefit of pan-cancer patients to ICB. The combination of SIGP and TMB provides a potential better prediction of the response to ICB.

EACR22-0363

Identifying novel SMYD3 interactors on the trail of cancer hallmarks.

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Introduction

SMYD3 overexpression in several human cancers highlights its crucial role in carcinogenesis. Nonetheless, SMYD3 specific activity in cancer development and progression is currently under debate. Taking advantage of a library of rare tripeptides (P1-P19), which we first tested for their *in vitro* binding affinity to SMYD3 and then used as *in silico* probes, we recently identified BRCA2, ATM, and CHK2 as direct SMYD3 interactors.

Material and Methods

We performed a comprehensive *in silico* analysis of all protein sequences identified by screening the whole human proteome for our previously tested tripeptides (P1-P19) in order to gain insight into novel SMYD3 cancer-related roles. In particular, we focused on tripeptide distribution and characterized the biological function of each identified protein to find the most relevant SMYD3 interactor candidates involved in cancer hallmarks. Surprisingly, this computational analysis allowed us to identify *in silico* various critical players implicated in cancer processes as novel SMYD3 interactors. These interactions were subsequently validated in several gastrointestinal cancer cell lines.

Results and Discussions

To gain insight into novel SMYD3 cancer-related roles, here we performed a comprehensive *in silico* analysis to cluster all potential SMYD3-interacting proteins identified by screening the human proteome for the previously tested tripeptides, based on their involvement in cancer hallmarks. Remarkably, we identified mTOR, BLM, MET, AMPK, and p130 as new SMYD3 interactors implicated in cancer processes. Intriguingly, SMYD3 appears as a central pivot around which two large functional clusters develop: one involved in DNA processing (DNA repair, telomere maintenance, and transcription) and the other implicated in sustaining cancer-related signaling (cancer metabolism and cancer proliferation).

Conclusion

Further studies are needed to gain insight into the functional mechanisms underlying these novel SMYD3 interactions in cancer hallmarks. An in-depth understanding of these processes may be useful to devise novel therapeutical approaches based on the combined pharmacological inhibition of SMYD3 and its newly identified interactors, which may have a

synergistic pro-oncogenic effect (e.g., MET). Indeed, this strategy may prove more effective by targeting distinct biological functions of cancer cells, such as DNA damage checkpoints (with SMYD3i) and cancer metabolism (with compounds targeting the AMPK/mTOR axis).

EACR22-0491

AI-powered pan-species computational pathology

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Introduction

Cancers occur across species. Understanding what is consistent and varies across species can provide new insights into cancer initiation and evolution, with implications for animal welfare and wildlife conservation. AI-powered models can contribute to the study of veterinary cancers and personalised medicine. However, the transfer and adoption of modern AI and computational pathology approaches in veterinary medicine have been slow.

Material and Methods

We conducted the first pan-species study of computational comparative pathology using a previously validated supervised convolutional neural network algorithm trained on human samples. We applied the AI model on tumour tissue sections from 20 species, including two transmissible cancers (mammalia=13, reptilia=4, aves=2, and amphibia=1). We validate the model's predictions of single-cell classification with 14570 annotations made by two veterinary pathologists. We project multivariate morphological spaces (morphospace) for each species using dimension reduction techniques based on cell morphology and staining features. We quantify morphospace overlap between human and non-human cells to explain the model's performance.

Results and Discussions

Although the algorithm was trained on human samples, it could distinguish three major cell types with remarkable accuracy in most species (19/20 species reached an accuracy $\geq$ 70%). Morphological conservation across species dictates that cells can be detected and correctly classified by a human specimen-trained AI, fostering our endeavour to develop pan-species computational pathology.

The algorithm achieves high accuracy in measuring immune response through single-cell classification for two transmissible cancers (canine transmissible venereal tumour, 0.94; Tasmanian devil facial tumour disease, 0.88), which have a high morphological overlap with human cells (morphospace overlap(%) = 82.6, and 72.2, respectively). Furthermore, in 18 other vertebrate species, accuracy (0.57-0.94) was influenced by morphospace overlap across different taxa, tumour sites, and variations in the immune compartment.

Conclusion

Our study revealed that AI technologies can generalise well to non-human tissues, by exploiting cell morphological similarities across species. Morphological overlap between cells

provides the biological foundation for developing and applying artificial intelligence and computational pathology to non-human species, revealing new opportunities and challenges for comparative oncology research and clinical veterinary care.

EACR22-0521

Cancer-type-specific thresholds reduce the high proportions of unexplained cases with homologous recombination deficiency detected by machine-learning classifiers

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Introduction

Homologous recombination deficiency (HRD) is the inability of a cell to repair damage due to double-stranded breaks and it leads to tumorigenesis. Despite the success of identifying HR-deficient patients based on testing for pathogenic mutations in HR-related genes, there are patients sensitive to PARP inhibitors with a similar phenotype to HR-deficient tumors but are lacking the necessary mutation in those genes. New machine-learning tools, CHORD and HRDetect, can successfully identify tumors' HRD status based on the whole-genome sequencing (WGS) data. Studies have shown that these tools have a high sensitivity in detecting patients with biallelic inactivation in HR-related genes across all cancer types. These studies have also suggested that more than 40% of cases labeled as HRD do not have a known cause explaining their HRD status. The goal was to find cancer-type-specific thresholds and investigate the impact of lack of promoter methylation of *BRCA1* and *RAD51C* (known causes of HRD but are rarely measured) for both classifiers to reduce the high proportion of unexplained cases.

Material and Methods

We collected published CHORD and HRDetect probability scores for 828 samples from breast, ovarian and pancreatic cancer from previous studies alongside the evidence of biallelic inactivation, DNA alterations, or promoter methylation, in HR-related genes. To assess the accuracy of each classifier for each cancer, we performed a ROC curve analysis. Tenfold nested cross-validation was used to find the optimal threshold values for both classifiers within each cancer.

Results and Discussions

HRDetect has higher sensitivity in the detection of biallelic inactivation in HR-related genes than CHORD and a higher proportion of unexplained cases with a universal threshold. When cancer-type-specific thresholds were applied, HRDetect reduced the number of unexplained cases by ~10% without reducing the 96% sensitivity. As for the CHORD, the unexplained cases were reduced by 1% while sensitivity increased from 85% to 94%. Lack of promoter methylation in ovarian cancer increased the proportion of unexplained cases by ~22% for HRDetect and by 26% with CHORD. The same was observed for breast cancer. The proportion of unexplained cases in pancreatic cancer may have been reduced if promoter methylation was analyzed in HR genes where monoallelic events had already occurred.

Conclusion

This study highlights the importance of promotor methylation data and the adjustment of WGS-based HRD classifiers thresholds for each cancer.

EACR22-0601

Interrogating the precancerous evolution of gene expression and pathway dysregulation in lung squamous cell carcinoma using GEPReLUSC, a new bioinformatic application

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Introduction

Lung squamous cell carcinoma (LUSC) is a type of lung cancer with a dismal prognosis that lacks adequate therapies and actionable targets. This disease is characterized by a sequence of low and high-grade preinvasive stages with increasing probability of malignant progression. Increasing our knowledge about the biology of these premalignant lesions (PMLs) is necessary to design new methods of LUSC early detection and prevention, and to identify the molecular processes that are key for malignant progression. To facilitate this research, we have designed GEPReLUSC (Gene Expression of Premalignant LUSC), an open-source application based on the shiny app interface that integrates the most extensive transcriptomic databases of PMLs published to this date.

Material and Methods

GEPReLUSC databases (GSE114489, GSE108124, GSE33479 and GSE109743) contain gene expression data of PMLs with known stage of progression and potential for progression into more advanced stages. GEPReLUSC contains multiple interconnected functions that will enable users to stratify samples using multiple parameters and interrogate PML biology in multiple manners, such as two and multiple group comparisons, interrogation of genes of interests, geneset enrichment analysis and the interrogation of user-defined gene signatures. The application can be downloaded and installed from <https://gitlab.com/cruk-mi/carlos-geprelusc>. Results can be downloaded for external analysis using packages not included in GEPReLUSC.

Results and Discussions

Using GEPReLUSC, we have carried out a comparative study of the potential role of chromosomal instability (CIN)-associated transcriptional signatures as a biomarker of PML progression and mapped the onset of the most relevant LUSC pathways (oxidative stress response, squamous differentiation, PI3K/Akt and CDK2/6 transcriptional signatures) to the sequence of LUSC developmental stages. CIN-signatures performed differently depending on the lesion stage and signature considered. As for the onset of relevant LUSC pathways, we observed an increase in CDK2/6 activity in low-grade PMLs, whereas activation of the other pathways occurred in high-grade lesions. These data suggest that genetic alterations which activate CDK2/6 activity (e.g. CDKN2A mutations) occur early in the natural history of LUSC.

Conclusion

GEPReLUSC is new bioinformatic tool that will critically facilitate new research for the identification of early detection biomarkers and acquire a better understanding of the LUSC precancerous stages.

A comprehensive investigation of histotype-specific microRNA and their variants in early stage ovarian cancer

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Introduction

microRNAs dysregulation it's known to play a key role in the pathological origin of cancer, moreover, the intensive application of sequencing technologies revealed the presence of multiple variants of mature miRNAs (called isomiRs). IsomiRs can be the result of the presence of SNPs in miRNA sequences or they can derive from alterations in the miRNA maturation process. In this study we explore the repertoire of expressed isomiRs in early stage epithelial ovarian cancer (EOC). Stage I EOC is a heterogeneous group of diseases from a histological and pathological point of view, the different histological subtypes are different entities with different molecular features. Exploiting isomiRs expression data we focus on the identification and characterization of histotype-specific biomarkers.

Material and Methods

Our cohort is composed of 215 stage I Ovarian Cancer samples. Small RNA sequencing was performed and reads were processed with the mirtop pipeline in order to quantify the expression of miRNA and isomiRs. Shallow whole genome sequencing (sWGS) was performed to obtain the copy number profiles which are then integrated with expression data exploiting statistical models.

Results and Discussions

We identified 971 expressed isomiRs, 36% are canonical miRNA and 64% are miRNA variants. The majority of these modifications (54%) is accounted for the alteration at their 3'-end while 12% for alterations at their 5'-end that could potentially change the seed sequence responsible for target recognition. We found a total of 42 markers, 9 of which are specific to Serous High, 3 to Serous Low, 7 to Endometrioid, 16 to Mucinous and 7 to Clear Cell subtypes. We identified new markers as well as our previously identified canonical markers (miR192/194 and miR30a-3p/5p) for mucinous and clear cell subtypes, however we can observe that they all express at least one variant that results to be a marker itself. Integrating miRNA expression with CNV and TF activity data, we were able to reconstruct regulatory networks, suggesting active roles of specific TFs in the definition of histotype specificity.

Conclusion

Our work represents the first effort to identify expressed isomiRs in stage I EOC. We found new histotype markers, some of which resulted to be variants of previously identified canonical markers, demonstrating the advantages of this new approach.

Unravelling microenvironment heterogeneity of ovarian cancer: a portray of innate and acquired platinum chemoresistance

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Introduction

Epithelial ovarian cancer (EOC) is one of the most frequent diagnosed cancers in woman and the major cause of mortality. High-grade serous EOC (HGS-EOC) represents its most aggressive subtype. Effective therapy includes a primary surgical cytoreduction, followed by a chemotherapy based on platinum analogues, but, despite an initial positive response, the majority relapse and have a poor outcome. RNA-Seq data gave us the power to investigate differential gene expression, immune gene signatures and pathways to define tumor microenvironment (TME) and immune profile of this EOC. This study aims to identify the transcriptional changes occurring in longitudinal biopsies of HGS-EOC by the comparison of samples from primary surgery (naïve tumor) and relapse after chemotherapy.

Material and Methods

The dataset includes 18 samples from 9 patients, collected at a primary surgery (FS) and a second surgery (SS) biopsy after the chemotherapy treatment. Patients were all sensitive before treatments, and only 4 developed the resistance after chemotherapy. After transcriptome reconstruction and quantification, measurements of stromal and immune infiltration along with tumor cellularity, have been computed through ESTIMATE, ConsensusTME and CIBERSORTx. Then, scores from ssGSEA of cancer hallmarks were analyzed through PCA. Appropriate tests were performed on the results: paired (FS vs SS, FS-SS-Sens vs SS-Sens and FS-SS-Res vs SS-Res) and not-paired (SS-Sens vs SS-Res).

Results and Discussions

By the comparison of matched samples taken at FS and SS, we studied the longitudinal genomic and transcriptomic landscape of HGS-EOC. Through the analysis of the overall cell composition and the immune cells component, we identify differences between FS and SS underlining the role of TME in tumor progression and relapse. Moreover, the hallmark pathway analyses show difference between FS and SS in sets promoting tumor invasion.

Conclusion

With RNA-Seq, we investigate the heterogeneity of patient samples before and after chemotherapy, trying to evaluate the differences which underline resistance. TME and immune system take part to the process of tumor progression and make a substantial difference in the outcomes. Our further studies will characterize these components, taking advantage of single-cell technique for an overall view of cell composition at single-cell resolution.

EACR22-1151

Non-canonical neoantigens are the largest fraction of peptides presented by MHC class I in mismatch repair deficient murine colorectal cancer

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Introduction

Immunotherapy based on checkpoint inhibitors is highly effective in mismatch repair deficient (MMRd) colorectal cancer (CRC). Systematic classification of the neoantigen repertoire in MMRd CRC is lacking. Analyses based on mass-spectrometry peptidomics highlighted the existence of MHC class I bound peptides originating from non-coding DNA regions. Based on these premises we investigated DNA genomic regions responsible for generating MMRd induced peptides.

Material and Methods

We exploited mouse CRC models in which MMR gene *Mlh1* was genetically inactivated. Briefly, mouse CRC isogenic lines CT26 *Mlh1*^{+/+} and *Mlh1*^{-/-} were inoculated in immunocompromised and -competent mice. Whole Genome Sequencing (WGS) and RNA sequencing (RNAseq) data were generated from samples obtained before and after injection in murine hosts. First, all-six frame peptide databases (DBs) were built from transcriptomes of MMR proficient (MMRp) and MMRd cells. A database of peptides lost after injection in immunocompetent mice was specifically generated, since those sequences were assumed to be edited by the immune system. DNA regions of MHC-I bound peptides were finally identified by liquid chromatography-mass spectrometry (LC-MS) and the matched transcriptome DB and whole genome data.

Results and Discussions

WGS analyses revealed an unbalanced distribution of putatively edited alterations across the genome in CT26 *Mlh1*^{-/-} after injection in immune competent mice. Specifically, untranslated (UTR) and coding regions exhibited the largest fraction of immune edited mutations. Moreover, the integrated computational and LC-MS analyses revealed the origin of MHC-I bound peptides in both MMRp and MMRd models, indeed only a small fraction of neoantigens originated from canonical exonic regions. On the contrary, CT26 *Mlh1*^{-/-} showed a strikingly different repertoire of mutated peptides, mainly originated from UTRs and out-of-frame coding regions.

Conclusion

Several lines of evidence suggest that neoantigens could be generated unconventionally from the genome. Our results suggest that MMRd CRC generates a significantly higher number of non-canonical mutated peptides that bind the MHC class I compared to MMRp CRC. These results reveal the importance of evaluating the diversity of neoepitope repertoire in MMRd tumors to identify novel neoantigens as therapeutic targets and further understand the complexity that rules cancer cell immunogenicity.

EACR22-1183

A new tool for investigating biomarker of hepatocellular carcinoma diagnosis and prognosis: Fusion transcripts*

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Introduction

Liver cancer is the 6th most common cancer in the world and HCC is the most common type of liver cancers. In the past decade, progressions in “-omics” studies and technological developments have enabled detection of biomarker candidates related to diseases. Nevertheless, hepatocarcinogenesis has still unclear mechanisms and progression processes. Early diagnosis options are very limited for HCC so existence of reliable biomarkers is crucial to its clinical practice. Fusions play a critical role in cancer progression and fusion transcripts (FTs) are frequently used as biomarkers in the diagnosis of hematologic malignancies, but there are limited studies focusing on fusions in HCC. In this study we aimed to investigate HCC-related fusions using bioinformatics approaches.

Material and Methods

In our study, we re-analyzed 8 RNA-seq datasets from NCBI SRA (Sequence Read Archive). This meta-dataset contained 164 primary HCC tumors, 164 adjacent normal samples. Briefly: we used STAR-fusion for the determination of FTs and Venny and for intersection and, ChimerDB 4.0 and UCSC Xena Browser for *in-silico* validation of lists and analyzing of correlations with expression patterns of 3' and 5' partners.

Results and Discussions

We identified FTs in all primary HCC tumors and their adjacent normal. Then we determined that tumor specific and also clinical feature-related (such as cirrhosis and HBV status) fusions. The 8 datasets were also evaluated at the transcriptome level and the correlations with the expression levels of the 3' and 5' partners of the specific fusions were analyzed. By using the ChimerDB 4.0 database we analyzed the TCGA LIHC cohort and 777 different fusions have been determined as HCC-related. Functional characteristics of 332 fusions were identified; 129 of them are tumor suppressor, 73 of them are oncogenic, 36 of them are kinase, and 94 of them are transcription factors.

This study provides the opportunity to combine data from a large number of samples and our results point to the importance and feasibility of identifying an HCC-specific and fusion-focused global candidate or a specific transcript profile. Finding biomarker FTs will provide a great advantage in terms of ease of detection and low-cost of analysis.

Conclusion

This study will provide new information to identify the roles of FTs in hepatocarcinogenesis and offers a strong and novel perspective.

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EACR22-1193

Timing Key Events in the Evolution of Testicular Germ Cell Tumours

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Introduction

Testicular germ-cell-tumours (TGCT) are the most common malignancy in men aged 15-34 years (y) and a major cause of life-years-lost of patients dying-of-cancer. The incidence rates of testicular cancer have been increasing in many countries since the middle of the twentieth century with the highest incidence of TGCT in Northern Europe. TGCTs are typically characterised by frequent mutation of *KIT* and *KRAS* oncogenes, near-universal whole genome duplication (WGD) and gains of chromosome 12p, predominantly through isochromosome 12p formation. However, a deep whole genome sequencing study of these cancers has not yet been performed.

Material and Methods

In partnership with Genomics England and using one of the largest genomic datasets in the UK, we analysed whole genomes from 60 samples across 57 patients (age 17-77y; median 35y) including multiple primary tumour regions sampled from a single participant. Sampled tumours represented a range of histological subtypes including pure seminomas, non-seminomas, and mixed germ cell tumours as well as a rare case of somatic transformation. All classes of somatic alterations, including coding and non-coding drivers, mutational signatures and copy number aberrations were identified through a range of extensively validated pipelines. A probabilistic timing model was used to reconstruct the chronological order of acquisition of recurrent genomic aberration across all testicular germ cell tumours and across different subtypes.

Results and Discussions

Through joint analysis of this cohort, together with data from recent studies including TCGA, we found evidence for new putative drivers. Molecular timing analysis confirmed that WGD likely occurs during the earliest stages of tumour development. However, we were also able to identify copy number events preceding WGD, potentially involved in tumour initiation. Patterns in mutational signature evolution during tumour development were inferred by clonal architecture analysis, and exemplified by an increase of SBS35, linked with chemotherapy treatment, and SBS18, linked with oxidative stress, in subclonal mutations.

Conclusion

These WGS data offer an invaluable resource for gaining novel disease and patient-related insights in TGCT. Identification of key enriched CNAs arising during early tumour development could provide opportunities for potential targeted intervention and ultimately, improved outcomes for this under-investigated patient group.

Using network biology and treatment-resistant *in vivo* models to study lethal prostate cancer

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Introduction

Prostate cancer (PC) is the malignancy with the highest incidence worldwide in males. Although localized disease can be effectively treated and usually has a favorable prognosis, progression to metastasis results in high mortality rates as current treatment modalities are not yet curative. The standard of care for advanced PC is hormonal therapy, termed Androgen Deprivation Therapy (ADT). Unfortunately, ADT typically elicits the emergence of a lethal phenotype, termed Castration Resistant Prostate Cancer (CRPC), which is treatment-resistant and frequently gives rise to a highly aggressive variant with features of neuroendocrine differentiation, known as Neuroendocrine PC (NEPC).

Material and Methods

We used Genetically-Engineered Mouse Models (GEMMs) of CRPC to perform a forward genetic screening using the Sleeping Beauty (SB) transposon system. Specifically, we selected a GEMM with loss of function of both Pten and Trp53, since we previously demonstrated that this model is able to transdifferentiate to NEPC tumors under ADT pressure. Then, we generated a mouse strain in which the SB transposase expression is under the control of a tamoxifen-inducible Cre allele driven by the prostate-specific Nkx3.1 promoter. SB activation generated tumors with an accelerated growth phenotype, when compared to non-activated controls, that present histological features of NEPC. To elucidate these tumors' regulatory program, we harvested SB-barcoded DNA-Seq and RNA-Seq and performed an integrative analysis that links genomic alterations to downstream regulatory programs, identifying also Master Regulator (MR) proteins that are associated with NEPC cell identity. Next, we analyzed a cohort of > 300 metastatic CRPC patients to reverse-engineer both regulatory network of Transcription Factors (TFs) and regulatory dependencies between signaling proteins and TFs activity.

Results and Discussions

Our analysis resulted in a regulatory network of NEPC-associated candidate MR proteins and their upstream genomic events, that are likely to be responsible for this treatment-resistant phenotype. Cross-species computational analysis of patient-specific MRs activity conservation between metastatic human CRPC and our mice cohorts, aligned NEPC patients to mice tumors with NE differentiation features, corroborating our study's clinical relevance.

Conclusion

In summary, we used *in vivo* models of prostate cancer to elucidate molecular drivers of NEPC, a lethal variant that is treatment-resistant. We are in the process of validating these results.

Kinase activity inference from murine phosphoproteomics data-sets

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Introduction

Abnormal cellular signaling is widely accepted as a hallmark of cancer, and the role of protein phosphorylation herein is paramount. Phosphoproteomics offers by means of the characterization of the protein phosphorylation state of a tissue, an unbiased charting opportunity of aberrant protein kinase activity. The extent and complexity of these data types, however, demand advanced dedicated methods for the inference of kinase activities. All efforts to this end can, to this day without exception, only be applied to human tissue data-sets. This also includes InKA, a very robust kinase activity inference method developed in our group (Beekhof *et al.*, Mol.Syst.Biol. 2019, PMID:31126969). Consideration that much of fundamental cancer research involves murine samples, underlines the urgent need for an equally advanced method for inferring kinase activity in these animal tissues.

Material and Methods

We chose a similar algorithmic architecture as in our previously developed InKA kinase activity score for human tissues. Calculation of non-zero InKA scores requires knowledge of murine kinase amino-acid sequences but also the corresponding activation-loop sequences. These were generated from murine Uniprot protein sequences and the Phomics resource. Even more important for deriving non-zero InKA scores, is knowledge of kinase-substrate (KS) relations, which is already limiting in the human case. In our human implementation an experimentally validated PhosphoSitePlus (PSP) kinase-substrate set is supplemented with a NetworkKIN (NWK) predicted set of KS relations to extend phospho-proteome coverage. As experimentally validated KS relations in the murine realm are about only 30% in extent compared to the human case, we included in our murine implementation an additional KS source besides a NWK predicted KS relation set. This source was generated by mapping human experimental KS relations to the mouse substrates using sequence homology.

Results and Discussions

Our resulting murine InKA method can infer the activity of a total of 362 kinases in murine datasets. Inclusion of homology mapped experimentally validated human KS expanded the kinase scope by 40 additional kinases. Application on a murine data-set showed differential kinase activity.

Conclusion

The murine InKA pipeline extends of a robust kinase activity inference method to murine phospho-proteomics experiments, allowing for extraction of detailed kinase activity changes under experimentally controlled conditions that are unattainable with humans.

EACR22-1255

The impact of tumour morphology landscape on molecular signatures

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Introduction

We investigate the intra-tumor heterogeneity of colorectal cancer and its impact on gene-based classifiers. While the consensus molecular subtypes (CMS) (Gunnery, 2015) identify some major vectors of inter-tumor variability, they are subject to extraneous noise, such as

tumor sampling strategy (Dunne, 2017), which may result in under-representation of some morphological patterns (e.g. tumor stroma, mucinous), possibly obscuring additional subgroups. Our own results identified correlations between dominant gene expression signal and morphological patterns.

Material and Methods

From 110 colon tumors, 203 whole-genome expression profiles corresponding to different morphological patterns (complex tubular (CT), desmoplastic (DE), mucinous (MU), papillary (PP), serrated (SE), solid trabecular (TB), tumor-adjacent and stroma) and whole-tumor were generated using Clariom D microarray. CRC gene module scores were computed as in (Budinska, 2013), cell population mixtures were estimated using xCell and CMS predicted using CMScaller. Five prognostic gene scores were computed and comparisons between whole-tumor and region-based were performed using rank correlation. PERMANOVA was used to test for the effects of individual (tumour), morphology and batch on the gene profiles.

Results and Discussions

30 whole-tumor and 173 regions were profiled (44 CT, 61 SE or PP, 17 DE, 19 MU, 5 TB, 27 others). Morphology had both the strongest effect on gene expression variability as represented by gene modules and different composition of cell types as estimated by xCell ($p < 0.001$), followed by the effect of patient ($p = 0.014$, $p = 0.017$). Important differences in signature ranks between morphological regions and the corresponding whole-tumor values were observed directly depending on the proportion of major morphological region.

Whole tumor CMS1-3 had good concordance with the subtype of at least one of its corresponding regions but CMS4 was less stable. CTs were mostly labeled as CMS1 and CMS2, DEs as CMS4 and CMS1, SEs as CMS2 and CMS3, MUs as CMS4. Canonical Wnt was mostly activated in MU, SE and stroma regions. The EMT signature had highest values in DE, MU and stroma regions.

Conclusion

Morphological regions may have a different molecular subtype than the whole tumor, indicating the presence of additional subtypes. The gene expression classifiers are sensitive to tumor sampling and clear specification of region(s) used for RNA profiling is necessary. Multi-region sampling opens the possibility for refining and stabilizing the classifiers.

EACR22-0088

Automated prediction of drug response in HCC cell lines with deep learning-based bioactivity modeling and experimental validation

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Introduction

Having prior knowledge about the response of tumors to chemotherapeutic agents is challenging in personalized medicine. Due to the high cost and long durations of drug screening processes, only a small part of the pharmacogenomic space can be screened. With the utilization of computational approaches, new treatment options can be inferred, which rely on testing and validating target-based drug response predictions. Due to the

higher complexity of the cell compared to a protein target, the translation of target-based inhibition knowledge to the phenotypic level is problematic. As a result, available target-based predictive methods fail to become parts of real-life biomedical applications. This study aims to propose a high-performance artificial intelligence-based computational system that predicts small molecule inhibitors directly against cancer cell lines (circumventing the prediction of interactions with target proteins), primarily focusing on hepatocellular carcinoma (HCC).

Material and Methods

The prediction model takes cell-line multi-omics-based features as input together with drug molecular features and processes them via deep learning architectures. The trained and optimized model is used to predict new inhibitors for 8 different HCC cell lines for anti-cancer drug repurposing. Predictions are filtered based on their pIC₅₀ values (>6) and specificity for minimum 6 different HCC cell lines. Compounds were tested in vitro via sulforhodamine B (SRB) assay. Annexin-V assay was performed to detect induction of cell death in HCC cell lines. Sorafenib was used as a control.

Results and Discussions

Among a large group of predicted inhibitors, 4 compounds, 2 investigational drugs (Loperamide N-oxide and Eprinomectin), which were not studied on HCC before, and 2 PI3K/mTOR pathway inhibitors (Rapamycin and ZSTK474), which were previously studied on HCC cell lines, were selected for in vitro validation studies. Predicted IC₅₀ values of all drugs were consistent with the IC₅₀ values obtained from the SRB assay. Moreover, these drugs inhibited cell growth and induced cell death in HCC cell lines. All drugs exhibited their cytotoxic effects in lower concentrations compared to Sorafenib.

Conclusion

Our prediction model successfully predicts specific small molecule drugs for HCC cell lines. With additional in vitro validation experimentation, we hope to identify new inhibitors that have the potential to be repurposed against HCC.

EACR22-0126

Personalised Medicine for Colorectal Cancer Using Mechanism-Based Machine Learning Models

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Introduction

Gaining insight into the mechanisms of signal transduction networks (STNs) by using critical features from patient-specific mathematical models can improve patient stratification and help to identify potential drug targets. To achieve this, these models should focus on the critical STNs for each cancer, include prognostic genes and proteins, and correctly predict patient-specific differences in STN activity.

Material and Methods

Focusing on colorectal cancer and the WNT STN, we used mechanism-based machine learning models to identify genes and proteins with significant associations to event-free patient survival and predictive power for explaining patient-specific differences of STN activity. First, we identified the WNT pathway as the most significant pathway associated with event-free survival. Second, we built linear-regression models that incorporated both genes and proteins from established mechanistic models in the literature and novel genes with significant associations to event-free patient survival. Data from The Cancer Genome Atlas

and Clinical Proteomic Tumour Analysis Consortium were used, and patient-specific STN activity scores were computed using PROGENy. Three linear regression models were built, based on; (1) the gene-set of a state-of-the-art mechanistic model in the literature, (2) novel genes identified, and (3) novel proteins identified. The novel genes and proteins were genes and proteins of the extant WNT pathway whose expression was significantly associated with event-free survival.

Results and Discussions

The results show that the predictive power of a model that incorporated novel event-free associated genes is better compared to a model focusing on the genes of a current state-of-the-art mechanistic model. Several significant genes that should be integrated into future mechanistic models of the WNT pathway are DVL3, FZD5, RAC1, ROCK2, GSK3B, CTB2, CBT1, and PRKCA.

Conclusion

Thus, the study demonstrates that using mechanistic information in combination with machine learning can identify novel features (genes and proteins) that are important for explaining the STN heterogeneity between patients and their association to clinical outcomes.

EACR22-0196

Machine learning-based tissue of origin classification for cancer of unknown primary diagnostics using genome-wide mutation features

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Introduction

Tumor tissue of origin (TOO) is an important factor for guiding treatment decisions. However, TOO cannot be determined for ~3% of metastatic cancer patients and are categorized as cancers of unknown primary (CUP). As whole genome sequencing (WGS) of tumors is now transitioning from the research domain to diagnostic practice in order to address the increasing demand for biomarker detection, its use for detection of TOO in routine diagnostics also starts becoming within reach. While proof of concept for the use of genome-wide features has been demonstrated before, more complex WGS mutation features, including structural variant (SV) driver and passenger events, have never been integrated into TOO-classifiers even though they bear highly characteristic links with tumor TOO.

Material and Methods

Using a uniformly processed dataset encompassing 6756 whole-genome sequenced primary tumors from the Pan-Cancer Analysis of Whole Genomes consortium (PCAWG) and metastatic tumors the Hartwig Medical Foundation (HMF), we have developed Cancer of Unknown Primary Location Resolver (CUPLR), a random forest based TOO classifier that employs 511 features based on simple and structural somatic driver and passenger mutations.

Results and Discussions

Our model can distinguish 35 cancer (sub)types with an overall accuracy of 90% and 89% based on cross-validation (n=6082) and test set (n=674) predictions. We found that SV derived features increase the accuracy and utility of TOO classification for specific cancer types. With CUPLR, we could determine the primary cancer type for 82/141 (58%) of CUP patients. Although CUPLR is based on machine learning, it provides a human interpretable graphical output with detailed feature explanations.

Conclusion

The comprehensive CUPLR output complements existing histopathological procedures and assists diagnostic usage to improve diagnostics for patients with CUP.

EACR22-0620

Pre-treatment CT radiomics predicts the atypical molecular subtype for locally advanced HNSCC

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Introduction

Patients with head and neck squamous cell carcinoma (HNSCC) may benefit from more personalised treatment based on biomarkers that allow for differentiating between tumours. We assessed if radiomics features from pre-treatment CT of locally-advanced HNSCC patients treated with primary radiochemotherapy (pRCTx) can be used to differentiate between four distinct molecular subtypes of HNSCC: atypical, basal, classical, and mesenchymal [1]

Material and Methods

A multicentre cohort of 206 patients from the German Cancer Consortium - Radiation Oncology Group (DKTK-ROG) with locally advanced HNSCC was divided into training and validation (122 and 84 patients, respectively). Gene data was derived from primary tumour biopsies using GeneChip® human transcriptome arrays (Affymetrix) and used to assign one of four molecular subtypes to each patient [2]. Radiomics features were derived from the

primary gross tumour volume (GTV) on the treatment-planning CT. They were filtered for robustness and afterwards clustered. Subsequently, four models, one to classify each subtype, were created within a machine-learning framework. Logistic regression models were developed on the training cohort and validated on the validation cohort using selected features. Model performance was measured using the area under the curve (AUC) with 95% confidence interval. Subsequently, the atypical model was re-evaluated for patients with human papilloma virus (HPV) negative tumours

Results and Discussions

110 patients (69 in training and 41 in validation) were assigned to one of four subtypes, while the remaining patients had an inconclusive assignment or insufficient correlation. Atypical subtype tumours could be well differentiated from the others with an AUC of 0.68 [0.52-0.79] in training and 0.69 [0.53-0.83] in validation. They have more regular shapes and are macroscopically more homogeneous, which is related to the identified radiomics features that evaluate volume density and non-uniformity of grey levels. The model also performed well for HPV-negative tumours, with a training AUC of 0.70 [0.53-0.84] and a validation AUC of 0.74 [0.56-0.89]. The other subtypes could not be inferred using CT radiomics features

Conclusion

Radiomics can distinguish the atypical subtype using pre-treatment CT features, also for the patients with HPV-negative HNSCC. Results will be validated in the upcoming prospective HNPrädBio study of the DTK-ROG.

References

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EACR22-0880

Quantitative descriptors of the tumour microenvironment predict immunotherapy response

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Introduction

Antitumor immune response is coordinated by complex mechanisms involving different cell types, intracellular networks, and cell-cell communication. In a recent study (Lapuente-Santana et al. 2021), we have used quantitative features derived from RNA sequencing (RNA-seq) to describe several facets of the tumour microenvironment (TME): cell type abundances, pathways and transcription factor activity, ligand-receptor and cell-cell interactions. Starting from these features, we used machine learning to identify biomarkers of immune response and predict response to immunotherapy in different cancer types. Here, we expand our approach by integrating spatial information extracted from tissue-slide imaging and cytokine signalling activity inferred from RNA-seq.

Material and Methods

We quantified from RNA-seq data 13 TME cell types (Finotello et al. 2019; Yoshihara et al. 2013) and the activity of 43 cytokines (Jiang et al. 2021). We considered different types of spatial features derived using computational pathology (Diao et al. 2021; Saltz et al. 2018). We trained cancer type-specific machine learning models to predict ten state-of-the-art scores of immune response, using multi-task regularised linear regression on the The

Cancer Genome Atlas (TCGA) data. Predictive performances were evaluated using cross-validation on the TCGA data and independent validation on eight datasets (encompassing four cancer types) from patients treated with immune checkpoint inhibitors.

Results and Discussions

Integration of spatial features improved the predictive power of the models (p -value < 0.05) on some cancer types (lung cancer, stomach cancer) but not on others (melanoma). The most important spatial features were the proportion of lymphocytes with respect to tumour cells in epithelial tissue and the cluster dispersion of lymphocytes. Cytokine signalling activities showed high predictive performance in the cross-validation and in the independent test sets. INFG, IL27, and MCSF were among the top cytokine biomarkers positively associated with immune response, and were validated in the validation cohorts. Finally, we showed that RNA-seq-derived features are robust to the number of measured genes, showing potential for being derived from expression assays targeting a few hundred genes.

Conclusion

Our approach provides a high-level representation of the TME from commonly available data (RNA-seq and pathology slides), allowing us to obtain interpretable biomarkers covering different sets of mechanisms behind patient response to treatment.

EACR22-1126

Predicting mismatch repair deficiency in ovarian carcinomas

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Introduction

Endometrioid (EOC) and Clear Cell Ovarian Carcinomas (CCOC) are two challenging ovarian cancer histotypes due to their bad prognosis in advanced stages, mainly related to resistance to conventional chemotherapy and lack of alternative options. These tumours present higher incidence of somatic alterations inactivating the DNA mismatch repair (MMR) system. Furthermore, immunotherapy has been recently granted approval for treatment of any tumour type with MMR deficiency. Therefore, accurate identification of MMR deficiency in EOCs and CCOCs is relevant because of its prognostic and predictive value.

Immunohistochemistry and PCR-based methods are the gold-standard techniques for the detection of MMR deficient tumours. Here, we aimed to explore the performance of genome-based biomarkers to detect MMR deficiency compared to standard methods. We hypothesized that this approach could provide gains in predictive capacity.

Material and Methods

Using FFPE tumour samples collected from a multicentre study ($n = 180$) and NGS data from a customized gene-panel enriched in microsatellite sequences, we combined gene-derived information (such as the presence of potential pathogenic mutations in the MMR genes), together with genome-based data (such as the Tumour Mutation Burden, the Microsatellite Instability burden, copy number variations and mutational signatures), to develop an algorithm to detect MMR deficiency.

Results and Discussions

The integration of different genomic biomarkers for the detection of MMR deficiency resulted in a synergistic combination, which significantly improved the prediction. Hence, this

approach may serve to enhance decision-making for referring patients to immunotherapy or chemotherapy.

Conclusion

An efficient clinical tool able to detect MMR deficiency for stratifying EOC and CCOC patients would be a serious breakthrough towards personalized medicine, but more important, for the treatment of these types of tumours, often tackled ineffectively with standard chemotherapy.

EACR22-0257

Identifying tumor cells at the single cell level

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Introduction

Tumors are highly complex tissues composed of cancerous cells, surrounded by a heterogeneous cellular microenvironment. One of the critical cell type annotation challenges is identification of tumor cells within single cell or spatial sequencing experiments. This is a critical limiting step for a multitude of research, clinical, and commercial applications. Here, we propose Ikarus, a machine learning pipeline aimed at distinguishing tumor cells from normal cells at the single cell level. We have tested Ikarus on multiple single cell datasets to ascertain that it achieves high sensitivity and specificity in multiple experimental contexts.

Material and Methods

We have implemented a two step approach for solving a problem that is perceived as simple: discriminating tumor cells from normal cells. In the first step, Ikarus pipeline integrates multiple expert labeled datasets to extract gene sets which discriminate tumor cells from normal cells. In the second step, Ikarus uses a robust gene set scoring along with adaptive network propagation for cell classification. By using robust gene set scoring and network propagation we have mitigated two common problems in single cell analysis: the influence of batch effects on sample comparison and parameter optimization during clustering.

Results and Discussions

We trained Ikarus on scRNA-seq datasets of colorectal and lung carcinomas. The algorithm was further tested on the following cancer types: lung, head & neck cancer, and neuroblastoma. Ikarus achieves an average balanced accuracy of 0.98 which is substantially higher than other competing machine learning methods.

Conclusion

We have implemented a two step approach for solving a problem that is perceived as simple: discriminating tumor cells from normal cells.

Automatic, parameterless discrimination between tumor cells and the surrounding tumor-associated tissue has multifactorial utility. Tumor cells can be streamlined into algorithms for neoepitope prediction, thereby enabling direct, clinically relevant insights. Furthermore, the increasing availability of multi-omics measurements would enable automatic genetic characterization of tumor subpopulations, and the subpopulation based recommendation of best therapeutic course of action

Predicting different tumor types from molecular data using feature extraction and machine learning approaches

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Introduction

Cancer is a complex disease, with patterns of genomic alterations showing intra-cancer heterogeneity and cross-cancer similarity. Molecular data spanning most cancer types can shed light on tumor molecular mechanisms and refine prevention, diagnosis and treatment in the personalized medicine era. Machine learning (ML) represents a powerful tool to capture relationships between molecular alterations and cancer types and build accurate methods to extract biological information. Starting from TCGA data, we developed a ML model aimed at distinguishing cancer types with high accuracy based on molecular lesions. This could improve cancer diagnosis by using specific DNA alterations, embedded in a replicable easy-to-use automated technology.

Material and Methods

Data of 9927 samples spanning 32 different cancer types were downloaded from cBioportal. To create datasets, calls for somatic point mutations (SNP, DEL, INS and ONP) and copy number alterations at chromosome arm-level were considered as predictive features of cancer types. Preprocessing and XGBoost classifier models were applied.

Due to imbalance in the dataset ascribable to different number of cases for each tumor, the following two strategies were considered: -set a percentage cut-off threshold to remove from the analysis less represented cancer types, -divide cancer types into different groups, and training a specific XGBoost model for each of them.

Results and Discussions

Using different cut-off thresholds, the XGBoost classifier achieved the best performance (accuracy of 77%, AUC score of 97%) on a dataset containing only 10 out of 32 tumour types.

By removing poorly represented cancer types and dividing tumors into 3 different groups (endocrine-related carcinomas, other carcinomas and other cancers), a total of 18 different tumor types were analyzed and the 3 analysis models achieved 78%, 71% and 86% accuracy respectively, with AUC scores above 96%.

Another global dataset was created to build a XGBoost model capable to link each group to a specific cancer type and used to technically confirm the appropriateness of the model, reaching an accuracy of 81% on the holdout data and a 94% AUC score.

Conclusion

A new accurate ML model discriminating among different cancer types based on somatic alterations was developed. Although further analyses to confirm the performance of this promising model as well as extract information of biological relevance are required, this approach could have potential clinical application in terms of cancer diagnosis improvement.

Massive Machine Learning driven mechanistic analysis of drug repurposing landscape in rare tumors

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Introduction

Mechanistic models provide a causal connection between omics measurements of gene activity and the consequences they have in the phenotypes through the modeling of their functional implications in biological processes. In turn, machine learning methods (ML) can learn new biological relationships from massive data, thus can be used to expand the current biological knowledge on molecular functional relationships.

There is a need for the identification of combinations of drugs that induce more efficient responses in rare tumors. Merging the knowledge about mechanisms of disease and drug action, together with the arising machine learning (ML) methods, here we present a mechanistic model approach to massively identify and define the disease influence map, gaining insight into the functional landscape of relevant genes in rare tumors, and the impact that drugs will have on that landscape.

Material and Methods

We used the KEGG database (DB) for the construction of each disease map. We built the disease map by extracting all the circuits (receptor-effector subpathways) containing the genes responsible for each rare tumor according to ORPHANET DB. Using GTEx expression data, we obtained circuits activity from Hipathia method to implement Multi-Output Random Forest (MORF) regression, backed by a repeated cross-validation strategy and unbiased SHAP (SHapley Additive exPlanations) values, to infer the effect of the approved drug targets from DrugBank DB over the activity of the constructed disease map. Finally, we analyzed the relevant targets by performing enrichment and network analyses to obtain relations among all the analyzed rare tumors.

Results and Discussions

We have obtained the mechanistic map of 28 rare tumors, with relevant approved drug targets for all of them. Besides, we have analyzed the functional landscape of relevant targets obtaining those genes relevant for most of the rare tumors, as well as those more specific. We also obtained the impact that each of these drug targets will potentially have on the individual regions of the disease map, pathways and functions, and analyzed each rare tumor in terms of hallmarks of cancer, showing the functional network of rare tumors in terms of potentially relevant drugs.

Conclusion

We presented a new approach in the use of mechanistic models to obtain potential drugs suitable for the treatment of rare tumors, that usually have a lack of funding and of a sufficient number of patients to perform massive analyses, thus accelerating the evaluation of putative new treatments

EACR22-0871

Automated classification of Circulating Tumor Cells (CTCs) using Convolutional Neural Networks (CNNs)

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Introduction

Clinical studies have demonstrated that CTC enumeration by CELLSEARCH® is a strong predictor of cancer prognosis and can provide an important tool to assess disease progression. CTCs are extremely rare cells and their counts, with respect to cut-offs, emphasize the importance of assigning these events accurately. Unfortunately, detection by human reviewers is often associated with high variability (Zeune L.L. Cytometry 2018), as well as being rather laborious. CNNs offer the opportunity to make enumeration less time-consuming and more reproducible and have already provided remarkably good results in various applications. For research use only, we developed a novel algorithm based on CNNs to detect and classify cells in images captured with fluorescence microscope.

Material and Methods

Blood samples, derived from metastatic prostate and breast cancer patients, were processed with CELLTRACKS® AUTOPREP®. Our CNN was trained to classify cells into CTC and non-CTC categories, based on fluorescent images acquired by CELLTRACKS ANALYZER II®. The algorithm was designed to integrate a new segmentation method with a classification CNN based on recent studies (Coumans F. ACTC 2021; Zeune L.L. Nature MI 2020) and it was trained on prostate patient samples. A pool of 5 expert reviewers performed a blind labeling of 23 patient samples, generating the ground truth (GT) by majority voting. The performance of the algorithm was compared with other 4 reviewers using F1 score.

Results and Discussions

The test dataset contained 5516 events in total, of which 1909 CTCs identified by the GT review. The algorithm's F1 score was 0.89, falling within the range of reviewers' performance (F1 = 0.85 - 0.95). These results would likely improve further by extending the training of the algorithm with additional samples from different tumor types. In addition, the estimated execution time per sample was on average about 18 minutes for manual review and 3 minutes for the automated method. This time saving could be increased by processing multiple runs in parallel.

Conclusion

In conclusion, the performance of the proposed algorithm is in line with that of individual reviewers, but with a six-fold time reduction. These preliminary results show the potential of applying automated methods to cell classification to remove reviewer subjectivity, improve standardization and save time.

EACR22-0885

Deep learning predicts somatic BRCA 1/2 genes mutational status from histopathology of epithelial ovarian cancer: a hypothesis generating study

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Introduction

Mutational status of BRCA 1/2 genes is gaining more and more weight in the therapeutic algorithm of high grade serous epithelial ovarian cancer (HGSOC) at first diagnosis.

Nevertheless, the evaluation of the mechanisms of homologous recombination deficiency (HRD) pathway, of which BRCA1/2 mutations are well-known causes, is progressively overcoming BRCA testing. However, HRD testing is not already integrated in clinical practice and it is not refundable in many countries. Histologic phenotypes have been recognized to somehow reflect the genetic alterations in cancer tissues. Since hematoxylin and eosin (H&E)-stained tissue slides are ubiquitously available for cancer patients, mutations prediction from the tissue slides could be a time-saving and cost-effective method to characterize patients and address them to the most appropriate genetic testing afterwards.

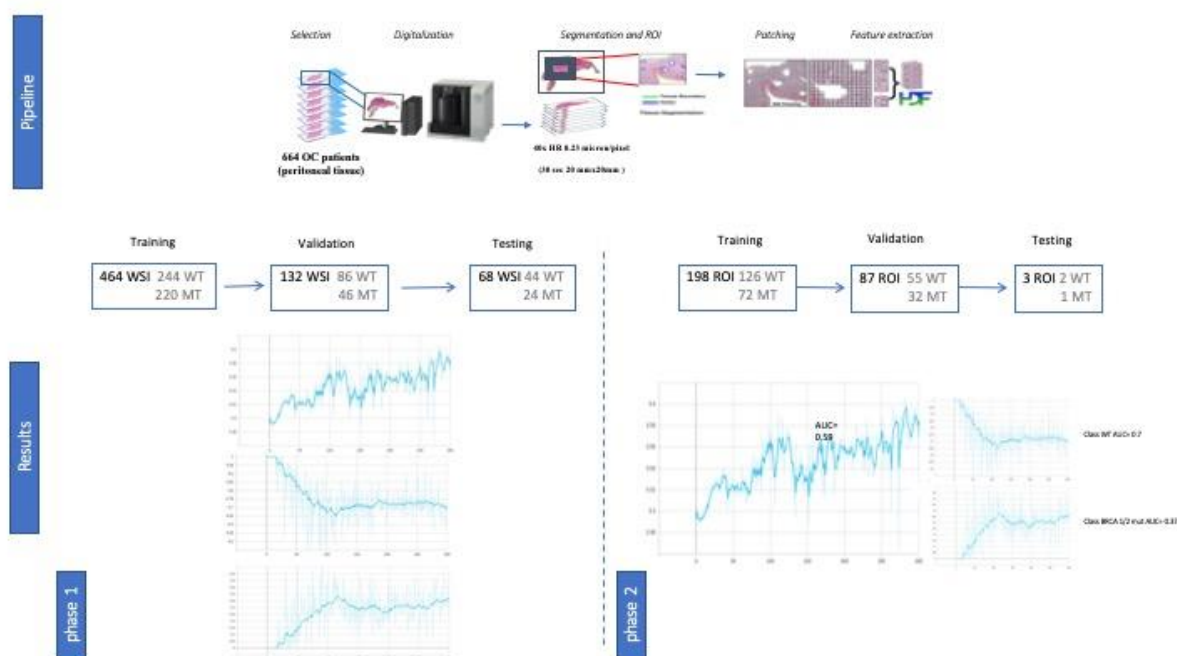
Material and Methods

We aimed at exploring whether somatic BRCA1/2 genes status can be predicted using ubiquitously available H&E histology. This could allow clinicians to select patients who are more likely to benefit from HRD assessment (BRCA 1/2 WT) at an early stage of the diagnostic process thus optimizing genomic profiling resources.

A recently published clustering constrained- attention multiple-instance learning (CLAM) as a high-throughput deep-learning framework was adopted.

Results and Discussions

We applied the model to a cohort of 664 digital H&E slices of HGSOE patients (Training set: n =464, Test set: n =132; 233 somatic BRCA1/2 mutated patients) from Fondazione Policlinico Universitario Agostino Gemelli IRCCS achieving an area under the curve of 0.7 in the training set and 0.55 in the testing set. We further refine the model by manually identifying areas of interest in half cases. We used 198 images for training (126/72) and 87 images for validation (55/32). At the end of training, the model reached a zero classification error on the training set, but the performance of the predictive model was 0.59 in terms of validation AUROC, and 0.57 validation accuracy.



Conclusion

Given the encouraging preliminary results in terms of feasibility, further developments are well worth carrying out.

On the one hand, we are already exploring the hyperparameters space for training optimization, eventually tweaking the learning algorithms and the neural networks architecture for better suiting the task of somatic BRCA 1/2 status identification. On the other, we are also adopting on the same dataset a different approach based on persistent homology.

EACR22-0177

A deep learning image analysis approach to improve high-throughput and multiparametric organoid drug screenings.

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Introduction

Patient-derived tumor organoids (PDOs) are the most advanced 3D cancer model that resemble both the pheno- and genotype of the parental tissue. PDOs are revolutionizing basic and translational cancer research, with one of the most promising applications in predicting clinical response *ex vivo* for personalized cancer treatment. However, the current repertoire of assays for high-throughput organoid analysis is critically limited, and the gold standard relies on a rudimentary, single-time point, viability assay. This has severely impeded the routine use and potential of organoids for basic research, clinical practise, and pharmaceutical applications.

Material and Methods

Here, using a convolutional network machine learning approach, we developed a high-throughput, automated organoid analysis platform that allows for kinetic monitoring of organoid growth and therapy response using live-cell imaging: **Organoid Brightfield Identification-based Therapy Screening (Orbits)**. Using Orbits, we performed a screening of several chemotherapeutics and targeted therapies on lung PDOs in 384-well plates and compared our method with current standards using a Tecan Spark Cyto. Informed consent was obtained, and this study was approved by the UZA Ethical Committee.

Results and Discussions

Not only could we monitor PDO growth, health, and varying degrees of therapy response in real-time, solely from brightfield imaging, when combined with a fluorescent cell death marker, our analysis further delineated cytostatic from cytotoxic therapy responses. Our results highly correlated with the readout from the gold-standard CellTiter-Glo 3D assay, and due to its machine learning approach, the Orbits analysis method could be easily applied to various PDO types in different culturing methods (e.g. matrix domes, Gri3D®-96 well plates). Finally, by implementing kinetic analysis, growth rate metrics (e.g. GR50 or normalized drug response) can be applied to drastically reduce inter-well and inter-patient variability.

Conclusion

Taken together, we have developed an advanced live-cell imaging drug-screening platform which i) offers high-throughput, real-time, and label-free PDO analysis with ii) the ability to provide insight into the mechanistic drug action and iii) flexibility to adapt for various applications and iv) compatibility with conventional live-cell imaging instruments (e.g. Tecan Spark Cyto, IncuCyte system). Finally, a university operated web app is being developed to make our image analysis software available (www.orbits-oncology.com).

EACR22-0379

Highly multiplexed tissue image analysis using superpixel-based segmentation

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Introduction

Ultra-high plex tissue imaging platforms, such as PhenoCycler™-Fusion, are transforming spatial biology by allowing up to 100 proteins to be detected on a single intact tissue section covering millions of cells. Quantitative digital image analysis plays a critical role in characterizing the complexities of the tissue microenvironment to understand the molecular and cellular mechanisms that underlie disease development, differentiation, and responses.

Material and Methods

The general analysis workflow consists of cell segmentation, followed by calculations of average protein expression from the corresponding immunofluorescence images, which are then used in cell phenotyping and spatial analysis. This workflow lacks precision, due to inaccuracies in cell segmentation algorithms and signal spillover. Moreover, rigorous quantification of certain proteins expressed in pseudopods, which are arm-like membrane projections from macrophages, microglia, endothelial cells or secreted proteins, can currently not be performed. To address this gap, we present an alternative approach that characterizes the spatial organization of the tissue microenvironment at the protein level rather than the cellular level. Here, the spatial entity is a protein fragment represented by a superpixel, which is a group of pixels that share similar perceptual features. A superpixel-based analysis therefore characterizes the tissue by protein-protein interaction rather than cell-cell interaction and thus provides a higher level of technical details. The proposed method consists of three main steps. First, unsupervised superpixel segmentation using simple linear iterative clustering (SLIC) is applied to the composite image of each pair of protein images. Then, the average intensity of each protein is calculated for each superpixel and unsupervised clustering is performed. Finally, spatial analysis is performed using the locations of the superpixel clusters.

Results and Discussions

To assess the effectiveness of our approach, we perform cross-correlation analysis of protein expression from superpixels and cell segmentation masks on different tissue types and evaluate the output based on a list of distinct and non-overlapping proteins.

Conclusion

We provide a practical and conservative method for the quantification of the spatial organization of proteins based on superpixel segmentation. The results of our study show that this method could improve the accuracy and predictive capabilities of computational models used in multiplex image analysis.

EACR22-0389

RNA_MUTECT2: somatic RNA-seq single nucleotide and indels variants discovery pipeline.

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Introduction

Mutations accumulate over time and generate clonal and subclonal populations causing intratumoral heterogeneity (ITH). Bioinformatics pipelines have been developed to calculate ITH from somatic variants found in DNA bulk sequencing data. However, not all somatic events that are detected in DNA sequencing data have a functional effect. We hypothesize that studying jointly DNA and RNA ITH somatic events will broaden the landscape of ITH. Somatic RNA variant calling is more challenging due to RNA-specific features such as coverage fluctuations, RNA editing, high number of duplicated reads, alternative splicing, and here we have extended upon the work of Yizhak *et al.*, 2019 (Science).

Material and Methods

RNA-Mutect2 Variant Calling: RNA-MuTect2 pipeline uses STAR-aligned RNA-seq BAMs files as input. We follow the same steps as Yizhak *et al.*, with GRCh38 aligned bam. An additional RNA editing detection was performed to tag suspected RNA editing events. Polyadenylation events were also highlighted and compared to a polyadenylation database. Whitelisting was applied with Cancer Genome Interpreter curated dataset. The pipeline was written in python3.

Data: A subset of 241 samples were taken from The Cancer Genome Atlas (TCGA) as a training set. The panel of normals were generated with 11,653 samples from The Genotype-Tissue Expression (GTEx) project.

Results and Discussions

We developed RNA-MuTect2 (RM2), extending upon RNA-Mutect method from Yizhak *et al.*, 2019. Using a subset of 241 tumours from The Cancer Genome Atlas (TCGA) we have compared our RNA-Mutect2 platform with the previous version and more than 80% detected single-nucleotide variant (SNV) overlap with the two methods. The SNVs that were found in previously and not detect in the RNA-MuTect2 calls, were filtered by the new pipeline due to low quality reasons and changes between the variant calling algorithms in the two mutation calling pipelines. RNA editing modelling was performed to tag suspected RNA editing events based on VAF, annotation and mutation type. Patterns of indels and RNA editing events display a novel landscape of the cancer transcriptome.

Conclusion

Transcribed somatic SNVs and indels can be accurately detected with the new RNA-MuTect2 pipeline. Our calls match with the previously published pipeline (Yizhak *et al.*, 2019 (Science)) and also detect small insertions and deletions. In addition, RNA-MuTect2 detects and predicts RNA editing events and polyadenylation events. These results will be used in future intratumoral heterogeneity studies.

EACR22-1117

ProDe: Protein interaction informed analysis of Cancer Dependencies

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Introduction

Cancer mutations may trigger tumors dependency on specific genes that can in turn expose new therapeutic opportunities. Whole genome screens (shRNA or sgRNA-based) on cancer cell lines assess the impact of single gene genetic perturbation on cell fitness. Data analysis of screenings performed on cells harboring vs. lacking a specific mutation can nominate putative dependencies (*single-gene* approach). However, different experimental set-ups and inter-cell line biological heterogeneity hamper the identification of reproducible hits. Based on previous literature, we hypothesized that the integration of protein-protein interaction network (PPIN) information in the single-gene approach analysis may lead to solid and

reproducible dependencies. To test our hypothesis, we implemented ProDe, an analysis approach that performs a protein interaction informed analysis of cancer dependencies.

Material and Methods

Genomics and CRISPR-KO screening data of 370 cell-lines across 21 tissues from CCLE and DepMap project, as well as PPIN from STRING database, were interrogated to identify putative dependencies. ProDe performance was tested against a commonly used *single-gene* approach leveraging a selection of literature derived cancer dependencies, pharmacological screens from GDSC database and hits from publicly available and *in-house* generated iso-genic (9p21 genomic locus) CRISPR-KO screens.

Results and Discussions

ProDe first ranks the screened genes accordingly to their higher essentiality in mutated compared to not-mutated models (two cell lines groups) and next, for each gene and a given PPIN, all relevant genes are jointly assessed for their ranks within the full gene list. When classifying literature-derived cancer dependencies, ProDe showed significantly higher AUC and recall values (Wilcoxon, p-value < 0.001). Pharmacological inhibition of ProDe nominated hits was overall more effective than in the case of *single-gene* approach nominated hits (Wilcoxon p-value < 0.001). Last, ProDe nominated dependencies better recapitulated hits derived from iso-genic screens (ROC test, p-value < 0.05) including known MTAP-RIOK1 dependency further supported by in-house screen findings.

Conclusion

Our workflow suggests better performance than standard approaches in detecting literature-derived dependencies and potential therapeutic opportunities. By detecting hits derived from iso-genic screens, it further showed improved identification of cancer dependencies exploiting data from cell-models with heterogeneous genetic backgrounds.

EACR22-0488

Mathematical modelling of tumour growth using breast cancer patient-derived tumour xenografts (PDXs).

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Introduction

Within the diversity of existing preclinical models to study cancer, patient-derived tumour xenografts (PDXs) represent one of the approaches that could best inform about patient-specific characteristics of the original disease. Ordinary differential equations (ODE) have been widely implemented to quantify and model tumour growth. Different ODE have been shown to model tumour growth successfully, however, whether the rate of tumour growth and the assumptions of each ODE that best models these are affected by the initial amount of tumour cells implanted is less well understood.

Material and Methods

We aimed to evaluate the fitting performance of several ODEs on PDTXs from five breast cancer samples. Single-cell suspensions derived from these tumour samples were subcutaneously transferred into immunodeficient mice, and tumour volume was routinely measured. Three different doses of injected tumour cells were tested in each PDTX model: 5×10^5 , 1×10^5 and 2×10^4 . We tested the fitting performance with exponential, logistic, generalised logistic, Gompertz, and Power-Law models and determine the existence of a correlation between tumour growth rate and the number of cells injected.

Results and Discussions

For this work, we model, compare and evaluate each of the five ODEs to describe the growth rate across all PDTXs models. After having estimated the growth rates and related parameters for each model, we found that the exponential model was overall the best to fit tumour volume measurements. In our experimental setting, tumours were not subjected to drug treatment and did not have limiting carrying capacity, which is essential to take into consideration since, in these other conditions, other models might perform better. Furthermore, varying the number of cells injected to establish tumours for each PDTX model allowed us to assess the positive or negative correlation of these two variables. Interestingly, this association will reveal if the growth rate is independent of the number of cells injected and also if this correlation is specific to the PDTX model studied.

Conclusion

In conclusion, we show detailed comparisons of mathematical models of tumour growth from PDTXs and test the association between growth rate and starting number of cells used to establish the xenograft. This work will be necessary to assess experimental variables and manipulations on tumours grown in animal models to understand tumour biology better.

EACR22-1250

Mechanistic models for survival study and in silico prediction of Sunitinib on Soft-tissue Sarcomas patients

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Introduction

Soft-tissue Sarcomas (STS) account for less than 1% of adult and 15% of pediatric tumors. These rare tumors are hard to treat which, in addition to the lack of research due to their low incidence, has contributed to an average life expectancy remaining at 1.5 years over the past 40 years. The search for systemic treatments that improve overall survival is an urgent need. Within the context of systems biology applied to drug discovery, we have developed a method to in silico evaluate the individual response of a patient to a drug. The method relies on the accumulated knowledge stored in biological databases to mechanistically predict the effect that the drug will have over the disease pathways, as well as to detect potential side effects. Using this method, we have evaluated the accuracy of the predicted response results with a data set of STS patients treated with Sunitinib at different time points, with the actual data on response.

Material and Methods

We used DrugBank for drug-target information and KEGG for signaling pathway networks. We normalized expression data from 102 STS samples using HTG Molecular platform and calculated the circuit activity by applying the HiPathia algorithm. Then, we simulated the effect of the drug over the mechanistic map by performing in silico knockdowns (KD) of the

drug target genes and recalculating the circuit activity values with the KD. Using the clinical response information, we selected non-/respondent patients based on tumor progression. Then, we evaluated the effect of the simulated drug, Sunitinib, on patients at basal time compared to those that respond to treatment at week 13.

Results and Discussions

We obtained 14 signaling circuits that are significantly altered by the in silico drug simulation. Our analysis yields values that are concordant with the observed in vivo deregulation. The main affected pathway is the VEGF-signaling pathway, where effectors such as BAD and CASP9 are upregulated by both real and simulated treatment, while PLA2G4B, RAC1, and PTK2 remained downregulated.

Conclusion

In silico simulation on mechanistic models arises as a powerful tool to evaluate the effect that drug/s may have on the signaling circuits of patients. Mechanistic models of diseases have proven to effectively connect gene expression profiles with functional cellular outcomes, making them a promising tool to evaluate and accelerate the discovery of new treatments.

EACR22-0125

Personalised Medicine for Colorectal Cancer Using Mechanism-Based Machine Learning Models

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Introduction

Gaining insight into the mechanisms of signal transduction networks (STNs) by using critical features from patient-specific mathematical models can improve patient stratification and help to identify potential drug targets. To achieve this, these models should focus on the critical STNs for each cancer, include prognostic genes and proteins, and correctly predict patient-specific differences in STN activity.

Material and Methods

Focussing on colorectal cancer and the WNT STN, we used mechanism-based machine learning models to identify genes and proteins with significant associations to event-free patient survival and predictive power for explaining patient-specific differences of STN activity. First, we identified the WNT pathway as the most significant pathway associated with event-free survival. Second, we built linear-regression models that incorporated both genes and proteins from established mechanistic models in the literature and novel genes with significant associations to event-free patient survival. Data from The Cancer Genome Atlas and Clinical Proteomic Tumour Analysis Consortium were used, and patient-specific STN activity scores were computed using PROGENy. Three linear regression models were built, based on; (1) the gene-set of a state-of-the-art mechanistic model in the literature, (2) novel genes identified, and (3) novel proteins identified. The novel genes and proteins were genes and proteins of the extant WNT pathway whose expression was significantly associated with event-free survival.

Results and Discussions

The results show that the predictive power of a model that incorporated novel event-free associated genes is better compared to a model focusing on the genes of a current state-of-the-art mechanistic model. Several significant genes that should be integrated into future

mechanistic models of the WNT pathway are DVL3, FZD5, RAC1, ROCK2, GSK3B, CTB2, CBT1, and PRKCA.

Conclusion

Thus, the study demonstrates that using mechanistic information in combination with machine learning can identify novel features (genes and proteins) that are important for explaining the STN heterogeneity between patients and their association to clinical outcomes.

EACR22-0490

Constructing an oncometabolic map for allosteric inhibition and drug repurposing in glioblastoma multiforme

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Introduction

In the era of precision oncology, clinical management of glioblastoma multiforme remains a major challenge, indicating an urgent need for novel drug targets and companion biomarkers, in a multidisciplinary therapeutic strategy mode. We and others appreciate that the elusive characterization of hypoxic and radioresistant glioblastoma cells is an important impediment to personalized radiotherapy.

Material and Methods

We focused on disease-centric and at the same time, drug-centric repurposing, looking for allosteric inhibitors. Emphasis was put on bioenergetics and oncometabolism. For this, we employed a. pharmacophore-based virtual screening and b. molecular docking-based virtual screening. Homology models were obtained based on structural information. Pharmit (Molport), CavityPlus and MetaPocket2.0 were used. Upon data filtering, data analysis was performed by Data Warrior v.5.2.1. Similarity charts were obtained based on FragFP (substructure fragment dictionary-based binary fingerprint descriptor). Molecular docking was performed by AutoDock Vina. Blood brain barrier permeability was predicted by LightBBB. Machine learning was employed for drug repurposing information coming from DrugBank (8,476 entities), including approved drugs, experimental drugs and drugs in clinical trials.

Results and Discussions

Driver nodes and key interactions were determined. Out of 13,178 candidate allosteric inhibitors, fifty-five chemical entities survived our strict criteria and were predicted to cross the blood brain barrier, forty-eight hit molecules were selected for efficacy and ADMETox screening in patient-derived glioblastoma cell lines.

Conclusion

Our strategy has resulted in datasets that allow for knowledge management hierarchy and map construction. Our disease-centric and at the same time, drug-centric pipeline leads to medium-to-high throughput outcomes.

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EACR22-0335

The secretome of highly metastatic cells as a source of biomarkers and metastatic effectors in colorectal cancer

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Introduction

Colorectal cancer (CRC) is the second leading cause of cancer death in developed countries, mainly as a consequence of metastatic spread. Secreted proteins are essential to communicate the cancer cells with the tumor-microenvironment, favoring tumor progression and metastasis. The cellular secretome, including exosomes, has demonstrated to be a rich source of metastatic effectors and biomarkers of metastasis in different tumors. Our goal was to identify proteins in the secreted fraction of highly metastatic cells in order to determine altered biological pathways and potential biomarkers of prognosis.

Material and Methods

A quantitative label-free proteomic analysis was carried out on the secretome of KM12SM and KM12L4 highly metastatic CRC cell lines versus poorly metastatic cell line SW620 from different genetic background. Proteomic results were validated using unbiased transcriptomic analyses. Hazard ratios and log-rank tests for the differentially-secreted proteins were determined in four different external datasets to select the proteins with clinical relevance for further analysis. Gene ontology was performed to determine the biological processes altered in highly metastatic cells.

Results and Discussions

After proteomic and transcriptomic analyses, 221 differentially-secreted proteins were found to be significantly associated to metastasis. Extracellular matrix constituents involved in cell adhesion and migration were overrepresented in the highly metastatic fraction. After survival analysis in a large combined meta-dataset, selected genes were able to predict the overall and progression free survival in stage II and stage III CRC patients. In addition, they showed higher expression in the stem cell-like and CMS4 subtypes of CRC, associated to worse prognosis. These genes were also associated to deficient mismatch repair and BRAF mutation. Remarkably, a significant number of altered proteins (NPC2, LDLR, LIPA, PCSK9) were involved in cholesterol metabolism, suggesting an enhancement of the LDL-associated cholesterol uptake and metabolism in highly metastatic cells.

Conclusion

The secretome of highly metastatic cells is enriched in proteins involved in essential pathways for metastasis such as adhesion, migration and lipid metabolism. In addition, several proteins with a robust and significant prognosis value were selected for further validations. Finally, cholesterol metabolism appears as a relevant target in metastasis treatment.

The proteasome regulator PSME4 abrogates response to immunotherapy by promoting a cold-tumor signature

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Introduction

The mammalian proteasome is estimated to degrade ~70% of all intracellular proteins and plays a key role in antigen processing for MHC presentation and modulation of inflammatory signals. Proteasome composition has been shown to be an important prognostic marker in response to immunotherapy in many cancer types, such as melanoma. Recently we have developed a system for proteasome footprinting by mass-spectrometry analysis of proteasome-cleaved peptides (MAPP; Wolf-Levy et al 2018). MAPP offers, for the first time, a direct analysis of the proteasome landscape in cancer. Utilizing this system, we have analyzed a clinical cohort of Non-Small Cell Lung Carcinoma (NSCLC) tumor samples and adjacent tissue controls to study the tumor degradome.

Material and Methods

The cohort included lung adenocarcinoma samples with matching adjacent tissue from nine patients, obtained from the Midgam biobank. MAPP was performed as previously described by cellular crosslinking followed by immunopurification of cellular proteasomes with nascent cleaved peptides. Peptides were then eluted from proteasomes and analyzed by MS/MS. We expanded our findings through computational analysis of transcriptomics (TCGA and CPI1000+ cohorts) and proteomics (CPTAC). Finally, we utilized a murine model of lung adenocarcinoma (KP1.9) where we depleted PSME4 and profiled immune response.

Results and Discussions

By examining the proteasome makeup of Non-Small Cell Lung Cancer tumors, we found that PSME4, an understudied regulator of the proteasome, is increased in response to smoking-induced DNA damage. Through analysis of patient outcomes upon immune checkpoint inhibition (ICI) across cancers, we find that PSME4-high tumors have reduced ICI responsiveness. Detailed proteomics analysis revealed that PSME4 reduces the inflammatory state of the tumor through inhibition of the immunoproteasome. This, in turn, reduces tumor secretion of IL-6, which promotes a cold tumor environment as observed in scRNA-seq.

Conclusion

Our findings suggest that PSME4-mediated regulation of proteasome activity is a novel mechanism of immune evasion in non-small-cell lung carcinoma and might be targeted therapeutically for restoring anti-tumor immunity.

Biomarkers in Tissue and Blood

ORAL PRESENTATION

The use of liquid biopsy for monitoring molecular residual disease and recurrence in head and neck squamous cell carcinoma

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Introduction

The development of personalised diagnostic assays capable of detecting and analysing circulating tumour-derived DNA (ctDNA) has allowed assessment for molecular residual disease following curative therapy. Despite efforts invested into biomarker developments for head and neck squamous cell carcinoma (HNSCC) over the past decade, the 5-year survival rate of patients with HNSCC remains below 50%. Early relapse and recurrent metastasis are some of the primary reasons for the dismal prognosis associated with HNSCC.

Material and Methods

We conducted a single-centre prospective cohort study to assess ctDNA collected from plasma and saliva in patients (n=60) with HNSCC (Stages I-IVb) who received primary surgical treatment with curative intent. In addition, we included cases who received definitive radio- or immunotherapy for disease recurrences. Samples were collected pre- and postoperatively as well as during clinical follow-up. Whole exome sequencing (WES) was performed on formalin-fixed paraffin-embedded tumour tissue to a median depth of 250x. For each patient, tumour-specific variants for personalised assay design (RaDaR, Inivata) were selected to analyse serial samples for evidence of molecular residual disease or recurrence.

Results and Discussions

In 37 patients analysed to date, we were able to detect ctDNA ranging from levels of 10.8% variant allele fraction (eVAF) to as low as 0.0005% eVAF. ctDNA was detectable in 100% of cases (8/8) with confirmed clinical recurrences with lead times between 56 to 265 days. This included a case with two synchronous tumours detected, one in the hypopharynx and a second in the oropharynx. WES revealed that these tumours were molecularly distinct since only 7.8% of the detected variants were shared. There was no overlap in the variants used in the RaDaR assays designed for these two tumours. ctDNA levels remained undetectable for the hypopharyngeal tumour. In contrast, ctDNA was detected at all timepoints pre- and postoperatively for the oropharyngeal tumour, with a lead time of 260 days from ctDNA detection to clinical progression when liver metastases were histologically confirmed.

Conclusion

Optimised biomarkers of molecular residual disease and recurrence are required as a means of guiding management decisions and as an addition to current diagnostic procedures for patients with HNSCC. Incorporating measurement of ctDNA from a minimal invasive liquid biopsy has therefore significant potential to guide treatment decisions and improve prognosis for this patient cohort.

EACR22-1218

TOP ABSTRACT

Profiling of the circulating cell-free DNA methylome for detection, monitoring and characterisation of cancer

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Introduction

Analysis of circulating cell-free DNA (cfDNA) is a powerful, minimally invasive approach for molecular characterisation of tumour genomes. Whilst the detection of cancer-specific mutations is becoming routine for directing treatment decisions and disease monitoring, using somatic mutations for cancer detection has limitations including a lack of recurrent mutations making the development of sensitive, universally applicable assays challenging. DNA methylation, an epigenetic modification may provide an alternative biomarker as cancer-specific methylation changes typically occur early in tumour development and with higher frequency than mutations. As well as enabling detection and monitoring, DNA methylation can also potentially provide information on the biology of cancers, including molecular subtyping. Here, we describe a robust workflow for genome-wide DNA methylation and demonstrates its clinical utility in the detection and subtyping of small cell lung cancer (SCLC).

Material and Methods

We have developed a genome-wide cfDNA methylation assay (T7-MBD-seq), that combines an novel NGS library preparation method with a methyl-binding domain enrichment approach that tags cfDNA molecules with adapters containing sample barcodes and unique molecular indexes allowing sample pooling. This enables low DNA input for individual samples and reproducible methylation enrichment. We have used this approach to profile DNA methylation in over 1,300 samples, including 593 cfDNA samples.

Results and Discussions

Methylation profiles generated from preclinical models and cfDNA samples from SCLC patients and non-cancer controls were comparable to previously described SCLC methylation patterns. A tumour/normal classifier, based on 4,061 genomic regions detected as being hypermethylated in SCLC correctly assigned 93% and 100% cfDNA samples from patients with limited and extensive stage SCLC respectively. To determine whether cfDNA methylation profiling could subtype SCLC patients, we next built a subtype classifier, based on methylation signatures derived from 59 SCLC cell lines and applied the classifier to cfDNA samples from 56 patients. The cfDNA samples were classified with a distribution of subtypes correlating closely to previously published IHC data and 10/11 samples with known subtypes were correctly classified.

Conclusion

Our data reveal the potential clinical utility of cfDNA methylation profiling as a universally applicable liquid biopsy approach for sensitive detection and monitoring of cancer and molecular subtyping.

EACR22-0118

Monitoring NRAS congenital melanocytic nevi patients by liquid biopsy

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Introduction

Congenital melanocytic nevi (CMN) are rare benign skin tumours caused by the acquisition of mutations mainly in the *NRAS* gene. Patients with larger-sized CMN or multiple CMN are at higher risk for extracutaneous complications, including a variety of neurologic complications and melanoma development in cutaneous and non-cutaneous localization. Here, we aimed to analyse the clinical utility of minimally invasive liquid biopsies for monitoring CMN patients at risk of developing melanoma.

Material and Methods

We analysed the presence of *NRAS* mutations in cell-free DNA (cfNRAS) of 14 patients with an *NRAS* mutated CMN, using the droplet digital PCR technology and two commercial assays designed to detect 13 mutations in codons 12, 13 and 61 of *NRAS*.

Results and Discussions

First, we examined ten patients with no CMN-associated comorbidities, and no cfNRAS was detected in any of their plasma samples (n=12). Next, we evaluated four patients who had CMN-associated comorbidity. Patients 1 and 2 had a localized central nervous system (CNS) melanoma and congenital brain abnormalities, respectively, and no mutated cfNRAS were detected in their samples. Patient 3, affected by giant CMN with neurological impairment (hydrocephalia), was under laser-hair-removal treatment of her CMN at the time of plasma collection. We found 12.9% of mutated cfNRAS copies in her plasma sample, even though she had no suspicious cutaneous or extracutaneous malignancy at the time of plasma collection or after two years clinical follow-up. We obtained a new sample 18 months after the last laser session and, in this case, no mutated cfNRAS was detected. Lastly, patient 4 had a metastatic melanoma harbouring the same *NRAS* mutation as the CMN. Serial samples of this patient were obtained during the disease course. While no mutated cfNRAS was detected when he was free of disease, progressively increasing levels of mutated cfNRAS were detected during disease progression, ranging from 0.6% to 82%, just before dying.

Conclusion

In CMN patients, cfNRAS is only detected in the presence of metastatic disease in organs other than the CNS. The analysis of plasma cfDNA might be a robust and reliable biomarker for monitoring CMN patients. However, external factors, such as laser therapy, might affect the detection of mutated cell-free DNA.

EACR22-0176

Emergence of RAS mutations after first-line (1L) treatment in metastatic colorectal cancer (mCRC) patients (pts) assessed by two liquid biopsy (LB) techniques: PERSEIDA study (NCT02792478)

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Introduction

CRC harbors a considerable heterogeneity, with temporal and spatial differences in genetic mutations. The prevalence of emergent *RAS* mutations after 1L therapy has not been extensively studied in mCRC pts. A recent publication reports smaller frequencies after 1L when compared to subsequent lines of treatment (Parseghian, ASCO2021). Here, we present the emergence rates of *RAS* mutations at disease progression (DP) in 1L mCRC pts as assessed by 2 LB techniques

Material and Methods

Prospective, observational, multi-center study in mCRC pts with baseline *KRAS* and *NRAS* wild-type (*RAS*_{wt}) according to tissue biopsy and treated following clinical practice. The circulating free DNA (cfDNA) *RAS* mutation status was analyzed at baseline and at DP by 2 LB techniques: the BEAMing (Sysmex-Inostics) in Cohort 1 (C1) (with 3 different mutant allele fraction [MAF] cut-offs [$\geq 1\%$; $\geq 0.1\%$; $\geq 0.02\%$]), and the Idylla™ (Biocartis) tests in Cohort 2 (C2) (MAF cut-offs: 0.4% [*KRAS*]; 1.5% [*NRAS*]). Additionally, the cfDNA of 8 pts of C2 that progressed after 1L chemotherapy + panitumumab (C-P) was analyzed using next-generation sequencing (NGS) (AVENIO® ctDNA expanded panel and Illumina NextSeq 550) containing 77 genes

Results and Discussions

A total of 65/64/58 (MAF $\geq 1\%$ / $\geq 0.1\%$ / $\geq 0.02\%$ [C1]) and 45 (C2) pts were evaluable (*RAS*_{wt} at baseline as per solid and liquid biopsies and blood sample available at DP), of which 58/57/51 (C1) and 40 (C2) received C-P. Table shows the prevalence of emergent *RAS* mutations at DP

Cohort	Treatment	MAF cut-off (%)	N evaluable	<i>RAS</i> mutant at DP, n (%)
1	Total	1	65	1 (1.54%)
		0.1	64	10 (15.63%)
		0.02	58	11 (18.97%)
	Panitumumab	1	58	1 (1.72%)
		0.1	57	9 (15.79%)
		0.02	51	9 (17.65%)
2	Total	0.4 (<i>KRAS</i>) 1.5 (<i>NRAS</i>)	45	6 (13.33%)
	Panitumumab	0.4 (<i>KRAS</i>) 1.5 (<i>NRAS</i>)	40	6 (15.00%)

Additionally, NGS-LB results found subclonal genomic variants (V) potentially associated with anti-EGFR resistance in 6 pts (75%). These included single nucleotide V

in *EGFR*, *ERBB2*, *KRAS* (2 pts), *FBXW7*, *PIK3CA*, *BRAF* (2 pts); gene copy number V in *MET* (3 pts), *EGFR* (3 pts), *ERBB2* (2 pts) and alternative splicing in *KDR* and *FBXW7*

Conclusion

After 1L treatment, a significant percentage of mCRC pts presented emergent *RAS* mutations at DP, partially explaining the observed secondary resistance. Similar prevalence were observed irrespective of the 1L treatment. However, a complex mutational process driving resistance is suggested based on NGS-cfDNA analysis. Further investigation in larger numbers of pts is warranted

Study supported by Amgen S.A.

EACR22-1078

Genomic and transcriptomic characterization and prognostic value of circulating exosomes in metastatic prostate cancer liquid biopsies.

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Introduction

Liquid biopsy allows non-invasive tumor monitoring and genomic profiling. Circulating tumor DNA (ctDNA) and circulating tumor cells (CTCs) have prognostic and predictive value in metastatic prostate cancer (mPC). We studied circulating exosomes as a complementary source of tumor material for molecular studies.

Material and Methods

Exosomes were isolated, followed by DNA (exoDNA) and RNA (exoRNA) extraction from: 1) human PC cell lines (PC3, LnCap, 22Rv1 and C4-2 and; 2) plasma from 22Rv1 and C4-2 mouse xenografts. Next, we prospectively collected longitudinal plasma from 36 mPC patients along therapy with androgen-receptor signaling inhibitors (ARSi); response was defined as PSA>50% fall at week 4. The cell-free vs exosomal origin of DNA/RNA in patients was confirmed through enzymatic protection assays. Copy number alterations (CNA) profiles obtained by shallow whole genome sequencing (sWGS, 0.4x) were used to infer tumor fraction (TF) in ctDNA and exoDNA. Mutation calling derived from a customized targeted-panel. We also pursued characterization of exoRNA by RNAseq and differential expression analysis.

Results and Discussions

In-vitro, in-vivo and in mPC patient's plasma, the yield of exoDNA was lower than for cfDNA, but exosomes contained bigger fragments of DNA (100-4000bp).

By sWGS, we demonstrated that exoDNA contains the entire genome. ExoDNA CNA profiling matches tumor cell CNA both in-vitro ($r=0.7-0.9$) and in-vivo ($r=0.8$).

In mPC patients, sWGS of exoDNA and ctDNA showed high correlation of CNA profiles ($r=0.82$, $p<0.001$). Somatic mutations were detected in exoDNA targeted seq, matching paired tumor and ctDNA next-generation sequencing (NGS). ExoDNA-TF was prognostic: exoDNA-TF>5% associated with shorter time to radiographic progression (HR 3.6,

p=0.0005). ExoDNA-TF kinetics reflect clinical response and tends to be higher at progression (average 1.5-fold increase). Moreover, exoDNA-TF on therapy was lower in responding vs non-responding patients (p=0.03).

Transcriptomic analysis of circulating exosomes in-vivo (C4-2 xenograft) treated with ARSi enzalutamide shows decrease in expression of androgen response elements. In mPC patient's plasma samples, exoRNAseq was feasible and correlated with tumor tissue RNAseq.

Conclusion

Tumor DNA and RNA can be isolated from circulating exosomes. ExoDNA NGS recapitulates tumor genomic features in vitro, in vivo and in patient's samples. ExoDNA-TF has prognostic value in mPC. ExoRNA analysis captures modulation of AR signaling upon AR-targeting therapy.

EACR22-1240

Early detection of relapse in resectable colon cancer patients by longitudinally following a personalized molecular signature from a blood test: initial results from the THRuST trial

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Introduction

More than half colon cancer (CC) patients are diagnosed when the disease is amenable to surgical resection leading potentially to curative treatment. Adjuvant treatment is currently administered to all stage III and high-risk stage II patients. Tumors shed cell free DNA (cfDNA) into the blood stream. The analysis of tumor informed DNA in plasma samples could enable precision oncology in CC, and identify patients with micrometastatic disease who need more aggressive adjuvant therapies, as well as those who are cured by surgery alone and may be spared the toxicity of prolonged or combinatorial chemotherapy regimens.

Material and Methods

The THRuST study is a prospective, multicenter study on stage II and stage III CC patients (<https://www.transcanfp7.eu/index.php/abstract/thrust.html>). A molecular signature of selected genetic alterations is defined for each CC patient based on next-generation sequencing of tumor exome at diagnosis. Individual genetic signatures will be assessed by the IntPlex method, including quantitative cfDNA parameters such as the total concentration of cfDNA, the mutant allele fractions of cfDNA, and the cfDNA fragmentation index.

Results and Discussions

Out of the 178 CC patients initially screened across four centers, 56 were stage III and 72 were stage II, with a median follow-up of 11 months. At time of writing, a subset of 29 stage III cases underwent tumor whole exome analysis to identify patient unique molecular alterations. A total of 2086 unique genetic variants were identified. APC, LRP1B, TP53, MUC4 and KRAS were the most frequently mutated genes, consistently with the genomic landscape of CC. Other clinically actionable less frequent alterations were also identified potentially informing therapeutic strategies at time of patient relapse. Analyses of patient specific mutations in plasma at baseline will be presented alongside longitudinal samples collected after surgery (two timepoints) and during follow-up (every three months).

Conclusion

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EACR22-0195

Clinical utility of liquid biopsy in colorectal cancer

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Introduction

There is an increasing interest in the use of liquid biopsy in the management of colorectal cancer (CRC) patients. Liquid biopsy has several advantages as compared with tissue biopsy, including the low invasive procedure, the fast turnaround time and the ability to provide a more comprehensive molecular portrait of the disease as compared with the analysis of a small fragment of a single tumour lesion.

Material and Methods

Our experiments involved 16 colorectal tumours using Archer® VariantPlex® Solid Tumor Kit which simultaneously detects and characterizes single nucleotide variants (SNVs), copy number variations (CNVs), insertions and deletions (indels) of 67 genes associated with solid tumors. In addition, 16 plasma samples (9 in pairs with solid tumours) were sequenced using LIQUIDPlex™ ctDNA 28 Kit which is an advanced solution for targeted next generation sequencing of circulating cell-free tumour DNA of 28 genes commonly associated with solid tumours. The two panels has an overlap in 22 genes which enables a more comprehensive view of tumour- plasma mutational status.

Results and Discussions

Mutations in TP53, APC and KRAS genes were observed in the majority of tumour samples. Examining the relationship between clinical parameters and mutations, a significant correlation was observed between mutation of NOTCH1 and FOXL2 genes, and T3 stage tumours ($p=0.032$). Seven plasma samples harbour 13 mutations in 5 genes (TP53; KRAS; NTRK3; SMAD4; BRAF); 9 samples (56.2%) did not show mutations of the tested genes. Majority of the mutations were missense mutations ($n=11$, 84.6%) and two were nonsense (15.4%). Notably, we could isolate significantly more cfDNA from patients' plasma with metastasis than patients with primary tumours. From 9 paired plasma samples 4 harboured mutations, but only 1 plasma and tumour pair harboured the same mutations. Interestingly, 5

out of 9 solid tumour exhibited gene mutations in the *TP53*, *KRAS* or *MET* genes which ones not revealed by the cfDNA Kit.

Conclusion

In summary, evidence suggests that liquid biopsy could provide relevant prognostic and predictive information in different phases of CRC progression, from early diagnosis and identification of minimal residual disease to the assessment of predictive biomarkers and the evaluation of the response to treatment and of the clonal evolution of the disease.

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EACR22-0549

Monitoring of minimal residual disease in patients with metastatic colorectal cancer

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Introduction

Examination of free circulating tumor DNA (ctDNA) in peripheral blood in the form of liquid biopsies shows increasing application in oncology. Due to minimal invasiveness, high specificity and sensitivity, this testing is of clinical importance especially in the early detection of disease recurrence, monitoring of response to treatment and estimation of prognosis in patients with advanced disease. In addition to quantification of ctDNA, it is also possible to perform targeted genotypic analyzes that allow rapid and sensitive detection of resistance to administered therapy and associated disease recurrences. These tests are important especially for diagnoses that are immediately life-threatening in patients, which undoubtedly include metastatic colorectal cancer (CRC).

Material and Methods

The most frequently mutated genes in CRC are *KRAS*, *BRAF* and *NRAS*. The presence of mutations in these genes in CRC generally predicts a worse prognosis and indicates resistance to biologic therapy aimed at inhibiting EGFR. We used ddPCR to determine the presence of *KRAS*, *BRAF* and *NRAS* mutations in ctDNA obtained from the blood serum of patients with CRC before surgery and tested their use as a screening method for rapid detection of the disease.

Results and Discussions

We achieved 75% agreement for the determination of *KRAS* gene mutations and 50% agreement for *BRAF* and *NRAS* gene mutations in ctDNA by ddPCR compared to sequencing analysis of genomic DNA isolated directly from tumor tissue.

Conclusion

From our current results, it can be concluded that ctDNA analysis is suitable for the detection of the presence of *KRAS* mutations. The aim of our further work is to verify whether this procedure can be used as a screening method for early detection of CRC recurrence in patients after surgery. Moreover, in case of a successful project outcome, we will then focus on developing new technologies based on electrochemical biosensors for the detection of these point mutations, which would be simple, cheap and fast.

EACR22-1077

Liquid biopsy of cerebrospinal fluid in a comparative study on patients with suspected leptomeningeal metastasis

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Introduction

Leptomeningeal metastases (LM) are diagnosed in up to 10% of metastatic cancer patients, the most frequent cancers being carcinomas of breast and lung or melanoma. Due to a dismal prognosis and an overall median survival of only four months new approaches for molecular analysis could help to identify individual treatment options.

Material and Methods

Cerebrospinal fluid (CSF) for molecular analyses was collected from patients (n=39) with different primary tumors but suspected LM. One part of CSF was analyzed by the standard neuropathological cytomorphology and immunocytology workflow while the second part was used to enrich circulating tumor cells (CTCs) by using the CellSearch® CTC Kit and subsequent single cell isolation by micromanipulation. Additionally, cell-free DNA (cfDNA), released from cancer or normal cells, was isolated from the supernatant (Nucleic Acid Kit, QIAgen) and amplified by an adapted library protocol. Where available, formalin-fixed and paraffin embedded (FFPE) tissues from primary tumors and systemic metastases were dissociated and stained with the DEPArray™ FFPE SamplePrepKit (Menarini Silicon Biosystems, MSB) and subpopulations isolated with the DEPArray™ technology. Isolated CTCs as well as subpopulations of tumor and stromal cells were amplified using the Ampli1™ Whole Genome Amplification (WGA) method. Finally, copy number variations (CNV) were identified by applying the Ampli1™ LowPass protocol on the Illumina MiSeq™ platform.

Results and Discussions

We confirmed the malignant origin of all putative CTCs (n=208) by aberrant CNV profiles while the CD45-positive cells (n=28) showed balanced CNV profiles. CNV results of cfDNA (n=18) showed aberrant profiles in 39 % (7/18), while 50 % (9/18) led to a balanced profile. 21 FFPE embedded tissues, derived from primary tumor (n=5) and systemic metastases (n=16) could be dissociated and separated in pure tumor and stromal cell populations. Molecular comparison of data derived from the different tissues can be compared to assess heterogeneity and clonal evolution for the disease of individual patients.

Conclusion

With this proof-of-concept study we could show, that our liquid biopsy protocol gives not only the opportunity to detect CTCs in the CSF but also to reach important molecular data from both CTCs and cfDNA. With the comparison to the primary tumor or metastatic tissue we try to track the evolution of metastases in order to define novel treatment options for patients with LM.

EACR22-1271

MET amplification testing on liquid biopsy of patients with EGFR mutated non-small cell lung cancer: incidence and biomarker behavior under antiEGFR therapy.

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Introduction

EGFR-tyrosin kinase inhibitors (EGFR-TKIs) have achieved remarkable outcomes in the treatment of patients with EGFR-mutant non-small cell lung cancer, but acquired resistance is still the main factor restricting their long-term use. Recent studies suggest that a key mechanism of resistance to a third generation EGFR-TKI, such as osimertinib, may be MET amplification and/or protein overactivation, especially when they are used as the first line treatment.

In this study, we retrospectively evaluated the incidence of ampMET in patients treated with osimertinib, at different time points to assess its expression pattern and behavior under different treatment regimens.

Material and Methods

17 NSCLC EGFR mutated patients were included in the study. EGFR mutations were analyzed using the QX200 Droplet Digital PCR System (Bio-Rad) platform, as well as MET amplification (ampMET), expressed as CNV (copy number variations).

Cell free DNA was extracted from plasma using QIAamp Circulating Nucleic Acid Kit.

All biomarkers were studied at three time points: t0: at inclusion in the study; t2 at progression to first line antiEGFR and t3 at progression to osimertinib (2nd line antiEGFR).

Results and Discussions

8 patients had at least one positive result for ampMET (CNV >2.7). For those, the evolution was as shown on the table.

- Patient 21 was positive at progression to 1st line, however after osimertinib ampMET was negative at 21.21.
- Patient 31 was positive at progression to osimertinib. After 3rd line cisplatin/pemetrexed, 31.10 was negative.
- Patient 41 never received osimertinib. 41.4 was ampMET positive.
- Patient 45 was included during erlotinib treatment and had a positive result at 45.2. At 45.18 ampMET was negative. Eventually at 45.20 ampMET was again positive.
- Patient 50 was positive at inclusion. At progression to 1st line ampMET was negative. Osimertinib was started at 50.5; ampMET retesting at progression to osimertinib was borderline.
- Patient 73 was positive at inclusion. Retesting at erlotinib progression was negative for ampMET; osimertinib was started and further analysis were negative.

- Patient 98 was positive at inclusion. Osimertinib was started and ampMET was higher at 98.4.

Conclusion

Resistance to targeted therapy is very complex to address as by switching off one route and alternative one might be activated. The results here presented, are an indicator that positivity in liquid biopsy alone at one time point, might not be enough reason to add a MET inhibitor to treatment regimen. Further studies are necessary.

EACR22-0715

TOP ABSTRACT

Circulating tumor cell-derived explant models reveal DNA damage response-based therapeutic opportunities in non-small cell lung cancer

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Introduction

DNA damage and genome instability contribute to non-small cell lung cancer (NSCLC) malignancy. However, their therapeutic exploitation is disappointing. CTC-derived eXplants (CDX) offer systems for mechanistic investigation of CTC metastatic potency and biology-driven therapeutic testings. We perform in-depth molecular and functional characterization of CDX models and demonstrate that targeting DNA damage response (DDR) defects impedes CTC-driven metastasis in NSCLC.

Material and Methods

CTCs were enriched from 30mL blood samples of 56 advanced NSCLC patients and implanted subcutaneously into Nod/Scid-IL2Rγ^{-/-} (NSG) mice. Tumors were palpable within a median of 108 days. Among the 4 CDX models established, 3 CDX-derived cell lines (GR-CDXL1, GR-CDXL3, GR-CDXL4) were obtained. CDX and cell lines were characterized by immunofluorescence (IF), immunohistochemistry and whole-exome sequencing (WES). Chromosomal instability (CIN) and DDR activity were evaluated by IF and western blot. Tumorigenic potential of CDX-derived cell lines was assessed in the chick embryo CAM and NSG mice. IC₅₀ was assessed using CellTiter-Glo®.

Results and Discussions

Four CDX models and three CDX-derived cell lines were established from NSCLC CTCs and recapitulated patient tumor histology and response to platinum-based chemotherapy. WES analysis showed considerable mutational landscape similarity between the CDX, corresponding patient tumor biopsy and single CTCs. Truncal alterations in key DDR genes were prevalent across models and assessed as therapeutic targets *in vitro*, *in ovo* and *in*

vivo. GR-CDXL1 presented homologous recombination deficiency linked to bi-allelic *BRCA2* mutation, *FANCA* deletion and unrepaired DNA lesions post-mitosis. GR-CDXL1 cells were sensitive to PARP inhibitor (PARPi) olaparib, despite chemoresistance, challenging the current clinical rationale claiming that chemosensitive NSCLC patients should respond to PARPi. Centrosome clustering promoted targetable CIN in GR-CDXL3 cells. In GR-CDXL4, olaparib sensitivity was dictated by SLFN11 overexpression, which also correlated with neuroendocrine marker expression in patient tumor, suggesting a predictive value of SLFN11 in histological transformation of NSCLC into SCLC.

Conclusion

This study unravels distinct DDR profiles as a central mechanism underpinning CTC metastatic potency. Our CDX models provide a robust platform for *ex vivo* drug testing of DDR-targeted strategies to expand patient categories that may benefit from precision medicine in metastatic NSCLC.

EACR22-0584

New insights the analysis of circuloma: targeting PIK3CA actionable mutations in metastatic breast cancer

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Introduction

Liquid biopsy represents a powerful tool for cancer patients management. An integrate approach, inclusive of Circulating tumor DNA (ctDNA), Circulating Tumor Cells (CTCs) and Circulating Extracellular Vesicles (EVs) (Circuloma) is becoming a major point since it may provide additional and repeatable data complementary with tissue genotyping. However, parallel and comparative molecular studies of these biomarkers is still lacking.

In advanced breast cancer (BC), hormone receptor (HR) positive, HER2 negative BC cases harboring *PIK3CA* actionable mutations (40%) can be treated with combination of PI3K inhibitor Alpelisib and Fulvestran.

This proof of concept study investigates *PI3KCA* mutations in the different circulome components with the goal to evaluate which information on molecular, targetable, profiling could be recollected in each of them, in comparison also to the corresponding tumor tissue.

Material and Methods

Four metastatic HR+/HER2- *PI3KCA* mutated BC patients candidate to be treated with Alpelisib were selected and corresponding tumor tissues sequenced with custom comprehensive panel. CtDNA, CTCs and EVs were retrieved from blood/plasma and further characterized with specific protocols: ctDNA was extracted by cfDNA LV kit; CTCs were enriched by negative selection and recovered with DEPArray; EVs were isolated by size exclusion chromatography, followed by DNA purification. Mutational profiles were assessed by a tagged-NGS panel (Oncomine Breast cfDNA AssayV2) for ctDNA and CTCs and by Droplet Digital PCR for EVs-DNA.

Results and Discussions

Our study shows that it is possible to recover in parallel mutational information from ctDNA, CTCs and EVs. A complete concordance of *PI3KCA* patients' specific mutations (H1047R, R1047L, E542K individually identified in three patients, E542K and E545K co-occurring in the fourth patient) was found on tissue and ctDNA of all the 4 patients. In 3/4 cases, those mutations were found also in CTCs on both classical CTCs population (large CD45-/EpCAM+/- cells) and in a novel "non conventional" CTCs subpopulation (smaller CD45-/EpCAM-/CD44+). DNA EVs mutational profiles were consistent with the CTCs ones, suggesting that mutated EVs are released by CTCs.

Conclusion

This study generated proofs of concept data on molecular content of the different circulating markers and put in evidence a new CTCs sub-population to be considered for a more comprehensive mutational characterization of the disease.

EACR22-1226

A prognostic four-gene signature based on circulating tumor cells predicts recurrence free survival of patients with bladder cancer

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Introduction

Bladder cancer is the most common genitourinary malignancy, associated with substantial morbidity, mortality and cost. Currently there are no reliable specific serum markers for diagnosing and evaluating risk profiles of bladder cancer. Our study aims to establish a reliable prognostic signature closely related to the recurrence that can predict prognosis and possibly help with individual monitoring of bladder cancer patients.

Material and Methods

Among 55 patients with diagnosis of bladder cancer, CTCs were isolated from the peripheral blood before TURBT using a micro-pore size selection method (ScreenCell®) and diagnosed microscopically. The Cancer Genome Atlas (TCGA) dataset was used to identify genes modulated in bladder cancer respect to normal tissues. Thus, a selected panel of 15

genes was evaluated through the digital droplet PCR and analyzed by stratifying patients in low or high tumor grade. Data established the presence of two well-defined clusters of strong association. Among the involved genes, thanks to univariate and multivariate cox regression analysis, we identified the candidate prognostic genes and construct the risk score model. Kaplan–Meier analysis, time-dependent receiver operating characteristic (ROC) curve were used to evaluate the prognostic power and performance of the signature.

Results and Discussions

A prognostic signature consisting of four prognostic-related genes was constructed. Cox regression analysis (univariate and multivariate) demonstrated that the gene signature is an independent prognostic biomarker for predicting the recurrence free survival of bladder cancer patients. The 4-gene signature significantly grouped patients in high and low-risk groups in terms of recurrent free survival (hazard ratio, HR = 2.704, 95% confidence interval: 1.010-7.313, Log-rank $p < 0.050$). ROC curves revealed that the 4-gene prognostic signature achieved a good performance (AUC = 0.6863) in predicting recurrence for bladder cancer patients.

Conclusion

Our pilot study suggested a new and reliable prognostic signature that has a significant implication in predicting recurrence free survival for bladder cancer patients and may help with early diagnosis and making effective clinical decisions regarding potential individual treatment.

EACR22-0751

CTC gene expression analysis to identify response biomarkers in luminal metastatic breast cancer patients

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Introduction

The selective dual inhibitor CDK4/6 (CDKi) in combination with endocrine therapy (ET) have increased progression-free survival (PFS) in luminal metastatic breast cancer (mBC) patients. Nevertheless, not all patients respond to polytherapy and those who respond, eventually develop resistance. We aim to decipher resistance and response mechanisms to CDKi plus ET therapy throughout the analysis of Circulating tumour cells (CTCs) from blood. CTCs have been considered potential biomarkers of therapy response, although none preclinical study has addressed CDKi plus ET response analysing these tumour cells so far.

Material and Methods

Peripheral blood samples (7.5 ml) were extracted at diagnosis of metastasis (before therapy initiation), and after the first cycle therapy in luminal mBC patients (n=18). Luminal mBC were classified as no responders if progression occurs 6 months after starting the combinatorial therapy, and responders if they continue with the therapy after that time. Besides, the latter group was divided among initial responders and long responders (considering the cut-off of the median time to progression, 2 years). CTC fraction was negatively enriched with RosetteSep (Stem Cell Technology) and RNA was extracted using RNeasy Micro Kit (Qiagen). Gene expression analysis was performed with the nCounter technology (PanCancer Pathways Panel) and RT-qPCR. Expression data were normalized to the reference gene (B2M), and relativized to paired PBMC expression to minimize the

expression from contaminating blood cells isolated unspecifically. Statistical analysis was performed using RStudio 4.0.4.

Results and Discussions

For the gene expression analysis of CTCs, we first performed a discovery study using the nCounter PanCancer Pathways Panel in a 6 patients. Next, 45 genes were validated by RT-qPCR in a larger cohort of mBC patients in a longitudinal way. Gene expression analysis revealed that the CTC population has a hybrid epithelial-mesenchymal phenotype regardless of polytherapy or patient response. It was observed differential gene expression among responders and non-responders patients both before and after therapy. Thus, we identified a group of genes able to discriminate among non-responder, initial responders, and late responder patients.

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Conclusion

With this approach, we have shown that the study of gene expression from CTCs of metastatic luminal breast cancer is feasible to decipher potential biomarkers of therapy response in this group of drug-resistant patients.

EACR22-0875

Development of a circulating tumor cell-based test to identify cancer patient candidates for PARP inhibitor therapy

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Introduction

Tumors harboring mutations in genes involved in the Homologous Recombination Repair (HRR) pathway harbor genomic instability and are intrinsically sensitive to DNA damaging drugs, such as PARP inhibitors (PARPi). Genetic and genomic tests are being used to identify tumors with HRR deficiency (HRD). However, they often lack the capacity to detect functional HRR restoration. To this end, our lab developed an assay based on the detection of RAD51 nuclear foci as a biomarker of HRR functionality and PARPi response in formalin-fixed paraffin-embedded tumor samples. Tumor biopsies are invasive and not always feasible. Therefore, circulating tumor cells (CTCs) are emerging as a surrogate source of tumor tissue for the prediction and monitoring of treatment responses. Hence, we aimed to optimize the RAD51 test to be used in CTCs.

Material and Methods

We developed a protocol using breast cancer cell lines that were spiked into human blood and enriched by RosetteSep or Parsortix. Different conditions of pre-extraction, fixation and permeabilization were tested on cancer cell lines before staining with two different antibodies against RAD51 and Geminin, and mounting with DAPI. RAD51 scoring in cell suspensions was compared to a previously validated methodology in FFPE cell pellets.

Results and Discussions

RosetteSep allowed the recovery of more CD45⁺ cells than Parsortix. The highest staining quality was achieved with a pre-extraction step, with home-made solutions for fixation and permeabilization, and by using a monoclonal RAD51 antibody. Filters were used to concentrate the cells but they increased the background signal and, thus, cells were directly placed on a slide and let air-dry. This staining methodology provided comparable RAD51 scores to the ones obtained in FFPE cell pellets.

Conclusion

We have optimized a protocol for real-time functional HRD testing by RAD51 in peripheral blood. Ongoing work is examining the performance and value of RAD51 testing in CTCs to predict response to PARPi treatment in metastatic breast cancer patients.

EACR22-0967

Definition of an EMT-related gene signature to track circulating metastasis initiating cells in lung cancer

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Introduction

Lung cancer Metastasis Initiating Cells (MICs), identified as CD133⁺/CXCR4⁺, are a specific cellular subpopulation endowed with mesenchymal properties and high metastatic potential which is associated with poor clinical outcome. Detection of MICs within circulating tumor cells (CTCs) isolated from patients' blood could therefore inform on metastatic risk and prognosis. Through single cell RNA profiling we previously identified a MIC-specific signature based on 157 Epithelial-Mesenchymal Transition (EMT) related genes (*Rothwell et al. BMC Genomics 2014*). Our aim here was to refine this MIC-related EMT-gene signature in biological samples used for CTC analysis.

Material and Methods

Public repositories of gene expression data were mined to refine the signature. MICs were sorted from A549 and LT73 lung cancer cell lines using MACSQuant® Tyto® Cell Sorter. MICs and non-MICs were spiked in PBMCs from healthy donors processed with the same immunomagnetic depletion procedure based on the AutoMACS ProSeparator used to enrich CTCs from blood of lung cancer patients. Digital PCR (dPCR) analysis using TaqMan® gene expression assays was performed to detect MIC-related genes. CK7 expression was used to confirm presence of cancer cells in all spike-in experiments. Specific siRNAs were used to silence selected MIC-related genes to evaluate their functional relevance. MICs content was assessed by flow cytometry.

Results and Discussions

We identified six MIC-related genes with undetectable expression in PBMCs from lung cancer patients and healthy donors as tested by both RT-PCR and dPCR (MMP3, MMP10, PRRX1, CDH11, GSTA1, ACTC1). dPCR analysis specifically detected the expression of all selected EMT genes in samples spiked with 10-20-50 MICs. Overall, considering analyses performed for all genes, we observed a significant difference ($p < 0.001$) in MIC-spiked samples (normalized transcript count, median= 0.98; IQR:0.36-2.04) compared with non-MIC (median=0; IQR:0-0). However, no linear correlation was observed between the number of spiked cells and the transcript copies detected by dPCR. Silencing experiments demonstrated a significant reduction of MICs in samples treated with siCDH11 (fold-decrease=4.5; $p=0.01$) and siMMP10 (fd=2.86; $p=0.04$).

Conclusion

Our analysis with dPCR confirms the efficacy of a refined EMT-gene signature to specifically detect MICs in processed PBMC samples. Furthermore silencing experiments evidence that selected genes could impact MICs maintenance and may represent novel MIC's specific targets.

EACR22-1007

Development of a novel approach for multiparametric characterization of CTCs from colorectal cancer

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Introduction

Circulating tumour cells (CTCs) are widely studied as prognostic and monitoring biomarker in most solid tumours, including colorectal cancer (CRC). CTCs analysis requires an isolation step before enumeration and characterization. Different protocols were developed for the former, but the latter is still done in a time-consuming manual manner, impeding to analyze several markers simultaneously. Here, we developed a novel approach consisting in the combination of two existing technologies: one for the semiautomatic CTCs isolation using the IsoFlux microfluidic system and another for the phenotypic characterization with the ImageStream imaging flow-cytometry platform.

Material and Methods

Protocol optimization was done using CRC cell lines (HT-29 and SW480) and peripheral blood from healthy donors. CTCs from early CRC patients were extracted before (N=16) and one month after surgery (N=9) using red blood cell lysis buffer and the IsoFlux protocol optimized in our lab. CTCs were fixed and permeabilized with 1.7% paraformaldehyde and 0.2% triton respectively and treated with papain (1U). Immunostaining for a 5-antibody panel was done for 20 minutes, cells were acquired in an ImageStream and data was analysed using the IDEAS software and our customized analysis template.

Results and Discussions

Cell recovery efficiency was 69.75% and limit of detection was at least 4 cells/ml. CTCs were identified based on cytokeratin (CK) expression and had significantly ($p<0.0001$) greater size than CK negative cells. CTC counts had a median of 37 cells (IQ 13.0-85.5) and 117 cells (IQ 24.5-359.0) for baseline and follow-ups respectively. CTCs heterogeneity based on 4 subpopulations was defined according to BRAF^{V600E} and PD-L1 expression, which frequencies varied among patients and time. Double negative population was the most frequent (100%), followed by the double positive (88%), allowing to classify patients into two groups. Post-surgery samples revealed an increase of CTCs in 55.6% (5/9) of patients and changes in CTC subpopulations responded to 3 models: gain of PD-L1, gain of PD-L1 and BRAF^{V600E} or loss of both markers.

Conclusion

We optimized an antibody panel to evaluate BRAF^{V600E} and PD-L1 expression on CTCs from early-stage CRC patients and we developed an efficient automatic analysis method, highlighting the importance of evaluating multiple parameters such as size, expression levels or protein localisation to identify intra and inter individual tumour heterogeneity.

EACR22-1021

Resistance Mechanism to BRAF inhibition Identified by Single Circulating Tumor Cell molecular profiling in BRAF-mutant Non-Small-Cell Lung Cancer

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Introduction

Analysis of liquid biopsy components such as circulating tumor cells (CTCs) and cell-free (cf) tumor DNA is crucial to develop the implementation of precision medicine in the treatment of non-small cell lung cancer (NSCLC), particularly in the resistance setting. In *BRAF*^{V600E}-mutant NSCLC, combination therapy with dabrafenib and trametinib demonstrated robust activity, but its resistance mechanisms are poorly known. Here, we used molecular profiling of single CTCs from 7 patients with *BRAF*^{V600E}-mutant NSCLC to carry out a pilot study to identify resistance mutations at failure to dabrafenib and trametinib treatment and compare mutations detected in CTCs to mutations found in cf-tumor DNA and tumor biopsies.

Material and Methods

Patients were enrolled in the MATCH-R trial (NCT02517892). Single CTC isolation strategy included RosetteSep enrichment, immunofluorescent staining (Hoechst33342/CD45/cytokeratins) and fluorescence activated cell-sorting. The process to identify CTC mutations and copy number alterations (CNAs) included Ampli1 whole-genome amplification, quality controls, multiplex targeted PCR with the Ampli1 CHPCustomBeta cancer panel and lowpass kit (Menarini Silicon Biosystems) followed by NGS. Cf-DNA was analyzed using InVisionFirst-Lung. Matched tumor biopsies were available for four patients.

Results and Discussions

All patients had adenocarcinoma histology. Most of the patients were treated with dabrafenib and trametinib in second line setting (71%). *BRAF*^{V600E} was detected in four matched-tumor biopsies [VAF, 28%-41%] and in four out of seven cf-tumor DNA samples [VAF, 0.07%-5.28%]. Median of isolated CTCs (Hoechst+/CD45-/CK+ cells) was 20 [8-28] in 30 mL blood. *BRAF*^{V600E} was identified in one patient. Multiple mutations in various off-target genes were predominantly identified in CTCs. TP53 pathway was mutated in 6 out of 7 patients [VAF, 12%-100%] with several variants. Various mutations in receptor tyrosine kinases, cell cycle or DNA repair pathways were also identified. A higher degree of mutational and CNA diversity was observed in CTCs compared to bulk samples (tumor biopsies, cf-DNA).

Conclusion

Single CTC profiling reveals for the first-time a wide spectrum of off-target alterations in multiple oncogenic pathways from patients with *BRAF*-mutant NSCLC. Single CTC

sequencing analysis may provide important complementary information to bulk tumor samples to assess heterogeneous resistance mechanisms and to guide precision medicine in *BRAF*^{V600E} NSCLC.

EACR22-0160

Association of Circulating Markers of Gut Barrier Damage and Inflammation with Colorectal Neoplasia Stage

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Introduction

A disturbance in the microbiome facilitated by gut barrier dysfunction and inflammation can influence development of colorectal neoplasia. One of the indicators of gut barrier status is lipopolysaccharide (LPS). Increased permeability of the gut barrier leads to extensive translocation of microbes into the lamina propria, which is associated with higher circulating levels of LPS. Additionally, increased serum concentrations of inflammatory cytokines have been associated with impaired gut integrity and its progressive dysfunction.

Material and Methods

We assessed markers of gut barrier dysfunction and inflammation in two different patient cohorts; Irish (discovery) and Czech (validation). ELISA assays were used to assess circulating concentrations of LPS binding protein (LBP) and C-reactive protein (CRP) in serum from patients with CRC (n=30), colorectal adenoma (n=40), and negative after colonoscopy/blood donor controls (n=35). Circulating levels of inflammatory cytokines (TNF α , IFN γ , IL1 β , IL2, IL4, IL6, IL8, IL10, IL12p70, IL13) and calprotectin were assessed using a MSD platform (Mesoscale Discovery, United States). Differences between pathology groups were assessed using the Kruskal Wallis or Mann-Whitney U test. Receiver operating curve (ROC) analysis was implemented to identify single and combinations of biomarkers capable of discriminating between CRC patients and controls with maximum sensitivity (SE) and specificity (SP) (combiROC package, RStudio).

Results and Discussions

In the discovery cohort, levels of LBP, TNF α , IL6, calprotectin and CRP were significantly higher in CRC patients than in controls (p=0.02, p=0.03, p=0.006, p=0.01, p=0.006, respectively). The area under the ROC curve (AUC) was 0.76 (95% CI: 0.64–0.87, p = 0.003) for LBP, 0.69 (95% CI: 0.55-0.82, p = 0.007) for calprotectin and 0.87(95% CI: 0.74–1.00, p = 0.003) for CRP. The optimal biomarker combination for discriminating between CRC and controls was CRP and calprotectin (SP= 1.00, SE = 0.40). In the validation cohort, levels of calprotectin and TNF α , were significantly higher in cancer compared to control (p=0.05, p=0.02, respectively), confirming the findings in the discovery cohort. IFN γ and IL10 were also significantly elevated in cancer compared to control (p= 0.02, p=0.003, respectively).

Conclusion

LBP, CRP and calprotectin may have potential as diagnostic biomarkers for CRC. Gut bacterial and barrier wall modulation and stabilisation has potential for preventing colorectal adenomas and colorectal cancer.

Immunohistochemical profile of myoepithelial cells in salivary gland tumors

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Introduction

Salivary gland tumors (SGTs) comprise a heterogeneous group of lesions that correspond to approximately 3-6% of head and neck tumors and present different histological characteristics. These different histological patterns have been attributed to the presence of myoepithelial cells, implying difficulties in the classification of these tumors. The carcinoma ex-pleomorphic adenoma, myoepithelial carcinoma, and basal cell adenocarcinoma, originate from the intercalated duct of the salivary gland and present a distinct myoepithelial component. Thus, the evaluation of the expression of a protein panel is important to ascertain the composition of these neoplasms, allowing the understanding of the molecular basis of these lesions, facilitating their classification and diagnosis. The aim of this study is to evaluate the expression of smooth muscle actin, calponin, caldesmon, cytokeratin 14, E-cadherin, beta-catenin, vimentin, p63 and Ki-67 proteins in the SGTs mentioned, using immunohistochemistry technique.

Material and Methods

Fifteen samples (8 carcinoma ex-pleomorphic adenoma, 4 myoepithelial carcinoma and 3 basal cell adenocarcinoma) were retrospectively selected from patients undergoing surgery from 1980 to 2020 at A.C.Camargo Cancer Center.

Results and Discussions

Demographic, clinical and pathological characteristics were assessed, and the majority of the patients are above 40 years old and are female. The most frequently affected site was the parotid and present low histological grade. Most cases of carcinoma ex-pleomorphic adenoma had no expression of smooth muscle actin and calponin, with expression being observed in the other tumor types; p63 protein, cytokeratin 14 and Ki-67 were partially expressed and caldesmon and vimentin were mostly expressed in the cases. Beta-catenin and E-cadherin proteins were expressed in all cases of all tumor types. Hierarchical cluster analysis demonstrated the formation of 2 groups, one composed mainly by carcinoma ex-pleomorphic adenoma cases and the other group presented a variety of histological types. Statistical analysis demonstrated an association between the absence of cytokeratin 14 and p63 expression and the occurrence of metastasis.

Conclusion

The proteins were expressed in a varied pattern in the cases studied and an association was observed between the loss of cytokeratin 14 and p63 expression and the occurrence of metastasis.

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Introduction

Cytokines are small glycoproteins discovered more than 50 years ago and they are produced by diverse cells of the immune system including tumors. Cytokines act on other cells, and thus provide communication between various cell subpopulations. In tumor stroma interactions play an important role because they can modulate tumor cell stimulation, cell activation, cell growth but can inhibit the immune system necessary in tumor elimination.

Material and Methods

To understand the relationship between cytokines and tumors, it is necessary to observe cytokines in the cells of the tumor environment as well as in the tumor itself. To respond to this complex interaction between tumor micro-environment, cytokines in tumor and micro-environmental cells need to be analyzed using a variety of methods adapted to the appropriate tissue type including serum, tissue lysate, separated cells, or gene material isolated from cells.

Results and Discussions

Initial results showed that in the serum of patients in the advanced clinical stage there is an imbalance of cytokines in favor of pro-inflammatory cytokine and reduced ratio of stimulatory cytokines estimated by single ELISA assay or multiplex platforms that allow testing of up to 21 cytokines simultaneously. The production of cytokines from effector cells in tumor environment tissues shows a decrease in the production of cytokines like IFN- γ , IL-2, IL-12, indicating decrease capacity in tumor cell elimination. Contrary to this, tumor cells can produce enormously certain cytokines including TNF- α , TGF- β , IL-6 or other growth factors necessary for tumor proliferation and growth like VEGF, EGF, TGF which has also been shown up regulated at the genetic level using RT-PCR or real time PCR methods. However, to understand the heterogeneity of tumor cells and the complex response between tumors and the micro-environment, it is necessary to examine single-cell cytokines as well as the use of computer models to interpret complex and bulky data obtained on the principles of multiomics.

Conclusion

Multiomic research platforms that include cytokine analyses on genetic level, epigenetic cytokine regulation in comparison to cellular and receptor-level analyses can together help to understand tumor immunology and give hope for more successful individual tumor immunotherapy in cancer patients.

EACR22-0418

Association of circulating markers of Gut Barrier Integrity and Inflammation with Pancreatic Ductal Adenocarcinoma

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a highly lethal malignancy with five-year survival rates of approximately 10%. Disturbances to normal gut microbiota (dysbiosis) and the consequent alterations in microbial products are increasingly implicated in the

development of cancers of the gastrointestinal tract and accessory organs including the pancreas. Dysbiosis has been associated with inflammatory processes and reduced gut barrier integrity potentially allowing bacterial products such as lipopolysaccharide (LPS) to enter circulation (endotoxemia). Intestinal fatty acid binding protein (iFABP) leaks from enterocytes and enters circulation following gut barrier damage. Objective: To investigate if dysbiosis driven gut barrier damage is associated with PDAC development.

Material and Methods

Concentrations of circulating inflammatory cytokines and gut barrier protein biomarkers were assessed in serum from a PDAC case (n=90) -control (n=60) cohort from the Czech Republic. TLR4, ZO1 and iFABP were assessed by ELISA. Calprotectin and IFN γ , IL1 β , IL2, IL4, IL6, IL8, IL10, IL-12p-70, IL13 and TNF α were assessed by mesoscale assay

Results and Discussions

Markers indicating impaired gut barrier function were elevated in PDAC cases relative to controls; iFABP (p=0.026), TLR4 (p=0.030) and ZO1 (p=0.0003). Calprotectin was also elevated in PDAC cases relative to controls (p=0.042). Five of the ten assessed inflammatory cytokines were elevated in PDAC cases relative to controls; IFN γ (p=0.025), IL10 (p=0.002), IL6 (p=0.027), IL8 (p=0.0009) and TNF α (p=0.045). No significant differences were detected for IL1 β , IL2, IL4, IL-12p-70 or IL13.

Conclusion

These findings suggest that a reduction in gut barrier integrity and attendant intestinal/systemic inflammation is associated with PDAC development. Next steps will be to assess correlations between these data, tumour microbiome profiles and survival outcomes.

EACR22-0514

Recombinant antibodies for cancer biology research and diagnostics

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Introduction

Recombinant monoclonal antibodies (rAbs) are increasingly common in the commercial antibody market as tools for basic cancer biology research and as reagents for diagnostic purposes. Recombinant technology creates antibodies that can be fully characterized at the sequence level and have dependable performance and supply. These features make rAbs particularly attractive when compared to traditional polyclonal and hybridoma-generated monoclonal antibodies, whose limitations and poor consistency have been described. To address this issue, GeneTex has focused on developing a recombinant antibody production platform and, in conjunction, has expanded its validation protocols. The goal is to make reliable recombinant antibodies for reproducible performance in all fields of cancer research and diagnostics.

Material and Methods

GeneTex's recombinant rabbit antibody production approach is based on work published by Starkie *et al.* (2016). This utilizes a multi-parameter FACS-based methodology to select antigen-specific IgG+ memory B cells from an immunized animal, with cloning of the antibody variable region genes from that isolated cell into an IgG backbone and subsequent antibody expression in mammalian cells. This protocol is particularly advantageous as both

the heavy and light chains from the same B cell are cloned, thereby preserving natural pairing. This protocol, which can be completed in perhaps eight weeks, also allows application-specific testing (e.g., for WB, IHC, ELISA, ICC/IF, etc.) of clones during the production process. Antibody validation is performed inhouse and, where possible, in collaboration with academic institutions where appropriate samples are more readily available.

Results and Discussions

The production platform described above was used to create well-validated antibodies for a number of targets relevant to cancer biology research. This includes reagents to detect specific RAS mutants, E-cadherin, TSG101, ZO-1, p63, and NRF2, among others. Additional reagents against the androgen, estrogen, and glucocorticoid receptors, as well as various EGFR mutants, have also been generated. Notably, a new PD-L1 antibody that does not cross-react with PD-L2 and whose fidelity has been rigorously assessed was recently added. Many of these reagents are now being tested on clinical samples as described above.

Conclusion

GeneTex has developed a recombinant antibody production mechanism to produce and assiduously characterize antibody reagents for both bench and clinical cancer biology settings.

EACR22-0660

LincRNA as Liquid Biopsy Marker of Brain Cancer

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Introduction

Medulloblastoma (MB) is the most common malignant pediatric brain tumor. The ability to monitor and detect brain tumor markers in real time enables access to tumor biology and may allow highly personalized treatment for each patient. Long non coding RNAs (lincRNAs) are functionally defined as transcripts longer than 200 nucleotides in length with no protein coding potential. lincRNA involvement in human cancers etiology is being increasingly proved. Tumor-secreted long non-coding RNAs in exosomes are emerging mediators of cancer-host cross talk communication in tumor microenvironments.

Material and Methods

We analyzed RNA sequencing of 170 Medulloblastoma tumor samples and quantified the genome wide long non coding RNAs (lincRNA) expression levels. MB has four core subgroups: wntless (WNT), sonic hedgehog (SHH), Group 3 and Group 4. We identified the differentially expressed lincRNA in the different MB core groups. Next, we tested the selected markers in normal brain and other types of brain tumors. We explored the tumor immune microenvironment in tumors of selected markers. Finally, we detected the markers in the blood of MB patients.

Results and Discussions

We identified a lincRNA that is distinctively highly expressed in group 4 (MB4). MB4 expression was further examined in microarray analysis on a larger cohort of medulloblastoma patient samples and a large cohort (n=1405) of patient samples that include normal brain and different types of brain tumor samples. MB4 proved to be highly expressed in group 4 Medulloblastoma and with a unique tumor immune microenvironment. MB4 was detected in the plasma of medulloblastoma patients with active disease, or

subtotal resection. MB4 was not detected in patients that their tumors were resected. Furthermore, MB4 is not detected in the serum of patients with medulloblastoma type SHH, penioblastoma, ewing sarcoma and neuroblastoma.

Conclusion

MB4 lincRNA is a highly specific medulloblastoma tumor biomarker that can be quantified from a blood test. MB4 can be a good diagnostic marker, and in future may also be a good target for therapy and impact immune therapy.

EACR22-0782

Large scale mRNA analysis in lung cancer patients reveals frequent high expression of *ROS1* and *RET* not associated with gene fusions

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Introduction

Around 3-5% of patients with lung cancer (LC) harbor fusions involving ALK receptor tyrosine kinase (*ALK*), *RET* proto-oncogene (*RET*) or *ROS* proto-oncogene 1, receptor tyrosine kinase (*ROS1*), which associate with response to targeted therapy. However, little is known about the RNA expression levels of these genes in wild-type (wt) LC tumors.

Material and Methods

A custom nCounter panel (NanoString® Technologies) was used to detect fusions and mRNA levels of *ALK*, *ROS1* and *RET* in formalin-fixed paraffin embedded (FFPE) LC samples from Spain and Brazil. The counts corresponding to the 3' probes were normalized using the negative and the housekeeping probes. Cut-off values for very high expression were established as the average counts for each gene plus three times the standard deviation in wt samples.

Results and Discussions

A total of 637 stage I-IV LC Spanish patients were retrospectively analyzed. We found very high expression of *ALK* mRNA in 82 patients. Of them, 70/82 (85%) were positive for *ALK* fusions. Twenty-four patients showed *ROS1* very high expression, and only 5/24 patients (21%) harbored a concomitant *ROS1* fusion. Among the remaining 19 patients with very high *ROS1* mRNA, six were *ALK* positive, seven harbored mutations in *EGFR* and two were non-smoker females with unknown drivers. Regarding *RET*, very high expression levels were found in 14 patients but only 2/14 (7%) showed a *RET* fusion. Six of the 14 patients presented neuroendocrine features and seven patients lacked of known drivers. These results were validated using an independent cohort of 175 Brazilian non small cell lung cancer patients. Very high expression of *ALK* mRNA was found in 28 patients; 24 of them (86%) harbored an *ALK* fusion. Four patients showed *ROS1* very high expression; all of them were *ROS1* negative but three showed a concomitant *ALK* fusion while the remaining patient was a non-smoker female without known drivers. Finally, very high

expression of *RET* was detected in 5 patients, only one of them (20%) harbored a *RET* fusion while the rest were pan-negative for known drivers.

Conclusion

Very high levels of *ALK* mRNA in LC are associated with *ALK* fusions. In contrast, the majority of patients with very high *ROS1* or *RET* mRNA levels are fusion negative. Preclinical research is being conducted to determine the clinical relevance of this finding.

EACR22-0816

Circulating microRNAs as predictive biomarkers in familial-hereditary breast cancer *C. Compagnoni¹, V. Zelli¹, E. Ricevuto¹, C. Ficorella¹, T. Sidoni², A. Di Marco³, A. Bianchi³, F. Zazzeroni¹, E. Alesse¹, A. Tessitore¹*

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Introduction

Circulating microRNAs (miRNAs) are considered potential and promising diagnostic and predictive biomarkers in cancer. Here, we performed a circulating miRNA profiling in hereditary-familial breast cancer (BC) cases in order to identify differentially expressed miRNAs with putative value of non-invasive biomarkers and to deepen the molecular mechanisms involved in the pathogenesis of familial-hereditary BCs.

Material and Methods

Plasma miRNAs of a series of 21 familial BC cases, including 14 *BRCA1/2* positive (BRCA) and 7 *BRCA1/2* negative (non-BRCA) BCs, were analyzed by miRNAsequencing. Three age-matched healthy controls were also included in the study. A bioinformatic pipeline comprising Bowtie1 tool for alignment to miRBase v.22 and the reference genome GRCh38, as well as DESeq2 package for differential expression analysis was used. Differentially expressed miRNAs were filtered based on a log2 fold change <-1 or >1 (down/up-regulated miRNAs respectively), and an FDR adjusted p-value ≤0.05. *In silico* analysis of the target/pathway genes of differentially expressed miRNAs was performed using the on-line DIANA and miRNet tools (<http://diana.imis.athena-innovation.gr>; <https://www.mirnet.ca>).

Results and Discussions

miR-320e and miR-486-3p emerged as the most relevant miRNAs able to discriminate BC cases and controls, showing down-regulation in BCs compared to healthy controls. Consistent with these data, tumor suppressor function was ascribed to these two miRNAs, as reported in several studies. Different expression levels of mir-486-3p were also observed related to BC molecular subtypes. A total of 20 differentially expressed miRNAs, 10 up-regulated and 10 down-regulated, emerged in the comparison between BRCA and non-BRCA cases, particularly relevant for the non-BRCA group. *In silico* analysis of target/pathway genes of the differentially expressed miRNAs revealed an enrichment of genes involved in different cancer types (e.g. prostate, lung, melanoma) and key biological processes, including cell cycle, apoptosis, focal adhesion and growth factor signaling pathways.

Conclusion

Overall, these preliminary results suggest that the analysis of the expression levels of circulating miRNAs in familial-hereditary BCs, based on the *BRCA1/2* germline mutational status, could provide, once extended and confirmed on a larger number of patients, important information for characterizing and putatively distinguishing familial BRCA and non-BRCA BCs, and might be considered as potentially applicable in screening and prevention programs.

EACR22-1192

Proteome analysis of gallbladder cancer secreted EVs and its relevance as reservoir of potential biomarkers and therapeutic targets.

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Introduction

Gallbladder cancer (GBC) is the most common biliary tract malignancy characterized by a late diagnosis, poor prognosis and high prevalence and incidence rate in Latin America and Asian region. To date, an insufficient number of therapeutic target and early detection biomarkers are available. There are continued efforts to identify novel circulatory biomarkers with improved sensitivity for detection of GBC. A new source of circulating biomarkers are extracellular vesicles (EVs), which are nanometer-sized membrane vesicles secreted by different cell types and released into different body fluids under normal and pathological conditions.

Material and Methods

To characterize the potential role of the GBC derived EVs proteome as a new source of biomarkers, we carried out a quantitative proteomic analysis using eight GBC cell lines. EVs were isolated from conditioned medium by serial ultracentrifugation. Subsequently, their morphology and abundance were characterized by Nanotracking Particle Analysis and Transmission Electron Microscopy. Proteins identified by LC-MS/MS analysis were classified according to the biological process, molecular function and subcellular location using an integrative bioinformatic analysis.

Results and Discussions

GBC-derived EVs showed a variability in size, abundance and protein composition across the all GBC cell lines analyzed. The proteomic analysis of total EVs-proteins led to the identification of 1600 unique peptides coresponding to 1200 proteins. Among the differentially expressed proteins, we found membrane proteins and extra and intracellular proteins involved in EVs transport, cellular communication and signal transduction. Recognized exosomal markers (GTPases, Tetraspanins, FLOT1, FLOT2, TSG101) and potential therapy targets (MIF, HSP90, S100A2) were identified by Western blot and immunohistochemistry.

Conclusion

EVs represent a new source to identify markers and potential therapy targets in GBC. Many proteins identified in this study are functionally relevant in tumor progression or have been known to be differentially expressed in GBC tissues. EVs could be obtained from body fluids as a non-invasive strategy for early diagnosis, therapy and clinical following of GBC.

EACR22-0161

Diagnostic potential of miR-135b gene expression in tongue cancer

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Introduction

High incidence of regional recurrences still presents a challenging problem in the treatment and survival of patients with tongue cancer. Existing pathohistological analysis used in the discrimination of tongue cancer from non-cancerous tissue needs improvements by introducing a more sensitive molecular approach. MicroRNAs (miRNAs) are small non-coding RNAs that act as posttranscriptional negative regulators of gene expression and are usually deregulated in cancer. Due to their tissue stability, miRNAs might be used as diagnostic molecular biomarkers. Over-expression of *miR-135b* was confirmed in numerous cancers. Our study aimed to estimate the relative expression of *miR-135b* in tongue cancer and non-cancerous tissue, investigate diagnostic potential of *miR-135b* expression and its association with clinicopathological characteristics.

Material and Methods

In the study were included 25 patients with tongue cancer. RNA was isolated from tongue cancer tissues and adjacent non-cancerous counterparts. RNA was converted into cDNA which was used for relative quantification of *miR-135b* gene expression normalized to RNU6B. *miR-135b* relative expression was reported as $2^{-\Delta Ct}$. The diagnostic potential was estimated by the receiver operating curve (ROC) analysis. Expression of *miR-135b* in cancer tissue was dichotomized as low or high by the median. The association of *miR-135b* expression with clinicopathological features was estimated by χ^2 test.

Results and Discussions

mir-135b expression was significantly increased in tongue cancer tissue (3.275 ± 4.095) compared to non-cancerous counterparts (1.389 ± 1.827), $p=0.001$, Wilcoxon test. Results of ROC analysis revealed that *miR-135b* might be considered as a biomarker for discrimination of tongue cancer from non-cancerous tissue (AUC=0.664, 95% CI=0.513-0.815, $p=0.047$). The optimal cut-off value was 1.575 estimated by the Manhattan distance model (sensitivity=56%, specificity=76%). *miR-135b* expression was higher than the median in 12 (48%) tongue cancer patients. None of the clinicopathological features were associated with *miR-135b* expression.

Conclusion

Obtained results indicate that *miR-135b* gene expression might be used as a potential diagnostic biomarker for sensitive discrimination of tongue cancer from non-cancerous tissue.

EACR22-0749

Detection of the ovarian cancer biomarker in body fluids using nucleic acid aptamers as diagnostic probes

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Introduction

Ovarian cancer is the deadliest gynecological cancer. The majority of the patients are diagnosed in advanced stages, with a 5-year survival ranging from 15 to 40 %. Currently, there are no easy early-stage and screening methods available, so late diagnosis remains a main factor contributing to poor prognosis. Recently, small synthetic nucleic acids, called *aptamers*, have emerged as potential diagnostic tools to detect cancer biomarkers. Here, we present high-affinity DNA aptamer probes for the detection of ovarian cancer biomarker in body fluids and its implication in diagnostics.

Material and Methods

Human Epididymis protein 4 (HE4) is a protein overexpressed in ovarian cancer and a valuable clinical biomarker. As a single blood biomarker, HE4 has the highest specificity and sensitivity in detecting ovarian cancer. The anti-HE4 DNA aptamers for diagnostic applications are selected from a random, highly diverse DNA library using *High-Fidelity Systematic Evolution of Ligands by EXponential enrichment (Hi-Fi SELEX)*, a method based on digital droplet Polymerase Chain Reaction (ddPCR), which ensures amplification of rare specific sequences. The sequences that are able to recognize HE4 are identified by Next-Generation Sequencing followed by bioinformatics.

Results and Discussions

Bioinformatics analysis on selected DNA sequences allowed the identification of anti-HE4 aptamer sequences that specifically target biomarker HE4. The characterization between candidate DNA probes and HE4 in body fluids is still undergoing, with the aim to evaluate binding affinity and specificity in blood and urine. To further investigate the diagnostic potential of these aptamers, the most potent anti-HE4 probe is used for the development of the *Enzyme-Linked OligoNucleotide Assay (ELONA)* for the detection of HE4 in surrogate samples of healthy individuals, low malignant potential ovarian masses, early stage (I/II) and late-stage (III/IV) ovarian cancer. ELONA offers a sensitive approach for detecting very low levels of HE4 in body fluids.

Conclusion

This prospective method can be used to detect HE4 in patient samples such as standard blood or non-invasive urine samples. Due to high-affinity and specificity, the identified anti-HE4 aptamer can offer cheap alternative to conventional antibodies in diagnostic tests. Therefore, it can be utilized as molecular probe in the various diagnostic methods, biosensors or point-of-care devices, and could be an additional tool towards reducing the high mortality of ovarian cancer.

EACR22-0136

DVL1, TWIST1, SNAIL and SLUG as nuclear markers of meningioma progression

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Introduction

Meningiomas are mostly benign tumors, but in small portion can develop malignant characteristics through progression to atypical and anaplastic grades. Those grades are more difficult to treat with classic therapies, therefore there is a continuing quest for molecular candidates for targeted meningioma treatments. Our focus was on discovering if Wnt signaling pathway and epithelial–mesenchymal transition (EMT), which are known to be involved in meningioma progression, can offer new molecular candidates for meningioma prognosis.

Material and Methods

We performed DAB-labelled immunohistochemical reactions on formalin-fixed paraffin-embedded meningioma sections with antibodies that detect active beta-catenin, DVL1, E-cadherin, N-cadherin, TWIST1, SNAIL&SLUG. Involvement of genetic changes in protein expression was tested using standard Sanger sequencing which gave us insight into exon 3 mutation hotspot of *CTNNB1* (beta-catenin) and PDZ domain of *DVL1*.

Results and Discussions

We discovered that DVL1, a central mediator of Wnt signaling pathway, as well as EMT transcription factors TWIST1, SNAIL and SLUG could be a good candidates as nuclear markers of meningioma progression. The most pronounced result showed that the nuclear expression of DVL1 was significantly correlated with a higher expression of active β -catenin ($p = 0.029$) and a higher meningioma grade ($p = 0.030$). This was also true for SNAIL and SLUG expression, which was significantly associated with higher meningioma grades ($p = 0.001$). TWIST1 didn't show statistical correlation with higher grades but was pronounced in most samples and its increased expression followed the increase of SNAIL and SLUG expression ($p = 0.033$). The high protein expression of EMT transcription factors could be the reason for observed low levels of E-cadherin in our samples. Interestingly, beta-catenin, although active, was found only in cytoplasm and was absent from nuclei. Genetic analysis showed that exon 3 of *CTNNB1* was mutated in 22.2% of samples and was absent in anaplastic samples, suggesting to be driving mutations of meningioma and not related to progression. On the other hand, the PDZ domain of *DVL1* was severely mutated and usually led to lower expression of DVL1.

Conclusion

Our study confirmed the role of key actors of conserved Wnt signaling pathway and EMT in intracranial meningioma progression. Furthermore, it suggests new molecular candidates for diagnostics and the treatment of meningioma patients.

EACR22-0192

Higher systemic Serum Amyloid A 1/2 levels are associated with a worse triple-negative breast cancer prognosis

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Introduction

Past research has identified the family of Serum Amyloid A (SAA) proteins to be intricately involved in inflammatory signaling and various disease pathologies. In cancer, SAA proteins are upregulated in the blood of cancer patients and tumor tissues, correlating in many instances with disease progression. However, the exact role of SAA proteins in cancer pathology is incompletely understood. We hypothesized that higher circulating SAA1 and SAA2 (SAA1/2) levels promote cancer by modulating cellular programs such as the cell cycle, apoptosis, and cell migration in tumors.

Material and Methods

C57BL/6 wild-type (WT) mice and C57BL/6 mice lacking the SAA1/2 orthologs (SAADKO) were injected with the EO771 cell line to establish triple-negative breast cancer (TNBC) allografts. Subsequently, tumor growth was monitored and modeled, species survival determined, the inflammatory profiles of mice assessed with a multiplex assay, and tumor molecular biology and histology characterized with western blotting and H&E histological staining.

Results and Discussions

Our results show no significant differences in tumor growth and species survival. However, as expected, blood plasma from control and tumor-bearing SAADKO mice had significantly less SAA than WT mice. Interestingly, this led to tumor-bearing SAADKO mice having higher MCP-1 and TNF α concentrations in plasma but lower IL-10. Tumors from SAADKO mice showed characteristics of apoptosis inhibition (decreased caspase cleavage) and DNA repair (PARP synthesis), while histological sections showed less tumor necrosis. Additionally, SAADKO tumors characteristically showed increased epithelial (E-cadherin, β -catenin) and decreased mesenchymal (vimentin, snail) marker expression, associated with a reduced metastatic phenotype. Together, these results support our hypothesis that higher circulating SAA1/2 levels promote cancer by modulating cellular programs, including apoptosis, necrosis, and cell migration.

Conclusion

Our study implicates increased systemic SAA1/2 in TNBC progression and aberrant signaling to a worse TNBC prognosis. As such, SAA1/2 could serve as therapeutic targets in the treatment of TNBC.

EACR22-0302

Role of Activin and Colony Stimulating Factors in melanoma TAM pro-tumoral phenotype

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Introduction

Melanoma cells dynamically interact with monocytes/macrophages to subvert their differentiation into tumor associated macrophages (TAMs). However, the pathways and microenvironmental cues governing their protumoral deviation are not completely understood. Here we explored the involvement of key myeloid differentiation cytokines in the acquisition of pro-tumoral functions.

Material and Methods

Co-culture assays were performed to determine the expression of the macrophage differentiation cytokines Activin A and Colony Stimulating Factors (CSFs) GM-CSF, M-CSF, and G-CSF. Tumor 3D invasion, proliferation and immune-suppression assays were

achieved to explore their specific functional roles. Allojection murine models were used to study the in vivo functional role of Activin A; and confocal screening of a paraffin embedded collection of 80 primary human melanomas to ascertain the differential expression of each cytokine in the tumor microenvironment.

Results and Discussions

We found that Activin A and several CSF play an instrumental role in the crosstalk between melanoma cells and monocytes/macrophages, which results in the upregulation of distinct tumor-sustaining genes and the achievement of pro-invasive and immunosuppressive functions of TAMs. Blockade of Activin prevents the acquisition of protumoral functions in vitro, and facilitates human melanoma allojection by transferred human lymphocytes in vivo. Notably, screening of a cutaneous primary melanoma collection showed that Activin A and specific CSFs are enriched in TAMs and/or melanoma cells from patients with worse outcome and constitute new and independent prognostic markers.

Conclusion

We identify Activin and specific CSFs, as key players in the pro-tumoral and immunosuppressive functions of TAMs, with significant potential as disease biomarkers and immunotherapeutic targets. Our results in primary melanoma suggest that pro-metastatic TAMs show a prominent pro-inflammatory profile, differing from the general assumption that tumor-supporting TAMs display an anti-inflammatory deviation.

EACR22-0308

Investigating β -catenin-driven inflammation in patients with desmoid tumor to identify prognostic biomarkers

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Introduction

Desmoid tumor (DT) is a rare locally aggressive tumor with high recurrence rate and unpredictable clinical course. Standard of care for DT patients is active surveillance, however, 30% of DT patients progress and shift to active treatment. Biomarkers of aggressive DT are not available and identification of progressing patients remains challenging. DTs are often characterized by mutations in β -catenin gene and transcriptomic studies showed that β -catenin-mutated DT surgical specimens have an 'inflammatory phenotype'. Cancer-associated inflammation is detectable in circulating immune cells, which are promising prognostic biomarkers for DT patients. The aim of this study is to investigate molecular profile of DT biopsies and phenotypic/functional features of blood immune cells, to dissect DT β -catenin-driven immune features, identify DT patients at risk of progression and guide tailored therapeutic approaches.

Material and Methods

This is a prospective study enrolling DT patients under active surveillance (n=80). Tumor biopsies transcriptomic analysis and blood phenotypic/functional/transcriptional studies will be performed, mutated β -catenin ctDNA levels will be evaluated and 3D-cultures will be assessed to test drugs combinations.

Results and Discussions

Blood immune-profile analysis shows altered myeloid and lymphocytic profile in DT patients, compared to healthy donors. Increase in immunosuppressive granulocytes, m-MDSC and e-MDSC subsets is observed. Progressing patients present peculiar immunosuppressive granulocytes and inflammatory cyto/chemokines profiles. Increase of PMN-MDSC is observed only in DT harboring T41A mutation. DT patients show altered frequencies of Th1, Th17 and Th2 T cells. Th22 and Th9 T cell subsets are higher in regressing patients, while TIM3 is overexpressed in progressing patients, that present also high percentages of activated T cells. Preliminary transcriptomic data show differential expression of immune and fibrosis-related genes in biopsies from progressors compared to biopsies from regressors. Further investigations of immune cells infiltrating the tumor site are ongoing.

Conclusion

These preliminary findings indicate that the inflammatory status detected at tumor site is reflected at systemic level in peripheral blood of DT patients. Peripheral immune-modulation of myeloid and lymphocytic subsets supports the hypothesis of immune system involvement in DT onset and it may represents a disease aggressiveness marker. Supported by Italian Ministry of Health (RF-2016-02362609).

EACR22-0462

E-Cadherin and β -Catenin Expression Minimize the Risk of Metastases in NSCLC Patients Treated with Chemoradiation

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Introduction

Non-small cell lung cancer (NSCLC) patients radically resected have a significant risk to progress for distant metastases of 40% overcoming the multifunctional cell process of the epithelial-mesenchymal transition (EMT) characterized by loss of cell-cell adhesion, detachment from the basement membrane, cytoskeletal rearrangement, and migration into the provisional matrix. These phenotypic alterations are accompanied by significant changes in molecular expression and signal transduction within cells, including downregulation of E-cadherin and its downstream target Wnt/ β -Catenin. NSCLC standard of care is based on simultaneous chemoradiation for limited stages and on chemotherapy alone or chemotherapy combined with anti-PD-L1 checkpoint inhibitors for extensive stage. In this study, we analyze two EMT-related proteins, E-cadherin and β -catenin, as promise biomarkers to select NSCLC patients that could benefit from personalized therapy.

Material and Methods

The expression of E-cadherin and β -catenin were examined in 120 surgically resected samples of NSCLC from patients with pathological stages I, II, IIIA. 60 patients received chemoradiation or at least one these treatments after surgery. During follow-up, 31 patients developed distant metastases. E-cadherin and β -catenin protein expression were detected by immunohistochemistry staining, and their expression quantified by QuPath.

Results and Discussions

A strong correlation was found between β -catenin and E-cadherin expression ($p=0.617$; $P<0.001$). β -catenin was higher in squamous histology compared to non-squamous histology ($P=0.02$), the same as observed for E-cadherin ($P=0.001$). In patients treated with chemoradiation, β -catenin expression tended to be higher than those without chemoradiation (mean, 2195 cell/mm² vs 1509 cells/mm²; $P=0.05$). Multivariate Cox analysis showed significant low risk of metastases in patients treated with local chemoradiation, whose tumors expressed E-cadherin>2119 cells/mm² and β -catenin $\leq$ 1945 cells/mm².

Conclusion

We inferred that a high expression of E-cadherin and low expression of β -catenin in tumor, minimize the risk of invasion and metastases in early pathological stage of NSCLC in patients treated with chemoradiation, prolonged overall survival. However, further investigations are needed for the establishment of E-cadherin and β -Catenin as molecular markers, affecting treatment response and survival.

EACR22-0472

MAP17 as a prognostic and predictive biomarker in pancreatic cancer

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Introduction

Pancreatic adenocarcinoma has been the seventh major cause of cancer-related deaths worldwide in 2020. Although this type of cancer does not present a high incidence in the population, its aggressive behavior leads to a high mortality with a life expectancy of ~5% at 5 years. Despite the development of novel screening and diagnostic techniques in the last years, the majority of patients are diagnosed in either locally advanced or metastatic stage (80-85%). In addition, the prognosis of these patients has not improved over the last two decades, being surgical resection the only chance to patients, when possible, since less than 20% of patients are diagnosed with resectable disease. Indeed, high rates of systemic (80%) and local (20%) recurrence are found within the first two years after surgery. The addition of chemotherapy in the adjuvant setting has been shown to improve survival rates and is routinely recommended after resection of pancreatic carcinoma of any stage. However, the option for metastatic pancreatic cancer involves systemic chemotherapy, with a limited benefit.

Therefore, the lack of effective therapies and the worse prognosis of patients make necessary to study and understand the molecular mechanisms mediating therapy resistance to identify new targets or biomarkers of response to improve the treatments.

Material and Methods

We studied the expression of MAP17 in a cohort of paraffin-embedded tissue samples from 97 patients with pancreatic cancer from the biobank of HUVR-IBIS and correlated this expression with different clinicopathological features. We overexpressed MAP17 in two

different pancreatic cancer cells lines and tumorigenesis was analyzed using different functional assays and measuring several stem cell markers by FACS. Finally, we performed IC50 assays with different drugs and correlated them with MAP17 expression.

Results and Discussions

We found that MAP17 is upregulated in tumor samples in the HUVIR-IBIS cohort of pancreatic patients. In addition, different levels of MAP17 expression stratify patients on risk of relapse. Then, we observed that MAP17 overexpression increases the tumorigenic and stemness properties of cells in two different pancreatic cancer cell lines. Indeed, MAP17 overexpressed cells showed a high sensitivity to different drugs commonly used in the clinics.

Conclusion

MAP17 could serve as a prognostic and predictive biomarker in pancreatic cancer to determine which patients are going to benefit from the different treatments to improve their response and survival.

EACR22-0556

Development of a prognostic risk model for clear cell renal cell carcinoma by systematic evaluation of DNA methylation markers

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Introduction

Current risk models for renal cell carcinoma (RCC) based on clinicopathological factors are sub-optimal in accurately identifying high-risk patients. Here, we perform a head-to-head comparison of previously published DNA methylation markers and propose a potential prognostic model for clear cell RCC (ccRCC).

Material and Methods

Promoter methylation of *PCDH8*, *BNC1*, *SCUBE3*, *GREM1*, *LAD1*, *NEFH*, *RASSF1A*, *GATA5*, *SFRP1*, *CDO1*, and *NEURL* was determined by nested methylation-specific PCR. To identify clinically relevant methylated regions, The Cancer Genome Atlas (TCGA) was used to guide primer design. Formalin-fixed paraffin-embedded (FFPE) tissue samples from 336 non-metastatic ccRCC patients from the prospective Netherlands Cohort Study (NLCS) were used to develop a Cox proportional hazards model using stepwise backward elimination and bootstrapping to correct for optimism. For validation purposes, FFPE ccRCC tissue of 64 patients from the University hospital of Leuven and a series of 232 cases from The Cancer Genome Atlas (TCGA) were used.

Results and Discussions

Methylation of *GREM1*, *GATA5*, *LAD1*, *NEFH*, *NEURL*, and *SFRP1* was associated with poor ccRCC-specific survival, independent of age, sex, tumor size, TNM stage or tumor grade. Moreover, the association between *GREM1*, *NEFH*, and *NEURL* methylation and outcome was shown to be dependent on the genomic region. A prognostic biomarker model containing *GREM1*, *GATA5*, *LAD1*, *NEFH* and *NEURL* methylation in combination with clinicopathological characteristics, performed better compared to the model with clinicopathological characteristics only, in both the NLCS and the validation population. However, the biomarker model had limited added prognostic value in the TCGA series.

Conclusion

In this study we performed a head-to-head comparison of potential prognostic methylation markers for ccRCC using a novel approach to guide primers design which utilizes the optimal location for measuring DNA methylation. Using this approach, we identified five methylation markers that potentially show prognostic value in addition to currently known clinicopathological factors.

EACR22-0634

Translocator Protein (TSPO) as Prognostic Biomarker in Head and Neck Squamous Cell Carcinoma

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Introduction

TSPO is a small 18-kDa protein located on the outer mitochondrial membrane. It has been proposed to take part in wide variety of cellular functions. The role of TSPO in cancer is not very well known. We have previously shown that radiotherapy (RT) increases the uptake of a TSPO-specific PET tracer (¹⁸F]F-DPA) in head and neck squamous cell carcinoma (HNSCC) cell lines and xenografts. The RT-induced ¹⁸F]F-DPA uptake was inhibited by silencing TSPO. This study aims to evaluate whether TSPO expression correlates with the overall survival (OAS) and treatment resistance in HNSCC.

Material and Methods

A validated HNSCC tissue microarray (TMA) cohort was created from patients treated in Turku University Hospital region during 2005-2010. The TMA was stained against TSPO and graded by the staining intensity into no staining, low/medium or high staining groups. The log-rank method was used to analyse the Kaplan-Meier survival estimations. Two HNSCC sub-cell lines (FaDu RR and Cal33 RR), with different levels of radioresistance (RR), were used to evaluate the relationship between ¹⁸F]F-DPA uptake and the level of RR. Cell lines were kindly provided by Prof. Anna Dubrovskaya (OncoRay, Dresden, Germany). Cells were seeded in 6-well plates, irradiated with 5x2 Gy on consecutive days and measured for ¹⁸F]F-DPA uptake.

Results and Discussions

A trend (p=0.08) towards a correlation between high TSPO expression and worse 5-year OAS was found in primary tumours (n=185). When oropharyngeal tumours, that are often p16-positive, were excluded from the analyses the trend became significant (p=0.006,

n=143). A similar result was seen in p16-negative tumours (p=0.008, n=142), whereas an opposite association was seen in p16-positive tumours (p=0.0179, n=26). The latter finding must be interpreted with caution due to the low number of patients. A second HNSCC/TMA (patients treated during 2011-2015) will be analysed for TSPO expression to further study the correlation between p16-status, tumour sub-site and treatment modality. After RT the uptake of [¹⁸F]F-DPA was higher in Cal33 RR cells (more RR) compared to the less RR FaDu RR cells indicating a role of TSPO in radiosensitivity. The molecular mechanisms that regulate TSPO after RT is unclear, and will be further investigated.

Conclusion

Our results support previous suggestions that TSPO is a potential prognostic biomarker. We further hypothesize that TSPO might be predictive for RT response in HNSCC. Identification of a biomarker that could predict RT response would be highly valuable.

EACR22-0636

LINE-1 hypomethylation is associated with poor outcomes in both HPV16-positive and HPV16-negative locoregionally advanced oropharyngeal cancer

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Introduction

Human papilloma virus (HPV) positivity represents a strong prognostic factor for improved survival in patients with oropharyngeal squamous cell carcinoma (OPSCC). However, a subset of HPV-positive OPSCC patients experience poor outcomes. Furthermore, HPV-negative OPSCC patients are still lacking prognostic biomarkers. This study assessed the impact of LINE-1 methylation level on progression-free survival (PFS) and overall survival (OS) of OPSCC patients, with a focus on the relationship between LINE-1 methylation levels and p53 protein expression level. Finally, differences in genome-wide/gene-specific DNA methylation were investigated and evaluated for their potential regulation by LINE-1 elements.

Material and Methods

163 stage III-IVb OPSCC patients managed with curative intent were retrospectively reviewed. Methylation of LINE1 repetitive sequences was evaluated by real-time quantitative methylation-specific PCR in formalin-fixed paraffin-embedded tissues. Immunohistochemistry staining was performed to determine p53 expression. DNA methylation analysis was conducted by using MethylationEPIC BeadChip).

Results and Discussions

LINE-1 hypomethylation was directly associated with poor prognosis and decreasing survival rates. When LINE-1 methylation was dichotomized as <55% vs. ≥55%, HPV16-negative OPSCC patients with LINE-1 methylation <55% reported the worst prognosis. Multivariable analyses further showed an increased risk of progression or death in patients with LINE-1

methylation <55%, regardless of the HPV-status. Tumor protein p53 gene is often mutated, and overexpressed in HPV-negative OPSCC. Since p53 has been reported to repress LINE-1 promoter, the association between p53 protein expression status and LINE-1 methylation levels was analyzed. Results indicated that among HPV16-negative patients with p53 $\geq$ 50%, LINE-1 methylation levels declined and remained stable at approximately 43%; any HPV16-positive patient reported p53 $\geq$ 50%. Finally, genome-wide methylation analysis revealed that a significantly lower content of cytosine-phosphate-guanine methylation could be found in relapsing OPSCC patients. Notably, the majority of the top hypomethylated genes contained LINE-1 elements in their promoter sequences.

Conclusion

LINE-1 hypomethylation was associated to poorer OS and PFS in OPSCC regardless of HPV16 status. Future research is needed to understand whether p53 may affect retrotransposon activity in OPSCC cells, and to elucidate the role of the differentially methylated regions identified herein.

EACR22-0682

Circulating microRNAs to monitor response to single agent immunotherapy in advanced NSCLC patients

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Introduction

Response to immune-checkpoint inhibitors (ICIs) is observed in almost 25% of advanced non-small cell lung cancer (NSCLC) patients, but about 80% of patients relapse due to acquired resistance mediated by both tumor intrinsic and extrinsic factors. The identification of systemic biomarkers to monitor the response to treatment would thus be clinically relevant. We have previously demonstrated that a circulating microRNA (miRNA) signature classifier (MSC) was associated with the response to ICIs independently of PD-L1 tumor expression. This study was aimed at evaluating if the modulation of circulating miRNA at baseline and during treatment is able to increase the predictive power of clinical variables.

Material and Methods

From year 2015 to 2020, plasma samples and clinico-pathological information, including blood cell count and PD-L1 tumor expression, were collected from advanced NSCLC undergoing ICI treatment in our Institution. Circulating miRNA profiling was measured using miRCURY LNA Custom Panel at the baseline, time of best response and possibly every year up to progression (PD). For the selection of predictors, a penalized regression model was fitted. The Cox model is used for overall survival (OS) and the logistic model for progression free survival at 6 months (PFS-6m). To validate the models, the C-index for discrimination ability and calibration plots were estimated.

Results and Discussions

Overall, 211 advanced NSCLC patients treated with ICI single agent were enrolled in our study. Before 6 months 142 (67%) patients progressed, while median OS was 9.24 months (95%CI:8.09-12.8). For PFS-6m the model with clinical variables only showed a C-index of

0.86 (0.80-0.92). Including miRNA, the C-index increased to 0.89 (0.84-0.96). Regarding OS, the inclusion of miRNA increased the C-index from 0.77 (0.74-0.81) to 0.79 (0.75-0.83). Similar results were obtained when considering the subset of 78 patients treated with ICI as first line: the C-index for PFS-6m increased from 0.81 (0.70-0.92) to 0.87 (0.78-0.96), while for OS from 0.77 (0.67-0.85) to 0.81 (0.71-0.90). For 82 out of the 91 (90%) patients with stable or responsive diseases as best response, longitudinal plasma samples were also available. Further analyses are ongoing to evaluate the longitudinal modulation of miRNA and their impact on patients' outcome.

Conclusion

Measuring circulating miRNAs at the baseline and during treatment could be an evaluable tool to help monitoring the response to single agent ICIs in advanced NSCLC.

EACR22-0798

Exploration of the Prognostic Efficacy of a Combined OncoMasTR and Immune Marker Signature in Early Stage Breast Cancer Using Multiplex Immunofluorescence

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Introduction

Traditionally, many women with lymph node-negative breast cancer (LN-BC) are unnecessarily treated with chemotherapy. This highlights the importance of accurately predicting patient prognosis. We previously identified OncoMasTR, a novel prognostic gene panel of master transcriptional regulators that predicted recurrence risk for LN-BC patients better than current prognostic signatures. Validation of OncoMasTR at the protein level in two independent breast cancer tissue microarrays cohorts using chromogenic immunohistochemistry (IHC) has shown promising results. Since the role of different immune cell populations as key prognostic biomarkers in breast cancer is well established, we hypothesise that the integration of immune status information will improve the prognostic capacity of OncoMasTR.

Material and Methods

Using one of these cohorts (n=498), we describe the immunofluorescence-based multiplex IHC analysis of three selected markers from the OncoMasTR panel (UHRF1, ATAD2, and TCF-19), and three immune markers (CD4, CD8, and FOXP3) using the Opal staining technique.

Results and Discussions

Survival analysis of OncoMasTR markers showed that high expression of UHRF1 (HR=2.0, p=0.03) and ATAD2 (HR=1.7, p=0.07), and low expression of TCF19 (HR=0.47, p=0.01), are individually correlated with reduced distant recurrence-free survival (DMFS). A double-positive population (ATAD2+ UHRF1+) with significant prognostic association (HR=1.9, p=0.031) was also identified. For immune markers, high intratumoural (HR=0.47, p=0.04),

stromal (HR=0.56, p=0.04), and whole core (HR=0.34, p<0.001) CD8+ cell density showed an association with increased DMFS. High intratumoural CD4+ (HR=0.48, p=0.01) and Treg cell (CD4+FOXP3+) density (HR=0.26, p=0.04) were also found to be linked with better DMFS, whereas no prognostic association was found for either cell population in stromal area. Furthermore, the close proximity of CD4+ cells and tumour cells were also found to have a significant association (HR=1.8, p=0.02) with reduced DMFS.

Lastly, a combined signature from immune, OncoMasTR and clinical markers (tumour size, node involvement) was identified using a bootstrap selection analysis. It showed significantly higher (HR=7, p<0.001) prognostic efficacy than the best performing model containing OncoMasTR and clinical markers only (HR=3.7, p=0.007). **Conclusion**

This demonstrates the promise of a multiplex IHC biomarker panel containing both OncoMasTR and immune markers, potentially offering a better prognostic prediction for early-stage BC.

EACR22-1080

Progression of adult diffuse hemispheric astrocytoma is associated with a gene network driven by the myeloid transcription factor SPI1

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Introduction

Low-grade astrocytomas (LGA) comprise 20% of primary brain tumours in adults. Their response to therapy is poor and they invariably progress to more aggressive lesions. Understanding this transformation could help significantly in the clinic. The tumour microenvironment (TME) is heavily infiltrated by glioma associated macrophages and microglia (GAMs) which promote tumour growth. Increased infiltration of blood borne macrophages might drive transformation and a derived signature could be used as a prognostic biomarker. Single-cell (Sc) RNA sequencing has been used to study cell types and states in glioma. This data can define transcription factor (TF) networks (regulons) which determine macrophage phenotypes. The activation of regulon subsets can then be used as a proxy for functional state and provide potential biomarkers.

Material and Methods

Sc-RNA sequencing was performed on four LGA patients with suspected transformation and different IDH status and histological grade. Tumour core and transforming areas were identified pre-operatively and sampled separately. Peripheral blood was sampled and processed in parallel. Regulons were inferred using the SCENIC algorithm. Regulon enrichment score in individual cells was calculated using AUCell. The TCGA LGG and GBM projects and GLASS data sets were used as external validation sources.

Results and Discussions

A GAM compartment common to all patients was found by clustering based both on gene expression and regulons suggesting that GAM profiles are homogeneous across astrocytoma types. Further regulon analysis identified two modules within the GAM compartment: a microglial AP-1 driven network and a SPI1 driven BDMC/macrophage module. This was possible thanks to the selective sampling and the regulon analysis

performed. Since regulons consist of gene sets associated with specific TFs, we could show differences in the SPI1 module between GAMs and blood borne cells. Furthermore, we found that the SPI1 blood borne signature correlated with poorer prognosis in public data.

Conclusion

Myeloid cells adopt a GAM phenotype in the TME associated with TF networks related to microglia and peripheral macrophages. A SPI1 blood macrophage signature correlates with poorer outcomes, which supports reports that link increased peripheral blood macrophage infiltration to poorer outcomes. However, the increased resolution of our approach suggests that an upregulated SPI1 blood signature in GAMs rather than actual infiltration of peripheral blood cells is responsible for this phenomenon.

EACR22-1148

SAMHD1 expression in residual disease is a Prognostic factor after neoadjuvant chemotherapy in early breast cancer.

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Introduction

The sterile human α motif and protein 1 containing the HD domain (SAMHD1) is a dTNP triphosphohydrolase that plays a role in cell metabolism and thus, has been linked to distinct diseases, including cancer. Neoadjuvant treatment (NAT) has become widely used in early breast cancer. Beyond the known clinical benefits of NAT, and the prognosis value of the pathological complete response (pCR), no other informative biomarkers exist. Thus, the development of new tools and better molecular targets that predict the prognosis of patients (pt) with residual disease after NAT is a matter of research. Here, we identified SAMHD1 expression as a prognostic biomarker after NAT in breast cancer (BC).

Material and Methods

Tumor biopsies from 182 p were collected from pt diagnosed with stage II-III BC were candidates for NAT between 2002 and 2012. Expression of estrogen receptor, progesterone receptor, basal cytokeratins, HER2, Ki67 and SAMHD1 was performed by immunohistochemistry in tissue microarrays. The percentage of SAMHD1 stained cells was evaluated and tumors were classified as positive ($\geq 1\%$) or negative. Univariate and multivariate analysis for overall survival (OS) and time to progression (TTP) were performed.

Results and Discussions

SAMHD1 was positive in almost 31% of evaluable pt. SAMHD1 positivity correlated with grade III tumors ($p=.023$), and higher levels of Ki67 post-NAT ($p=.017$) suggesting that SAMHD1 was indeed affecting cell proliferation capacity. SAMHD1 positivity was also correlated with reduced clinical response in pt treated ($p=.016$). SAMHD1 positive patients presented shorter TTP (log Rank $p=.002$), with a median TTP of 4.58 years for SAMHD1

positive pt, whereas it was not reached for SAMHD1 negative pt. Thus, to gain insight into the putative role of SAMHD1 as a prognostic factor in BC tumors treated with NAT anthracycline-based regimens with residual disease at surgery, we evaluated OS according to SAMHD1 status. Median OS was 10.78 years (95%CI, 4.17-17.39) for SAMHD1 positive pt compared to an estimated 17.75 years (95%CI, 0 to 14.6) for SAMHD1 negative pt ($p=.016$). For OS the multivariate analysis showed that negative SAMHD1 and negative nodal status were associated with prolonged OS after NAT ($p=.004$ and $p=.041$).

Conclusion

SAMHD1 expression was significantly associated with shorter PFS and OS after NAT. Further evaluation of additional cohorts, in clinical studies including combination therapies that modulate SAMHD1 expression and function, will prove its value and potential clinical applications.

EACR22-1270

ASCL2 transcription factor expression is associated with progesterone and oestrogen receptors in breast cancer

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Introduction

Achaete-scute-like 2 (Ascl2) is a transcription factor of the basic helix-loop-helix family associated with tumor processes. Its overexpression induces intestinal stem cell hyperplasia and depletion leads to their loss. We studied the expression of Ascl2 in a cohort of 189 patients with breast cancer of Algerian origin.

Material and Methods

Ascl2 expression was studied by immunohistochemistry (IHC) on 189 micro-biopsy samples from patients with breast cancer and its transcript level by quantitative RT-QPCR on 38 cytopuncture samples from the primary tumor and/or the metastatic lymph node. Relative transcript expression was related to its expression in colorectal lines SW620 and SW480 known for high and low/negative expression, respectively. The data were analyzed by the SPSS tool (Statistical Package for the Social Sciences) aiming to define whether there are correlations between the expression of Ascl2 and the clinicopathologic characteristics of subjects. We performed in silico analyses on the correlation of Ascl2 expression and survival rate in people with breast cancer.

Results and Discussions

IHC results show that the Ascl2 protein is more expressed in triple negative cancers (88.6%) compared to luminal A and B type cancers where expression is found respectively in 41.4% and 50% of cases. In line with these data, the RT-QPCR results show a heterogeneous relative expression of Ascl2 in the cytopunctures with low expressions close to the SW480 line or high expressions close to the SW620 line. A great heterogeneity was also observed in the metastatic lymph nodes. Statistical analysis using SPSS software and through the Pearson Ki-2 test shows that high Ascl2 expression is associated with a low level of

estrogen receptors ($p < 0.001$) and progesterone receptors ($p < 0.001$) as well as with the molecular subtypes of breast cancer ($p < 0.001$). However, no association was observed between Ascl2 expression and tumor size ($p = 0.5$) or proliferative activity assessed by Ki67 expression ($p = 0.938$) or with the presence of metastatic lymph nodes ($p = 0.246$). In Silico analysis by RNA Microarray (Kaplan Meier Plot platform) show that probability of survival of subjects with high expression of Ascl2 is reduced compared to those with low expression ($p = 6.10^{-6}$).

Conclusion

Ascl2 expression is associated with hormonal receptors expression in breast cancer in an Algerian cohort. In addition, this expression is higher in triple-negative cancers, suggesting that Ascl2 expression could be a marker of aggressiveness and a therapeutic target.

EACR22-0247

No association between BRAF V600E mutations and aggressive clinicopathologic features in Croatian patients with papillary thyroid carcinoma

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Introduction

BRAF V600E mutations are the most common genetic alteration in patients with papillary thyroid carcinoma. Their prognostic value and association with aggressive features in patients with papillary thyroid carcinoma have been extensively studied but with conflicting results. The aim of this study was to examine association of BRAF mutations with aggressive clinicopathologic features in a cohort of Croatian patients with papillary thyroid carcinoma.

Material and Methods

In this study we analysed thyroid papillary cancer samples from 177 patients. DNA was isolated from formalin-fixed paraffin-embedded tissue and assessed for presence of BRAF V600E mutation using competitive allele-specific TaqMan polymerase chain reaction. Association between BRAF mutations and aggressive clinicopathologic features was examined using appropriate statistical test (Chi-square, Fisher's exact or Mann-whitney test). This research was funded by Croatian Science Foundation project IP-2019-04-1130.

Results and Discussions

BRAF V600E mutation was found in 110 (62%) out of 177 patients. Patients with BRAF mutation were significantly older than patients without BRAF mutation (median age 50 vs. 39 years; $p = 0.0004$). BRAF mutation was found in 52 (66%) out of 79 patients with localised disease, 42 (63%) out of 67 patients with lymph node metastases, and in 16 (52%) out of 31 patients with distant metastases. There was no significant association between BRAF mutations and metastatic status ($p = 0.197$). We also found no statistically significant association between presence of BRAF V600E mutation and gender ($p = 0.128$), tumour diameter ($p = 0.404$), capsular invasion ($p = 0.496$), intrathyroidal dissemination ($p = 0.641$), extrathyroidal extension ($p = 0.911$), lymph node metastases ($p = 0.330$), and distant metastases ($p = 0.097$).

Conclusion

Our study showed no association between BRAF V600E mutation and clinicopathologic features of aggressive papillary thyroid carcinoma.

EACR22-0249

Clinical Significance of SOX2 expression in early head and neck tumorigenesis and late stages of disease progression

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Introduction

The pluripotency transcription factor SOX2 is frequently overexpressed in cancers, including head and neck squamous cell carcinoma (HNSCC). The tumor-promoting activity and involvement of SOX2 in tumor progression has been extensively demonstrated, thereby emerging as a promising therapeutic target. However, the clinical significance of SOX2 as a biomarker in HNSCC progression and also its role in early stages of tumorigenesis and the possible contribution to malignant transformation remain undetermined.

This study investigates the clinical relevance of SOX2 protein expression in early tumorigenesis as a cancer risk biomarker, and also its impact at late stages of HNSCC progression on patient prognosis and disease outcome

Material and Methods

SOX2 expression was evaluated by immunohistochemistry in a large series of 94 patients with laryngeal precancerous lesions (dysplasias) and a cohort of 348 surgically-treated HNSCC and correlated with clinicopathological data and patient outcome.

Results and Discussions

Nuclear SOX2 expression was frequently detected in 38 (40%) laryngeal dysplasias, whereas stromal cells and normal adjacent epithelia showed negative expression. Univariate Cox analysis showed that SOX2 protein expression ($p < 0.001$) but not histological grading ($p = 0.432$) was significantly associated with laryngeal cancer risk, and found to be the only significant independent predictor of laryngeal cancer development in multivariate analysis (HR = 3.531, 95% CI 1.144 to 10.904; $p = 0.028$). SOX2 expression was observed in 105 (30%) HNSCC tissue samples, and strongly correlated with NANOG expression. Patients harboring positive SOX2 expression exhibited higher disease-specific survival (DSS) and overall survival (OS) rates. However, these differences did not reach statistical significance.

Conclusion

These findings underscore the relevant role of SOX2 in early HNSCC tumorigenesis rather than in late stages of tumor progression. More importantly, SOX2 expression emerges as a strong independent predictor of laryngeal cancer risk in patients with precancerous lesions beyond current WHO histological grading.

EACR22-0345

Co-examination of SPARC and WNT3A expression identifies distinct subsets of tumor

stroma in NSCLC with impact as predictive and prognostic biomarkers

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Introduction

Among the molecular subsets of NSCLC recently described, the gene SPARC driven carcinomas subtypes and response to target therapy. As a regulator, SPARC can enhance the expression of its downstream target WNTs signaling pathway, activating cysteine-rich glycoproteins carried on the surface of secreted exosomes that migrate to target cells, promoting cancer cell motility, and metastatic potential. However, different methodology for quantification impacts discordant results as predictive and prognostic markers. We aimed to explore clarify the relationship between SPARC and WNT expressions in tumor stroma and their clinicopathological implications for NSCLC patients.

Material and Methods

We enrolled 120 surgically resected samples of NSCLC (79% in early-stage). We used QuPath software to quantified SPARC and WNTs protein expression in tumor stroma were detected by immunohistochemistry staining. The outcome was survival time until death from recurrent lung cancer.

Results and Discussions

SPARC high expression in tumor stroma (>14.3%) was observed in 41% NSCLC. For WNT3A the high expression (>19.2%) was detected in 47% patients and correlated with T₁₋₂ stage (P=0.04), and early-stage (P=0.05). SPARC expression was correlated with WNT3A stroma expression (p=0.27, P=0.003). Multivariate Cox model analysis demonstrated that adjuvant radiotherapy was related to survival time (P=0.04), but quantitative staining of the tumor stroma for SPARC, with WNT3A as co-dependent, added prognostic information (P=0.03) and were more strongly prognostic than radiotherapy. A cutpoint at the mean of expression for SPARC and WNT3A divided the tumor stroma into two groups with distinctive survival times (P=0.04, for both). Those with SPARC ≤14.3% and WNT3A ≤19.2% had a median survival time of just 65.1 and 60.9 months, respectively. While SPARC >14.3% and WNT3A >19.2% had a median survival time of 98 and 96.7 months, respectively. We inferred that a high proportion of SPARC and WNT3a stabilize the tumor stroma in early-stage of NSCLC, controlling cell motility and thus avoiding regional and distant metastases.

Conclusion

SPARC and WNT expression could be helpful to stratify tumor stroma for a selection of a subset of patients who would benefit from targeted therapies and consequently better prognosis. Thus, in the current emergent scenario, personalized treatments will become effective, and the implementation of like SPARC and WNT3A biomarkers detection will be useful for the selection of patients who can benefit from them.

EACR22-0441

Membrane-Intercalated Glycosaminoglycans and Collagen Stroma Mitigate Invasion and Metastases, and Prolonged Survival of Patients with NSCLC

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Introduction

The fibrotic stromal response of the tissue to tumor development and invasion is a ubiquitous phenomenon in non-small cell lung cancer (NSCLC), called tumor desmoplasia. Tumor desmoplasia is the result of complex interactions between NSCLC cells and stromal cells. The response of the cells to these changes must involve integral cell surface molecules that recognize their ligands and initiate intracellular transmission of signals. Glycosaminoglycans (GAGs) like heparan sulfate (HS) and chondroitin sulfate (CS), bind to interstitial matrix components such as basement membrane and interstitial collagen types. We analyze the relationship between these GAGs and collagen types with stroma and their clinicopathological implications for NSCLC patients.

Material and Methods

We enrolled 120 surgically resected samples of NSCLC. HS and CS protein expression were detected by immunohistochemistry and by immunofluorescence for collagens (Col) types I, III, IV, and V. We used QuPath to quantify their expression.

Results and Discussions

In our cohort, low expression of HS was detected in 56% of NSCLC cases and correlated with T_{1,3} stage, contrasting with HS high expression in 44% of the samples in T_{2,4} stage (P=0.04). Low Col I expression occurred in 65% of NSCLC in T₁₋₃ stage while high expression occurred in 35% of tumor stroma in T₄ stage (P=0.04). Low Col IV expression was associated with tumor size (P=0.05), 56% in tumors ≤3 cm, and 44% for >3 cm. Patients who received chemotherapy tended to present tumor stroma with low expression of Col V (70% of cases, P=0.07). Multivariate Cox analysis showed that T₁₋₂ stage and adjuvant chemotherapy were associated with low risk of death (P<0.05 and P=0.09, respectively), but the HS expression added prognostic information (P=0.04) and was more strongly prognostic than T stage and chemotherapy. Patients that presented HS expression ≤1.5% had a median survival time of 5.8 months, while those who presented HS>1.5% had a median survival time just of 4.9 months. We inferred that a low proportion of GAGs associated with Col I and V in tumor stroma, as well as Col IV in the basement membrane, mitigate invasion and metastases in T1-T2 stage and early pathological stage. These findings suggest that adjuvant chemotherapy decreasing the risk of death and prolonged overall survival.

Conclusion

In the emergent scenario, membrane-intercalated GAGs and collagen stroma like HS, Col I, and Col V can be implemented as biomarkers for the selection of patients being helpful to personalized treatments.

EACR22-0687

Transcriptomic characterization to find prognostic biomarkers and therapeutic targets in Malignant Pleural Mesothelioma patients

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Introduction

Malignant pleural mesothelioma (MPM) is a rare and fatal disease for which platinum-based chemotherapy has been the standard treatment for decades. To date, the most reliable predictors of better outcome are still histological subtype (epithelioid MPM is associated with the best prognosis) and clinical parameters, such as early stage disease, younger age and female gender. At genetic level, gene mutations have a limited prognostic role and many of them are not druggable. This study aimed to discover new deregulated pathways to develop novel drugs and identify new biomarkers.

Material and Methods

MPM patients with available FFPE tissue were retrospective and prospective enrolled at Fondazione IRCCS Istituto Nazionale dei Tumori of Milan and Azienda Sanitaria Universitaria Giuliano Isontina of Trieste and recruitment is already ongoing, within the granted Italian MOH project, GR-2016-02361229. RNA was automatically extracted and quantified by fluorometer. Transcriptome was assessed through 3'DGE method. Patients were divided into 3 groups based on months of survival: long survivors (LS) >36 months, normal survivors (NS) between 12 and 36 months and short survivors (SS) < 12 months. Comparison of gene expression levels was performed among these groups.

Results and Discussions

One hundred sixty-six patients with available tissue, collected from 2006 to 2021, were enrolled. For 151 patients (91%) RNA was successfully extracted and sequenced, and survival information were available. Quality check showed no differences among the three runs of sequencing and even between the two different participating centres. 35 patients were LS, 58 were NS and 46 were SS. Twelve out of 151 patients were not classifiable because still in follow up. According to a preliminary analysis, SS patients displayed 21 down regulated genes compared to NS patients; five of these genes, were down regulated in SS also when compared to LS. Moreover, SS patients displayed 25 up regulated compared to NS patients; among them 8 genes, were up regulated also when compared to LS. Further analyses are ongoing to also consider clinical predictors, to perform pathway-level comparisons, and to correlate expression level to survival as continuous variable.

Conclusion

Our data showed a different expression level of some genes in patients with worse prognosis. In particular, the major interest may lie in over expressed genes for which it would be conceivable a treatment aimed at their inhibition to achieve a better clinical outcome.

EACR22-0707

Assessing strategies for counting CD8+ tumour infiltrating lymphocytes with a semi-automated method

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Introduction

Tumour Infiltrating Lymphocytes (TIL) are components of the tumour microenvironment and are involved in the local endogenous immune response to cancer. TILs present prognostic and predictive value in different tumour types and its evaluation is gaining increasing interest. Not only the type of TIL population but also its localization within the Tumour Microenvironment (TME) is the key for control of tumour progression. The different impact of TILs located exclusively within the tumour islets (intraepithelial TILs) or both, within the tumour islet and tumour stroma (intratumoral TIL) has been highlighted. However, scoring TILs in these two differentiated compartments may be challenging. We aim to evaluate the performance and ease of application of a semiautomated image analysis-based TIL scoring in both tissue locations. For that, in a proof of concept assessment, we have analysed CD8+ TILs in Endometrioid (EOC) and Clear Cell (CCOC) ovarian carcinomas.

Material and Methods

Cores from 302 EOC and CCOC tumours were arrayed in 9 TMA, tissue sections were immunostained using an anti-CD8 antibody and generated images were digitalized. Using the Zeiss Zen 3.3 software an expert pathologist screened cores for a hotspot of CD8+ TILs, delimited a 0.55 mm-circle diameter and delineated the tumour islets with the circle area. Then we run an automated algorithm generated to differentiate stained cells and obtained CD8+ TILs count in the tumour islet (intraepithelial TILs) and in the whole circle section (intratumoral TILs). The Ovarian Tumour Tissue Analysis consortium (OTTA) criteria was used as TIL scoring reference method to evaluate performance of our semiautomated procedure.

Results and Discussions

Our preliminary results on 302 tumours indicate a greater than 80% agreement between our semiautomated method and the reference manual scoring. While the intraepithelial scoring, which involves disregarding TILs in the stroma or abutting tumour cells, was more time consuming, the intratumoral scoring was fast and easily implemented.

Conclusion

Our semiautomated method combines the accuracy ensured by the involvement of a specialist pathologist with the inter-sample reproducibility and objectivity provided by automated counting. If further validated, it may help to simplify TIL reporting, in particular for tumours where lymphocytes infiltrating both, tumour and stromal compartments, are those showing clinical implications.

EACR22-0719

Relevant biological processes in high-risk clear cell renal carcinoma patients defined by Bio-miR

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Introduction

Renal carcinoma is the sixth most common cancer in men and the eighth in women. Two-thirds of patients have localized disease and an additional 16% have a locoregional disease at diagnosis. A significant proportion of all these patients are going to relapse. However, there is not a specific treatment or an established biomarker in this disease. Bio-miR is a dichotomist signature based on the expression of 9 microRNAs, recently communicated in ASCO, which divides clear cell renal carcinoma (ccRCC) patients into two groups with different risk of relapse.

Material and Methods

One hundred and eighty patients with ccRCC were analyzed by high-throughput proteomics. Proteins were extracted from FFPE tissue. Data-independent acquisition mass spectrometry analysis was performed on an Orbitrap Fusion, equipped with a Digital PicoView source, and coupled to an M-Class UPLC (Waters). Protein quantification was performed in Spectronaut using the default settings. Protein data was analyzed using Significance Analysis of Microarrays (SAM) and probabilistic graphical models, which allow the identification of relevant biological processes between groups of tumors.

Results and Discussions

3091 proteins were identified, of which 2026 meet quality criteria, and were used for subsequent analysis. SAM identified 124 differentially expressed proteins between low-risk and high-risk groups defined by Bio-miR. Proteins overexpressed in the high-risk group were mainly related to focal adhesion, metabolism, and angiogenesis. Adjuvant angiogenesis inhibitors have shown modest benefit at the cost of high toxicity in medium-high risk localized ccRCC unselected patients. A tailored selection of patients could help design new clinical trials involving second-generation anti-angiogenic drugs. Probabilistic graphical models defined eight functional nodes related to specific biological processes. Differences between low- and high-risk tumors were detected in Complement activation and Translation functional nodes. Complement immunotherapy has been proposed as an alternative to immune-checkpoint inhibitors in some oncologic scenarios.

Conclusion

Promising therapeutic targets, such as angiogenesis, metabolism, or complement activation, were found deregulated in high-risk ccRCC patients defined by Bio-miR. These processes need to be further investigated.

EACR22-0969

Clinical and pathological impact of neuroendocrine lineage-specific transcription factors in adenocarcinoma of the colon.

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Introduction

Colon adenocarcinomas with neuroendocrine differentiation (ADC-NED) are frequent and have been associated with a poor prognosis. In pure neuroendocrine cancer models, such as small cell lung cancer, neuroendocrine phenotype is mediated by the alternative

activation of transcriptional programs, but their role in ADC-NED has not been investigated, nor are their associated molecular characteristics. The aim of our study was to analyze the gene expression of transcriptional regulators of neuroendocrine differentiation in ADC-NED in correlation with the mutational profile and pathological characteristics.

Material and Methods

Two case series were analysed: a) set 1 (95 cases) with homogeneous genotype (all BRAF-mutated); set 2 (43 cases) with heterogeneous genotype as assessed using a 22 gene colon cancer panel. In both series the presence of neuroendocrine differentiation was assessed by means of synaptophysin immunohistochemistry. In all cases, the expression of 8 genes involved in the transcriptional regulation of neuroendocrine differentiation (ASCL1, DLL3, INSM1, MYCL1, NEUROD1, NOTCH1, POU2F3 and YAP1) was assessed by using real time PCR. Gene expression levels were correlated with histological grade, pT and pN stage and presence of mutations.

Results and Discussions

In set 1, cluster analysis segregated all but one synaptophysin-positive cases within a major cluster significantly enriched for NOTCH1, POU2F3, ASCL1 and DLL3 that were also positively reciprocally correlated. By contrast, INSM1, MYCL1 and YAP1 were significantly overexpressed in the cluster containing almost all synaptophysin-negative cases. Moreover, NOTCH1 and POU2F3 overexpression was correlated with a lower response to treatment, and low YAP1 expression was associated with mucinous phenotype. In set 2, synaptophysin-positive cases were more heterogeneously distributed, with an association with INSM1 and NEUROD1 overexpression. NOTCH1 reduced expression was associated with positive nodal status. None of the genes was correlated with any specific genotype.

Conclusion

Our data show that: i) ADC-NED are associated with specific profiles of transcriptional regulation; ii) the expression of these genes is influenced by the tumor genotype; iii) the association of some of these genes with clinical and pathological features support their potential role as novel biomarkers.

EACR22-1105

ASCL2 transcription factor expression is associated with progesterone and oestrogen receptors in breast cancer

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Introduction

Achaete-scute-like 2 (Ascl2) is a transcription factor of the basic helix-loop-helix family associated with tumor processes. Its overexpression induces intestinal stem cell hyperplasia and depletion leads to their loss. We studied the expression of Ascl2 in a cohort of 189 patients with breast cancer of Algerian origin.

Material and Methods

Ascl2 expression was studied by immunohistochemistry (IHC) on 189 micro-biopsy samples from patients with breast cancer and its transcript level by quantitative RT-QPCR on 38 cytopuncture samples from the primary tumor and/or the metastatic lymph node. Relative transcript expression was related to its expression in colorectal lines SW620 and SW480 known for high and low/negative expression, respectively. The data were analyzed by the SPSS tool (Statistical Package for the Social Sciences) aiming to define whether there are correlations between the expression of Ascl2 and the clinicopathologic characteristics of subjects. We performed in silico analyses on the correlation of Ascl2 expression and survival rate in people with breast cancer.

Results and Discussions

IHC results show that the Ascl2 protein is more expressed in triple negative cancers (88.6%) compared to luminal A and B type cancers where expression is found respectively in 41.4% and 50% of cases. In line with these data, the RT-QPCR results show a heterogeneous relative expression of Ascl2 in the cytopunctures with low expressions close to the SW480 line or high expressions close to the SW620 line. A great heterogeneity was also observed in the metastatic lymph nodes. Statistical analysis using SPSS software and through the Pearson Ki-2 test shows that high Ascl2 expression is associated with a low level of estrogen receptors ($p < 0.001$) and progesterone receptors ($p < 0.001$) as well as with the molecular subtypes of breast cancer ($p < 0.001$). However, no association was observed between Ascl2 expression and tumor size ($p = 0.5$) or proliferative activity assessed by Ki67 expression ($p = 0.938$) or with the presence of metastatic lymph nodes ($p = 0.246$). In Silico analysis by RNA Microarray (Kaplan Meier Plot platform) show that probability of survival of subjects with high expression of Ascl2 is reduced compared to those with low expression ($p = 6.10^{-6}$).

Conclusion

Ascl2 expression is associated with hormonal receptors expression in breast cancer in an Algerian cohort. In addition, this expression is higher in triple-negative cancers, suggesting that Ascl2 expression could be a marker of aggressiveness and a therapeutic target.

EACR22-1115

Identification of chemotactic profile of glioblastoma multiforme by integration of transcriptomic and protein-based data

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Introduction

Despite successive advances in understanding the immunobiology of glioblastoma multiforme (GBM), this most common primary malignant brain tumor remains essentially incurable. Existing evidence suggests that the type of infiltrating immune cells in the tumor microenvironment (TME) is associated with prognosis in GBM. Selectively increased tumor infiltration with immunosuppressive cells and decreased migration of cytotoxic lymphocytes appear to be dictated by the characteristic profile of chemokines and chemotactic compounds in glioma TME. Here, by integrating transcriptional and proteomic data, we identified a profile of factors responsible for glioblastoma chemotacticity.

Material and Methods

We performed mapping of chemokine expression in GBM using RNA sequencing (RNAseq) of the matched primary tumor tissues and primary tumor-derived cell cultures and combined it with bulk and single-cell RNAseq analysis of TCGA and CGGA repository data.

Chemokine expression results were verified by Luminex method in primary GBM cell culture secretome. Finally, we assessed the prognostic value of chemokines in glioblastoma and created a risk score based on MDK and CXCL2 expression.

Results and Discussions

Analysis of over 800 samples from tumor tissues, cell cultures and available repository cohorts revealed a consistent picture of the chemokine expression profile in GBM, with a predominance of midkine, CXCL(10,12,14,16), CX3CL1, CCL(2,4). We further identified the cellular sources of chemokines with the highest expression in the GBM microenvironment and found that midkine, CCL2, CCL5, XCL2, and CXCL16 were associated with poor overall survival. The two best candidates for multivariate prognostic role were MDK and CXCL2, implicating their synergistic role in promoting angiogenesis, supporting the glioma stem cells (GSCs) functionality, and aiding the tumor growth.

Conclusion

Our study provided new insights into chemotactic communication between cellular constituents in the GBM microenvironment, identifying key molecules responsible for modulation of tumor infiltration and growth-promoting signals. We have recognized the chemotactic factors that may serve as potential targets for specific inhibitors or to improve the homing of adoptive cell therapies to the tumors.

EACR22-1243

The Prognostic Significance of Neural Markers in Colorectal Cancer

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Introduction

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer-related deaths worldwide. Although the TNM (tumor-lymph node-metastasis) staging classification system is still regarded as one of the main prognostic factors, clinical outcome can vary considerably for patients diagnosed in similar stages. Recent findings indicate that neurons and glia within the CRC tumor stroma could affect clinical outcome in CRC patients. In this study, we aim to investigate the prognostic value of the neuronal marker neurofilament (NF) and the glial marker glial fibrillary acidic protein (GFAP).

Material and Methods

Formalin-fixed, paraffin-embedded (FFPE) tumor tissues from the Netherlands Cohort Study on diet and cancer (NLCS) were immunohistochemically stained for NF (n=487) and GFAP (n=447). Two independent observers assessed the presence of NF or density of GFAP staining within the tumor. Associations between NF or GFAP expression and multiple clinical and pathological characteristics were investigated using Pearson chi-square test, long-rank

tests and Kaplan-Meier. Multivariate analyses were performed with Cox proportional hazard models, adjusted for potential confounders and known prognostic factors.

Results and Discussions

NF positive nerves and GFAP positive glial cells were found within the tumor stroma. In the multivariate analysis, adjusted for age at diagnosis, sex, tumor location, differentiation grade and TNM/dukes stage, NF was associated with a poorer cancer specific survival (HR 2.38; 95%-CI 1.37-4.13, $p = 0.002$). On the other hand, high-density GFAP staining was associated with a better survival compared to negative staining (HR 0.56; 95%-CI 0.33-0.95, $p=0.030$). Low-density GFAP staining showed the same trend, but was not statistically significant (HR 0.83; 95%-CI 0.53-1.29, $p = 0.396$). When combining both markers, negative NF staining together with positive GFAP staining was strongly associated with a better survival (HR 0.49; 95%-CI 0.28-0.86, $p=0.012$).

Conclusion

NF is significantly associated with a poorer survival in CRC whereas high-density GFAP staining is associated with better survival in CRC. However, validation of these results in other cohorts is needed. Combining both markers, negative NF together with positive GFAP staining shows the best survival. These findings provide new evidence for a potential prognostic role of neural markers in CRC and could supplement on the existing evidence of the biological role of neural structures in CRC.

EACR22-0853

ShRNA-based loss-of-function screening to find novel combinatorial drug targets and biomarkers in CRC

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Introduction

Colorectal cancer (CRC) is the second cause of cancer deaths in Europe. In advanced disease, patients are treated among others, with combinations of DNA-damaging agents such as 5FU and irinotecan (FUIRI). However, intrinsic and acquired resistance involve treatment failure. Novel therapies as well as reliable predictive biomarkers are needed to improve patients' outcomes. Chromatin factors directly interact with DNA, facilitating or impairing the activity of such drugs by modifying chromatin conformation. We aimed to uncover if certain chromatin regulators are responsible of FUIRI resistance and may be used as novel drug targets and/or predictive biomarkers.

Material and Methods

We performed a loss-of-function screening (LOFS) using a shRNA library targeting more than 900 chromatin factors in a pool-approach in HT29 CRC cell line. Cells were treated with 0.1 μ M 5FU + 0.055nM SN38 (irinotecan's active metabolite) during 4 weeks consecutively. After NGS comparison between nontreated and treated cells, genes that increase FUIRI sensitivity or resistance were selected using R based package (FDR>0.5). Individual validation was performed: knockdown efficiency was checked by Western blot (WB) and RT-qPCR; response to treatment was analysed by viability assays using flow cytometry. 38 genes showed significant changes according to the concordance trend of at least 6 of 8shRNA for each gene. We selected 25 genes for expression analysis using Nanostring

technology in 92 FFPE samples of advanced CRC patients treated with first-line 5FU+SN38 combinations. Associations with response rate and progression-free survival were assessed by chi-square and log-rank tests, respectively. Combinations of FUIRI + 5μM of PDD00017273 (PARG inhibitor) were studied by MTT following Chou-Talalay method.

Results and Discussions

We selected 14 genes for functional studies and 11 were individually validated. PolyADP-Ribose Glycohydrolase (PARG) and Histone Methyltransferase Complex Regulatory Subunit (DPY30) were selected for further studies. PARG and DPY30 downregulation decreased up to 50% and increased 50% cell sensitivity to FUIRI treatment, respectively. High DPY30 mRNA levels were associated with a higher response rate in patients ($p < 0.0001$). Combinations of 5FU, SN38 with PDD00017273 were synergistic *in vitro*.

Conclusion

LOFS strategy demonstrates to be a useful tool to identify chromatin regulators as possible drug targets (PARG) and/or predictive biomarkers (DPY30). Further research is ongoing and will be presented at the EACR meeting.

EACR22-0933

ORAL PRESENTATION

High prevalence of potentially druggable molecular alterations in lung carcinoids with high proliferative index.

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Introduction

Lung carcinoids with high mitotic count (>10 mitoses in 2mm²) and/or high proliferation index (>20%) (LC-HPI) have been recently recognized and coded in the WHO classification of lung tumors as a separate group, with peculiar pathological and clinical features, including a poorer prognosis. However, the genomic features of these tumors are very poorly characterized. We therefore aimed at investigating the genomic background of LC-HPI, with special reference to alterations in genes of potential therapeutic relevance.

Material and Methods

Twenty-eight cases of LC-HPI were selected at the Pathology Units of “San Luigi” and “Città della Salute e della Scienza” Hospitals, Turin. Genomic profiling was assessed by means of wide targeted-next generation sequencing using the Oncomine Comprehensive V3 panel (ThermoFisher) and Gene Studio Prime S5 Ion Torrent apparatus. All variants with an allelic frequency >5%, a coverage >300, coded as deleterious by Ion Reporter II software and classified as pathogenic or likely pathogenic by Varsome free database, were considered significant. All significant alterations were further validated by Alamut software (Sophia Genetics). All gene fusions detected were validated by FISH and/or immunohistochemistry.

Results and Discussions

Twenty-six cases overall (93%) showed significant molecular alterations (from 1 to 10) in the genes analyzed. Twelve cases (43%) harbored mutations (single or co-occurrent) in genes that are potential therapeutic targets. Genes involved in DNA repair mechanisms were mutated in 9 cases (32%), including 6 (21%) affecting the mismatch repair. Among tyrosine-kinases ERBB2/HER2 showed the highest mutation rate (4/28, 14%). Gene fusions were

detected in 3 cases (11%), including 1 EML4/ALK, 1 KIF5B/RET and 1 ETV6/NTRK2. Additional potentially druggable single nucleotide variants (all missense mutations, and not exclusive) were detected in RET, FGFR1, NTRK1 (in one sample, each) and KRAS G12C (in two samples).

Conclusion

The high prevalence of potentially druggable molecular alterations in LC-HPI paves the way to individualized therapeutic approaches in these rare tumors characterized by a well differentiated morphology but a clinically aggressive behavior.

EACR22-0133

ORAL PRESENTATION

Ultra-sensitive monitoring of leukemia patients using superRCA mutation detection assays

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Introduction

The ability to observe, evaluate, and count even extremely rare nucleic acid sequence variants in biological samples is a frequent need in biology and medicine. In particular, rare tumor-specific mutations in patient samples serve as excellent markers to monitor the course of malignant disease and responses to therapy in clinical routine, and improved assay techniques are needed for broad adoption. We describe herein - superRCA assays - which provides for rapid and highly specific detection of DNA sequence variants present at very low frequencies in DNA samples. Using a standard flow cytometer we demonstrate precise, ultra-sensitive detection of single-nucleotide mutant sequences from malignant cells against a 100,000-fold excess of DNA, to follow the course of patients treated for acute myeloid leukemia (AML).

Material and Methods

Sequence of interest are first enriched by targeted PCR amplification from a patient sample. The amplified sequences are next converted to DNA circles that are subjected to rolling-circle amplification (RCA). Padlock probes specific for mutant or wild-type sequences are then used to probe the repeated sequences of the RCA products with exquisite specificity, followed by RCA of the circularized probes. The large DNA clusters that result from each starting DNA circle are referred to as superRCA products.

Results and Discussions

With the spike-in samples, we have successfully demonstrated that superRCA assay can reliably detect IDH mutation at 1 in 100,000 levels, as comparison, the ddPCR assays sensitivity was limited at 1 in 1000 levels due to high false positive event rate and high standard deviations. In 3 longitudinal AML patient studies, these patients were initially high with IDH mutations at the diagnosis of AML, then after the stem cell transplantation treatment, the mutation levels was decreased to undetectable levels both with superRCA and ddPCR assay. But with superRCA assay, we were able to detect IDH mutation at 1 in 10,000 before the patient were fall complete remission, while with ddPCR assay it was impossible to detect such intermediate conditions for the patient due to the limited sensitivity of ddPCR assay.

Conclusion

The superRCA assay procedure is suitable for routine use by virtue of its high sensitivity and simplicity. The 3-hr protocol only requires a sequence of five additions to a DNA sample, separated by incubations, before reaction products are analyzed using a standard flow cytometer.

EACR22-0414

TOP ABSTRACT

Signatures of chromosomal instability as therapeutic biomarkers

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Introduction

Chromosomal instability (CIN) is a hallmark of cancer and results in the accumulation of whole or partial chromosome changes. CIN has been associated with disease stage, metastasis, poor prognosis, and therapeutic resistance. However, as the causes and consequences of CIN are complex, CIN has generally been used as a broad umbrella term. Here, we present a systematic framework to comprehensively characterise the diversity, extent and origins of CIN and define how different types of CIN relate to clinical phenotypes.

Material and Methods

Using 6,335 genome-wide copy number profiles across 33 cancer types, we derived 17 pan-cancer copy number signatures. These signatures encode patterns of copy number change representing different underlying causes of CIN. We integrated over 40 independent genomic and clinical datasets to propose signatures aetiologies. Using these signatures we predicted response to 44 targeted therapies and identified 104 novel drug targets across CRISPR, RNAi and drug perturbation of 297 cell lines. We also derived two signature-based clinical classifiers to predict sensitivity to platinum-based chemotherapy and resistance to doxorubicin.

Results and Discussions

We discovered that our 17 pan-cancer copy number signatures characterised different causes of CIN including multiple types of mitotic errors, replication stress, impaired homologous recombination (HR), impaired non-homologous end-joining and extrachromosomal DNA (ecDNA) amplification. Using overall survival as a surrogate for treatment sensitivity, we trained cox-proportional hazards models using our impaired HR signature-based clinical classifier and showed that we could predict sensitivity to platinum-based chemotherapy treatment in ovarian cancer (HR:0.54, $p < 0.001$, $n = 545$), validation cohort (HR:0.54, $p = 0.039$, $n = 71$). We could even distinguish sensitive from resistant cases within BRCA1 germline mutant carriers (HR:0.28, $p = 0.015$, $n = 36$). We also found that a second classifier using ecDNA signatures could predict resistance to doxorubicin treatment (HR:5.2, $p = 0.001$, $n = 24$).

Conclusion

Our results illuminate a fundamental structure underlying CIN and highlight the potential of CIN signatures to improve the treatment of patients with extreme CIN tumours. By developing our signature-based classifiers into companion diagnostic assays, we might administer therapies in a more targeted manner, allowing resistant patients to avoid toxic side effects, and healthcare systems to reduce the cost burden of ineffectual treatment.

Novel biomarkers for oncolytic virotherapy

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Introduction

Oncolytic viruses (OV) are a promising approach to combat advanced staged cancer, including pancreatic adenocarcinoma (PDAC). OVs from many virus families with various mechanisms of infectivity are currently being developed. However, to date direct comparisons between different OVs and molecular biomarkers to predict therapeutic outcomes are rare. Our project aims at elucidating this lack of knowledge by searching for biomarkers predictive of OV response in patient-derived PDAC cultures.

Material and Methods

A recently established set of 14 primary PDAC cultures reflecting intra- and interpatient tumor heterogeneity was used as an in vitro model system. The cultures were screened for sensitivity towards 5 OVs from different virus families currently investigated in clinical trials (measles virus, parvovirus, reovirus, adenovirus and the EMA-approved OV talimogene laherparepvec). Dose-dependent response kinetics were obtained via cell viability assays (XTT) and cytotoxicity assays (LDH release). Viral replication was evaluated in (RT-) qPCR; automated analysis methods (IncuCyte and xCELLigence systems) were used to monitor viral infection in real time.

In addition, baseline transcriptome profiles of all cultures were obtained by RNA microarray analysis of each culture.

Phenotypic cell viability data retrieved from the screen was weighted using z-score calculation and summarized in a sensitivity matrix.

Results and Discussions

The 14 cultures showed distinct OV sensitivity profiles. Culture- and virus-specific response patterns were reproducible in two independent viability assays.

The transcriptome profiles correlated with viral susceptibility as measured by the viability assays. Differential gene expression of two distinct clusters of cultures with similar transcriptome profiles revealed several pathways associated with viral sensitivity.

In a second approach, correlation analyses between gene expression and experimental phenodata were performed for each virus individually. Previously described host factors were retrieved in this analysis, validating our approach. Furthermore, potential novel host factors were identified that are suitable biomarker candidates.

Conclusion

Our findings illustrate the sensitivity spectrum of primary PDAC cultures towards OV and thus underline the need for pre-therapeutic patient stratification. To this end, our project delivers a broad, descriptive, and comparative gene expression profiling data set, which provides a rich source of biomarker candidates warranting future validation.

EACR22-0240

Co-analysis of ASCL1 and DLL3 expression identifies distinct subgroups in resected neuroendocrine carcinomas of the lung with impact on tumour stage and prognosis

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Introduction

Small cell lung carcinomas (SCLCs) and large cell neuroendocrine carcinomas (LCNECs) are high-grade neuroendocrine carcinomas of the lung with very aggressive behavior and poor prognosis. Their therapeutic protocol as well as the histological classification remain the same in the last years. The transcription factor Achaete-scute homolog-1 (ASCL1) is involved in the tumorigenesis of SCLC, and can enhance the expression of its downstream target Delta-like protein 3 (DLL3). Thus, SCLC patients then could benefit from DLL3 target therapy. Regarding LCNECs, they characterize a heterogeneous group of tumour, which can exhibit distinct molecular subtypes, questioning their clinical managing as NSCLCs or SCLCs. We aimed to clarify the relationship between ASCL1 and DLL3 expressions and their clinicopathological implications for SCLCs and LCNECs patients.

Material and Methods

We enrolled 31 surgically resected samples that were obtained by archival formalin-fixed paraffin-embedded of SCLC (N=22) and LCNEC (N=9). ASCL1 and DLL3 protein expression was detected by immunohistochemistry staining and quantified by QuPath

Results and Discussions

DLL3 expression was positive in 21 (95.5%) cases of SCLC, and 4 (55.6%) cases of LCNEC. ASCL1 was positive in both SCLC and LCNEC (mean 86 positive cells/mm² vs 79 positive cells/mm², respectively). DLL3 high expression was observed in 51.6% patients and was correlated with tumour size and lymph node metastasis ($p = 0.048$, $p = 0.028$, respectively). ASCL1 high expression was detected in 54.5% of SCLC and 44.4% of LCNEC. A positive correlation was observed between ASCL1 and DLL3 expression ($p = 0.371$, $p = 0.04$;). In addition, an association between DLL3 expression and patients overall survival (OS) was observed. Patients that presented high-expression of DLL3 had a better prognostic with a higher OS rate than those presented DLL3 low-expression (21.46 months vs 3.25 months, respectively; $p = 0.001$, Log Rank, Mantel-Cox). Although univariate analysis showed that patients with DLL3 high expression had a better OS, multivariate cox regression analysis showed neither ASCL1 nor DLL3 expression was an independent prognostic factor. Thus, analysis of ASCL1/DLL3 expression could be more significant in selection of patients suitable for target therapy.

Conclusion

A new scenario is emerging, which, if personalized treatments are effective, will also lead to the implementation in practice of ASCL1 and DLL3 biomarkers detection for the selection of patients who can benefit from target therapies.

EACR22-0252

The Spatial Biology Revolution: Whole Transcriptome Digital Spatial Profiling Combined with Sub-Cellular Spatial Molecular Imaging Provides High Sensitivity, Multi-omic Tissue Profiling

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Introduction

Next-Generation sequencing of bulk samples revolutionized life science research, including our ability to discover biomarkers and understand cancer biology. Single-cell sequencing (sc-Seq) extended our ability to characterize samples by enabling deep profiling of sample heterogeneity and specific characterization of rare-cell populations. However, sc-Seq approaches do not provide information about the location of the cells in the context of a tissue, making it difficult to understand how cell classes are spatially organized and how they function in the context of tissue architecture. Thus, there is a demand for technologies that can perform high-plex molecular profiling of bulk and sc-Seq but do so while retaining the spatial relationships of the original tissue. We have developed two such platforms: the GeoMx Digital Spatial Profiler and the CosMx Spatial Molecular Imager which work independently and in concert to enable spatially resolved high plex, multi-analyte profiling of tissues.

Material and Methods

The assay utilizes affinity reagents (antibodies or in-situ hybridization probes) coupled to oligonucleotide tags which are either released from discrete regions of the tissue using focused UV exposure and then digitally counted at unlimited multiplex (ex-situ via GeoMx) or imaged directly in space by the CosMx Spatial Molecular Imaging (SMI). This enables profiling of RNA and protein from challenging tissue types, including formalin-fixed paraffin embedded (FFPE) tissue.

Results and Discussions

Data will be presented highlighting the utilization of GeoMx Whole Transcriptome Atlas (~18,500 probes) to understand Tissue and Cancer Biology. Data will also be presented demonstrating the utility of CosMx with a 1,000 plex assay to provide single cell and subcellular RNA and protein profiling from FFPE tissue. To date, the GeoMx platform has been used in >100 and large-scale datasets for both GeoMx and CosMx are available at www.nanostring.com.

Conclusion

Together, the GeoMx and CosMx spatial biology platforms have the potential to transform the study and understanding of all areas of life science research, including discovery, translational, and clinical drug development.

CDKN2A status and p16 expression in external auditory canal squamous cell carcinoma.

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Introduction

External auditory canal squamous cell carcinoma (EACSCC) is a rare cancer with an incidence of 0.1-0.6 cases/100.000 inhabitants per year and poor overall survival. Due to the presence of unspecific symptoms, it is often detected in advanced stages. The low number of patients makes difficult to find clear etiologic or prognostic factors and to assess better treatment strategies which are currently based on surgery and radiotherapy. The gene CDKN2A codifies p16 which regulates cell proliferation by the inhibition of G1-S transition, and is frequently altered in cutaneous and head and neck squamous cell carcinomas. The aim of this study was to evaluate the value of CDKN2A and p16 as prognostic and therapeutic marker in a cohort of EACSCC.

Material and Methods

The cohort consisted of 43 EACSCC samples. CDKN2A gene mutations and copy number losses were studied by Sanger-PCR sequencing and Multiplex Ligation-dependent Probe Amplification (MLPA), respectively. Expression of p16 was evaluated by immunohistochemistry (IHC) and graded according to the percentage of tumour cells with cytoplasmic or nuclear staining: grade 0 (0-10%), grade 1 (10-30%), grade 2 (30-70%) and grade 3 (70-100%), for statistical purposes, also dichotomized into low (grades 0 and 1) and high (grades 2 and 3). All results were correlated to clinical and follow-up data.

Results and Discussions

Three of 38 analyzed samples (8%) carried CDKN2A mutations in exon2, affecting two T1 and one T2 cases. Gene copy number loss appeared in two of 35 analyzed samples (6%), both stage T4. Gene alterations did not correlate with prognosis or survival. IHC positivity of p16 was observed in 14% (5/36) grade 0, 36 % (13/36) grade 1, 31% (11/37) grade 2, 19% (7/36) grade 3. CDKN2A gene mutation and copy number loss did not correlate with p16 staining. Our results indicate an important role for CDKN2A and p16 alterations in EACSCC and are in agreement with a previous study on 10 cases EACSCC (Sato et al, Cancer Science 2020). Low p16 staining (grade 0-1) occurred significantly more frequently in stage T4 cases than in stages T1-T2-T3 (77%, 13/17 versus 26 %, 5/19 samples), Fisher's Chi2 p=0.007. However, p16 expression was not associated with worse overall or disease-free survival.

Conclusion

Neither CDKN2A gene status nor p16 expression showed prognostic value in EACSCC. Nevertheless, patients with advanced T stage cases and low p16 expression could benefit from treatment with CDK4/6 inhibitors.

LncRNA based antigen load enables prediction of patient's survival and immunotherapy outcomes in Melanoma

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Introduction

ICB (immune checkpoint blockade therapy) is one of the promising treatments for melanoma. However, ICB response varies among patients, emphasising the importance of identifying genomic biomarkers to predict likely responses in advance of treatment. LncRNAs are previously associated with cancer-specific epitopes. We aim to establish the association of lncRNA based immunogenicity scoring (lnc-IM) with tumor immune microenvironment (TIM), predicting ICB responses and prognostic value in melanoma.

Material and Methods

The data used includes TCGA-SKCM (n=101), and ICB treated melanoma cohorts UCLA (n=27) and MSKCC (n=21). Each patient was assigned a lnc-IM score based on the total number of peptides that are strong MHC-I binders as per patient's HLA genotype. A logistic regression-based classifier was used to predict ICB responses based on lnc-IM scores.

Results and Discussions

The survival analysis showed better survival of patients with low lnc-IM counts (HR= 0.26, p=0.0012) in the TCGA-SKCM cohort. TIM is an indicator of tumor progression and immunotherapeutic responses. We compared immune cell scores (calculated using xCell algorithm) with lnc-IM scores. CD8-T cells showed a correlation of -0.33 (p=0.0008) with lnc-IM scores. Furthermore, we divided immune cells into the anti-tumor (B cells, M1 macrophages, activated dendritic cells, CD4 -memory T cells, Th1 cells) and pro-tumor group (Th2 cells, T regulatory cells, M2 macrophages). Anti-tumor immune cells showed significant differences among low and high lnc-IM groups, while no such association was apparent among pro-tumor group. The checkpoint proteins PD1 and CTLA4 also showed significant differences among the two groups.

We further explored if such scoring can help improve the prediction of ICB efficacy. lnc-IM count (derived in this study) and the neoantigen load (derived as a part of the trial) were used to assign each patient a "combined antigen score" among ICB treated cohorts. We hypothesised that such scoring could help predict ICB outcomes for tumors that are not hypermutated but can benefit from this therapy. We demonstrated that such a classifier improved the predictions, yielding an AUC of 0.8 with an accuracy of 0.83, precision 0.77 and recall 1. Survival analysis showed a significant association of combined antigen scoring with survival outcomes .

Conclusion

lnc-IM scores are associated with elevated anti-tumor immune responses and can be used as a potential biomarker to improve ICB efficacy predictions after validation in bigger cohorts.

EACR22-0412

Long-term prognostic factors for a high-risk oral squamous cell carcinoma population

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Introduction

Oral squamous cell carcinoma (OSCC) comprises most of head and neck neoplasms and is one of the highest-ranking and lethal cancers in Pakistan due to prevailing mouth habits. Hormonal receptors act as prognostic markers and targets for therapy in some cancers, but their application in OSCC is largely unexplored. This study aimed to evaluate the expression

of hormonal receptors in OSCC patients and correlate it with 10-year, overall and disease-free survival.

Material and Methods

Immunohistochemistry for Her-2, AR, ER and PR was performed on 100 formalin-fixed paraffin-embedded primary OSCC specimens from the years 2005-2010. Receptor expression was correlated with mouth habits and clinicopathological features and patient survival was analyzed using Kaplan-Meier method and Cox regression univariate analysis.

Results and Discussions

In 100 patients, there were 57 males and 43 females. Immunopositive Her-2 expression was observed in 21% of patients, AR in 13%, ER in 3% and 0% for PR. Patients with betel quid/areca nut mouth habits had significantly absent Her-2 expression ($P=0.035$). Also, Her-2 negative patients were also negative for AR expression ($P=0.002$). Her-2 positive patients had poor 10-year survival ($P=0.041$). A trend of low survival and high recurrence rate was observed in AR positive patients, but this was not significant ($P=0.072$). No statistically relevant correlations were seen in the case of ER and PR.

Conclusion

Her-2 is a valuable marker for predicting long-term prognosis and Her-2 positive OSCC patients may benefit from anti-Her-2 targeted therapy.

EACR22-0632

MASTL KINASE MODULATES THE RESPONSE TO RADIOTHERAPY IN HEAD AND NECK SQUAMOUS CELL CARCINOMAS

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Introduction

Head and neck squamous cell carcinoma (HNSCC) is a subtype of cancer arising from the epithelial cells of the oral cavity, pharynx and larynx. These patients are generally treated with surgery, chemo- and/or radiotherapy, yet the success of these treatments remain low due to toxicity, resistance development and lack of predictive biomarkers. Microtubule-associated serine/threonine kinase-like (MASTL) is an essential kinase for the control of cell division that also participates in the DNA damage response. This kinase has been shown to be overexpressed and promote tumor progression in some cancer types, such as breast and colon carcinomas, and there are some evidences suggesting that its inhibition cooperates with genotoxic treatments. However, its role in HNSCC remains unknown

Material and Methods

MASTL expression was analyzed by immunohistochemistry in a cohort of 355 HNSCC samples from patients who underwent surgery with no previous treatment, and in a second panel of 64 HNSCC samples from patients who received induction chemotherapy. We have also generated MASTL overexpressing and knock out HNSCC cell lines to further examine

the effect of MASTL levels on cell proliferation and on the response to ionizing radiation and/or cisplatin, the main chemotherapeutic agent used for HNSCC treatment

Results and Discussions

We found that MASTL overexpression correlates with good prognosis in HNSCC, specifically in those patients with pharyngeal tumors who received adjuvant radiotherapy. In contrast, we did not find any correlation of MASTL expression with the response to chemotherapy. In cell lines neither the knock-out nor the overexpression of MASTL affect the proliferation of HNSCC cells and, in agreement with patient data, we did not detect any difference in the sensitivity to cisplatin. However, our preliminary data indicates that overexpression of this protein enhances the response to ionizing radiation. Interestingly, this correlates with a higher rate of DNA double strand breaks reflected by increased detection of γ -H2AX and 53-BP1 foci in MASTL-overexpressing cells when receiving ionizing radiation

Conclusion

These findings demonstrate that MASTL is not essential for the proliferation of HNSCC cells and does not modulate the response to cisplatin, but sensitizes cells to radiotherapy, presumably by modulating specific DNA damage repair pathways. Therefore, MASTL might have therapeutic value as a predictive biomarker for radiation therapy

EACR22-0712

Correlation between drug response and tumor microenvironment in early-stage Lung cancer

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Introduction

Non-small cell lung cancer (NSCLC) accounts for about 85% of all lung cancers. There is a strong rationale for incorporating PD-1 checkpoint inhibitors into the treatment of early-stage NSCLC, given the breakthrough results in advanced-stage NSCLC. Most of the efforts so far to identify clinically useful biomarkers do not preserve spatial information and leave us blind to the critical source of information revealed in the cell-to-cell biology of the tumor microenvironment (TME). This work presents a quantitative image analysis based on multiplexed immunohistochemistry and machine learning to interrogate complex dynamic information from the tumor microenvironment and find predictive insights into treatment response for NSCLC.

Material and Methods

Tumor tissues samples from resected NSCLC (adenocarcinoma and squamous cell carcinoma) tumors further treated with adjuvant pembrolizumab therapy were used. Patient frozen tissue sections were prepared and stained using a panel of 20 immune or tumor-related antibodies. The stained cells were detected and analyzed with the Hyperion imaging system. Further classification and spatial analysis were performed using proprietary workflow to identify which cells are influencing the treatment response.

Results and Discussions

Tumor samples of NSCLC with similar clinical characteristics at the T1N0M0 had different cells types, including CD4⁺ T cells, CD8⁺ T cells, CD20⁺ B cells, CD14⁺ macrophages, CD56⁺ natural killer cells, granulocytes, etc. More immunosuppressive cells were found in

the sample of patient that did not respond to immunotherapy illustrating that differences in the tumor microenvironment of samples may contribute to different patients' resistance to immunotherapy or find predictive insights into treatment response. Furthermore, the abundance of the drug target was found to be higher in the CR patient demonstrating that in addition to the higher occurrence of the immune system activity in the responder patient, the same patient had the drug target highly expressed in the TME.

Conclusion

High-multiplexed proteomics analyses using the Hyperion imaging system help identify and analyze the tumor microenvironment heterogeneity of NSCLC and explain the factors leading to different prognoses for NSCLC patients despite the same TNM stage. Furthermore, additional information for screening drugs for targeted therapy was found, that could greatly improve NSCLC treatments' individualization.

EACR22-0728

Screening of miRNAs that function as tumor biomarkers in head and neck cancer

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Introduction

Head and neck cancer (HNC) is a very heterogeneous disease which exhibit different characteristics depending on the origin of the tumor. Chemotherapy with cisplatin together with tumor resection remains the main treatment for patients with advanced and localized disease. However, survival of patients in advanced stages is very low and relapses after treatment are one of the most common causes of death, occurring in 60% of the cases. Therefore, the search for new biomarkers that improve the therapeutic outcome is fairly needed.

microRNAs (miRNAs) have been proved to play a crucial role in pathological and physiological processes through the regulation of proliferation, differentiation, metabolism and cellular apoptosis. Nowadays it is known that there is an abnormal expression of miRNAs in several cancer types, where they can act as oncogenes, tumor suppressor genes or modulators of the cancer stem cell pool. Thus, miRNAs could function as plausible biomarkers of diagnosis and prognosis of tumors, as predictors of the response to the treatment and, even, as molecular targets for new therapies. In this work we seek to determine which miRNAs can be used as biomarkers in head and neck squamous carcinoma (HNSCC).

Material and Methods

We used a bioinformatic approach to make a first screening for miRNAs that could serve as biomarkers in HNC in available patient databases. Then, we corroborated the correlation of the selected candidates with the stemness phenotype comparing their expression in cancer stem cells vs. tumor cells in different HNC cell lines.

Results and Discussions

We found 16 possible candidates through the bioinformatic screening and, after testing them in our HNC cell lines, we selected 7 miRNAs as possible biomarkers of HNC. Most of these miRNAs have already been studied in multiple types of tumors and one of them has shown to present a deregulation of its levels in patients with HNSCC with early development due to the use of tobacco.

Conclusion

In the present, the study of miRNAs as biomarkers for tumors and other diseases is a promising and expanding field. Therefore, in this work we have proposed a rationale for the discovery of miRNAs that could be used for that goal. Thanks to this strategy, we found a few interesting candidates and we are currently validating their suitability as prognostic markers in HNC. Altogether, our results will contribute to shed light on the use of microRNAs as a prediction tool.

EACR22-0746

Objective, Single-Instrument Microsatellite Instability Analysis using High-Resolution Melt

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Introduction

Microsatellite instability (MSI) is a genomic alteration in the length of microsatellites provoked by a deficient DNA mismatch repair system. MSI is known to occur in several types of cancer, e.g., colorectal cancer (CRC), and identification of MSI in tumors is important as a predictive factor of response to different therapies, in particular for immune checkpoint inhibitors. The gold standard for analysis of MSI is currently done using assays comprising five different loci of mononucleotides. The usual procedure for MSI-testing involves amplification of purified tumor DNA by PCR, after which the lengths of the amplified DNA fragments are compared to wild type DNA fragments by capillary electrophoresis. In the present study, the MicroSight® MSI Panel is compared to the gold standard technique. The panel analyzes five mononucleotide microsatellite sequences: BAT25, BAT26, NR22, NR24, and MONO27, by real-time PCR and high-resolution melt (HRM), evaluating microsatellite length via PCR product melting profiles.

Material and Methods

Genomic DNA extracted from tumor and non-tumor formalin-fixed paraffin embedded tissues of 185 CRC patients was analyzed using both the PlentiPlex™ MSI Panel, which uses the fragment analysis method, and the MicroSight® MSI Panel. The results from the two panels were compared and the results from the fragment analysis were used to calculate sensitivity and specificity for the MicroSight® MSI Panel.

Results and Discussions

The MicroSight® MSI Panel makes it possible to determine the MSI status of CRC samples without the need of an electrophoresis fragment analyzer. The usage of real-time PCR followed by HRM analysis allows correct classification of the MSI status of tumor samples using a universal reference instead of a paired non-tumor DNA sample. For all loci, specificity and sensitivity were 100% and between 97-100%, respectively, using paired samples. The specificity and sensitivity varied between 91.2-100% and 94.1-100%, respectively, for all loci for the samples analyzed with a universal reference. Using both

paired and universal reference, all samples obtained the same MSI-classification as the fragment analysis.

Conclusion

A standardized, single-instrument, fast, and objective method to analyze a patient's MSI status is presented. The MicroSight® MSI Panel showed 100% agreement of the MSI status with the fragment analysis method when both utilizing paired samples and when using a universal reference.

EACR22-0830

Combined circulating Effector Memory T cell subsets and tumor-specific mutations predict clinical response to PD-1 blockade in patients with non-small cell lung cancer

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Introduction

Immune therapy by blockade of PD-1 or PD-L1 (PD-1 blockade), with antibodies, has revolutionized treatment options for non-small cell lung cancer (NSCLC) patients whose tumors do not carry targetable mutations. However, the benefit of immune therapy is limited only to a subset of the patients. The current study aims to identify biological markers of clinical response in a discovery cohort of patients early on during treatment, to distinguish patients clinically responding to PD-1 blockade from those who do not respond using both immunological and genetic markers.

Material and Methods

Blood drawn from NSCLC patients, before starting PD-1 blockade and after four consecutive treatment cycles was analyzed using a clinical flow cytometric assay for changes in the frequencies and concentration of immune cell populations. Further, next generation sequencing (NGS) was performed on the extracted DNA from archival tumor biopsies from the same patients. The iRECIST criteria was used to evaluate the clinical responses with a cut-off at 9 months.

Results and Discussions

Patients that responded to PD-1 blockade had a significantly increased frequency of activated effector memory CD4 and CD8 T cells, and decreased frequency of central memory CD4 and CD8 T cells in circulation post-treatment compared to pre-treatment. Further, we observed an increase in effector CD8 T cells that expressed intermediate levels of CD45RA in patients responding to PD-1 blockade. Although similar patterns of changes in immune cell populations was also evident in non-responder patients, they were not significant. NGS analysis of the tumor tissue identified pathogenic and likely-pathogenic mutations in *TP53*, *KRAS*, *NOTCH-1* and *STK11* in the patients that responded to PD-1 blockade. Finally, a multivariate analysis, combining the immune and genetic parameters could discriminate responders from non-responders for this discovery cohort of patients.

Conclusion

We report that combined analyses of selected immune cell and genetic parameters could predict clinical response to immune therapy and could in the future after validation be useful for precision medicine efforts.

EACR22-0898

Study of BRCA1-foci as a possible predictive biomarker for platinum and olaparib response in ovarian carcinoma preclinical models.

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Introduction

Half of the ovarian carcinoma (OC) patients harbors defects in homologous recombination (HR) pathway. Platinum- and poly ADP-ribose polymerase inhibitor (PARPi)-based therapies are very effective in OC; however, some patients do not respond and/or develop a resistant disease. Predictive biomarkers of response are needed to better stratify patients who could benefit from these therapies. We showed that the basal level of RAD51-foci positive cells predicted olaparib (a PARPi) sensitivity in a xenobank of OC-patient-derived xenografts (PDX). Herein, we explored the potential of the basal level of BRCA1-foci in predicting the response to therapy in the same models.

Material and Methods

The study was undertaken in 35 PDX models, whose *in vivo* sensitivity to cisplatin (DDP) was known; in 17 PDXs olaparib response was also available. The basal % of BRCA1-foci positive cancer cells were quantified in formalin-fixed-paraffin-embedded (FFPE) PDX samples by immunofluorescence. The co-staining with geminin (GMN) allowed foci quantification only in S/G2 cells. Foci-score (% of GMN positive tumor cells with at least 5 BRCA1-foci per nucleus) was calculated in at least 50 GMN positive nuclei per sample. Samples with BRCA1/GMN+ cells >35% were considered BRCA1-foci positive.

Results and Discussions

The mean of BRCA1/GMN positive cells was 39%, with a median of 35% (min 0%, max 93%), with 48.57% (17/35) of the PDXs classified as BRCA1/GMN positive. The % of BRCA1/GMN positive cells significantly associated with BRCA1 mutational status (p-value=0.001, Kruskal-Wallis test) and correlated with the % of RAD51/GMN positive cells in the PDX analysed (Spearman correlation index=0.656). Statistical analysis showed significant association between the basal BRCA1-foci score and DDP and olaparib responses (p-value=0.0445 and p-value=0.014, respectively, Kruskal-Wallis test). The positive and negative predictive values (VPP and VPN) for DDP were 88,89% and 47,06%, respectively (accuracy 68,57%). For olaparib, the VPP and VPN values were 54,55% and 100%, respectively (accuracy 73,68%).

Conclusion

Our data demonstrated that the basal level of BRCA1-foci can be easily quantified in FFPE OC samples and predicts both the response to DDP and olaparib. While these data need to be corroborated in OC specimens with known sensitivity to DDP and olaparib, they represent a breakthrough in predicting drug response considering that this is a low-cost immunofluorescence-based assay, potentially easily translatable in a clinical setting.

Biomarkers of response to immune checkpoint inhibitors in poorly differentiated sinonasal tumors.

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Introduction

The sinonasal area is the site of a wide variety of rare cancer types. Histopathological classification can be challenging, especially when tumors are poorly differentiated. Despite advances in surgery and radio-chemotherapy, the 5-year survival rate is still low and ranges between 20% and 70%, depending on the histology and tumor stage. Thus, there is an unmet clinical need for new therapeutic options. We aimed to analyze microsatellite instability (MSI), PD-L1 expression, and CD8+ tumor-infiltrating lymphocytes (TILs) as predictive biomarkers for immunotherapy.

Material and Methods

Using immunohistochemistry we analyzed 62 poorly differentiated sinonasal tumors, including 32 undifferentiated carcinoma (SNUC), 8 neuroendocrine carcinoma (SNEC), 13 olfactory neuroblastoma (ONB), 4 poorly-differentiated squamous cell carcinoma (PD-SNSCC), and 5 high-grade non-intestinal-type sinonasal adenocarcinoma (HG-non-ITAC), for expression of MSI markers MLH1, MSH2, MSH6 and PMS2, for PD-L1 on tumor cells and CD8 on TILs.

Results and Discussions

Four of 32 (13%) SNUC, but none of the other tumor types displayed complete absence of all four MSI markers. PD-L1 expression was observed in 6/32 (19%) SNUC, 1/8 (13%) SNEC, 1/13 (8%) ONB, 1/4 (25%) PD-SNSCC and 2/5 (40%) HG-non-ITAC. CD8+ TILs were present in 6/32 (19%) SNUC, 2/8 (25%) SNEC, 1/13 (8%) ONB, 0/4 (0%) PD-SNSCC and 2/5 (40%) HG-non-ITAC. Among SNUC, MSI was mutually exclusive with PD-L1 and CD8+ TIL positivity, and together these markers indicated 13/32 (40%) of cases as immunogenic tumors where treatment with immune checkpoint inhibitors may be promising. Based on PD-L1 and CD8+ TIL positivity, also a subset of HG-non-ITAC and SNEC could benefit from immunotherapy. The least immunogenic sinonasal tumor subtypes were ONB and PD-SNSCC.

Conclusion

Immune checkpoint inhibitors such as pembrolizumab and nivolumab are emerging as new options for treatment of many tumor types. The data in this study indicate that among poorly differentiated sinonasal tumors, especially SNUC and to a lesser extent also HG-non-ITAC and SNEC may respond well to this promising new therapy.

profiling (CTMP)

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Introduction

CTMP via NGS is a powerful tool in precision oncology, in particular for clinical trial selection. The aim of this study was to evaluate the frequency and spectrum of non-recurrent oncogene mutations identified through routine CTMP.

Material and Methods

Analysis was performed across patients, who underwent CTMP. Tumor samples (FFPE) were sequenced employing NGS-based 409 genes panel assay. Downstream analysis was restricted to mutations within 40 oncogenes with known mutation hotspot sites and associated with sensitivity to any cancer therapy via gain-of-function mutations.

Results and Discussions

Across 270 patients referred for CTMP (20% - CRC; 14% - Pancreas; 10% - NSCLC; 10% - Breast; 46% - Other) a total of 1426 somatic mutations were detected (in average, 5.2 mutations per patient). After excluding patients with high tumor mutational burden (4%), a total of 412 mutations in 40 oncogenes associated with sensitivity to any cancer therapy were identified. Of them, 71 (17%) mutations among 46 (17%) patients (20% - CRC; 20% - NSCLC; 15% - Breast cancer; 6% - Head and Neck cancer; 6% - Ovarian cancer; 33% - Other) were located outside of known mutation hotspot sites, were not annotated in COSMIC and were not annotated as activating in any mutation database (CGI, JAX, OncoKB). Of these, 16 (22%) were detected in patients with no other mutations in oncogenes/tumor-suppressor genes and 61% in patients with 1-2 additional mutations in oncogenes/tumor-suppressor genes. Across 71 non-recurrent mutations, 53 (74%, p-value < 0.01) were located within structured protein domains, while 15 (21%, p-value < 0.01) were located within kinase domains. In addition, 43 of the identified non-recurrent mutations were located within highly conservative protein positions (according to CADD, SIFT, Provean predictions). Finally, 17 non-recurrent oncogene mutations identified in 16 patients (6%) could be suspected to be gain-of-function genetic variants

Conclusion

Non-recurrent oncogene mutations are frequent (17%) across patients referred for CTMP. Majority of them may be suspected to be activating and associated with antitumor therapy sensitivity. These mutations would be missed in case of clinical interpretation of NGS results is performed via mutation Knowledge Bases and, thus, molecular tumor boards would be required. Further in silico/in vitro analysis would be required to confirm their gain-of-function status and/or oncogenic significance.

EACR22-1118

Embryonic T-box transcription factor Brachyury in androgen-dependent prostate cancer models

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Introduction

Brachyury (TBXT), a transcription factor of the T-box family, has a pivotal conserved role in vertebrates' embryonic development. More recently, it has been recognized for its role in several neoplasms. In line with studies in other types of tumors, our group reported that Brachyury is aberrantly overexpressed in prostate cancer (PCa), being associated with aggressiveness *in vitro* and chemotherapy resistance. Furthermore, Brachyury is an important regulator of epithelial-to-mesenchymal transition (EMT) and an androgen receptor (AR) modulator. Given this, we hypothesized that Brachyury could modulate the therapeutic response to antiandrogens in PCa.

Material and Methods

To test our hypothesis, we chose two androgen-dependent models, LNCaP and 22RV1, and genetically modulated these cells to overexpress Brachyury. First, AR expression was evaluated after Brachyury overexpression (OE) through western blot. Secondly, Brachyury's effect on the response to first-generation (flutamide and bicalutamide) and newly (enzalutamide and abiraterone) antiandrogen therapies were evaluated *in vitro* through MTS assay. Additionally, we evaluated the proliferative capacity of the cells *in vitro* and *in vivo* with a xenograft mouse model. Lastly, to further validate our previous results concerning the role of Brachyury in EMT, we analyzed the expression of some EMT- and focal adhesion-related molecules through qPCR and western blot.

Results and Discussions

In agreement with the previous findings of the group, we demonstrated that not only 22RV1, but also LNCaP Brachyury-overexpressing cells, had upregulated levels of AR, and curiously in 22RV1 cells was also observed an increase in the expression of the AR splice variant (ARv). Overall, these first results gave strength to our initial hypothesis; however, no effects were observed in PCa cells' response to the antiandrogenic therapies used. We also assessed proliferation *in vitro* and tumor growth *in vivo*, to exclude possible proliferation variations of the cells that might have influenced the previous results, and no differences were observed. Finally, we obtained results consistent with a partial EMT induction in LNCaP cells after Brachyury OE.

Conclusion

Brachyury expression is associated with an increase in full-length AR and ARv in LNCaP and 22RV1 cell lines, yet is not sufficient to confer therapy resistance in a short exposure timeframe.

EACR22-1150

Development of a patient-derived tumoroid drug screening platform for predicting clinical therapy response in cancer patients

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Introduction

There is an unmet clinical need for a novel model that can identify neoadjuvant chemotherapy (CT) and chemoradiation therapy (CRT) (non-)responders to save gastrointestinal (GI) cancer patients from over- and undertreatment. Patient-derived tumor

organoids (PDOs), 3D *ex vivo* models, have emerged as a promising predictive biomarker to fill this gap. With our project we aim to improve the analytical and clinical validity of PDO drug screenings to enable their implementation in the clinic.

Material and Methods

Here we focus on overcoming two important limitations that hamper the full potential of PDO drug screenings for CT/CRT response prediction: *ex vivo* drug panels/treatment schedules not consistent with the treatment received in clinic and a lack of robust and high-throughput organoids screenings assays. For this, PDOs, generated from GI tumor resections or biopsies, are screened with a variety of treatment regimens. Monitoring of the PDO drug response is performed with our in-house developed 3D image analysis software that provides a multiparametric read-out, in combination with live-cell imaging (Tecan Spark Cyto) and an automation infrastructure for a highly innovative high-throughput PDO drug screening platform.

Results and Discussions

A variety of GI PDOs have successfully been established and validated, among which e.g. esophageal adenocarcinoma and rectal adenocarcinoma. Our *ex vivo* drug screenings on GI PDOs, with e.g. CROSS and FLOT, show that differences in treatment schedules have a significant impact on the *ex vivo* drug responses. For instance, when comparing *ex vivo* single-dose vs. fractionated radiotherapy, fractionated gave better in *ex vivo* synergy with CT. Also, with our platform we can differentiate cytotoxic from cytostatic responses and identify resistant subclones. Our results suggest that using relevant *ex vivo* treatment regimens combined with our novel screening platform may drastically improve the prediction of a durable clinical response.

Conclusion

We have established *ex vivo* treatment regimens that better resemble the standard clinical practice and have set up a screening platform that allows kinetic monitoring of organoid therapy screening. We have gotten insights in the ability of PDOs to distinguish synergistic effects and the optimal parameters for determining *ex vivo* drug response. In the next steps we will correlate PDO drug responses with the patient's clinical response in a multicentric prospective observational cohort study.

EACR22-1244

Predictive biomarker of pathological complete response to neoadjuvant treatment in HER2+ breast cancer.

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Introduction

In response to neoadjuvant therapy, pathological complete response (pCR; lack of residual disease in breast and lymph nodes), has been proposed as a prognostic marker for long-term clinical outcomes, such as disease-free (DFS) and overall survival (OS), in human

epidermal growth factor receptor 2 (HER2)-positive and triple-negative breast cancer. In clinical practice, recognizing those patients likely to achieve such responses proves challenging to the oncologist, making the identification of new useful biomarkers vital. Here, we searched for potential biomarkers that anticipate pCR to neoadjuvant therapy in HER2-positive, hormone receptor-negative breast cancer tissue samples.

Material and Methods

Patients with early and locally advanced breast cancer, diagnose as HER2-positive, hormone receptor-negative by immunohistochemistry (IHC) at the time of neoadjuvant treatment were included. We defined two groups: responder (R) and non-responder (nR). We analyzed transcript expression using ClariomD array. Differential expression between the two groups was analyzed using in-house R scripts. Gene validation was performed by qPCR using TaqMan Gene Expression Assay (Applied Biosystem).

Results and Discussions

Considering a fold change ≥ 2 and an adjusted p-value <0.05 as statistically significant, we found 53 differentially expressed transcripts. The RNA was annotated as non-coding RNAs in over 25% of the cases. The distribution of such molecules was as followed: 8 long non-coding RNAs (56%); 3 non-coding RNAs (22%) and 3 pseudo-genes (22%). Regarding protein coding transcripts, gene ontology analysis revealed an enrichment of terms associated to metabolic processes and response to toxic substance. Our data showed a significant downregulation of UDPglucuronosyltransferase 2B15 (UGT2B15; p-value=0.0173), which encodes a glycosyltransferase, involved in the metabolism and elimination of toxic compounds.

Conclusion

The ability to predict which patients will benefit from neoadjuvant therapy and achieve a pCR would allow for improved patient stratification and more personalized medicine. Here, we identified a set of transcript differentially expressed in patients that achieve pCR when compared with tissue samples with residual disease. After validation, our data show that UGT2B15 is consistently upregulated in nonresponder patients. Further work is needed to elucidate UGT2B15 role, but it is worth mentioning that an increased rate of glucuronidation has been associated with a loss of potency for the target drugs

EACR22-0199

Identification of biomarkers of response to a novel HER3-directed antibody drug conjugate (ADC) using breast cancer (BC) patient-derived xenografts (PDX) models

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Introduction

HER3 is overexpressed in 30-50% of breast cancers and has been associated with poor prognosis. Patritumab deruxtecan (HER3-DXd; U3-1402), a HER3-directed ADC with a potent topoisomerase I (TOP1) inhibitor payload, has shown promising antitumor activity in metastatic and treatment-naïve BC (NCT02980341, NCT04610528). Here, we aimed to study the activity of HER3-DXd in BC PDX models to identify biomarkers of response.

Material and Methods

The antitumor activity of HER3-DXd (10 mg/kg dosed once weekly, 4 doses) was assessed in 24 BC PDX models (17 HR+ and 7 triple negative) and compared to the antitumor activity of the clinically approved TOP1 inhibitor irinotecan (50 mg/kg dosed once weekly). HER3 expression was assessed by immunohistochemistry (IHC). mRNA expression of 72 BC-associated genes was measured using the nCounter platform to assess the proliferation score and PAM50 subtype classification. Quantification of DNA damage and DNA repair during the S-phase of the cell cycle (γ H2AX and RAD51 nuclear foci in geminin-positive cells) was performed in pharmacodynamic experiments by immunofluorescence (IF).

Results and Discussions

Eleven out of 24 (46%) PDXs exhibited a profound and sustained sensitivity to HER3-DXd, and HER3-DXd showed improved activity compared to irinotecan in 11/21 (52%) models. Correlation analysis showed an association between HER3-DXd long-term response and *TP53* mutation ($p=0.009$), or a basal-like subtype ($p=0.044$). Baseline levels of *HER3/ERBB3* were not positively associated with long-term response ($p=0.041$ and $p=0.002$, respectively). Relapsed models showed increased expression of genes related to chemotherapy-resistance (*MDM2*, *NAT1*, *MAPT*, *GRB7*, *BCL-2*). Mechanistically, treatment with HER3-DXd induced HER3 membrane downmodulation followed by a transient antiproliferative effect based on proliferation score and a significantly higher induction of S-phase DNA damage in those models that presented a sustained response to HER3-DXd as compared to those that relapsed.

Conclusion

HER3-DXd exerts a potent antitumor response in BC PDXs across baseline levels of HER3 expression. *TP53*-mutant, basal-like PDX models were more likely to show long-term responses to HER3-DXd than luminal B PDX models. Compared to irinotecan, HER3-DXd induced improved antitumor activity inducing higher and persistent levels of S-phase DNA damage.

EACR22-0205

BID upregulation shifts tumor cells to apoptosis in response to AURKB inhibition

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Introduction

BH3 interacting-domain death agonist (BID) is a member of the Bcl-2 family that plays a key role in the regulation of apoptosis. We previously showed that amplification of Chr22q11, leading to upregulation of the BID, associates with sensitivity to AURKB inhibition (AURKBi) in *EGFR*-mutant non-small cell lung cancer (NSCLC) cell lines.

Material and Methods

We used 24 established cell lines derived from lung, breast, melanoma, urothelial, prostate, head and neck and pancreatic tumors, together with 36 EGFR-TKI resistant clones derived from PC9 and 11-18 cells. The effects of AZD2811, a specific AURKB inhibitor, and other drugs were tested by MTT assays, flow cytometry and *in vivo* experiments using xenografts in athymic nude mice. Copy numbers were analyzed by FISH and Q-PCR; mRNA expression by nCounter and RT-Q-PCR and protein expression by Western blotting. Finally, a doxycycline-inducible pTRIPZ vector was used for ectopic expression while silencing was achieved by shRNA lentiviruses

Results and Discussions

Among the 60 tumor cell lines and clones, *BID* upregulation was consistently associated with sensitivity to AZD2811, independently of 22q11 amplification ($p < 0.0001$). Sensitive cases included breast, urinary bladder and *EGFR*-wt lung tumor cells; gene silencing systematically rendered these *BID*-overexpressing cells insensitive to AURKBi. In contrast, cell lines with low *BID* were uniformly resistant to AURKBi and ectopic expression of *BID* significantly increased AZD2811-induced cell death, particularly in absence of *KRAS* or *BRAF* mutations. In experiments using doxycycline gradients, we found that reduction in survival after AZD2811 treatment was dose-dependent on *BID* levels, following a Michaelis-Menten type kinetics ($R^2 = 0.91$). Western blotting experiments indicated that AZD2811 treatment reduces CASP-2 and MDM2 levels in most tumor cell lines, but only triggers activation of *BID* and CASP-3, leading to cell death, in *BID*-overexpressing cells. Analysis of the COSMIC database revealed a prevalence of 1% and 5% for Chr22q11 amplification and *BID* overexpression in human tumors, respectively. In patients from our institution, we found a frequency of 0.7% (1/143 samples analyzed) for 22q11 amplification and 6% (10/163) for high *BID* mRNA levels

Conclusion

BID upregulation is present in 5-6% of human tumors and associates with sensitivity to AURKBi in cancer cells of different origins, thus implying its potential use as a predictive biomarker for AURKB inhibitors in clinical trials

EACR22-0223

Targeted genomic and immunohistochemical analyzes of preoperative tumor biopsy to optimize pre-treatment stratification of patients with endometrial cancer

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Introduction

Endometrial cancer (EC) is the most common cancer of the female genital tract in developed countries and is the sixth most common cancer in women worldwide, with more than 400,000 new cases diagnosed in 2020. Both its incidence and its associated mortality are increasing. Therefore, one of the main goals of clinical research in recent years is to try to identify such procedures that will make it possible to refine the stratification of patients with

endometrial cancer according to the degree of risk of relapse and thus better apply treatment strategies.

Material and Methods

In our pilot project, we analyzed tumor samples from 60 patients with endometrial cancer (30 with recurrent disease, 30 without it) using a combination of genomic testing and immunohistochemical analysis.

Results and Discussions

The combination of targeted sequencing of relevant genes together with immunohistochemical determination of the expression level of selected proteins enabled the use of the newly designed molecular biological classification in clinical practice without the need for costly complex genomic sequencing.

Conclusion

The obtained pilot data encouraged us to plan a larger study of about 700 patients in order to optimize the preoperative classification of the tumor into the correct group according to the degree of risk of recurrence. With sufficiently large patient populations covering all stages of the disease, the adequacy of current surgical and oncological approaches in terms of adjuvant radiotherapy and / or chemotherapy can be analyzed. From today's point of view, these are untargeted and, as a result, cause either excessive and unnecessary radicality of treatment or, conversely, insufficient treatment for a particular patient.

EACR22-0269

Biomarkers characterization associated with stemness and therapy resistance in head and neck squamous cell carcinoma tumors

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Introduction

Head and neck squamous cell carcinoma (HNSCC) entails around 4% of all globally diagnosed cancers with more than 640,000 new cases per year and 355,000 annual deaths. Discerning the genetic alterations of HNSCC in early stages may help to infer the malignant potential of the premalignant lesions, and even the healthy mucosa, in patients exposed to risk factors. Accordingly, it could help to detect possible targets for effective chemoprevention strategies as well as possible therapeutic targets for patients developing invasive neoplasia. However, there are no clinical or molecular biomarkers currently validated in the standard practice. The objective of this study is to find transcriptional alterations in patients with oral cavity cancer that allow the identification of gene networks responsible for resistance and prognosis.

Material and Methods

We performed an analysis of 11 public transcriptional datasets to find genes whose expression may be highly deregulated in oral cavity tumors. The genes sets identified were subject to a Gene Set Enrichment Analysis (GSEA) to find clear correlations of the gene set

with Gene Ontology (GO) terms. Then, we analyzed the ability of these genes as prognostic markers of the oral cavity tumors using the SurvExpress portal. The expression levels of these genes of interest were validated in a cohort of 98 tumor samples collected from the Central University Hospital of Asturias (HUCA) by quantitative PCR.

Results and Discussions

In order to find possible future targets for treatment or prevention of these tumors, we identified two panels of genes that are recurrently miss-expressed in patients and that successfully stratify tumoral and non-tumoral samples based on expression levels, as well as patient survival.

The gene panels are mainly related to the cell cycle, DNA damage response, cytokine signaling and the immune system, but also with the embryonic stem cell core. Validation of these panels in an independent cohort led to the identification of three non-interconnected genes, *WDR66*, *SERPINH1* and *ZNF622* that are able to predict patient survival. These genes relate to the stem phenotype, being transcriptional targets of the pluripotency transcription factors Sox2 and c-Myc.

Conclusion

Our results suggest that *WDR66*, *SERPINH1* and *ZNF622* constitute a minimal signature able to predict the prognosis and response of HNSCC tumors.

EACR22-0357

Subtype-specific metabolomics profiles of breast cancer patients treated with neoadjuvant therapy

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Introduction

Breast cancer is the most diagnosed and the leading cancer death cause in women worldwide. Metabolomics allows the quantification of the entire set of metabolites in blood samples, making it possible to study differential metabolomics patterns related to neoadjuvant response in the breast cancer setting. Clinically, tumors are usually classified into four different molecular subtypes: triple-negative, HER2+, Luminal A, and Luminal B (HER2+ or HER2-). These tumor subtypes respond differently to systemic and local treatments and differ in their risk factors.

Material and Methods

One hundred and three plasma samples of breast cancer patients before receiving neoadjuvant treatment were analyzed through UPLC-MS/MS by Metabolon Inc. Clinical data regarding response to treatment (complete or partial) and tumor subtype were collected. Probabilistic graphical models, significant analyses of microarray (SAMs), classificatory algorithms, and class comparison analyses were used to establish differential metabolic patterns between conditions.

Results and Discussions

985 metabolites were identified, within which there are lipids, amino acids, peptides, nucleotides, carbohydrates, cofactors and vitamins, and xenobiotic compounds. Metabolomics data allow the identification of differences between groups in response to treatment. These differences are specific to each breast cancer subtype. Patients with HER2+ tumors showed differences in metabolites related to amino acids and carbohydrates pathways between the two pathological response groups. However, patients with triple-

negative tumors showed differences in metabolites related to the long-chain fatty acids pathway. Patients with Luminal B tumors showed differences in metabolites related to acylcarnitine, hexosylceramides, and sphingomyelins metabolism pathways.

Conclusion

It is possible to identify differential metabolomics patterns between complete and partial response to neoadjuvant therapy, being this metabolomic profile specific for each breast cancer subtype. These results pave the way to the implementation of blood-based tests in the clinical routine and help in the clinical decision-making process.

EACR22-0383

The application of genomic footprints for treating chromosomal instability

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Introduction

Up to 80% of all cancer patients present chromosomal instability (CIN), a hallmark of cancer that causes the accumulation of numerical and structural alterations across the genome. The lack of recurrent drivers in complex tumours limits the efficiency of the state-of-the-art gene-based precision oncology, being needed to develop new whole-genome approaches for tackling these aggressive cancers. Recent studies have shown that chromosomal instability consists of many different types, each of which leave characteristic footprints or signatures across the genome. We have developed a computational approach to identify and quantify different CIN types from tumour DNA sequence data. Our framework represents an opportunity for specifically targeting the pivotal vulnerability of these difficult-to-treat cancers.

Material and Methods

In this regard, we explored the utility of copy number signatures for therapy response prediction and drug target identification. We integrated data from 297 cancer cell lines from The Cancer Cell Line Encyclopedia (CCLE), including copy number profiling, genome-wide CRISPR knock-out screens, genome-wide RNAi screens, and the PRISM drug repurposing screen. We assessed correlations between signature activities, gene essentiality, and sensitivity to drug perturbation of the gene.

Results and Discussions

Copy number signature activity was significantly correlated with both genetic and drug perturbation of 40 targets, and thus can be used as biomarkers for predicting response of 44 existing therapies. Moreover, we identified 49 CIN-related target genes with druggable structures that currently have no targeted therapies in the clinic.

Conclusion

Our analyses reinforced the potential of copy number signatures to be developed into companion diagnostics for improved patient stratification and treatment selection, and to support the development of more efficient and rational therapeutic strategies to overcome complex tumours.

EACR22-0411

The effect of high cancer stem cells marker expression correlated with risk factor exposure in oral squamous cell carcinoma patients

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Introduction

Oral squamous cell carcinoma (OSCC) is a top-ranked cancer in the Pakistani population. Recent advances claim that cancer stem cells (CSCs), are responsible for tumour progression, treatment resistance and metastasis. This study investigated the impact of CSC markers expression on overall (OS) and disease-free survival (DFS) of OSCC patients.

Material and Methods

Immunohistochemistry was used to evaluate CD44, CD133, L1CAM and SOX2 expression in a well-characterised cohort of 100 Pakistani patients with primary treatment naïve OSCC. The immunoreactivity for each marker was correlated with patient clinicopathologic characteristics, oral cancer risk chewing habits and survival.

Results and Discussions

In this cohort of 100 patients, there were 57 males and 43 females. The median OS and DFS times observed were 64 and 52.5 months respectively. Positive expression for CD44, CD133, L1CAM and SOX2 was observed in 33%, 23%, 41% and 63% of patients. High CD44 expression correlated with decreased OS ($P=0.047$). While CD133, L1CAM and SOX2 had no effect on either OS or DFS.

Conclusion

Of the CSC markers investigated here, only CD44 was a predictor for poor OS. CD44 was also associated with advanced AJCC and T stages. Interestingly, CD133 was significantly lower in patients who habitually consumed oral cancer risk factors.

EACR22-0439

A novel 6-gene expression signature for prediction of response to checkpoint inhibitors in non-small cell lung cancer

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Introduction

Immune checkpoint inhibitors (ICIs) are associated with durable antitumor activity in advanced non-small cell lung cancer (NSCLC), however, selection of the best candidates for this type of therapy is a challenge. PD-L1 expression, the only adequately validated predictive biomarker of response to ICIs, is imperfect, and not completely discriminatory between responders and non-responders. Gene expression profile (GEP) is a promising way to evolve in this field. In the present study we aim to identify a GEP of immune-oncology related genes, predictive of response to ICIs in NSCLC.

Material and Methods

This study analyzed advanced NSCLC patients treated with ICIs in any line: a discovery cohort composed of 66 Brazilian patients and a validation cohort composed of 54 Spanish patients. Clinical data were collected from medical records. Total RNA was extracted from FFPE tumor tissue. GEP was assessed using the nCounter® PanCancer IO360 panel.

LASSO regression with Cox proportional hazards approach was used to define the new gene expression signature.

Results and Discussions

In the discovery cohort, median age 62.5 years (36 - 81), most patients were males (57.6%), stage IV (91.9%), ECOG-PS≤1 (90.7%), smokers/former smokers (83.3%) and adenocarcinoma histology (67.7%). The majority was treated with ICI in the first-line setting (42.4%) and nivolumab was the most frequently used drug (52.6%). At data cutoff, median follow-up for overall survival (OS - time from ICI initiation to death by any cause) was 27.7 months and for real-world progression-free survival (rwPFS - time from ICI initiation to disease progression as assessed by treating physician) was 28.2 months. The median OS and rwPFS were 17.7 months and 5.6 months, respectively. We identified a 6-gene signature (Lung Tumor Score – LungTS) that discriminate patients at low and high risk for death (median OS: 36.1 vs 15.5 months; $p<0.001$) and for disease progression (median rwPFS: 10.1 vs 4.2 months; $p<0.001$). Considering the expression of CD274 (PD-L1), the LungTS signature also discriminated two populations regarding OS and rwPFS ($p<0.0001$). In the validation cohort, which had clinicopathological characteristics similar to the discovery cohort, the OS for high versus low LungTS was 17.0 months and 9.0 months, respectively ($p=0.004$). Patients with high score also showed better rwPFS, although not statistically significant (median 10.0 versus 4.0 months; $p=0.11$).

Conclusion

Our study identified a novel 6-gene immune-signature predictive of response to ICI in NSCLC.

EACR22-0621

Elucidating the role of SCNAS burden as a biomarker of ICIs benefit in NSCLC

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Introduction

Non-small cell lung cancer (NSCLC) management has dramatically changed by the emergence of immune checkpoint inhibitors (ICIs). Nevertheless, many patients do not benefit from this type of treatment, thus biomarkers of benefit are of great interest. The role of somatic copy number alterations (SCNAs) burden remains elusive.

Material and Methods

We assembled a cohort of NSCLC patients treated with ICIs. For 89 patients we obtained the SCNAs profile using shallow whole-genome sequencing (sWGS) on tissue biopsy. For 60 patients, we obtained the transcriptional immune profile using a Nanostring panel. The overlap between the two cohorts was of 35 patients. We then studied the association of different SCNAs and tumor immune infiltration features with ICIs benefit using survival models.

Results and Discussions

We observed a negative association between the SCNAs burden and ICIs progression-free survival (PFS). This association was stronger when focusing on chromosome and arm level alterations. SCNAs burden showed differences between primary and metastatic lesions. SCNAs burden negatively correlated with the levels of several immune cell types in the tumor microenvironment, although when stratifying by sex, samples from males did not show this correlation. We validated our observations using lung adenocarcinoma data from The Tumor Cancer Genome Atlas.

Conclusion

SCNAs burden is a potential biomarker of ICIs benefit in NSCLC, although some nuances should be considered. Further studies will be needed to established its clinical utility.

EACR22-0647

Profiling the response to neoadjuvant chemoradiotherapy in locally advance rectal cancer using data-independent acquisition mass spectrometry (DIA-MS)

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Introduction

Neoadjuvant chemoradiotherapy (nCRT) has been widely used to improve the local control rate and survival rate in patients with locally advanced rectal cancer (LARC). Response to treatment varies among patients and it is still unknown who will have the best benefit from nCRT. The aim of this study was to discover the specific tissue molecular features that might lead to different responses to treatment.

Material and Methods

During the period 2018-2019, pretreatment FFPE tissue samples from 97 patients diagnosed with LARC (stage II/III) were gathered at the Institute for Oncology and Radiology of Serbia. All patients were treated with neoadjuvant chemoradiotherapy (5-FU/Leucovorin) and their response was evaluated using a tumor regression grade (TRG) system. Depending on TRG the cohort was divided into responders/non-responders (TRG1/2 vs. TRG 3-5, Mandard scale). Data independent acquisition mass spectrometry (DIA-MS) was performed on a carefully selected cohort with the aim of analyzing patients who had the most distinctly different response to therapy. Bearing this in mind, we selected a group of 20 patients (11 non-responders and 9 responders) and analyzed their proteomic profiles. DIA-NN was used for searching the spectra and the Perseus software was used for the statistical analysis of data, while enrichment pathway analysis was performed using Metascape.

Results and Discussions

In total 4849 proteins were identified in 20 rectal cancer FFPE samples. Principal Component Analysis (PCA) indicates that responders compared to non-responders have a significantly different proteomics profile. Statistical analysis using a two-sided t-test resulted in the identification of 871 differentially expressed proteins ($p < 0.05$) (235 overexpressed in responders and 636 overexpressed in non-responders). Fundamental cell processes (RNA processing, DNA processing, translation, and extension of telomeres), as well as neutrophil degranulation, were identified as the key characteristics of the responder's group by pathway enrichment analysis. The non-responder group, on the other hand, is characterized by signaling pathways related to intracellular/extracellular transport, and biomolecule metabolism (lipids, vitamins, cofactors, carbohydrates, nucleosides).

Conclusion

The DIA-MS approach offered unprecedented proteome coverage for FFPE samples. The differentially expressed proteins and biological processes constitute interesting findings that hold the potential for improving LARC patient management.

EACR22-0743

TRPV2 correlates with endometrial cancer aggressiveness and its activation improves chemosensitivity

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Introduction

Endometrial cancer (EC) is the most commonly diagnosed gynaecological malignancy in developed countries. EC comprises type I endometrioid EC and type II non-endometrioid EC, the most aggressive and with poor prognosis. Treatment options, hysterectomy, chemotherapy and combinations with radiation, have limited efficacy, and only limited options remain if the tumors relapse or metastasize. Transient Receptor Potential Vanilloid 2 (TRPV2) is often dysregulated in tumors and has been associated with altered cell proliferation, invasiveness and aggressiveness in different cancer cell lines and animal models. In this work we analysed TRPV2 expression in human type II EC biopsies and type I and II EC cell lines, and the TRPV2-dependent effects on cell migration and chemosensitivity *in vitro*.

Material and Methods

TRPV2 expression was evaluated in EC cell lines by RT-PCR and western blot and in EC biopsies by immunohistochemistry. TRPV2 correlation with Overall Survival (OS) and Progression-Free Survival (PFS) was evaluated by Kaplan–Meier analysis. TRPV2 transfection and activation was used to evaluate the modulation of migratory capacity, Akt expression and chemosensitivity, by wound healing, western blot and MTT assay.

Results and Discussions

Results show that EC malignancy correlates with high levels of TRPV2, that is associated with shorter PFS. In cells expressing low levels of TRPV2, TRPV2 transfection increased migration and Akt expression, confirming that TRPV2 increases aggressiveness. Chemosensitivity was increased in overexpressing TRPV2 cells and with a TRPV2 activator, mainly in TRPV2 transfected cells, probably because drug permeates directly through TRPV2 pore channel, like seen in another study with glioblastoma cells.

Conclusion

Data support the hypothesis that TRPV2 expression increases EC aggressiveness, therefore can be used as a marker for diagnosis and to optimize the therapy, with a TRPV2 agonist might being a strategy as adjuvant therapy.

EACR22-0773

Raf Kinase Inhibitory Protein (RKIP) protein as a theranostic marker in HPV-related cancers.

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Introduction

RKIP has been reported to be downregulated in multiple cancers and involved in tumor aggressiveness and, poor prognosis. Curiously, it was in Cervical Cancer (CC), that RKIP expression was found in lower percentage of patients', with a significant loss in pre-malignant lesions. However, in the world of HPV-related cancers, specifically for Head and neck cancer (HNSCC), little is known about RKIP. Thus, we aim to uncover RKIP role in HNSCC. Given RKIP role in the regulation of many signaling pathways related to therapy prediction, such as MAPK, we aimed to explore its putative role as a modulator EGFR targeted inhibitors, used for HNSCC management.

Material and Methods

To characterized RKIP expression in HNSCC, a series of samples (200) were evaluated for RKIP expression. To address RKIP role, biologically and as modulator of EGFR-targeted therapies response, RKIP was knocked out (KO) and overexpressed (OE), by CRISP-Cas9 and cDNA plasmids, respectively. Also, to study HPV as an RKIP regulator, the oncoproteins E6 and E7 were silenced using siRNA. Finally, to define an RKIP molecular signature in these tumors, RKIP KO cells were evaluated by NanoString.

Results and Discussions

By evaluating RKIP expression in HNSCC human samples, was found RKIP absence association with worse clinical outcome. *In vitro*, we showed that JHU28 RKIP KO cells had a higher migratory capacity, while, FADU and VU147T RKIP OE cells showed lower migratory capacity. Also, we found that RKIP KO cells had a higher capacity to form larger tumors *in vivo*, revealing an aggressive phenotype. Further, we observed that JHU28 RKIP KO cells present a higher IC₅₀ value for EGFR inhibitors when compared to control, while in CC cell lines no differences were observed. Interestingly, RKIP KO cells presented increased activation of EGFR, pointing to an EGFR activation modulated by RKIP. Additionally, we observed that, upon HPV oncoproteins silencing in CC and HNSCC cell lines, RKIP expression increases at protein and mRNA level. Lastly, through NanoString, we were able to identify common alterations between HNSCC and CC cell lines that together defined an RKIP-molecular signature.

Conclusion

We showed that RKIP is an important prognostic marker and, predictor of response to EGFR-targeted therapies in HNSCC. Further, by the definition of a common RKIP-molecular signature, in HNSCC and CC, we provided new targets to manipulate in order to reverse RKIP-associated phenotype. Importantly, we gave new insights about RKIP regulation mechanisms in HPV-related cancers.

EACR22-0845

Identification of genetic biomarkers in non-small cell lung cancer, to predict resistance or response to immune therapy

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Introduction

The currently available treatment options for non-small cell lung cancer (NSCLC) patients mainly depend on tumor stage, histology and molecular markers. Patients diagnosed with NSCLC can be treated with antibodies against PD-1/PD-L1, called immune checkpoint blockade (ICB). In spite of the success of ICB, and since only approximately 1 in 3 will respond, better genetic biomarkers are warranted to select patients most likely to respond. The success of the treatment so far has only been linked to a few genetic markers. These include the Tumor Mutation Burden (TMB), calculated by different methods and a few specific DNA-variants in onco- and/or tumors suppressor genes. This study aims to identify better genetic markers, than those reported to date, which can be useful to improve the selection of NSCLC patients who will benefit from immunotherapy.

Material and Methods

Thirty-eight NSCLC patients' stages III or IV, treated with immune checkpoint blockade (ICB), as the first- or second line treatment were included in the analysis. FFPE tumor samples and blood samples from each patient were sequenced, with a large next generation sequencing (NGS) panel including 597 cancer-related genes. Bioinformatics tools were used for calculation of TMB, calling of SNP and insertion/deletion variants and assessment of mutational signatures of the tumors. Clinical response to ICB was determined every 12 weeks based on immune related Response Evaluation Criteria in Solid Tumors (irRECIST).

Results and Discussions

In accordance with several other studies, we found no correlation between high TMB score and clinical response to ICB in our cohort of patients, while we found a statistically significant correlation between DNA-variants in *KRAS* and clinical response to ICB. Our data also highlights the importance of analyzing a combination of specific DNA-variants, which would strengthen the correlation to clinical response to ICB.

Conclusion

By a comprehensive analysis of the genetic landscape of the tumors, including analysis with several bioinformatics tools, correlation to clinical response was investigated. A significant correlation with clinical response was observed in tumors with DNA-variants in *KRAS*. Correlation to high TMB as a single genetic marker was not significant, but a combination of different variants and signatures showed correlation to clinical response to ICB. The results can contribute to personalized treatment of individual patient tumors.

EACR22-0903

Trabectedin-olaparib combination primes immunotherapy with HLA-independent killer lymphocytes against soft tissue sarcomas.

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Introduction

Advanced STS have a severe prognosis and no reasonable therapeutic options. We previously showed that PARP1 inhibitor OL in combination with the DNA-damaging agent TR was effective against STS preclinical models variable degrees. We now evaluate if STS cells surviving TR+OL would be tackled by HLA-independent immunotherapy with Cytokine-Induced Killer lymphocytes (CIK) and Natural Killer (NK) cells.

Material and Methods

We selected 3 groups of STS cells: leiomyosarcomas (DMR, MESSA), fibrosarcomas (HT1080, SW684), undifferentiated pleomorphic sarcomas (S018, S006,) and treated them with their relative IC75 for 72 h to obtain the 25% fraction surviving to TR+OL and we analyzed by flow cytometry the expression of CIK/NK ligands. Next, we tested CIK/NK killing activity against surviving cells (scaled effector/target ratios) and assayed cell viability both with CellTiterGlo™ and colony growth. In selected experiments, we performed RNA-sequencing analysis to dig on molecular mechanism evoking CIK/NK ligands after TR+OL treatment.

Results and Discussions

Different levels of CIK/NK ligands were expressed in STS: negligible levels of NK/CIK ligands in DMR; MIC A/B, (98±0.66%), ULBP2 (79%±11.26) and ULBP3 (57%±20) in MESSA; ULBP 2/5/6 (13.4%±1.56) in SW684; MIC A/B, (37%±8.6) and ULBP 2/5/6 (93%±2.5) in HT1080; MIC A/B (87%± 6.8) and ULBP2/5/6 (98%±0.5) in S018; MIC A/B (43±6.6%) and ULBP2/5/6 (74%±12) in S006. CIK/NK cells showed significant antitumor activity against all of these 6 cell lines after 72h. Next, we evaluated how TR+OL impinged on CIK/NK ligands observing that after 72h treatment in cells displaying a high level of these molecules remained high, whereas MIC A/B significantly increased in DMR and HT1080 (17.75%±4.9, 54%±5.11, respectively) and ULBP2 significantly increased in DMR and SW684 (19.8%±4.43 and 22.8%±3.7, respectively). Interestingly in these cells, TR+OL pre-treatment primed NK/CIK antitumor effects, also at minimal effector/target ratio, if compared

to the control (at effector:target=1:12, the killing activity was 93%, 78%, and 87%, in DMR, HT1080, and SW684, respectively; $p < 0.01$ vs. controls). Gene set enrichment and their interconnections with CIK/NK ligands in treated cells will be presented.

Conclusion

In conclusion, CIK/NK-based adoptive immunotherapy was effective against STS surviving TR+OL therapy. TR+OL primed the action of CIK/NK modulating the expression of their ligands on tumor cells. This integrated approach warrants further investigations both in vivo and in the clinical setting.

EACR22-0955

Genomic markers and response to chemotherapy in locally advanced gastric cancer patients

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Introduction

Gastric cancer (GC) is a complex disease that represents the fifth most common malignancy in the world and the third leading cause of cancer-related death worldwide. GC shows a high level of heterogeneity as well as a marked gender difference in incidence. Neoadjuvant chemotherapy (NCT), followed by surgery, represents the standard of care for locally advanced stages, but the efficacy of such treatments are still limited for many patients. In the present study, we aimed at defining whether gene alterations, microsatellite instability (MSI) and tumor mutational burden (TMB) are associated with pathological response to NCT.

Material and Methods

A retrospective cohort of nineteen GC patients who received NCT (12 males and 7 females; median age 69 years) was considered. DNA was extracted from bioptic chemo-naïve formalin-fixed paraffin-embedded tissue samples and genetic alterations of more than 500 genes, as well as MSI and TMB status, were characterized using Trusight Oncology 500 panel (Illumina) on NextSeq 550 sequencer (Illumina). Pathological response to NCT was evaluated on surgical specimens using tumor regression grade (TRG) according to Becker's criteria. Patients were grouped in responsive (TRG 1-2; n=9) and non-responsive (TRG 3; n=10) patients.

Results and Discussions

Pathogenic variants on *TP53*, *ARID1A*, *ZFX3*, *FGF3*, *GNAS*, *PIK3CA* and *RNF43* genes, as well as *FGF3* and *MYC* amplification were the most frequent alterations. Interestingly, *PIK3CA* mutations were more prevalent among non-responsive than responsive patients ($p=0.087$). Twenty-six percent of patients had high MSI ($\geq 20\%$ unstable MSI sites) and 32% high TMB (>10 mutations/Mb), but no significant associations with response were found. High TMB and MSI were significantly related

to *ARID1A*, *GNAS*, *PIK3CA*, *RNF43* and *ZFHX3* mutations. No significant associations were found with the main clinical-pathological parameters, except for the correlation between *TP53* variants and the intestinal-type GC ($p=0.018$), as well as for *MYC* amplification and the tumor localization in the body of the stomach ($p=0.040$).

Conclusion

Despite the small case series, the prevalence of *PIK3CA* variants in non-responsive patients warrants further investigation, considering the emerging of new targeted therapies against mutant *PIK3CA*. In the era of personalized medicine a wide genomic characterization of chemo-naïve tissues from GC patients can help stratifying patients for more tailored treatment. The study is supported by ERAPerMed 2019-275.

EACR22-0991

Hypoxia-induced changes in the composition of extracellular proteins in bladder cancer cells identifies candidates to explore as potential circulating biomarkers

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Introduction

There is a need to improve treatment outcomes for patients with bladder cancer. Tumour hypoxia is common and associated with treatment resistance and an increased risk of metastasis. Hypoxia can be targeted with evidence that only patients with the most hypoxic tumours benefit. Our group derived a 24-gene signature to assess hypoxia in bladder cancer that requires access to pre-treatment diagnostic biopsies. The need for biopsies limits the ability to carry out serial measurements. As the extracellular matrix has been used in the discovery of plasma biomarkers, we aimed to identify candidate proteins associated with a hypoxic cell derived extracellular matrix (CDM).

Material and Methods

Four bladder cancer cell lines (T24, UMUC3, J82, RT4) were grown in hypoxia (0.2% O₂) or normoxia (20.9% O₂) for 7 days. CDM was collected after decellularization with NH₄OH. Hypoxia was confirmed by western blotting of cell lysates (GLUT1) and CDM (FN). CDM samples were analysed using mass spectrometry (MS). Pre-treatment plasma samples from 10 bladder cancer patients were collected and also analysed by MS. Both datasets were analysed with R Studio *in silico*.

Results and Discussions

MS analysis of CDM samples identified 1,733 proteins, with 594 changing significantly in response to hypoxia (fold change >1, $p_{adj.} < 0.05$) in ≥ 1 cell line. Comparing *in vitro* (1733 proteins) vs plasma (794 proteins) samples identified 84 common proteins, 13 of which showed significant hypoxia-induced changes in cells (fold change >1, $p_{adj.} < 0.05$). A candidate circulating protein signature (HP, FKBP4, HK1) was identified and shown using unsupervised hierarchical stratification to separate hypoxic from normoxic CDM samples. Additionally, ontology analysis of the 84 CDM proteins detected in plasma showed significant enrichment ($p < 0.01$) with “cell adhesion molecule binding” and “cadherin binding” terms. Pathway analysis identified EEF1G and UTRN as proteins associated with those terms.

EEF1G and UTRN had significant change in their expression (fold change >1, p.adj. <0.05) in hypoxia and both were detected in plasma. The analysis suggests EEF1G and UTRN have a potential role in hypoxia promotion of metastasis in bladder cancer. Interestingly, EEF1G has already been associated with metastasis in prostate and breast cancer.

Conclusion

Our candidate circulating biomarker signature (HP, FKBP4, HK1) should be studied further in bladder cancer patients. The role of EEF1G and UTRN in promoting metastases in bladder cancer also warrants further investigation.

EACR22-0993

Splicing factor RBM22 is a novel prognostic biomarker and therapeutic target in prostate cancer.

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Introduction

Prostate cancer (PCa) is one of the leading causes of cancer-related deaths among men in developed countries. Thus, identification of new therapeutic approaches to tackle this pathology is urgently needed. In this context, we have recently reported that the dysregulation of splicing machinery components in PCa might represent an original source to identify novel diagnostic/prognostic biomarkers and therapeutic targets.

Material and Methods

Two internal well-characterized cohorts of patients and two *in silico* cohorts were used to evaluate the levels of the splicing factor RBM22 and the association with PCa characteristics. The functional and molecular consequences of RBM22 manipulation (e.g. overexpression) were assessed in different PCa-derived cell lines (LNCaP and PC-3) and preclinical *in vivo* mouse models (TRAMP and xenografts on immunosuppressed mice).

Results and Discussions

We demonstrated a profound down-regulation of RBM22 (at mRNA/protein levels) in human PCa samples vs. non-tumor control samples. Of note, RBM22 levels were inversely associated to key clinical aggressiveness features in PCa (e.g. extraprostatic extension and perineural invasion). These results were confirmed in two additional, independent *in silico* human cohorts. Overexpression of RBM22 in PCa cells decreased aggressiveness parameters *in vitro* (e.g. proliferation, migration, tumorspheres and colonies formation, etc.), and drastically decreased tumor development and progression *in vivo*, underling a relevant direct association of lower RBM22 levels with enhanced PCa progression. These results were corroborated using the TRAMP mouse model, wherein gradual reduction of RBM22 from prostatic intraepithelial neoplasia to moderately differentiated PCa and to poorly differentiated PCa was observed. RBM22-actions were mediated through the alteration of

key oncogenic signaling-pathways (i.e. cycle-apoptosis, PI3K pathways, etc.) and critical molecular regulators (i.e. MYC, MCYN and E2F), and may also involve the alteration of alternative splicing events of key genes involved in these pathways.

Conclusion

Conclusion: Together, we highlight RBM22 as a potential prognostic biomarker and an efficient therapeutic target in PCa, offering a clinically relevant opportunity worth to be explored in humans.

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EACR22-1067

Precise Spatial Multiplexing of Protein Biomarkers for Immune Profiling in Tissue Samples with ChipCytometry

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Introduction

Immunohistochemistry (IHC) is the most widely used diagnostic technique in tissue pathology. However, IHC is associated with several limitations including the labeling of just a few markers per tissue section and limited quantification of cell populations. As a result of plex limitations, key questions about tumor biology are missed, which could be important for advancing our understanding of tumor biology and ultimately improving patient outcomes.

Material and Methods

ChipCytometry is a novel image-based platform for precise spatial multiplexing that addresses these challenges by combining iterative immuno-fluorescent staining with high-dynamic range imaging to facilitate quantitative phenotyping with single-cell resolution. The platform enables simultaneous detection of dozens of markers on a single tissue section and enables accurate quantification of protein expression levels necessary to deeply profile single cells, understand interactions between key immune cells, and identify topographic biomarkers.

Results and Discussions

Here we demonstrate how standard FCS files are generated from multichannel OME-TIFF images, enabling identification of cellular phenotypes via flow cytometry-like hierarchical gating. Quantification of results reveal precise expression levels for each marker in the assay in each individual cell in the sample, while maintaining spatial information about each cell.

Conclusion

ChipCytometry has the potential to advance precision medicine in immuno-oncology and inform the discovery of novel biomarkers but enabling quantitative analysis of cellular phenotypes in the spatial context.

EACR22-1089

The implication of RKIP loss in primary resistance to EGFR inhibitors in NSCLC: a

novel predictive biomarker?

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Introduction

Raf Kinase Inhibitory Protein (RKIP) is a tumor suppressor protein that inhibits important signaling pathways such as MAPK, PI3K-AKT, JAK-STAT. It is found downregulated in a plethora of cancers, including non-small cell lung cancers (NSCLC). This loss of RKIP expression is correlated with higher TNM stage, presence of metastasis and resistance to conventional therapy. Curiously, little is known regarding the implication of RKIP's loss in the response to targeted therapy, like Epidermal Growth Factor Receptor (EGFR) inhibitors, which outstand in the treatment of advanced stages of NSCLC. As RKIP is a broad inhibitor of EGFR related pathways, its absence may lead to their constitutive activation, and being this phenomenon the main cause of resistance to therapies against the EGFR receptor, we hypothesize that the loss of RKIP expression may have a predictive role in the response to EGFR inhibitors in NSCLC.

Material and Methods

To explore this, two lung adenocarcinoma cell lines, PC9 and HCC827, were genetically altered to knockout (KO) (by CRISPR-cas9) and overexpress (OE) RKIP, respectively. The role of RKIP in the response to EGFR inhibitors was evaluated *in vitro* through cytotoxicity assays (MTS) and real-time cell analysis (xCELLigence), using Erlotinib, Afatinib, Allitinib and Osimertinib drugs. Moreover, 3D tumor spheroids were established to assess cytotoxicity and proliferation rates upon treatment. Western blot was done to assess the expression and/or activation of RKIP, its targets, and the signaling pathways. These results were validated *in vivo*, using the subcutaneous xenograft mouse model.

Results and Discussions

In general, when RKIP is knocked out, cells become less responsive to EGFR inhibitors, seen by the higher IC₅₀ values in PC9 RKIP KO cells, compared to the control. Further, *in vivo* assays showed that PC9 RKIP KO tumors were less responsive to the daily treatment with Afatinib, seen by lower tumor shrinkage upon treatment, as to PC9 CTR tumors. Such results were corroborated in HCC827 RKIP OE cells, in which the opposite effect was observed, as HCC827 RKIP+ cells became more responsive to EGFR inhibitors. When assessing the activation levels of EGFR related signaling pathways upon treatment, AKT pathway was overactivated in RKIP KO cells, thus shedding light on the possible mechanism of resistance behind RKIP's loss.

Conclusion

To sum up, these results show the potential of RKIP as a negative predictive biomarker to EGFR targeted therapy response in NSCLC.

EACR22-1219

Multi-omic profiling of rectal tumour and sera samples from rectal cancer patients demonstrates metabolomic alterations associated with cancer development and therapy response

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Introduction

Rectal cancer incidence is significantly increasing, with the majority of patients presenting at late stage. There is an urgent need to identify biomarkers to aid early diagnosis of rectal cancer. Furthermore, resistance to neoCRT is a significant problem in rectal cancer. Altered energy metabolism has been proposed to have a role in cancer development and therapeutic resistance. In this study, multiomic profiling was performed using rectal cancer and non-cancer rectal tissue, and the role of altered energy metabolism in rectal cancer was assessed.

Material and Methods

Pre-treatment rectal cancer biopsies and sera from consenting rectal cancer patients were obtained at diagnostic colonoscopy. Non-cancer rectal tissue biopsies were obtained from consenting patients undergoing routine endoscopy. Metabolomic profiling was performed on pre-treatment rectal adenocarcinoma tissue biopsies, tumour conditioned media (TCM) and sera, and on rectal tissue biopsies and non-cancer conditioned media (NCM) from non-cancer patients by LC-MS. Transcriptomic analysis was performed on pre-treatment rectal cancer and non-cancer rectal tissue using Lexogen QuantSeq sequencing and Ingenuity Pathway Analysis (IPA) software. MesoScale Discovery 54-plex ELISAs were used to assess the secretome of cancer and non-cancer rectal tissue.

Results and Discussions

In total, 23 metabolites significantly differed between rectal cancer and non-cancer rectal tissue. Four secreted metabolites were significantly different between TCM and NCM. Transcriptomic profiling identified 470 differentially expressed genes between cancer and non-cancer tissue. IPA analysis of transcriptomic data predicted alterations to cancer-related pathways, including cell cycle and DNA repair between cancer and non-cancer tissue. Ten inflammatory mediators were significantly altered in the secretome of rectal cancer. GLM analysis of the metabolome of pre-treatment sera from rectal cancer patients ($n=52$), demonstrated 16 metabolites significantly reduced with increasing tumour regression score (TRS).

Conclusion

We have demonstrated significant alterations in the metabolome, secretome and transcriptome of rectal tumours, when compared to non-cancer rectal tissue, highlighting a role for altered metabolism in the pathogenesis of rectal cancer. Importantly, we have identified sixteen metabolites significantly associated with response to neo-CRT in pre-treatment sera from rectal cancer patients, highlighting their potential as predictive biomarkers of treatment response.

EACR22-1169

POSTER IN THE SPOTLIGHT

Detection of DNA damage using STRIDE as a new PD biomarker in liquid biopsies and

FFPE tissue sections

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Introduction

Reliable and precise measurement of DNA damage levels is critical for the development of effective therapies that interfere with DNA repair machinery and/or directly induce DNA damage. However, application of commonly used antibody-based IF or IHC tools for assessing biomarkers in clinical samples, such as FFPE tissue sections or liquid biopsies, can be challenging.

Here we are presenting the new assay - STRIDE (SensiTive Recognition of Individual DNA Ends) that is being developed to address this challenge. STRIDE was shown to be a highly sensitive and specific technology for the direct detection of single- or double-strand DNA breaks *in vitro* (Kordon et al., 2020).

Material and Methods

Here, we discuss first data from an ongoing collaboration aimed at validating STRIDE as a functional PD biomarker in animal and human samples. STRIDE was used to selectively label single- and double-strand DNA breaks in cancer cell line models/animal xenografts, human FFPE tissue sections from various types of solid cancers, and human PBMCs.

Results and Discussions

The results obtained so far demonstrate the applicability of the STRIDE technology to measure PD effects of selected DDR inhibitors and other DNA damaging agents. These data also show the potential for the STRIDE to allow measurement of endogenous DNA break levels in a variety of tumor types, often with different genetic backgrounds.

Conclusion

Taken together, the initial assessment shows that STRIDE has the potential to provide a versatile method for selective DNA break detection in any type of biological material, including liquid biopsies and different tumor types. This proof-of-concept research will be followed by further method validation to support STRIDE as a technology for analysing DNA fragmentation caused by DNA damaging therapeutics and targeted DDR inhibitors in the clinic.

EACR22-0870

Identification of deregulated gene expression across all stages of Pancreatic Ductal Adenocarcinoma as a platform to develop pharmacogenomics biomarkers for early diagnosis and therapy

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the most frequently diagnosed form of pancreatic cancer worldwide. PDAC is associated with poor survival rates mainly due to the disease being usually diagnosed at late stages. We gathered publicly available gene

expression data from eligible studies to identify diagnostic biomarkers and investigate the molecular heterogeneity between the stages of PDAC tumors.

Material and Methods

We searched the NCBI GEO database for gene expression studies conducted on tumor tissue or blood samples from patients with PDAC who have not undergone treatment. We carried out Differential Gene Expression Analysis (DGEA) on the combined normalised samples, adjusted for study and tumor stage (AJCC system) to compare tumor and normal samples. The list of statistically significantly ($p_{adj} < 0.05$) differentially expressed genes (DEGs) was used to conduct enrichment analysis via active subnetworks and miRNA enrichment analysis. We then used results from these analyses to identify the most significant genes and pathways and map these to marketed drugs' pharmacological targets. The same process was repeated for studies with blood samples and results were compared to those from the tissue analysis.

Results and Discussions

The global expression matrix of tumor tissue samples consisted of 25699 genes and 448 samples (8%, 53%, 5%, 5% for stages 1,2,3,4 respectively) while the corresponding matrix for blood samples consisted of 31581 genes and 122 samples (63% tumors). For each stage, more than 8000 DEGs were found when tumor tissue samples were compared to normal samples and a DEG overlap of roughly 6000 genes across stages was found. Between PDAC blood samples and normal blood samples, 7145 DEGs were found. An overlap of 820 DEGs (with the same direction of dysregulation) between tissue samples of all stages and blood samples was reported. Active subnetwork analysis revealed enriched ribosome, spliceosome and adherens junctions KEGG pathways across all stages and blood samples. Nitrogen metabolism was the only pathway exclusively enriched in stage 1 samples compared to all other samples.

Conclusion

Importantly, PDAC tumors from different stages demonstrate significant molecular similarities. Gene expression deregulation begins at stage 1, covers a large number of genes and is consistent across all stages. We present a list of identified genes that could potentially act as a diagnostic tool to detect PDAC development early and/or to act as potential targets for efficient pharmacological interventions.

EACR22-1050

Biochemical and functional characterisation of patient pancreatic cyst fluid for the identification of a novel biomarker panel of patient cancer risk

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Introduction

Pancreatic cancer is one of the worst prognosis cancers with a 5-year survival rate <7%. Pancreatic cystic lesions (PCLs) are fluid-filled protrusions either on or inside the pancreas and can be benign or pre-malignant. Current guidelines to stratify patients based on cancer

risk are imperfect. Here, we characterised the proteome and transcriptome of pre-malignant cysts to identify a novel biomarker panel that stratifies patient cancer risk. Furthermore, we examined the functional influence of low- and high-risk cyst fluid on driving the hallmarks of cancer in normal and metaplastic cells *in vitro*.

Material and Methods

Patient pancreatic cyst fluid (PCF) was collected from 30 patients by EUS-FNA. Patients were stratified using the 2018 European evidence-based guidelines as low-risk (n=15) or high risk (n=15). PCF was sonicated and subsequently processed using SP3 paramagnetic beads prior to LC-MS. MS-generated label-free quantification intensity data were analysed in Perseus (v1.6.13.0). HTG microRNA whole transcriptome sequencing was run on whole PCF. MiRNA sequencing data were analysed using HTG EdgeSeq Reveal (v3.1.0). A normal (HPDE) and a metaplastic (hTERT-HPNE) human pancreatic cell line were cultured with either low-risk or high-risk patient PCF for 24h in serum-starved conditions. A variety of *in vitro* assays were employed to assess the hallmarks of cancer.

Results and Discussions

MS-analysis of PCF revealed eight proteins to be significantly upregulated in high-risk PCF compared to low-risk ($p < 0.05$, FDR=0.05, $s_0 = 0.1$). Whole transcriptome sequencing revealed three miRNA to be significantly upregulated in high-risk PCF compared to low-risk PCF (adj- $p < 0.05$, FDR=0.05, $s_0 = 0.1$). Unsupervised hierarchical clustering using the eight differentially expressed proteins and three miRNA gave a clustering accuracy of 95.8%. *In vitro*, several hallmarks, such as proliferation, were demonstrated to be statistically differentially modulated by low- and high-risk PCF in both HPDE and hTERT-HPNE cell lines indicating a functional influence of PCF on pancreatic disease.

Conclusion

Normal and pre-malignant pancreatic cellular biology can be affected by exposure to patient PCF. Multi-omic profiling of pancreatic cyst fluid revealed differentially expressed factors between low- and high-risk PCF, which could be utilised independently or as part of a multi-marker panel for the stratification of patients into high- and low-risk groups for malignancy.

EACR22-1082

Feasibility of sputum and plasma samples for digital detection of microRNA-based biomarkers as potential approach for early detection of lung cancer

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Introduction

Lung cancer is the deadliest cancer worldwide. Lung cancer screening programs have been applied for high-risk subjects (smokers; 50-75 yrs) to try to reduce lung cancer death rates in high-income countries. Low Dose Computed Tomography has been employed for lung cancer screening, but this approach is not available for all, especially in low-middle income countries. Biomarkers detection in body fluids may direct more precisely high-risk subjects to screen programs, decreasing health system load. However, this approach is not always feasible and usually takes time due to optimization procedures. **We aimed to evaluate the**

feasibility for digital detection of microRNA-based biomarkers in liquid biopsy samples.

Material and Methods

Plasma and sputum samples from high-risk subjects with noncancerous lesions (n=6) attended the mobile unit of the Barretos Cancer Hospital screening program. For microRNA isolation, we used miRNeasy Serum/Plasma kit for plasma samples and Plasma/Serum Circulating and Exosomal RNA Purification kit for sputum samples. From 100ng of RNA, we evaluated the expression of 827 clinically relevant miRNAs (nCounter Human V3 miRNA panel, NanoString). For quality control (QC), housekeeping genes were used to check genomic DNA (gDNA) contamination (ACTB, B2M, GAPDH, RPL19, RPLP90); positive control "E" (POS_E) counts must be higher than the mean of negative control plus 2*SD. For miRNA expression analysis, raw data were input into Rosalind software. We get the Multidimensional Scaling graph to evaluated dissimilarity between sputum and plasma.

Results and Discussions

Regarding QC, no QC flags were observed according to assay control, POS_E was higher than 22.74; and no gDNA contamination was detected (mean ACTB=25.6; mean B2M=14.3, mean GAPDH=9.16, mean RPL19=15, mean RPLP90=7.6). Sputum and plasma samples were grouped into two independent clusters. Hsa-miR-451a was exclusively expressed in plasma (mean=6339.3), and hsa-miR-23a-3p (mean=201.0), hsa-574-5p (mean=99.3), and hsa-let7b-5p (mean=135.6) were exclusively expressed in sputum. A subset of miRNAs were expressed in both fluids: hsa-miR-223-3p (101.8), hsa-miR-320e (mean=90.0), hsa-miR-4445 (mean=373.6), hsa-miR-7975 (mean=82.3), and hsa-miR-612 (mean=273.3).

Conclusion

The use of biofluids for the evaluation of miRNA-based biomarkers by digital detection is feasible and may be a potential approach for increasing sensitivity of lung cancer detection in screening programs.

EACR22-0005

Circulating miR-21 and miR-125b in women living with Human immunodeficiency virus: Dysregulated biomarkers for monitoring cervical carcinogenesis

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Introduction

As of 2018, the prevalence of Human immunodeficiency virus (HIV), and cervical cancer (Ca) attributable to HIV was higher in Africa than in other continents. Identifying individuals at a high risk of developing Ca among immunocompromised persons, using less invasive techniques, remains a major challenge. The objective of this study was to evaluate HIV infection dysregulation effects on Ca-linked oncomirs (miR-21, miR-146a, miR-155, miR-182, and miR-200c) and tumor suppressors (miR-let-7b, miR-125b, miR-143, miR-145, and p53 gene), in a bid to identify early indicators of genetic instability, and biomarkers for monitoring of high-risk individuals.

Material and Methods

This case-control study included 173 women without abnormal Pap smear; confirmed HIV seropositive women (HIV+ = 103) and HIV seronegative women (HIV- = 70). Relative expressions of miRNAs and p53 gene in blood and cervical cells were determined using

reverse transcriptase PCR and gel electrophoresis. T-test was used to compare the data from HIV+ and HIV- women. Significance was set at $p \leq 0.05$.

Results and Discussions

Similar pattern of miR-21, miR-146a, miR-182, miR-200c, miR-125b, and miR-145 expression was observed in both samples. Higher expressions of miR-155 and p53 gene were observed in cervical cells of HIV+ women compared with HIV- women ($p = 0.046$, and 0.033 , respectively) whereas lower expressions of miR-155 and p53 gene were observed in the blood of HIV+ women compared with HIV- women ($p = 0.539$ and 0.049 , respectively). In both blood and cervical cells, higher miR-21 expression ($p = 0.032$ and 0.198) and lower miR-125b expression ($p = 0.050$ and 0.004 , respectively) were observed in HIV+ women compared with HIV-women. Interestingly, in blood, a lower expression of miR-146a was observed in HIV+ women compared with HIV- women ($p = 0.036$) whereas in cervical cells, lower expressions of miR-182 and miR-200c were observed in HIV+ women compared with HIV- women ($p = 0.035$ and 0.045 , respectively). The low expression of miR-146a, miR-182 and miR-200c in HIV+ women could be due to immune exhaustion, viral immune evasive mechanism, or an indicator of viral latency while the higher expression of miR-21, and lower expression of miR-125b and p53 could be early indicators of genetic instability prior to epithelial transformation.

Conclusion

This study suggests that circulating high expression of miR-21 and low expression of miR-125b and p53 gene could be used in identifying individuals at risk of developing Ca, especially among immunocompromised patients.

EACR22-0227

One step to early diagnostic in ovarian cancer

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Introduction

Ovarian cancer is the most lethal gynaecological cancer. It is already named a silence killer. This is due to the difficulty in detecting early stages as well as the lack of effective therapeutic options for advanced ones. Currently, CA125, Human Epididymis protein 4 (HE4) and OVERA are the only markers for monitoring treatment, detecting disease recurrence or to test patients presenting ovarian mass

Material and Methods

Bearing ID8P53-/- tumours mice were blood analysed during all the tumour progression until ascites was present. We detected hematopoietic progenitors by flow cytometer, number of mature cells with a hematocounter and serum cytokines in a luminex analysis. Human immune secreted proteins were analysed against a specific hematopoietic progenitor signature and TCGA-OV public data.

Results and Discussions

Different progenitor types were mobilized at the beginning of the tumour growth. We also detected a later increase of neutrophils when ascites was apparent. Cytokines analysis showed us some specific molecules that are important at the beginning and at the end of the tumour process. We found interesting correlations between the hematopoietic progenitors signature and human immune secreted proteins in ovarian cancer, which are functionally related with our mouse model results. That give us the courage to deeper analysis through these cytokines and, in the future, to check in human samples.

Conclusion

Blood circulating cytokines related to hematopoietic progenitors could help us to detect early ovarian tumour formation

EACR22-0231

Breath-based early detection of lung cancer using a metabolic probe targeting tumour specific extracellular β -glucuronidase.

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Introduction

Lung cancer is a leading cause of mortality with 5-year survival less than 20%, largely due to many cases being diagnosed late. Early detection can increase cancer survival up to 13-fold underscoring the need for effective screening. Targeted low dose computed tomography (LDCT) can be effective but its impact to date has been limited due to slow adoption and variable uptake in high-risk populations.

Breath analysis represents a non-invasive screening approach either alone or alongside LDCT. Numerous studies have sought potential breath biomarkers of lung cancer. Many have produced promising results but to date, no validated biomarkers with clear connections to cancer metabolism have been revealed. We have explored an alternative, probe-based approach using Exogenous Volatile Organic Compound (EVOC) Probes. The probes target tumour associated extracellular β -glucuronidase, a glycosidase enzyme that normally resides within lysosomes.

Material and Methods

We use a hydrophilic non cell permeable substrate probe D5-ethyl- β D-glucuronide (D5-EtGlu) that upon hydrolysis by the target enzyme releases D5-ethanol, a unique volatile reporter molecule. This provides a readout of tumour associated enzyme activity using breath analysis.

Results and Discussions

Administering D5-EtGlu to mice resulted in tumour specific release of D5-ethanol, enabling discrimination between healthy and tumour bearing animals. Increased expression of β -glucuronidase in lung cancer tissue and the tumour microenvironment was confirmed with immunohistochemistry (IHC) in clinical samples. A phase 1a clinical trial administered D5-EtGlu to healthy individuals in a single ascending dose study to establish safety and background D5-ethanol levels in healthy individuals. This resulted in no adverse events and low/no D5-ethanol signal verifying the inaccessibility of D5-EtGlu to intracellular β -glucuronidase. The next stage, currently ongoing, is a proof of mechanism study in humans. D5-EtGlu is administered intravenously to lung cancer patients followed by breath analysis. D5-ethanol breath levels will be compared to cancer free individuals receiving the same D5-EtGlu dose.

Conclusion

Non-invasive breath testing has great potential for lung cancer diagnosis including a potential role in screening. Our current work is evaluating the use of an administered probe to stimulate tumour-specific enzyme activity producing marker detectable on breath. Continued success could result in a sensitive and highly specific method for lung cancer early detection.

EACR22-0547

miR-944/MMP10/AXL- axis Predict Lymph Node Metastasis in Early-Stage Tongue Cancer

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Introduction

Lymph node metastases among early-stage oral cancer patients with a clinically negative neck play a decisive role in prognosis. With an accurate prediction of occult lymph node metastases, 70% of the patients can be spared from elective neck dissection. Despite significant advances, no promising biomarkers exist to predict preoperative nodal status.

Material and Methods

We retrospectively analyzed 219 early-stage tongue cancer resected specimens for MMP10 expression obtained from patients before surgery, including the N-zero phase III clinical trial (NCT00193765) samples, by immunohistochemistry and real-time qPCR. To investigate the role of *MMP10* and its upstream/ downstream effectors in metastasis, tongue cancer cells overexpressing *MMP10* and *shMMP-10* were characterized using *in vitro* assays and orthotopic tongue cancer mouse model, followed by whole transcriptome analysis.

Results and Discussions

We found upregulation of *MMP10* in 86% of patients with lymph node metastases ($p < 0.0001$) that establish *MMP10* as a promising prognostic biomarker. We show that overexpression of *MMP10* induces tumor growth and nodal metastasis mediated through the AXL signaling pathway. Similarly, loss of *MMP10* suppresses nodal metastasis using mouse tongue orthotopic model with bioluminescent imaging. Furthermore, we show *miR-944*, which negatively regulates *MMP10* by targeting its 3'-UTR, is downregulated in patients with nodal metastases ($p < 0.05$).

Conclusion

Our findings establish *MMP10* as a candidate biomarker that could potentially aid in sparing a significant proportion of early-stage tongue cancer patients from mandatory elective neck dissection.

EACR22-0729

Targeting tumour-associated aldo-keto reductase activity with exogenous volatile organic compound (EVOC) probes to detect lung cancer

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Introduction

Early detection could improve cancer survival, particularly for lung cancer, but this requires accessible screening methods. Non-invasive breath tests targeting cancer metabolism could drastically improve detection. Tumour cell metabolism is adapted for survival in the tumour microenvironment. Aldo-keto reductase enzymes including AKR1B10 and AKR1B15 are upregulated in human lung tumours to reduce aldehydes, produced by lipid peroxidation, into corresponding alcohols. These aldehydes and alcohols are volatile and could possibly be detected in breath to detect cancer. Here we measure AKR1B10 and AKR1B15 activity in lung cancer cells by administering aldehydes as exogenous volatile organic compound probes (EVOC® Probes) and monitoring alcohol production through *in vitro* headspace analysis.

Material and Methods

A549 lung cancer cells were exposed to one of three treatments: tolrestat (AKR1B1/B10 inhibitor), JF0064 (AKR1B1/B10/B15 and ALDH1A1 inhibitor) or DMSO vehicle (control). Both AKR inhibitors have different substrate affinities but were expected to have similar overall effects. Subsequently aldehyde substrates (1 aromatic, 1 alkanal and 2 alkenals) were added and headspace samples collected at 1, 3 & 6h using HiSorb sorptive extraction and analysed with GC-MS. For evaporation control, a parallel experiment used VOCs analysed in cell culture medium (no cells).

Results and Discussions

Overall, DMSO control samples showed lower aldehyde and higher alcohol levels compared to evaporation controls suggesting that AKRs are active in cancer cells, activity that was reduced by AKR inhibition. At the 1h timepoint, aldehyde levels did not differ between treated and untreated cells. However, tolrestat treatment showed lower levels (75-100% reduction) of all expected alcohol products, while JF0064 treated cells showed lower levels of alcohols from aromatic aldehyde (~50%) and alkanal (~20%) metabolism but increased levels of alkenal products (>2-fold) relative to the DMSO control. Increased alkenols may be due to inhibition of ALDH1A1 by JF0064, an enzyme with higher affinity to unsaturated aldehydes. This may increase the availability of other aldehydes as substrates for other enzymes.

Conclusion

Using AKR inhibitors we demonstrate that alcohol/aldehyde ratio reflects AKR activity, suggesting the potential to use of these aldehydes as EVOC® Probes for cancer early detection on breath by targeting upregulated AKR activity in lung cancer tissue.

EACR22-1171

Blood biomarkers for detection of cancer-associated cachexia

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Introduction

Cachexia is a high prevalent syndrome in oncologic patients, which remains a key challenge in oncology today. This condition is frequently underrecognized and undertreated and have devastating consequences for patients. The understanding of the molecular mechanisms of cachexia has increased in recent years, and early assessment and intervention have been associated with improved patients outcomes. Proteomic technologies have emerged as powerful strategies for the discovery of protein biomarkers. However, its use in oncology has not been fully explored. The aim of this study was to investigate differential expression of blood proteins between cachectic and non-cachectic patients that could provide clues to gain knowledge on its pathophysiology, and eventually postulate new therapeutic strategies.

Material and Methods

A total of 13 subjects attending in the Medical Oncology Department of 12 de Octubre Hospital (Madrid, Spain) were included in this study: 6 cachectic and 7 non-cachectic patients. Blood samples were collected from all the participants, and plasma was separated and immediately stored at -20°C until analysis. We used 2D-DIGE and mass spectrometry to identify candidate biomarkers of cachexia. ImageQuant v5.1 and DeCyder v6.5 (GE Healthcare) was used for spots analysis. Differences between cachectic and non-cachectic patients were considered significant when satisfied the Student's test ($p < 0.05$). Data generated by image analysis was used in the construction of principal component analysis (PCA). Proteins of interest were classified in The Gene Ontology Resource by Gene Ontology terms based on a given protein's biological processes, molecular functions, or cellular components.

Results and Discussions

Using gel-based proteomics, we identified 6 proteins of interest as cachexia biomarkers. In the PCA analysis, it is observed that there is a main component that differentiates samples from non-cachectic and those from cachectic patients. Our results showed a diminished expression of TF, SERPINC1, SIDEROPHILIN and TRANSTHYRETIN, and an increased expression of HP and SERPINA1 in cachectic patients. All these proteins are involved in biological processes of acute inflammatory response.

Conclusion

Inflammatory processes are highly related to the triggering of cachexia in cancer patients. The control of inflammatory processes could be an initial therapeutic approach for those patients susceptible to develop cachexia. Additional studies are needed to fully characterize this pathological condition.

Carcinogenesis

EACR22-0590

Colibactin-producing *E. coli* induces a metabolic reprogramming of colonic epithelial cells toward a pro-carcinogenic metabolism.

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Introduction

Colonization of the colonic mucosa by colibactin-producing *E. coli* (CoPEC) could be involved in the development of colorectal cancer (CRC). Colibactin is a genotoxin produced by an enzymatic machinery encoded by the *pks* genomic island. Intracellular multiplication of CoPEC likely requires the exploitation of host resources to replicate and survive which could altered metabolic states of intestinal epithelial cells leading to transformation. Aim of this study is to investigate effects of CoPEC infection on the metabolic state of human intestinal epithelial cells.

Material and Methods

Human colorectal carcinoma T84 cells were infected with the 11G5 strain, a CoPEC strain or its mutant (11G5- Δpks). Intracellular metabolomes were investigated using ¹H-NMR and LC-MS in positive and negative modes (PFEM MetaboHUB). Extracellular metabolomes were investigated by targeted metabolomics analysis using LC-MS (Biocrates). Data were analyzed using XCMS package on Galaxy-W4M platform. Genotoxic properties of the CoPEC (γ H2Ax immunostaining) were measured in chronically infected and serine/glycine-depleted (SD) diet-fed mice.

Results and Discussions

Principal component analysis of intracellular metabolome revealed strong discrimination between the three groups (non-infected, 11G5 and 11G5- Δpks -infected cells). We identified increases of lactate and pyruvate in 11G5 and 11G5- Δpks -infected cells when compared to control cells suggesting induction of a Warburg-like metabolism in infected cells. Analysis of the extracellular metabolome supported this result. Discriminant metabolites between the 11G5 and its 11G5- Δpks mutant showed that colibactin affects mostly metabolites involved in host defense against DNA-damages and oxidative stress as increases of glutathione, UDP-GlcNAc and pantothenic acid and a decrease of serine levels. We showed that the 11G5 strain uses serine from the enterocyte activating the serine-utilization-genes, which allows it a growth advantage over a commensal strain. Furthermore, we highlighted in mice that a SD diet leads to a transient decrease in 11G5 bacterial colonization associated with a decrease of the genotoxic proprieties of the CoPEC.

Conclusion

Our work supports that CoPEC-induced metabolic reprogramming in colonic cells could participate to its pro-carcinogenic activity. We identified that serine seems to be a metabolite of interest, used by CoPEC to colonize the digestive tract and to exert its pro-carcinogenic properties.

EACR22-0425

EFFECT OF NUTRIENT AVAILABILITY ON THE TUMORIGENIC PROPERTIES OF HEAD AND NECK CANCER CELL LINES

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Introduction

NAD is an essential metabolite that plays an important role in metabolism. Tumor cells must increase NAD production by promoting NAMPT expression, the limiting-enzyme of the Salvage pathway of NAD. Within the tumor, malignant cells must compete with stromal and noncancerous cells for the same nutrient and energy resources. Tumor cells show the ability of reprogram their metabolism, depending on nutrients availability, to allow tumor proliferation, development and survival. Most of cancer cells rely on glycolysis rather than mitochondrial oxidative phosphorylation, although high energy efficiency and oxygen availability, what is known as the Warburg effect. Aerobic glycolysis shift requires a high availability of glucose. Cancer cells also depend on glutamine and NAD metabolism. However, nutrient limitation can affect the tumorigenic properties of cancer cells.

Material and Methods

We use two HNSCC cell lines (RPMI-2650, Detroit-562) with different phenotype and tissue origin. We studied how nutrients availability affect the proliferation and clonability formation of tumor cells with NAMPT expression reduced. We performed a growth curve and a clonability assay in different metabolic conditions: high glucose (glucose 4.5g/L, glutamine 0,5g/L, nicotinamide 0.004mg/L), low glucose (1g/L), free glucose, free glutamine and free nicotinamide (a NAD precursor).

Results and Discussions

The cell lines showed a different metabolism phenotype: RPMI-2650 was more dependent on glutamine, whereas Detroit-562 depended on glucose and nicotinamide metabolism. The proliferation and the clonability capacity of RPMI-2650 cells were significantly reduced in medium without glucose and were totally inhibited in medium without glutamine. The proliferation and the clonability capacity of Detroit-562 cells were decreased in medium without glutamine and were inhibited in medium without glucose or nicotinamide. On the other hand, the low glucose availability caused an increase in the growth rate of NAMPT-reduced cells. This increased cellular proliferation is probably due to a change in the metabolism of these cells. However, we did not observe significant variations in the levels of NAD in the different metabolic conditions.

Conclusion

The nutrients availability from the medium can affect the tumorigenic properties of cancer cells. Cellular proliferation and clonability ability depend on glucose, glutamine and nicotinamide availability. However, more studies are needed to understand how these nutrients affect the metabolism of tumor cells.

EACR22-0053

ORAL PRESENTATION

Mutant clones in normal epithelium outcompete and eliminate early oesophageal micro-tumours

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Introduction

Human epithelial tissues accumulate cancer-driver mutations with age, yet tumour formation remains rare. The positive selection of these mutations argues they alter the behaviour and fitness of proliferating cells. Hence, normal adult tissues become a patchwork of mutant clones competing for space and survival, with the fittest clones expanding by eliminating

their less-competitive neighbours. However, little is known about how such dynamic competition in normal epithelia influences early tumorigenesis.

Material and Methods

To answer this question we have developed an *in vivo* mouse model that recapitulates the mutational landscape of the normal human oesophageal epithelium. We have used transgenic mouse models, quantitative lineage tracing, ultra-deep DNA sequencing, whole-tissue confocal imaging and mathematical modelling to resolve the dynamics of mutant clones in this tissue, and their role in the development of emerging tumours.

Results and Discussions

Our results show that mutant clone dynamics in the normal oesophageal epithelium are defined by the relative fitness of clones competing for survival within the tissue confined space. In this environment, clones carrying particular mutations prevent early tumorigenesis by keeping in check more malignant clones (Colom B. et al. Nat. Gen 2020), and by directly outcompeting and eliminating emerging micro-tumours (Colom B. et al. Nature 2021). Importantly, manipulating the normal epithelium mutational landscape influences early tumour formation, an approach that may lead to the development of novel preventive strategies against cancer.

Conclusion

Mutant clones in normal epithelium have an unexpected anti-tumorigenic role in purging early tumors through cell competition, thereby preserving tissue integrity.

EACR22-0697

ORAL PRESENTATION

Elucidate the Role of Tumor Isolated *Enterococcus faecalis* and Its Metabolic Products on CRC onset and GVB Impairment

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Introduction

Colorectal cancer (CRC) is the third leading cause of cancer-related mortality worldwide and 50% of patients with localized disease ultimately develop distant metastases. Recently, deep-sequencing technology revealed a strict correlation between colonic dysbiosis and CRC development, suggesting a role of the microbiota in colorectal tumorigenesis. Members of the gut microbiota can have pro-tumorigenic features and contribute to CRC onset by impacting gut barrier integrity or via the activation of immune responses. Moreover, bacteria may take part in CRC progression, further enhancing carcinogenesis or even promoting metastasis formation.

Material and Methods

To better identify those bacteria that may be involved in CRC, we took advantage of the *APCMin/+C3aRKO* mice, a murine model of spontaneous colon tumorigenesis. These mice develop fewer tumors in the small intestine compared to the *APCMin/+* model but are characterized by increased tumor burden in the colon and an impaired gut vascular barrier (GVB), resembling human CRC. By whole metagenomic analysis we observed a statistically significant enrichment of *Enterococcus faecalis* (EFSC1) in the tumors of *APCMin/+C3aRKO* mice compared to the healthy mucosa. We wondered if the tumor

isolated EFSC1 might have a direct role in primary tumor onset as well as GVB impairment, which occurs early during carcinogenesis and represents one of the first steps in metastatic spreading.

Results and Discussions

We found that, in C57BL6/J WT mice, EFSC1 and its metabolic products impacted gut homeostasis, reducing the colonic mucus layer, and disrupting both epithelial and endothelial barriers, suggesting its possible role in carcinogenesis. To further verify this hypothesis, we treated APCMin/+C3arKO mice with EFSC1 or its metabolites and confirmed that either the bacterium or the metabolites increased tumor multiplicity in the colon. We validated the role of EFSC1 metabolites on colon tumorigenesis in the APCMin/+ model. In accordance with the results obtained in the previous model, we observed a higher number of neoplastic cells and selective recruitment of macrophages in the EFSC1 metabolites treated group compared to the control.

Conclusion

These results suggest that the metabolites produced by EFSC1 induce changes in gut homeostasis and, together with mutations in APC genes, boost the formation of primary tumors in the colon. We speculate that the identification of EFSC1 specific metabolites may be clinically relevant, opening to a new therapeutic intervention in CRC treatment.

EACR22-0018

Investigating the role of cancer stem cells in oral cancer metastasis using a microfluidic chip

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Introduction

300,000 cases of oral squamous cell carcinoma (OSCC) are diagnosed every year, and around 30% of patients exhibit metastatic spread. Metastasis is a complex process by which cancer cells travel to a secondary site to form a new tumour. Previous work has suggested that cancer stem cells (CSCs) are capable of driving metastasis by switching between epithelial and mesenchymal states. Furthermore, the tumour microenvironment and surrounding tissues also play a key role in metastasis. For instance, cancer cells can utilise the vascular system to travel to a distant site and metastasise. However, the role of CSCs in these interactions has not been established. Microfluidic devices are a promising new *in vitro* model to study these interactions, specifically due to their compartmentalised structure.

Material and Methods

Using photolithography and soft lithography techniques, we fabricated 3 channel microfluidic chip devices composed of a Polydimethylsiloxane (PDMS) block bonded to a glass cover slip. We added endothelial cells in one side channel and OSCC cells in the opposite one. The interactions between these two cell types were studied within a fibrin gel in the middle channel and assessed using immunofluorescence and automated confocal imaging.

Results and Discussions

So far, by using this model we have uncovered a two-way communication between cancer cells and the endothelial cells, that greatly changes the behaviour of both compartments. Most interestingly, the mode of cancer invasion undergoes a marked change. In the

presence of endothelial cells, invasion is much slower and epithelial to mesenchymal transition (EMT) is absent – instead invasion progresses through chains of epithelial cells. We have characterised CSC marker expression in these chains of OSCC cells via immunohistochemistry. Furthermore, we have studied the role of the endothelial cells in the formation of these OSCC chains of cells. The presence of endothelial cells appears to drive significant matrix re-organisation, with thicker fibres observed under the microscope. In future work we aim to establish the role of this endothelial-driven matrix remodelling in inducing the observed change in OSCC invasive phenotype, further characterise these OSCC chains of cells and validate this organ-on-a-chip model using formalin-fixed paraffin-embedded human oral tumour specimens.

Conclusion

We propose that our findings may represent the generation of a vascular niche that acts to modify the CSC phenotype in invasive carcinoma.

EACR22-0030

Docking protein p130Cas regulates acinar to ductal metaplasia during pancreatic adenocarcinoma development and pancreatitis

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Introduction

Acinar to Ductal Metaplasia (ADM) is considered the prerequisite for the initiation of Kras-driven pancreatic adenocarcinoma (PDAC) and candidate genes regulating this process are emerging from GWAS studies. Among the few candidates, the adaptor protein p130Cas emerged as a potential PDAC susceptibility gene and a Kras-synthetic lethal interactor in pancreatic cell lines, however its role in PDAC development has remained largely unknown. The study aims to validate the oncogenic potential of p130Cas in pancreatic cancer and to understand its contribution to Kras-driven signaling pathways.

Material and Methods

Human PDAC samples and murine Kras^{G12D}-dependent pancreatic cancer models of increasing aggressiveness were used to evaluate the expression of p130Cas during tumor development. Moreover, p130Cas was conditionally ablated in pancreatic cancer models to understand its contribution to Kras-induced tumorigenesis.

Results and Discussions

Here, we show that high expression of p130Cas is detected in the majority of PDAC patients and correlates with higher histologic grade and poor prognosis. Murine models showed increased p130Cas expression during Kras-induced ADM and subsequent tumor development. In a model of Kras-driven PDAC, loss of p130Cas inhibits tumor development and potentially extends median survival. Deletion of p130Cas suppresses acinar-derived tumorigenesis and progression by repressing PI3K-AKT signaling, even in the presence of a worsening condition like pancreatitis.

Conclusion

Our observations finally demonstrated that p130Cas acts downstream of Kras to boost the PI3K activity required for ADM and subsequent tumor initiation. This demonstrates an unexpected driving role of p130Cas downstream of Kras through the PI3K/AKT, thus indicating a rational therapeutic strategy of targeting the PI3K pathway in tumors with high expression of p130Cas.

Divergences between the genomic profile of advanced thyroid cancers in putative aggressive genes.

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Introduction

Thyroid carcinoma(TC) is the most common endocrine cancer. Some tumors of follicular cell origin such as papillary thyroid carcinomas with distant metastases (PTCs-DM), poorly differentiated thyroid carcinomas (PDTCs), and anaplastic thyroid carcinomas (ATCs) behave aggressively showing significantly reduced disease-specific survival rates. Though over the past 25 years several genes have been associated with tumor progression and aggressiveness, the differences or similarities concerning the mutational load of those genes in aggressive TCs with different degrees of differentiation are not clear.

Material and Methods

By genotyping 13 PTCs-DM cases (54 areas), 34 PDTCs cases (57 areas), and 36 ATC cases (69 areas) we sought to determine the prevalence of mutations in genes that have been associated with progression and aggressiveness in thyroid carcinogenesis (*EIF1AX*, *TERTp*, and *PIK3CA*). To assess intratumor heterogeneity, clonal nature, and implication in dedifferentiation different tissue areas within the same tumor were studied. In 22 cases it was possible to analyze matched metastatic samples. Genotyping was approached by PCR and direct sequencing

Results and Discussions

The overall prevalence of *TERTp*, *PIK3CA*, and *EIF1AX* mutations found in PTCs-DM was 69%, 15%, and 0% respectively. Among PDTCs the ratios were 39% at *TERTp*, 13% at *PIK3CA*, and 12% at *EIF1AX*. In ATCs the frequency of *TERTp*, *PIK3CA*, and *EIF1AX* mutations were 65%, 26% and 19% respectively. Concurrent activation of more than one gene was seen in 1 case of PTC-DM (8%), 2 cases of PDTc (6%), and 10 cases of ATC (28%). The PTC-DM case exhibited activation of *TERTp* and *PIK3CA*. In one of the PDTc cases coexisted *TERTp*+*EIF1AX* mutations, whereas in the other all of the 3 genes investigated were mutated. Between the 10 ATCs with at least 2 genes mutated 40% revealed *TERTp*+*EIF1AX*, 40% *TERTp*+*PIK3CA*, 10% *PIK3CA*+*EIF1AX* and 10% *TERTp*+*PIK3CA*+*EIF1AX*

Conclusion

Based on our results *EIF1AX* activation seems to be a late event, related to tumor dedifferentiation and progression. The ratio of *TERTp* mutations seen in PTC-DM compared with the average rate reported in the literature in unselected, low/intermediate-risk PTCs and the ratios found in PDTcs and ATCs suggest an important role for *TERTp* mutations in aggressiveness. The association of *PIK3CA* mutations with *EIF1AX* and/or *TERTp* mutations could represent a cooperating and enhancing event in dedifferentiation, progression, and aggressiveness. The results are relevant for tailored gene-targeted therapies applied to patients with aggressive TC

Endocrine therapies promote anti-proliferative effects on triple negative breast cancer cell lines.

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Introduction

Triple negative breast cancer (TNBC) accounts 20% of all breast cancers with poor survival rates. Thus, endocrine therapies present poor effectivity in it. However, *in vitro* studies suggested that these therapies alter tumor cells steroid hormone secretion affecting vital processes. The aim of this study is to determine the efficacy of some hormonal therapies in TNBC cell lines to clarify effectiveness of them.

Material and Methods

Dutasteride (5 α -reductase inhibitor), Anastrozole (aromatase inhibitor), and ASP9521 (17 β -HSD inhibitor) effects on IPC-366 cells were evaluated due to their high malignancy, high hormonal component and be a good research model for human disease. Sensitivity, proliferation, and migration assays and hormone determination of estrone sulfate (E1SO4), estradiol (E2) and testosterone (T) were performed.

Results and Discussions

The results obtained from the sensitivity assay to determinate the half-maximal effective concentration (EC-50), showed an effective *in vitro* concentration of 1 μ M for each compound, thus, this dose was used on subsequent assays. After proliferation and wound healing assays media was collected to determine hormone secretions by an amplified enzyme immunoassay. Results revealed that Anastrozole was slightly the most sensitive drug to IPC-366 cells. A significant cell viability reduction of 20% approximately with each compound was found. However, all treatments increased the migration ability. Regarding hormonal secretion in proliferation, Dutasteride and ASP9521 promote T secretion while Anastrozole diminished it. This can be related to an anti-proliferative effect. However, all drugs showed a reduction on E1SO4, acting as an estrogen reservoir to promote cell survival. Hormone secretion in migration assays showed a significant increase in E1SO4 levels with all compounds. Also, E2 levels are increased with Dutasteride and ASP9521, where migration was more advanced. Therefore, estrogens secretion, especially E1SO4, promotes cell migration increasing the metastatic capacity of these cells.

Conclusion

In conclusion, all therapies promote a reduction in cell proliferation by increasing androgen levels, but estrogens could reduce the endocrine treatment effectiveness. Besides, high E1SO4 levels promotes migration in IPC-366 cells. The combination of any of these drugs with E1SO4 secretion inhibitors may result in a promising treatment for TNBC.

EACR22-0796

TAZ associated lncRNA TAZ-AS202 promotes lung tumorigenesis through regulation of EPHB2 signalling

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Introduction

Lung cancer represents the main cause of cancer-related death world-wide, with an overall five years survival of 16%. Thus, better understanding of the molecular mechanisms at the basis of lung cancer pathology is crucial to ensure the most appropriate management for patients. Long-non coding RNAs (lncRNAs) have become important players in the regulation of several physiological and cancerous processes. In our laboratory, we discovered and characterized a novel lncRNA named TAZ-AS202 which is expressed in lung cancer cells. This transcript shares the promoter region and is transcribed in antisense orientation with the TAZ gene. TAZ is a transcriptional coactivator that enhances a gene expression program promoting cancer cells proliferation, migration, invasion and drug-resistance.

Material and Methods

To dissect TAZ-AS202 function we performed the following experiments in A549 and NCI-H23 lung cancer cells: siRNA experiments to perturbate target genes expression levels, followed by gene expression analysis by qRT-PCR and functional assays, including proliferation, migration and invasion. To dissect the pathways regulated by TAZ-AS202, we performed RNA-sequencing experiment. To study the expression of TAZ-AS202 in patients' specimens we used the RNA-scope technique.

Results and Discussions

TAZ-AS202 silencing strongly impairs lung cancer cells proliferation, migration and invasion, supporting the idea of a pro-oncogenic role for this lncRNA in the context of lung cancer. Moreover, its expression is upregulated in lung cancer tissue compared to surrounding normal tissue. TAZAS202 does not influence the expression and/or the activity of its cognate TAZ gene, implying an in trans molecular mechanism through which exerts its function. RNA-seq analysis shows that the EphA2 signalling gene EPHB2 is positively regulated by TAZ-AS202. TAZ-AS202 regulates EPHB2 through a transcriptional mechanism, involving a set of transcription factors that have been already described to enhance tumor progression. Strikingly, EPHB2 silencing mimics the silencing of TAZAS202 in lung cancer cells, indicating that EPHB2 modulation is the main target of TAZ-AS202.

Conclusion

Our data indicate that TAZ-AS202 supports lung cancer cells proliferation and acquisition of aggressive features by regulating ephrin signalling receptor EPHB2. The TAZ-AS202/EPHB2 axis may become a novel potential therapeutic target to counteract the progression of lung cancer.

EACR22-0866

Specificities in MYC and MET cooperation in hepatocellular carcinoma

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Introduction

Hepatocellular carcinoma (HCC) is the most common liver cancer and few therapeutic options are available. The transcription factor MYC and the receptor tyrosine kinase MET are associated with HCC pathology as both are frequently overactivated in patients. This study was designed to assess the cooperation of MYC and MET in inducing HCC, to analyse

their molecular features, and to highlight how their combinatorial targeting impacts HCC cell viability.

Material and Methods

Analysis of publicly available database was performed to document the expression of *MYC* and *MET* in HCC patients. Hydrodynamic tail vein injection in the *R26^{StopMet}* mouse genetic setting was performed to model enhanced *MYC* and *MET* levels in a subset of hepatocytes. Histological, immunohistochemical, and RT-qPCR studies were made on tumour samples. Cell viability and protein expression were measured after treatments targeting *MYC* and *MET*, through CCK-8 kit and western blot, respectively, on a panel of human HCC cells.

Results and Discussions

Available database analyses of independent cohorts identified a subgroup of HCC patients with high *MYC* and *MET* levels. Forced expression of *MYC* and *MET* in hepatocytes was sufficient to induce liver tumorigenesis, histologically characterised as HCC, even in the absence of pre-existing injuries associated with a chronic disease state. Intriguingly, by comparing *Myc-R26^{Met}* versus *Alb-R26^{Met}* tumours, we found that *MYC* switched the molecular features of *MET* tumours, exemplified by loss of AFP and OPN expression. The cooperativity of *MYC* and *MET* in HCC is also exemplified by the vulnerability of cells to agents targeting both signals, as illustrated by reduced cell viability *in vitro*.

Conclusion

Our results document the cooperativity of *MYC* and *MET* to induce and to sustain liver tumorigenesis. They also illustrate how *MYC* switches the expression of a set of molecular features in *MET* tumours.

EACR22-0273

The molecular regulation of autophagy in breast cancer: New insights into the role of Serum amyloid A

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Introduction

It has been reported that serum amyloid A (SAA), an acute phase protein normally secreted by the liver, is also synthesized by cancer cells and CAFs in the tumour microenvironment. SAA can activate the PI3K/Akt and MAPK signalling pathways, which are known modulators of the intracellular degradation process, autophagy. However, the relationship between SAA and autophagy in breast cancer has not yet been investigated. Therefore, the aim of this study was to investigate the role of SAA in the regulation of signalling pathways and autophagy in *in vitro* and *in vivo* models of breast cancer.

Material and Methods

For the *in vitro* model, MDA-MB-231, MCF7 and the MCF12A cells were transiently transfected with a control vector, pcDNA3, and the pcDNA3-hSAA1 plasmid to overexpress SAA1. An *in vivo* tumour-bearing mouse model with SAA1/2 knockout was established. Autophagy, proliferation, apoptosis and signalling pathway activation were assessed in these models.

Results and Discussions

SAA overexpression in the MCF12A, MDA-MB-231 and MCF7 cells resulted in an increase in cell viability and increased the expression of the proliferation marker, MCM2, in the MCF12A and MCF7 cell lines. SAA overexpression *in vitro* resulted in the inhibition of autophagy, while SAA1/2 knockout induced autophagy *in vivo*. The expression of the cargo recruiter, p62, was increased in the MCF7 cells as well as in SAA1/2 double knockout mice. SAA overexpression decreased MAPK and PI3K signalling pathway activation in the MCF12A cell line, activated these pathways in the MDA-MB-231 cell line, and inhibited Akt signalling in the MCF7 cells. SAA1/2 knockout resulted in the inhibition of the PI3K pathway protein, PKB/Akt, while increasing the activation of the MAPK, p38. SAA overexpression resulted in decreased colocalization of pERK and LC3-II in the MCF12A cell line and increased colocalization in the MDA-MB-231 cell line. Lastly, SAA1/2 knockout promoted resistance to apoptosis through the regulation of autophagy.

Conclusion

We have determined a novel role for SAA in autophagy in breast cancer cells, where SAA inhibited autophagy *in vitro*. Additionally, SAA promotes proliferation through the regulation of the PI3K/Akt and MAPK signalling pathways. As such, the double knockout of SAA1/2 induced autophagy, promoted tumour cell survival, and regulates the activation of the PI3K/Akt and p38 signalling pathways. SAA thus plays an important role in breast cancer tumourigenesis.

EACR22-0309

The impact of MCPIP1 protein on tumor initiation and cancer stemness.

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Introduction

Developing new cancer treatment strategies is extremely difficult due to the problem of identifying cancer stem cells (CSCs) which are responsible for tumor initiation and progression. The MCPIP1 protein (Monocyte Chemotactic Protein-1 Induced Protein) is involved in the negative regulation of inflammation due to its RNase activity. A growing number of publications suggest that the MCPIP1 protein may influence the development of cancer by regulation of factors involved in angiogenesis, proliferation and cell death. The main aim of our research is to study the importance of MCPIP1 for the presence of factors involved in tumor initiation as well as acquirement of stemness features.

Material and Methods

To examine the effect of MCPIP1 in normal epithelial cell line TCMK-1 we transduced cells to downregulate MCPIP1 (shCtrl, shMCPIP1) or to overexpress its mutated, inactive form (pLIX PURO, pLIX D141N). We analyzed cells proliferation and clonogenicity, as well as the levels of CSCs markers by western blot, NGS and qPCR. Next, cells were injected subcutaneously into NOD-SCID mice to check if low level or mutation of MCPIP1 will induce tumor growth *in vivo*. Mice blood was analyzed for the presence of circulating tumor cells (CTCs) with flow cytometry.

Results and Discussions

We have shown that the low level or mutation of MCPIP1 increases the proliferation and clonogenicity of TCMK-1 cells. Moreover, cells with decreased level of MCPIP1 protein or D141N mutation were characterized by higher levels of the c-Met receptor, CD44 and c-Myc phosphorylation, which are the markers of CSCs. In addition, low level of MCPIP1 results in increased expression of the factors involved in epithelial to mesenchymal transition:

vimentin, Twist and Snail. The obtained results suggest that the loss of the MCPIP1 protein causes the acquisition of the characteristics of neoplastic cells. Next, we checked whether MCPIP1 low level in normal cells predisposes them to proliferate when administered to mice. We found that, D141N mutation caused the growth of large tumors with higher expression of Cd44, Klf4 and Myc and number of CTCs, while the control cells injected into mice developed only local fibrosis.

Conclusion

We believe that the MCPIP1 protein may be a marker of tumor initiation and play a key role in neoplastic transformation by regulating the changes in cell phenotype and levels of CSCs markers.

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EACR22-0349

INVESTIGATING LUNG SQUAMOUS CELL CARCINOMA DEVELOPMENT USING GENETICALLY ENGINEERED PRIMARY HUMAN BRONCHIAL BASAL CELLS

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Introduction

Lung squamous cell carcinoma arises from the accumulation of genetic driver alterations in bronchial basal cells. LUSC genomes lack obvious actionable mutations and present with a high degree of inter-tumour heterogeneity. Tractable, patient relevant models are needed to study LUSC pathogenesis and investigate the role of recurrent genomic alterations in driving cancer progression. Therefore, we intend to develop a tractable human model of LUSC progression to increase our understanding of the disease.

Material and Methods

The co-operation of ubiquitous LUSC aberrations (*TP53* and *CDKN2A* loss) and commonly deregulated pathways (*SOX2/P63*, *PI3K/AKT* and *KEAP1/NRF2*) was investigated by genetic engineering of primary human bronchial basal cells. This culminated in eight basal cell mutants harbouring LOF alterations in *TP53* and *CDKN2A* combined with additional pathway alterations. We investigated changes in basal cell proliferation and cell cycle dynamics. Furthermore, air-liquid interface organotypic cultures were used to model histological changes in the bronchial epithelium driven by different combinations of mutations. These cultures were analysed for changes in histology indicative of LUSC progression and for changes in the expression of bronchial epithelium markers using immunohistochemistry.

Results and Discussions

Increases in proliferation were driven by inactivation of *TP53*, *CDKN2A* and *PTEN*, whereas *SOX2* overexpression inhibited mutant basal cell proliferation. LUSC alterations also drove striking phenotypic changes in 3D organotypic cultures of the bronchial epithelium. All mutant cultures displayed a thickening of the bronchial epithelium and significantly increased cellularity indicating hyper-proliferation. *SOX2* overexpression inhibited mucociliary differentiation and drove the appearance of a considerably disordered epithelium with clear cellular and nuclear pleomorphism, indicative of high-grade premalignant lesions.

Conclusion

We developed a basal cell model of recurrent LUSC genetic alterations. This approach is unique due to [1] the use of primary human cells and [2] the representation of interpatient heterogeneity and developmental stages by introducing different combinations of pathway alterations. Early results indicate that basal cells carrying cumulative alterations display increasingly complex phenotypes with features of low and high-grade premalignant lesions. Further work is required to assess the tumorigenicity of mutant cells.

EACR22-0513

Elimination of cancer phenotype upon expression of Meig1 in cancer cells

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Introduction

Each cancerous process has "driver" events that initiate the process and confer selective growth advantage to the cells. A "driver" event can be a gain of function mutation in an oncogene or a loss of function mutation in a tumor suppressor. Identifying "driver" events is a key to understanding cancer development and developing new therapeutic approaches. Meig1 is an evolutionarily conserved gene, first identified in mouse spermatocytes undergoing meiotic recombination. We found that MEIG1 is an important component of the DNA damage response, and that Meig1-KO mice are predisposed to developing tumors, suggesting it is a tumor suppressor that its absence might contribute to genome instability and cancer.

Material and Methods

A simple nuclease assay consisted of incubating plasmid DNA in either whole cell protein extract, cytoplasmic extract or nuclear extract, from various cancerous or non-cancerous cells, followed by gel electrophoresis to monitor plasmid degradation. For soft agar colonies assay, 5000 cells were seeded in 0.7% agar in medium supplemented with Kanamycin. 2 weeks later, number and diameter of colonies were unbiasedly analyzed using a code specifically written for this purpose.

Results and Discussions

In a nuclease assay, whole cell extracts from all cancer/tumor cells, invariably, degrade plasmid DNA, whereas extracts from non-cancer cells do not. Cytoplasmic extracts from all cells digest plasmid DNA, whereas nuclear extracts do not. Increasing amounts of nuclear extracts, added to cytoplasmic extracts, rescue plasmid DNA, suggesting nuclease inhibitory activity in the nucleus. Nuclear extracts from cancer cells have a significantly reduced inhibitory activity. Intriguingly, recombinant MEIG1, when added to whole cell extracts from cancer cells, or expressed in cancer cells, consistently abrogate plasmid degradation, suggesting that **MEIG1 can overcome the cancerous phenotype** of increased nuclease activity. Anchorage-independent growth ability is a prominent characteristic of cancer cells, and often used to determine oncogenicity. In our experiments, overall number, and average size, of colonies were significantly smaller in Meig1 expressing cancer cells, compared to control cancer cells, suggesting again that **MEIG1 can significantly reduce the cancerous phenotype** of cells.

Conclusion

Aberrant expression of Meig1 might be a "driver" event for cancer development, as expression of a normal form of the protein eliminates at least some of the characteristic cancerous phenotypes.

Identification of carcinogenesis and tumor progression processes in pancreatic ductal adenocarcinoma using high-throughput proteomics

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive disease with an overall 5 year-survival rate of just 5%. A better understanding of both the carcinogenesis processes and the mechanisms of progression of PDAC disease is mandatory.

Material and Methods

Fifty-two PDAC patients treated with surgery and adjuvant therapy, with available primary tumor and normal tissue, preneoplastic lesions (PanIN), and/or lymph node metastases were selected for the study. Proteins were extracted from small punches and analyzed by LC-MS/MS using data-independent acquisition. Protein expression data was analyzed using probabilistic graphical models, allowing functional characterization. Functional node activities were calculated as the mean of expression of those proteins related to the main function of each node. Comparisons between groups were done using linear mixed models.

Results and Discussions

Three proteomics tumor subtypes were defined. T1 (32% of patients) was related to adhesion, T2 (34%) had metabolic features, and T3 (34%) presented high splicing and nucleoplasm activity. Relevant biological processes related to carcinogenesis and tumor progression were studied in each subtype. Carcinogenesis in T1 subtype seems to be related to an increase of adhesion and complement activation nodes activity, whereas tumor progression seems to be related to nucleoplasm and translation nodes. Regarding T2 subtype, it seems that metabolism and, especially, mitochondria act as the motor of cancer development. T3 analyses point out that nucleoplasm, mitochondria and metabolism, and extracellular matrix nodes could be involved in T3 tumors carcinogenesis. Identified processes were different among proteomics subtypes, suggesting that the molecular motor of the disease is different in each subtype, and pointing out new actionable targets in each subtype. Additionally, these proteomics subtypes were validated in the PDAC TCGA cohort.

Conclusion

Three proteomics subtypes were identified in PDAC: an adhesion-related subtype (T1), a metabolic subtype (T2), and a nucleoplasm subtype (T3). Biological processes involved in carcinogenesis and tumor development were established, being different in each subtype. These differences can have implications in the development of future tailored therapeutic approaches for each PDAC proteomics subtype.

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POSTER IN THE SPOTLIGHT

Early events of low-grade serous ovarian cancers modelled with organoids and single cell sequencing

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Introduction

Ovarian cancers are the most common gynecologic malignancies. Low grade serous ovarian carcinoma (LGSOC) is a rare tumor, accounting for 5% of all ovarian cancer cases. LGSOCs are characterized by high fatality rates over the long term, with only 10-20% of women surviving 10 years after diagnosis, due suboptimal response to current chemotherapies. LGSOC harbours a relatively stable genome, with common activating mutations in *BRAF*, *KRAS* and *NRAS*. *NRAS* mutation (Q61R) co-occur with *EIF1AX* mutations (G8E), and the two mutated proteins functionally cooperate. Low incidence of this disease means it is poorly understood, and the resulting lack of available models further limits the study of the underlying mechanisms. We therefore propose to utilize organoid cultures, which consist of 3D multicellular units that resemble *in vitro* a tissue or organ of body.

Material and Methods

To reflect genetic background and cell of origin of LGSOC, *NRAS* Q61R and *EIF1A* G8E mutant proteins were overexpressed via lentivirus in primary normal human Fallopian tube tissues. Gene expression alterations were resolved with single-cell RNA sequencing (scRNA-seq). Cytological signs of transformation of organoids were assessed. Patient-derived tumor organoids (PDTOs) were cultured to assess how well our LGSOC-modelling organoids (LMOs) recapitulate the histological features of patient tumours.

Results and Discussions

LMOs showed cytologic signs of transformation such as increased nuclear/cytoplasmic ratio, prominent nucleoli, cellular pleomorphism. Papillary structures, a major histologic characteristic of LGSOC tumor were also observed. PDTOs showed similar cytological features and organization as LMOs. We identified genes up-regulated in double-mutant compared to single-mutant organoids such as *CA125* and *TACSTD2*. *CA125* is one of the earliest identified biomarkers for ovarian cancer and has remained to be the most useful serum marker despite limited sensitivity and specificity; *TACSTD2* overexpression correlates with a chemo-resistant, aggressive malignant phenotype.

Conclusion

Organoid culture and scRNA-seq is a powerful duo in studying early tumorigenesis events. We established a novel model that largely recapitulated LGSOC cytology by introducing common co-occurring mutations into normal Fallopian tube tissues. Genes upregulated in double mutants included well-characterized biomarker (*CA125*) and a potential biomarker or therapeutic target (*TACSTD2*). Our work will be crucial for developing more targeted treatment options.

EACR22-0294

TOP ABSTRACT

Single-molecule sequencing of neurons reveals a high burden of cell-cycle independent somatic mutations

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Introduction

In recent years, large scale sequencing efforts have yielded significant insights into the process of somatic mutagenesis in normal tissues and cancer development. However, these endeavors have been largely restricted to mitotic tissues, where it is possible to detect mutations in clonal cell populations or in in-vitro colonies derived from single cells.

To overcome these limitations, we developed nanorate sequencing (NanoSeq), a duplex sequencing protocol with an error rate of <5 per billion base pairs that calls mutations in single DNA molecules. In this study we use NanoSeq to explore the mutational landscape of neurons and cardiac muscle to investigate how somatic mutations accumulate in the absence of cell division.

Material and Methods

Frozen tissue samples were collected through collaboration with the Cambridge Brain Bank, Memorial Sloan Kettering and Papworth Tissue Bank. Neuronal populations were isolated by flow sorting using the NeuN marker from multiple brain regions from 93 donors ageing from 4-weeks post-birth to 100-years old. Cardiac muscle was isolated from 17 donors using laser capture microdissection.

Results and Discussions

NanoSeq sequencing of neurons and cardiac muscle reveals a pattern of somatic mutations very similar to dividing cells. We show that post-mitotic cells have comparable somatic mutation rates to rapidly cycling tissues. Signature decomposition shows the linear accumulation of the clock-like signatures SBS1 and SBS5 with age demonstrating that these processes can occur in the absence of cell division.

Tissue specific signatures were seen in both cardiac muscle and neurons. Neurons have a higher burden of indels than other somatic tissues, with an enrichment of indels that are longer than 1 bp in highly expressed genes. This pattern resembles a mutational process that has been observed in cancer genomes and is linked to both base-excision repair and topoisomerase activity. Cardiac muscle biopsies from the donors of smokers have somatic mutation burdens in excess of 4000 substitutions per genome due to a smoking-like C>A signature.

Conclusion

The sequencing of post-mitotic tissues highlights the ability of NanoSeq to sequence and study the mutational landscape of any cell type. Importantly, our work shows that dominant mutational processes can occur independently of cell division. In addition, post-mitotic tissues shed light on distinct mutational processes previously seen in cancers and normal dividing cells.

EACR22-0096

Marine-derived chromomycin A5: a novel strategy for the targeted treatment of TBX2-driven rhabdomyosarcoma

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Introduction

Rhabdomyosarcoma (RMS) accounts for the majority of paediatric cancers. To date, localized RMS can be cured with multi-modal therapy, however, overall survival for patients with metastatic or recurring RMS has shown little to no improvement over the last five decades². Thus highlighting the need for developing novel and targeted therapies to treat RMS^{2,3}. TBX2, a T-box transcription factor is overexpressed in RMS and is required to drive oncogenic phenotypes such as promoting proliferation and bypassing cellular senescence. Importantly, the depletion of TBX2 inhibits these oncogenic phenotypes⁶ which suggest that targeting TBX2 may be a promising anti-RMS strategy. Recently, we and others have reported that chromomycin A5 (CA5), the marine derived compound and anti-tumour antibiotic, interacts with TBX2¹ and exerts cytotoxicity against a panel of murine and human cancer cell lines⁵. This study investigates and describes the anti-cancer activity of CA5 in RMS.

Material and Methods

Cell lines representative of the major RMS subtypes, alveolar (RH30) and embryonal (RD) RMS were treated with CA5, and the effects were assessed with an array of molecular and cell biology techniques such as MTT cell viability, clonogenic, and scratch motility assays, western blotting, immunocytochemistry, and qRT-PCR.

Results and Discussions

We show that at 48 hr treatment of RMS cells and non-malignant FG0 fibroblasts, CA5 has IC₅₀ values of 5 nM in RMS cells and ~10 in FG0 fibroblasts. CA5 therefore displayed selective cytotoxicity towards RMS cells with a selectivity index of 2⁴. Furthermore, we show that in RMS cells, CA5 depletes TBX2 protein levels, induces DNA damage, exhibits long-term cytotoxicity, and induces cell death via mitotic catastrophe and the apoptotic pathway.

Conclusion

Collectively, our data shows CA5 to display great potential as a targeted treatment of TBX2-driven RMS.

EACR22-0140

Tousled like kinases as a target in cancer therapy

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Introduction

The Tousled like kinases 1 and 2 (TLK1 and TLK2) are evolutionarily conserved Ser/Thr kinases that are required for genome stability and normal development in numerous organisms. Both kinases contribute to histone deposition by targeting the histone chaperone ASF1 and are regulated by the DNA damage response.

TLKs expression is ubiquitous and often elevated in human cancers with suppressed immune gene expression. In some cancer types, elevated TLK levels correlate with decreased disease-free survival. In cultured cells, TLK depletion causes replication stress, DNA damage and altered chromatin maintenance. In vitro, TLK depletion synergizes with checkpoint and PARP inhibition, and elicits an innate immune response that is dependent on

the Alternative-lengthening of telomeres pathway. The main aim of this study is to explore the therapeutic potential of targeting TLKs in cancer.

Material and Methods

We generated conditional mouse models where we can modulate TLK2 expression in developing breast cancer. We assessed primary tumor growth and metastasis and characterized the tumor microenvironment.

Results and Discussions

We first used the well-characterized Mouse Mammary Tumor Virus-Polyoma Middle Tumor-antigen (MMTV-PyMT) mouse model and showed that acute depletion of TLK2 in already formed tumors stalled primary tumor growth and reduced lung metastases. We also observed an increased early immune infiltration that includes activated T cells and higher levels of PD-1, which could potentially sensitize these tumors to immune checkpoint blockade.

To test this possibility, we developed syngeneic breast and prostate cancer systems that will allow us to combine TLK2 depletion with immune checkpoint blockade or chemotherapy therapies in vivo. We will use these systems to test the therapeutic effects of combining TLK2 depletion with anti-PD-1 and PARP inhibition.

Conclusion

We suggest that TLKs may be a viable target for cancer therapy, as their loss leads to replication stress and innate immune activation that may sensitize tumors to immune checkpoint blockade and PARP inhibitors, facilitating further tumor regression.

EACR22-0188

The effect of benzopyrene-mediated cellular senescence on malignant change of breast cancer.

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Introduction

The risk of assessment of chemicals are mainly focused on cancer initiation and promotion. On the contrary, despite the fact that most cancer deaths are due to recurrence and metastasis (malignant change), there is a lack of knowledge about chemicals-induced malignant change of cancer. The relationship between chemicals and cancer prognosis, recurrence, and metastasis has also begun to be reported epidemiologically. In this point of view, analysis of the effects of chemicals on malignant change of cancer is critical issue. Here, we focused on breast cancer, for which the association between smoking and malignant change of cancer has been epidemiologically reported. Here, we tried to identify the effect of chemicals on malignant change of cancer and its mechanism by utilizing benzopyrene (BP), which is contained in exhaust gas and cigarette smoke, as a model chemicals.

Material and Methods

In this study, we used ER positive breast cancer cell line, MCF7. Cell viability was evaluated by MTT assay. Moreover, cell proliferation was assessed by labeling cells with CFSE and

analyzed by flow cytometry. The expression of senescence was evaluated by western blotting and SA- β -gal staining.

Results and Discussions

Firstly, we confirmed the effect of BP exposure on cell proliferation. MTT assay and CFSE staining revealed that the cell proliferation of MCF7 cells was slowed down by exposing 0.1 and 1 μ M of BP for 72 hours. Therefore, to examine whether BP exposure cause cellular senescence in MCF7 cells, we analyzed the expression of cellular senescence markers. We found the upregulation of senescence markers such as p53, p21 after 72 hours exposure of BP. Additionally, the intensity of SA β -gal was also increased after exposure of BP, indicating that exposure to BP induces cellular senescence in MCF7 cells. To further explore the biological effect of BP exposure, we removed BP from culture medium of MCF7 cells and incubated for several weeks, and analyzed cell proliferative capacity. After several weeks, senescent-experienced MCF7 cells began to proliferate again and the growing speed was faster than that before BP exposure. These findings indicate that BP exposure might induce cellular senescence and contribute to malignant change of cancer.

Conclusion

In summary, BP induced cellular senescence in MCF7 cells and further induced more aggressive phenotypes of MCF7 cells after several weeks of incubation without BP. Now, we are working on the mechanisms how senescent-experienced cancer cells acquire aggressive phenotypes.

EACR22-0356

Targeting the DNA-repair chromatin-modifier protein SMYD3 as a potential epigenetics-based therapy for gastric cancer

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Introduction

The histone methyltransferase SMYD3 has an oncogenic role in various cancer types, making it a potential target for drug discovery. Its overexpression correlates with poor prognosis, cancer growth, and invasion, especially in gastric cancer (GC). Recent studies suggest that its oncogenic activity might also be mediated by its interaction with non-histone proteins. Its emerging role in the regulation of DNA damage response by promoting homologous recombination (HR) repair, suggests that its inhibition is an attractive therapeutic strategy in combination with common cancer therapies.

Material and Methods

I performed extensive characterization of the functional interaction between SMYD3 and HR proteins in GC cell lines. I investigated by pharmacological inhibition of SMYD3 and PARP, GC cell lines features as cell death response and proliferation.

Results and Discussions

I found that SMYD3 is overexpressed in 25% of human GC (TCGA Pan-Cancer dataset) and that high-SMYD3 and HR-proficient GC cells seem to become sensitive to PARP inhibition when combined with SMYD3 inhibition. I hypothesized that combined inhibition of SMYD3 and PARP could represent a valid strategy to induce cancer cell death as a synthetic

lethality approach in GC cell lines. I analyzed SMYD3 expression levels in human GC cells and it was significantly overexpressed in AGS, NCI-N87, and HGC-27. Next, I characterized the somatic mutations and copy number alteration data of key HR-related genes to assess the mutational status of GC cells and to identify two different subsets: HR-proficient and HR-deficient. To study the effect of SMYD3 inhibition on the HR pathway, I treated cells with a SMYD3i and/or Olaparib for 72h and assessed cell death response in single and dual treatments. My results confirmed the efficacy of combined SMYD3 and PARP inhibition in HR-proficient GC cells with high SMYD3 levels (AGS and NCI-N87), whereas no significant difference was observed in low-SMYD3 KATOIII. In particular, I found that combined treatment promotes apoptosis by activation of caspases and consequent cleavage of PARP.

Conclusion

Devising a therapeutic approach for gastric tumors expressing high levels of SMYD3, based on combined treatment with SMYD3 and PARP inhibitors, could have multiple benefits. In particular, it might help mitigating potential drug toxicity by lowering the overall dose needed, while concomitantly promoting GC sensitization.

EACR22-0480

Comprehensive assessment of gene mutations revealed overlapping dependencies for PARPi and chemotherapy response in ovarian cancer

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Introduction

PARP inhibitors (PARPi) have revolutionised the therapeutic landscape of epithelial ovarian cancer (OC) treatment with outstanding benefits in regards to progression-free survival, especially in patients carrying either BRCA1/2 mutations or harbouring defects in homologous recombination repair. Yet, it remains uncertain which PARPi to apply and how to select responders using clinical and molecular characteristics, especially in forefront therapy when platinum sensitivity is still unknown.

Material and Methods

Through a systematic literature review in combination with the exploration of publicly available CRISPR-Cas9 library screens and Genomics of Drug Sensitivity in Cancer (GDSC) data, we identified potential gene dependencies predicting PARPi response. Functional assessment was performed in six constitutively Cas9 expressing OC cell lines and subsequent examination of 33 genes using a CRISPR-Cas9 mutagenesis assay together with various PARPi and carboplatin during a cell culture for up to 24 days.

Results and Discussions

We identified CDK12 as a common vulnerability for OC cell survival suggesting a potential new candidate for targeted therapy. Regarding altered PARPi response, ATM was the only tested gene inducing olaparib sensitivity in a cell line-independent manner. Additionally, acquired olaparib sensitivity was also observed for MUS81, NBN, RAD51/B/C, RNASEH2A, PALB2, XRCC1, and XRCC3 in at least 3 out of 6 cell lines. Taken together, our functional screen identified ten novel potential biomarkers for PARPi response. As the major survival benefit of PARPi treatment was reported in chemo-sensitive tumours, we next assessed top candidate genes in combination with olaparib, niraparib, talazoparib, and carboplatin. Interestingly, we observed almost identical dropout rates in a gene- and drug compound-independent manner supporting the strong correlation of cancer cell response to DNA damaging drugs.

Conclusion

Our data suggest a general mechanism-of-action of PARPi and chemotherapy demonstrated by various overlapping gene dependencies. Genes identified as functional linked with PARPi sensitivity by our screen have been reported to be altered in up to 40% of OC patients, suggesting that a genetic screen of those genes may allow better stratification of patients with increased benefit to this treatment.

EACR22-0578

Targeted NGS for hotspot mutations in patients with multiple primary tumors reveals

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Introduction

Multiple primary tumors (MPTs) are defined as the presence of two or more synchronous or metachronous cancer in one patient. Survival of patients with MPT varies according to tumor type, genetic factors, environmental and lifestyle factors. To meet these challenges, modern medical science needs in-depth studies of the molecular genetic mechanisms leading to the development of MPT.

Material and Methods

We collected FFPE tissue of six patients with multiple tumors in penis/kidney (MPT016), breast/endometrium (MPT021), lung/liver (MPT023), ovary/stomach (MPT024), bladder/prostate (MPT034), and appendix/bladder/endometrium (MPT043). DNA was isolated from tumor tissue and NGS was performed with HotSpot Panel v2 on the Ion PGM System platform. The results were analyzed by Torrents Suite and VarSeq software.

Results and Discussions

In total 13 tumor probes were analysed for variants in hot spot regions of 50 genes associated with development on cancer. Based on ClinVar, COSMIC and VarSome database the discovered variants were classified into three groups – Pathogenic (13.46%), VUS (26.92%) and Benign (59.61%). Pathogenic variants were identified in four genes; c.35G>T(KRAS) in MPT021; c.35G>A (KRAS) and c.3140A>G (PIK3CA) in MPT043; c.586C>T (TP53) in MPT016 , c.455delC (TP53) in MPT023; c.1633G>A (PIK3CA) and c.746C>G (FGFR3) in MPT034. Rare variants of unknown clinical significance, but strong in silico predictions for pathogenicity were found in 4 patients and 12 genes. Interestingly, 7 mutations were found in the kidney tumor of MPT016 in addition to TP53 pathogenic mutation - c.3814G>A, c.3516-3C>T(ALK), c.1742-3C>T(BRAF), c.1015C>T (CDH1), c.2638G>A (ERBB2), c.421G>A (ERBB4), c.2038G>A (PDGFRA). Two mutations in RET were found in ovarian tumor/MPT024- c.2633G>A and c.2695G>A (RET); The remaining mutations were spread in lung/MPT0023 - c.2180G>A (JAK3); liver MPT023/ - c.1036G>A (TP53); In the three localizations of MPT043 mutations c.1416A>T (KDR) and c.3029C>T(MET) were found, while only in the appendix in addition c.1136C>T (SMARCB1) was seen.

Conclusion

This pilot targeted NGS for hot spot analysis showed specific profiles of somatic mutations in patients with MPT. The obtained results will help to elucidate the specific molecular genetic profiles and respectively the influenced signal-transduction pathways and networks leading to the development of MPT.

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EACR22-1022

A Rare Case of Sarcoma in Burns Scar

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Introduction

Cancer arising in longstanding burns scar is well established. Carcinoma of cutaneous lineage like squamous cell carcinoma, basal cell carcinoma, and rarely melanoma, are documented. Cancer arising from burns scar behaves differently from conventional carcinoma. Globally, only 14 sarcomas arising in the burns scar have been literally reported so far.

Material and Methods

A sixty year old male patient with complaint of growth over right upper back from six months presented to us. He had significant history of burns to that region in his childhood and repeated skin breakdown. After thorough evaluation, it was confirmed to be malignant. He underwent wide local excision with 2cm margin and reconstruction of the resultant defect with the thoraco-dorsal artery perforator flap. He completed a course of radiotherapy and is still surviving.

Results and Discussions

Histopathological examination of the specimen showed that it was a sarcoma specifically malignant fibrous histiocytoma. Mesenchymal cells are present in deeper dermis and subcutaneous tissue. Due to loss of skin, which is a protective barrier, the mesenchymal cells become prone to trauma resulting in faster turnover leading to higher chances of mis-transcription of DNA.

Conclusion

Sarcoma, though rarely reported in burns scar, can be more invasive and may require a different therapeutic approach. Indicating the growing need to study in detail the evolution of sarcoma in burns scar.

EACR22-1202

Prevalence of Aflatoxin B1 and loss of BRCA2 expression in Adenocarcinoma of the colon in Southern Nigeria

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Introduction Mounting evidence shows that there is a high prevalence of late-stage, aggressive, and metastatic adenocarcinoma of the colon (AdCC) in Africa, with high mortality and low survival rate among patients. Literature finger food contaminants such as mycotoxin, especially Aflatoxin, and mutations in DNA repair genes as attributable factors to the high incidence of abdominal cancers. However, there is a paucity of studies to confirm the hypothesis. Hence, this study aimed to determine the prevalence of Aflatoxin B1 and Breast Cancer type 2 Susceptibility gene (BRCA2) mutation, evidenced as loss of its expression, in AdCC in Southern Nigeria. **Material and Methods**

This retrospective study included 83 cases of AdCC observed in two health care facilities in Southern Nigeria between 2017 and 2020. Tissues were stained using histochemical and immunohistochemical techniques, scored, and documented as positive (+1, +2, and +3) or negative (0; loss of expression) for Aflatoxin B1 and BRCA antigen. Data were analyzed using descriptive, T-test, and Chi-square/Fischer square analyses.

Results and Discussions

The mean age of patients with AdCC was 47.34 ± 14.73 years while the mean age of females (57.1%) was slightly higher than their male (42.9%) counterparts (46.00 ± 4.50 years and 43.50 ± 6.63 years, respectively; $p = 0.752$). The prevalence of well, moderately, and poorly differentiated were 42.9%, 35.7% and 21.4%, respectively. The prevalence of Aflatoxin B1 and loss of BRCA2 protein was 62.7% and 25%, respectively. The prevalence of +1, +2, and +3 scores among BRCA2 positive patients were 27.8%, 27.8% and 44.4%, respectively whereas the prevalence of +1, +2 and +3 scores among Aflatoxin B1 positive patients were 48.1%, 36.5% and 15.4%. No significant relationship was observed between tumour grade and Aflatoxin B1, and BRCA2 mutation ($p > 0.05$). The combined effects of both Aflatoxin B1 and loss of BRCA2 protein had a stronger association with tumour grade compared with the individual effects of the factors. Additionally, the prevalence of BRCA2 loss in this study is higher than the prevalence that has been reported in other studies.

Conclusion

This study revealed a high prevalence of Aflatoxin B1 and loss of BRCA2 gene among patients with adenocarcinoma of the Colon in Southern Nigeria. Individuals with both toxin and BRCA2 mutation should be closely monitored for the development of the disease.

EACR22-0219

Treatment with a next generation PARP1 selective inhibitor potentiates the efficacy of carboplatin in pre-clinical models of ovarian cancer

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Introduction

Despite their success in the clinic, treatments with currently approved poly(ADP-ribose) polymerase inhibitors (PARPi) lead to adverse events that have limited their therapeutic potential, especially when combined with already poorly tolerated chemotherapeutic agents.

Recently it has been suggested that the inhibition of only PARP1 is required for antitumor activity, while it appears that the action on other PARPs may be responsible for adverse effects.

This hypothesis led to the development of next generation inhibitors with improved selectivity for PARP1, among them the potent inhibitor and trapper AZD5305.

Here, patient-derived ovarian cancer xenografts (OC-PDXs) were investigated to assess whether malignant progression could be impaired by the combination of AZD5305 with carboplatin (CPT), a standard-of-care treatment for ovarian cancer patients.

Material and Methods

Three OC-PDXs with different response to platinum(pt)-based therapies were implanted subcutis or orthotopically in nude mice to evaluate the effect of treatment on tumour growth and metastatic spread in the peritoneal cavity. AZD5305 was administered orally (po) once daily (QD) 5 days ON/2OFF. CPT was dosed intravenously once a week for four weeks (Q7x4).

Plasma levels of AZD5305 were measured; the reduction of PARylation in tumours was investigated to confirm target engagement.

Results and Discussions

AZD5305 plus CPT combination therapy outperformed single agents efficacy in all the OC-PDXs tested. Specifically, subcutaneously growing HOC106 tumours experienced tumour regression that persisted for at least 4 weeks after therapy withdrawal. Notably, greater efficacy was also evident against the poorly pt-responsive HOC107 tumours, where the combination therapy stabilized tumour growth even at doses that on their own were not effective.

Most importantly, the combination therapy impaired the metastatic dissemination and significantly prolonged the lifespan of HOC22 bearing mice (orthotopically implanted). In this model, improved efficacy was particularly evident when CPT was administered at suboptimal doses, which were either poorly or not effective as monotherapy.

All the doses and schedules used in this study were well tolerated.

Conclusion

Our pre-clinical data demonstrate that the combination therapy of CPT and AZD5305 was well tolerated and drove superior efficacy compared to monotherapies of both agents, providing an opportunity to maximize clinical benefit for this class of anti-cancer agents.

EACR22-0761

Genome segmentation by DNA damage repair dynamics

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Introduction

Somatic mutagenesis results from the interplay between DNA damage and repair, both of which are influenced by genomic and chromatin features. Previous attempts to characterize somatic mutagenesis have used chromatin features as surrogates of mutation rate, thereby leading to genome segmentations encoding distinct chromatin states that can be associated with different mutation propensities. With the availability of DNA damage and repair sequencing at different time points after exposure to the mutagen, we are now in a position to directly characterize the damage and repair interplay that results in mutations.

Material and Methods

To explore this issue, we leverage publicly available data from experiments mapping UV light damage and repair at different time points after UV exposure (Hu et al. 2017, Adar et al. 2016). In our direct approach to studying damage and repair, instead of focusing on segmenting the genome by chromatin features, we propose an alternative approach to partition the genome into “repair states” according, solely, to the dynamics of the DNA repair. We take an autoregressive modeling perspective, resorting to Hidden Markov Models and Bayesian nonparametric extensions which allow for automatic inference of the most parsimonious set of repair states. We explored different models and sets of states by looking at different types of UV damage involving different di-pyrimidines to gain information on similarities and differences between how they are repaired.

Results and Discussions

The modeling uncovered the spectrum of repair dynamics differing by speed, the acceleration point of repair, and dynamics of the damage types. When we finally cross the states with different (epi)genomic features, we find that this spectrum of UV repair states reflects a continuum of the features’ changes. We explore and name the states starting at slow & late repair in repressed, packed, inaccessible regions and ending in fast & early repair in expressed, transcribed, active regions of the genome.

Conclusion

This work sets a framework for a comprehensive analysis of the interplay of DNA damage and repair and the epigenome and understanding how those complex mutational processes lead to mutations. While we focus on the UV data, in the future, this approach will allow us to explore if the damage caused by other mutagens and their subsequent repair dynamics can be explained by similar or different factors.

EACR22-1101

MEIG1 is an important component of the DNA damage response

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Introduction

A hallmark of cancer is genome instability leading to various genetic changes. In fact, each cancer patient has a unique combination of genetic changes, and even within a specific tumor, there is an intra-tumor heterogeneity. This, of-course, challenges the ability to apply one efficient treatment to different cancers. Meig1 is a highly conserved gene, first identified in mouse spermatocytes undergoing meiotic recombination. Meig1-KO male mice have an aberrant ability to properly repair the physiologically induced DNA breaks that enable recombination, leading to massive apoptosis and lack of mature sperm. Increased occurrence of tumors has been observed in Meig1-KO mice, and we hypothesized that this might be due to delayed or aberrant damage response that increases genome instability.

Material and Methods

Mouse NIH-3T3 fibroblasts, and CRISPR/CAS9-induced Meig1-KO fibroblasts, were exposed to different DNA damaging agents and the DNA damage response was compared between the two cell populations. Various ways were utilized to monitor DNA repair, including following γ H2AX and 53BP1 foci kinetics, damage site labeling analysis and by applying a NHEJ assay.

Results and Discussions

Cells were treated with Phleomycin (13 μ g/ml 2h) to cause DNA Double strand breaks, and we found that the kinetics of γ H2AX foci disappearance during recovery was significantly

slower in Meig1-KO cells, compared to control cells. Likewise, formation of 53BP1 foci was hampered, and foci disappearance was significantly delayed, in Meig1-KO cells compared to control cells. Using the damage site labeling technique one can follow the repair kinetics of oxidative damage by counting dots (damage sites)/Mbp of DNA over time. When this technique was applied to follow the repair of oxidative damage (H₂O₂ 100μM 5h) in Meig1-KO cells vs. Meig1-KO cells expressing recombinant Meig1, it was evident that repair was significantly more efficient in Meig1 expressing cells. In a NHEJ assay where a break was introduced into a GFP expressing plasmid in a way that GFP could be expressed in cells only upon repair of the break, we found that Meig1-KO cell had a significantly reduced ability to repair the plasmid, compared to control cells. Moreover, expressing recombinant Meig1 in these KO cells restored the repair ability to control levels.

Conclusion

The MEIG1 protein is an important component of the DNA damage response.

EACR22-0786

HPV integration drives local chromatin reorganization and oncogenic activation in cervical cancers.

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Introduction

Human papillomavirus (HPV) infections are the primary drivers of cervical cancers, and in most cases, the HPV DNA gets integrated into the host genome. Although the oncogenic impact of viral genes (such as E6/E7) expressed from the HPV genome has been well studied, the impact of HPV integration on host chromatin structure and gene regulation is not yet fully understood. In this study, we addressed this limitation through cancer genomics data analysis, combined with chromatin interaction assays.

Material and Methods

We analysed the HPV integration sites from available genome-wide profiling of cervical cancers, together with chromatin states information from cancer (HeLa) and normal (NHEK) cell lines, to study the genome-wide enrichment of HPV integration. Further by integrating the gene expression data from the matched tumor samples, we correlated the impact of HPV integration on neighboring gene expression. Further, 4C-seq experiments in HeLa cells (which have HPV18 integration) were done to identify the host regulatory regions that are interacting with the HPV DNA.

Results and Discussions

At first, we find that the HPV integration is preferentially enriched in active chromatin regions, and negatively enriched in repressed regions. Surprisingly, we observed that the transcriptional activity immediately adjacent to the HPV integration region is relatively higher (as compared to non HPV-integrated samples) regardless of whether the HPV integration was in active or inactive regions. This suggests that the HPV integration could alter the host chromatin state and enhance gene expression locally. In addition, we find that the HPV integrations are recurrent in certain topologically associating domains (TADs) and affect the expression of oncogenes (such as MYC, TP63, ERBB2) within them. To explore this further, whether the integrated HPV DNA itself have any cis-regulatory effect, we performed 4C-seq experiments in HeLa cells and observed looping interactions between integrated HPV DNA

and nearby MYC/PVT1 regions, leading to overexpression of these genes in an allele-specific manner.

Conclusion

Taken together, these results show that the integrated HPV DNA can promote oncogenesis through alterations in the local chromatin structure and dysregulation of host oncogenes, in addition to the oncogenic activity of HPV E6/E7 genes. Based on these findings, we proposed that the HPV integration is a strong driver and triggers multiple modes of oncogenic activation (both cis and trans) that promotes cancer progression.

EACR22-1122

Human papillomavirus 42 is an oncogenic driver in Digital Papillary Adenocarcinoma

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Introduction

Up to 15% of human cancers are caused by microbial pathogens, most notably by viruses that interfere with host signaling pathways. Prominent examples include oncogenic human papillomavirus (HPV) in cervical carcinoma and head and neck cancer or Merkel cell polyomavirus in Merkel cell carcinoma. International consortia such as The Cancer Genome Atlas (TCGA) and the International Cancer Genome Consortium have mapped the genomic landscape of common cancer types and analyzed the contribution of viral pathogens. Rare cancer types, however, have not been systematically screened for viruses, potentially missing viruses as oncogenic drivers.

Material and Methods

We screened 214 specimens from 19 rare skin tumor types for the presence of viral pathogens using a metagenomic classifier together with a database covering 6,453 viruses. To detect HPV42 and other HPV types in 33 common cancers, we assessed raw RNA-seq data of 11,091 tumor samples from the TCGA project. We assessed the oncogenic activity of HPV42 by performing transformation assays in primary cells, investigating the interactome of HPV42-E6 and -E7 oncoproteins, and profiling transformed cells in a multi-omics approach. To identify a conserved transcriptomic fingerprint of oncogenic HPV transformation, we employed a machine learning approach on differentially expressed genes between HPV+ and HPV- tumors across cervix and head and neck cancer.

Results and Discussions

We identified HPV42 in 96% of digital papillary adenocarcinoma (DPA) and showed that HPV42 integrates into the genome of DPA tumors, keeping the E6 and E7 oncoproteins intact. The HPV42-E7 protein shares the cellular targets RB1 and PTPN14 with oncogenic HPV types and is sufficient to induce transformation *in vitro* and tumor growth *in vivo*. We did

not identify HPV42 in any of 11,091 TCGA tumors, indicating a cell lineage-specific role of HPV42-driven transformation for DPA. Finally, we found a fingerprint specific for HPV-driven oncogenic transformation, which is present in DPA tumors and supports HPV42 as an oncogenic driver of DPA.

Conclusion

We identified HPV42, a virus previously described in benign anogenital warts and currently categorized as non-oncogenic (low-risk) HPV, as an oncogenic driver in DPA. HPV42 shares the molecular hallmarks, a transcriptomic fingerprint, and cellular targets with oncogenic HPVs and will serve as a strong diagnostic marker for DPA. HPV42 should be included in routine diagnostic HPV testing panels and future HPV vaccination programs.

EACR22-0134

The junctional protein Afadin: from mitotic spindle to intestinal organoid in a Wnt dependent manner

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Introduction

Colorectal cancer (CRC) is one of the deadliest human malignancies in which the high relapse rate is increased by the high frequency of cancer stem-like cells (CSC). Aberrancies in stem cell proliferation and epithelial polarity in the intestinal epithelium are key events in the generation of CSCs. The Wnt signaling pathway is a critical regulator of intestinal stemness, orchestrated by Wnt3-secreting Paneth cells acting as niche. Interestingly, recent studies identified a role for Wnt signals in oriented asymmetric stem cell division, suggesting an implication in self-renewal in the crypts. However, the Wnt-related molecular mechanisms governing intestinal stem cell (ISC) divisions and how they are deregulated in CRC remains largely unclear.

Material and Methods

I am using intestinal organoids grown from murine intestinal cells. Single cells are plated on matrigel with specific media to allow organoids' growth. To induce knock-down (KD) of Afadin, single cells are infected with lentiviruses expressing sh-RNA targeting Afadin. After 5 days, infected organoids are evaluated by qPCR analysis, fixed and live imaging to monitor growth and differentiation grade, and RNAseq analysis.

Results and Discussions

The ablation of Afadin leads to defective morphogenesis of intestinal organoids derived from wild-type mice. These defects are characterized by a significant decrease in the differentiation rate and a reduced proliferation. Consistently, transcriptional analysis of Afadin-depleted organoids shows differences in the regulation of specific pathways (such as apical junctions and mTOR pathway) suggesting that absence of Afadin influences the transcriptional profile of organoids. In addition, we observed impairment in the mitotic spindle orientation of dividing cells in the intestinal crypts, in agreement with the described role of Afadin in spindle positioning. Lastly, we obtain preliminary data showing that these defects are less pronounced in organoids derived from APC^{-/-} mice, in which the Wnt pathways is constitutively active, hinting at the possibility that Afadin could be implicated in correct differentiation of intestinal cells.

Conclusion

Our data suggest that Afadin plays a role in the molecular machinery orchestrating Wnt-dependent planar divisions and transcriptional programs in intestinal organoids. Importantly,

our results provide molecular information on niche-regulated ISC divisions that maintain epithelial morphogenesis under physiological conditions and are likely deregulated in neoplastic conditions.

EACR22-0318

Right on time to defeat cancer: investigating the connection between the circadian rhythm and lung tumorigenesis.

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Introduction

Lung cancer is one of the most prevalent reasons for cancer-related death worldwide. The circadian system is a mechanism allowing to anticipate changes in geophysical time. Emerging studies provide evidence in favor of an interconnection between cell cycle and circadian clock in mammalian cells. Upon malignant transformation, both the cell cycle and the circadian clock undergo significant changes, although the causality stays unclear in most cases. We thus aimed to identify molecular mechanisms underlying the connection between circadian clock disruption and lung tumorigenesis in humans.

Material and Methods

A549, A427, H1264 and H727 cell lines corresponding to different subtypes of non-small cell lung cancer (NSCLC) were analyzed for molecular clockwork and compared to normal human bronchial epithelial cells (HBEC3-KT). Cells were transduced with *Bmal1*-luciferase (*luc*) and *Per2*-*luc* lentivectors, synchronized in vitro with dexamethasone, and circadian bioluminescence was recorded at population and single cell levels. In parallel, confluence was monitored in an IncuCyte®. The impact of siClock-mediated clock perturbation and of clock modulators on cancer cells was evaluated by circadian bioluminescence recording, IncuCyte® live cell analysis and monitoring of the cell cycle by Fluorescence Ubiquitination Cell Cycle Indicator (FUCCI), as well as MTT assay.

Results and Discussions

NSCLC cells bore circadian rhythmicity that were distinct from the non-malignant counterpart and varied among different cell lines. A549 cells showed regular rhythm until they reached stationary phase leading to an abrupt drop in the expression of *Bmal1-luc* and *Per2-luc*. Knockdown of *CLOCK* gene in A549 cells decreased their proliferation, with certain clock modulators mimicking this effect.

Conclusion

The identified alterations in rhythmicity among various NSCLC cell lines underline the heterogeneity of lung cancer and demonstrate the need for a tailored approach to each lung cancer subtype and potential application of core clock alterations for diagnostics of lung cancer. Our data showed suppressed cancer cell growth following clock disruption and identified core-clock components as novel promising therapeutic targets. Further mechanistic studies in cell lines, as well as the characterization of core-clock component changes in primary lung cancer cells established from patient biopsies will have direct implications for current efforts on identifying novel therapeutic targets and on improving the diagnosis of these malignancies.

EACR22-0450

Investigation of common coregulators between the Serum Response Factor and the Androgen Receptor identifies novel therapeutic targets in castrate resistant prostate cancer.

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Introduction

Advanced prostate cancer (PCa) is managed using androgen ablation therapy (ADT), including anti-androgens such as enzalutamide which target the androgen receptor (AR). Although initially very effective, nearly all patients develop resistance to ADT. Therefore, novel therapies which do not target AR directly and therefore avoid the AR-related resistance, are urgently needed. One strategy is to target AR's co-regulators such as the Serum Response Factor (SRF). SRF is a transcription factor which plays an important role in many cellular mechanisms relevant to cancer, including cytoskeleton organization, cell invasion and migration. We previously showed a synergistic effect following concomitant SRF and AR inhibition and identified a crosstalk between SRF and AR. The aim of this study was to investigate the molecular determinants of the SRF/AR relationship.

Material and Methods

LNCaP Abl cells, a model of castrate-resistant PCa (CRPC), were treated with a combination of 3 SRF inhibitors (CCG1423, CCG257081 and Lestaurtinib) and enzalutamide. The cell cycle was studied using WB and flow cytometry. To identify the key players involved in the SRF/AR crosstalk, we performed co-immunoprecipitation (co-IP) of SRF and AR, followed by mass spectrometry (MS). Common interactors with both AR and SRF were identified using label-free quantified values.

Results and Discussions

Decreased S phase and cell cycle arrest at the G1 phase were observed following inhibition of SRF with all three inhibitors. This was confirmed by WB, which showed a significant increase in p27 and a decrease in CDK4. Biological replicates for SRF and AR pulldowns were run on a timsTOF MS. Downregulation of either SRF or AR, using siRNA, was used to exclude non-specific binding from the MS. A ratio of the protein of interest/knockdown was analysed using the label free quantification values. We got 140 hits for SRF and 101 for AR, with 51 being upregulated in SRF and 80 in AR. 9 proteins were identified which co-precipitated with both AR and SRF. These included nuclear-binding protein Ran and HSP90 alpha subunit. Ran is a cofactor of AR involved in AR nuclear transport and colocalises with

RhoA, which is a cofactor of SRF. HSP90 is an important AR chaperon, crucial for its transcriptional activity.

Conclusion

Our co-IP and MS approach identified common interactors between SRF and AR, which will explain the relationship between SRF and AR and provide a pipeline of future therapeutic targets for CRPC.

Cancer Cell Biology

EACR22-0494

ATIP3 deficiency sensitizes breast cancer cells to WEE1 inhibition

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Introduction

Breast cancer is a leading cause of death by malignancy in women worldwide. Although the 5-year survival rate is of 87%, some breast cancers are of bad prognosis. These cancers, classified as triple-negative breast cancers (TNBC), are heterogeneous, highly proliferative and metastatic tumors that lack expression of biomarkers, rendering them ineligible for targeted therapies. TNBC is only managed with chemotherapy that benefits to 30% of patients, the others being chemoresistant. It is therefore important to identify molecular targets that may be effectively targeted in such cancers. Our team has identified ATIP3 as a prognostic biomarker in breast cancers. ATIP3-deficient TNBC are of worst prognostic and represent the most aggressive breast cancers, as they have high levels of aneuploidy.

Material and Methods

In a quest for coupling this biomarker to a targeted therapy, we rationalized that coupling the deficiency of ATIP3 to the inhibition of a cell cycle kinase would cause massive genomic instability and cell death, rendering the ATIP3 population vulnerable to the inhibition of such a kinase. We screened a total of 28 different cell cycle kinase inhibitors on TNBC spheroids expressing or not ATIP3 by evaluating their effect on cell viability, enabling us to identify a WEE1 inhibitor as a differential hit. Then, using immunofluorescence and fluorescence microscopy methods, we evaluated the effects of WEE1 inhibition on ATIP3 deficient cancer cells. Using different molecular methods, we showed how ATIP3 deficiency sensitizes breast cancer cells to WEE1 kinase inhibition.

Results and Discussions

We identified AZD1775, a WEE1 inhibitor, as a differential hit that induces higher mortality rates in ATIP3 deficient TNBC spheroids. ATIP3 deficient cells entered mitosis massively and more rapidly in response to WEE1 inhibition. WEE1 inhibition also induced higher rates of mitotic errors leading to elevated cell death rates in ATIP3 deficient cells in comparison to cells that express ATIP3. In addition, we found that ATIP3 deficient cells present low endogenous levels of replication stress that are exacerbated by WEE1 inhibition. This replication stress coupled to an early entry into mitosis causes massive cell death of ATIP3 deficient TNBC cells.

Conclusion

Thus, this study first suggests targeting WEE1 kinase as potential targeted therapeutic approach for a ATIP3 deficient breast cancers, explores the effects of WEE1 inhibition and the mechanisms by which ATIP3 deficient cells are more sensitive to this inhibition.

EACR22-0496

Synergistic effect of sorafenib in combination with naproxen on hepatocellular carcinoma cell lines

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Introduction

Hepatocellular Carcinoma (HCC), which develops on a background of chronic liver inflammation, is one of the most prominent primary liver cancer types and a leading cause of cancer-related deaths worldwide. Due to the resistance to targeted therapies, combination therapy has become an important strategy to increase treatment efficacy. Recently, non-steroidal anti-inflammatory drugs (NSAIDs) possess anti-cancer activity in many cancer types. This study aimed to find a synergistic interaction between sorafenib and different NSAIDs and identify their mechanism of action.

Material and Methods

Cellular cytotoxicity of sorafenib in combination with naproxen, aspirin, ibuprofen, and flurbiprofen was determined by NCI-SRB assay on HCC cell lines. Results were analyzed by SynergyFinder tool, and synergistic doses were determined accordingly. Growth inhibition was monitored by real-time cell electronic sensing (RT-CES) system. Cells were stained with propidium iodide (PI) for cell cycle analysis or with Annexin-V/PI for cell death analysis using flow cytometry. Western blot analysis was performed to determine changes in the expression of proteins that play critical roles in HCC development and prognosis.

Results and Discussions

Among all combinations, sorafenib and naproxen synergistically inhibited the growth of HCC cell lines. Naproxen alone had no significant effect on cell viability in concentrations below 400 μ M. In both epithelial-like Huh7 cells and mesenchymal-like Mahlavu cells, the combination of 5-10 μ M sorafenib and 300-400 μ M naproxen resulted in significantly higher growth inhibition compared to the effect of sorafenib alone. Moreover, upon combined treatment, apoptotic cell death was increased, and G1 arrest in HCC cell lines was enhanced compared to cells treated with sorafenib or naproxen alone. Lastly, PI3K/Akt pathway activity was suppressed upon combinatory treatment, where phospho-Akt and its downstream elements were significantly decreased in western blot analysis.

Conclusion

Altogether, our results have shown for the first time that naproxen synergistically enhances cytotoxicity of sorafenib on HCC cell lines, which could be considered as a potential therapeutic option for HCC patients.

EACR22-1130

Role of the calcium channel TRPV6 in the development and progression of pancreatic cancer

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a major cause of cancer-associated mortality in Western countries. Prevention is exceedingly difficult since patients rarely exhibit symptoms and currently no sensitive and specific tumor markers are available for early diagnosis at a curable stage. PDAC is inherently linked to the unique physiology and microenvironment of the exocrine pancreas such as pH, mechanical stress, and hypoxia. On the other hand, Ca²⁺ signals are emerging to be particularly relevant in cancer. They mediate interactions between tumor cells and the tumor microenvironment to drive different aspects of cancer progression. Mutations or aberrant expression of key proteins that control Ca²⁺ levels can cause deregulation of Ca²⁺-dependent effectors to promote cancer progression. Increasing evidence suggests that the overexpression of the Ca²⁺ channel TRPV6 is a common event in cancers of epithelial origin. Here we studied the role of TRPV6 channel in PDAC cells.

Material and Methods

After analyzing the channel expression in several PDAC cell lines, we prepared four different stable clone models from Panc-1 cell line. These clones either under or overexpressed TRPV6. Our work sought to elucidate the potential role of TRPV6 in cell viability, proliferation, cell cycle progression and apoptosis resistance. In order to evaluate these, we used recognized techniques such as MTS cell survival assays, cell count and ATP quantification. Furthermore, we assessed apoptosis resistance through cell cycle assay (using sub-G1 peak quantification) and TUNEL assay.

Results and Discussions

We demonstrated that cancer cells deficient in TRPV6 have a less aggressive phenotype, through the analysis of cell proliferation and ATP levels. They are also more prone to cell death through apoptosis, which was studied using cell cycle assay, with focus on the sub-G1 phase, and TUNEL assay. Contrasting with these results, overexpression of the TRPV6 channel led to increased cell proliferation and cell viability, measured by cell count and MTS assay. Further studies will focus on the mechanistic actions by which TRPV6 is affecting PDAC cells, by both *in vitro* and *in vivo* studies.

Conclusion

Overall, our results suggest that TRPV6 plays a role in the modulation of PDAC cell phenotype on proliferation, cell survival and apoptosis resistance.

EACR22-0029

NOVEL SYMMETRIC DERIVATIVES OF CARBAZOLE WITH A DUAL ANTICANCER MECHANISM OF ACTION: INHIBITION OF DNA TOPOISOMERASE II α AND PROTEIN TYROSINE KINASES

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Introduction

In cancer therapy, protein tyrosine kinases and DNA topoisomerase II α are substantial anticancer targets. DNA topoisomerase II α is involved in replication, transcription, and chromatin remodeling, therefore it is a crucial target in fast proliferating cancer cells. Protein

tyrosine kinases play a vital role in the process of signal transduction and therefore are responsible for the regulation of the cell cycle, differentiation, growth, and apoptosis of cells. The control (normalization) of the oncogenic forms of protein tyrosine kinases can be essential in the treatment of cancer with molecular targeted therapy. This study aimed to determine the mechanism of action responsible for high cytotoxic properties of novel symmetric derivatives of carbazole.

Material and Methods

The cytotoxic activity of compounds was determined against human lung cancer cell lines (A549, H226, H460) and colorectal tumor (HCT-116) using the MTT test after 72h treatment with compounds. The cell cycle progression and apoptosis induction were defined using flow cytometry assays. Each carbazole derivative initially was tested by a tyrosine kinase assay kit to investigate the *in vitro* effect of compounds on phosphorylation of immobilized peptides mediated by total cellular protein tyrosine kinases (PTKs). To confirm *in vitro* results, the phosphorylation of PTKs was investigated by flow cytometry after treatment of non-small cell lung cancer A549 cells with studied compounds. The inhibition of human DNA topoisomerase II α was explored using *in vitro* DNA relaxation assay.

Results and Discussions

We found that examined compounds possess excellent cytotoxic properties against lung and colorectal tumors with an IC₅₀ value in the nanomolar range. Treatment with these compounds halts cell cycle progression in the G1 phase in the lung cancer cell line, respectively. Cell cycle arrest induced by tested compounds was followed by massive apoptosis. Results of the DNA relaxation assay revealed that carbazole derivatives inhibit DNA topoisomerase II α in the micromolar range. The total activity of protein tyrosine kinases was significantly inhibited both *in vitro* and *in vivo* by studied compounds in similar ranges.

Conclusion

The presented studies show that newly synthesized symmetric carbazole derivatives exhibit promising antitumor properties. Dual targeting crucial cancer features, like unlimited DNA replication (topoisomerase) and sustaining proliferative signaling (RTK) could contribute to the specific eradication of cancer cells.

EACR22-0097

POPX2 phosphatase and its interactome in regulating cancer cell biology

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Introduction

Protein phosphorylation regulates a myriad of signalling pathways which impact on many cellular functions. Here we discuss the role of POPX2 phosphatase in the regulation of cancer cell biology. POPX2 belongs to the PP2C family of serine-threonine phosphatases. The levels of POPX2 have been found to be higher in many invasive and aggressive cancers. Some of its known substrates including PAK and CaMKII kinases have also been implicated in the control of cell proliferation, migration and invasion. We are interested in the study of POPX2 function through its interactome. Thus far, using different approaches, we have identified POPX2 interacting proteins which are known regulators of the cell cycle, apoptosis and the Hippo pathway. We have now begun to piece together the different roles of POPX2 in cancer progression. In this report, we determine POPX2's role in cell cycle control through its interaction with Checkpoint Kinase 1 (Chk1), a kinase activated to regulate cell cycle checkpoint upon DNA damage.

Material and Methods

Using the bioinformatics approaches that we have developed and adopted, Chk1 was identified as a potential interacting partner of POPX2. We further validated protein interaction through pulldown, western blot analysis and immunoprecipitation of endogenous protein complex. *In-vitro* phosphatase assays were performed to determine if Chk1 was a substrate of POPX2. Cells were treated with a topoisomerase inhibitor, VP16, to induce DNA double stranded breaks and damage. We then monitored cell cycle progression of control, POPX2-over expressing and POPX2-knockdown cells in normal versus DNA damage conditions.

Results and Discussions

We have identified Chk1 as a POPX2-interacting protein. Chk1 is also found to be a substrate of POPX2. When the cells suffer DNA damage, the ATM/ATR kinases are induced to phosphorylate Chk1 at Ser317 and Ser345. Chk1 will then become activated and initiate the checkpoint at G1-S transition to prevent replication of the damaged DNA. In the presence of high POPX2 levels, Chk1 phosphorylation at Ser317 and Ser345 is reduced in response to VP-16 treatment. As a result, the G1-S checkpoint is mis-regulated.

Conclusion

In this study, we have proposed an innovative two-pronged bioinformatic approach to identify interacting partners of POPX2 phosphatase. We have also validated Chk1 as a substrate of POPX2. In cancer cell types with high POPX2 levels, Chk1's activation in response to DNA damage is affected and resulted in mis-regulation of the G1-S cell cycle transition.

EACR22-0417

Significance of the Q61R and Q61K HRAS mutations in thyroid cancer: from in silico discovery to in vitro and in vivo testing.

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Introduction

Thyroid cancer (TC) incidence and mortality are increasing globally. Combined analyses of different molecular data might drive the discovery of new markers for better discrimination of the low-risk disease that may evolve in high-risk disease. The RAS mutation is the second most genomic lesion in TCs, associated with a good prognosis in TC, and has been explored as a marker in TC nodules evaluation. In this study, we explore the biological difference of Q61K and Q61R mutation of HRAS. We found promising differences in silico, in vitro, and in vivo.

Material and Methods

We clustered (PMID: 33560368) 505 human samples of the THCA study from TCGA. We studied the DEGs between the natural clusters and got gene profiles for functional analysis. We applied the eeScore methodology (PMID: 29323298) to get the gene profile of Q61K versus Q61Rmutated samples and to uncover the transcription factors (TF) associated with the two mutations. In silico results were verified at molecular and phenotypic levels in immortalized rat thyroid follicular cells(FRTL5) reversibly over-expressing Q61R and Q61K mutated RAS (TET-ON system). The xenograft approach in zebrafish embryos has been used to verify differences in cancer progression.

Results and Discussions

We confirmed the canonical subgrouping of TCs in BRAF-like and RAS-like, but a new natural group of mixed samples emerged. A deeper study of the RAS-like cluster revealed two sub-groups: one collecting samples with no RAS mutation and the other with mutually exclusive NRAS and HRAS mutations. The last included only Q61 mutated in R (N=10) or K (N=3). Several DEGs between Q61K and Q61R suggested a possible difference in cancer progression, confirmed by reversible over-expression of both in FRTL-5. The increased proliferation of Q61R cells vs Q61K emerged only after 6 days of induction whereas the increase of the (S+G2-M) cell fraction in Q61R vs Q61K cells was evident also after 3 days. The thyroid differentiation markers (NIS, PAX8, etc.) were reduced in both cell lines, although more striking in Q61R cells. TF study suggested a role for GLI2, concordantly found increased in Q61R cells. Finally, in zebrafish embryo xenograft assay, a time-dependent increase of cell proliferation and migration was observed only in embryos injected with Q61R.

Conclusion

Although other experiments are needed, our data suggest exploring the molecular characterization of RAS mutation to better discriminate more aggressive RAS-mutated TCs.

EACR22-0455

Investigating NVP-BEZ235 on aggressive subtypes of Lung Cancer

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Introduction

Lung cancer is an aggressive and rapidly metastasising malignancy. It has an overall 5-year survival rate of only 15%. Lung cancer is classified into: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). While, NSCLC is the most common form of lung cancer, SCLC is the most aggressive type.

The PI3K/AKT/mTOR pathway is one of the most dysregulated cascades in cancer associated with cell proliferation, angiogenesis & metastasis. Furthermore, aberrant PI3K/mTOR signalling has been reported in lung cancer. NVP-BEZ235 is a PI3K/mTOR inhibitor that targets PI3K, mTORC1 and mTORC2. In this research, the effect of NVP-BEZ235 has been investigated in NSCLC and SCLC cells.

Material and Methods

NVP-BEZ235 was investigated on H460 (NSCLC) cells through a PrestoBlue® viability assay. NVP-BEZ235 was tested using concentrations ranging from 0-25µM over 72 hours. An ELISA was done to investigate the effect of NVP-BEZ235 on eIF4E, one of the downstream effectors of mTORC1. NVP-BEZ235 was also investigated on DMS79 (SCLC) cells through a PrestoBlue® assay. Given that the drug was so potent in inhibiting H460 cell proliferation, smaller concentrations were tested (0-4nM) over 72 hours.

Results and Discussions

In H460 cells, a dose and time dependent decrease in cell viability was observed with increasing concentrations of NVP-BEZ235 with an IC₅₀ of 0.05µM. This shows how potent NVP-BEZ235 is in inhibiting H460 proliferation. The ELISA confirmed that NVP-BEZ235 successfully decreased eIF4E levels, from 800pg/ml before treatment to under 100pg/ml after 5 hours of treatment with 0.05µM NVP-BEZ235. This confirms that the drug is inhibiting proliferation by targeting PI3K/mTOR signalling. eIF4E is important in the translation of various oncogenes including c-MYC, VEGF & MMP3. Therefore, by decreasing eIF4E, NVP-

BEZ235 may potentially also limit angiogenesis and metastasis.

Since NVP-BEZ235 was so potent in limiting proliferation in NSCLC, this drug was investigated on SCLC which has a higher inclination towards metastasis. However, lower concentrations of NVP-BEZ235 were not successful in limiting proliferation of DMS79 cells which may be explained since SCLC tend to be more aggressive.

Conclusion

Protein analysis is currently being implemented on the treated cells to investigate the effect of the drug on proteins related to the drug mechanism. Additionally, the drug's potential in reducing metastatic ability in such aggressive subtypes is also being studied using cell invasion assays.

EACR22-0473

Involvement of hormones and their receptors in the progression of low- and high-grade glioma

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Introduction

Gliomas are the most common primary brain tumors in adults. The WHO classifies these tumors according to their degree of malignancy (histological grade 1 to 4) and molecular subtype (IDH1 gene mutation, 1p19q codeletion): astrocytoma, oligodendroglioma and glioblastoma (IDHwt grade 4). Conventional treatment of glioblastoma consists in a concomitant radio-chemotherapy (temozolomide) and in some cases a surgical resection. Despite, the 5-year survival rate is less than 5 %. Few risk factors are clearly defined, but women are 1.5-fold less affected than men. Based on these observations, we hypothesized that hormonal and particularly estrogenic signaling have neuroprotective properties and addressed the key role of the G-protein coupled estrogen receptor GPER1.

Material and Methods

The association between patient survival and the expressions of GPER1 or its related genes was investigated through the mining of TCGA-LGG/GBM databases and a workflow of statistical and bioinformatics analyses. The impact of GPER activity on tumor growth was also explored in *ex vivo* cultured low- and high-grade patient samples and glioblastoma primary or established cell lines exposed to GPER1 agonist (G-1) and/or temozolomide and/or radiotherapy.

Results and Discussions

For the first time, we highlighted the association between a high GPER1 expression and patient survival and identified gender and tumor subtype specific related-gene signatures of good prognosis. *Ex vivo*, G-1 induced a significant decrease in glioblastoma cell proliferation. We observed a cell cycle arrest in G2/M phase caused by a disruption of the microtubular network. Finally, G-1 potentiated the efficacy of standard treatment, even in chemotherapy less-sensitive cells, and could lead to cell apoptosis.

Conclusion

Taken together, our results supported the hormone-sensitivity of low- and high-grade glioma and suggested that G-1, in combination with standard therapy, might be a promising way to limit the progression and aggressiveness of GBM. We also highlighted the need for a

gender-specific approach to both investigate the mechanisms that underly glioma progression and search for more efficient and tailored therapies of this still incurable disease.

EACR22-0587

HDAC7 influence proliferation and invasion of colorectal cancer cells by modulating E-Cadherin and Nur77

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Introduction

Colorectal cancer (CRC) is among the most diagnosed cancer world-wide. The 5-year overall survival is around 60%, that is dramatically reduced for advance/metastatic disease. Several efforts have been made to identified possible drivers and therapeutic target of CRC, however, only few of the identified genes, such as EGFR or VEGFR, has been successfully employed to design targeted therapy, but very often resistance occurs despite no further mutation are detected, suggesting a role for epigenetics in driving tumour progression and response to treatment. Preliminary data obtained by our group, identified the Histone Deacetylase 7 (HDAC7) to be overexpressed in CRC tissue as compared to the non-tumoral counterpart, suggesting that HDAC7 could play a pro- tumorigenic role in CRC. For all the above reason, the aim of our work is to investigate the role of HDAC7 in colorectal cancer.

Material and Methods

We used SW480 and SW620 CRC cell lines, from which we created HDAC7 over expressing and HDAC7 knock-down stable cell lines. We evaluate gene expression using QPCR. Proliferation was assessed with crystal violet assay, and protein expression was investigated by western blot. Invasion assay was performed using transwell support

Results and Discussions

Our data show that overexpression of HDAC7 increase cell proliferation in both cell models, while silencing of HDAC7 reduced cell proliferation. Furthermore, HDAC7 overexpressing cells have an increased invasive potential, while knock-down of HDAC7 significantly decrease CRC cells invasion rate. RNA analysis highlights that modulation of HDAC7 expression perturbate the expression of E-Cadherin and Nur77, both genes already described to have a role in cancer cell invasion.

Conclusion

In summary our results show that HDAC7 has a role in modulating CRC cells proliferation and invasion and strongly suggest a role of HDAC7 in CRC. Further experiments are needed in order to understand the exact mechanism of action and to identified genes that are under HDAC7 control and that could be directly involved in in CRC

EACR22-0851

The role of p21 and WEE1 kinase in differentiation of acute myeloid leukemia cells

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Introduction

Our previous study demonstrated that 5-aminoimidazole-4-carboxamide ribonucleoside (AICAr) induces differentiation of leukemia cells through inhibition of pyrimidine synthesis and activation of checkpoint kinase 1 (Chk1). *CDKN1A/p21(CIP1)* was among genes upregulated in both U937 cells and primary acute myeloid leukemia (AML) blasts differentiated with AICAr. In addition, AICAr-mediated differentiation was associated with an increase in inhibitory phosphorylation of cyclin-dependent kinase 1 (CDK1)/CDC2, and similar changes were observed in response to brequinar, another pyrimidine synthesis inhibitor, as well as cytarabine. WEE1 kinase phosphorylates and inhibits CDC2. WEE1 is known to regulate leukemia cell proliferation and survival, but the role of WEE1 in differentiation has not been investigated. Therefore, the aim of this study was to test for the role of p21 and WEE1 in differentiation of AML cells.

Material and Methods

Gene set enrichment analysis (GSEA) of GEO (series GSE128950 and series GSE145061) and ArrayExpress (series E-MTAB-9209) database data was performed using GSEA and R software. Human AML cell line U937 (ECACC Cat#85011440) was incubated in the presence of cytarabine (10, 100, 1000 nM), AICAr (0.2 mM), brequinar (0.5 μ M), and MK-1775 (125, 250 nM). The number of viable cells was determined by trypan blue exclusion. DNA content for cell cycle analysis and the expression of differentiation markers were determined by flow cytometry. Transfections were performed using Neon™ and siRNA targeting CDKN1A and WEE1. The levels of p21, WEE1, Ser-345-p-Chk1, Tyr-15-p-CDC2 and β -actin were determined by Western blot.

Results and Discussions

In samples treated with differentiation inducers, GSEA demonstrated upregulation of the hematopoietic cell lineage genes and downregulation of cell cycle pathway genes. Downregulation of p21 did not reduce differentiation of AICAr- and brequinar-treated U937 cells, although it diminished differentiation in cytarabine-treated cells, as previously described. MK-1775, a pharmacological inhibitor of WEE1 kinase, in a dose that effectively decreases Tyr-15-p-CDC2, significantly reduced differentiation effects of all agents tested. Downregulation of WEE1 decreased the number of viable cells and decreased the expression of differentiation marker CD11b in cells treated with pyrimidine synthesis inhibitors.

Conclusion

Cytarabine-mediated differentiation of AML cells depends on p21, while differentiation in response to pyrimidine synthesis inhibitors depends on WEE1 kinase.

EACR22-1039

miR-21 deletion enhances sensitivity of PDAC to CDK4/6 inhibition in vitro

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal and aggressive malignancies with limited therapeutic options. (miRs/miRNAs) are small endogenous non-coding RNA molecules, which are associated with cellular proliferation, invasion, apoptosis, and cell cycle regulation. The oncogenic miR-21 is involved in cell proliferation and metastasis; and knocking out miR-21 in vitro correlated to cell cycle arrest at G2/M phase alongside inhibition of cell proliferation. The scope of this study was to assess the mechanisms of action of CDK4/6 inhibitor Palbociclib, PD-0332991 and miR-21 gene editing (KO) on cell survival and death in PDAC.

Material and Methods

In the current study, using a CRISPR/Cas9 technology, we individually knocked out (KO) the expression of miR-21 in two PDAC cell lines (MiaPaCa-2 and Panc-1), to assess the mode of action of PD-0332991 (2 μ M, 24h) with miR-21 involvement, compared with miR-21 KO alone. Colony formation, cellular viability, caspase 3/7 activity and cell cycle analysis by using propidium iodide cell staining were assessed, to understand the potential of PD-0332991 treatment along with miR-21 KOs for PDAC therapeutics.

Results and Discussions

Our data indicated that: both miR-21 KO alone and in combination with PD-0332991, significantly induced cell cycle arrest and apoptosis, in Panc-1 and MiaPaCa-2 PDAC cells. PD-0332991 treatment significantly decreased colony formation, reduced cell viability by 50% and induced caspase 3/7 activity of both Panc-1 and MiaPaCa-2 miR-21 KOs, compared with miR-21 KO alone. G1 arrest was also enhanced in Panc-1 and MiaPaca-2 miR-21 KOs following PD-0332991 treatment, while S and G2 phases were significantly suppressed.

Conclusion

The data suggest that the CDK4/6 inhibitor, PD-0332991, poses a novel therapeutic strategy for PDAC, which could be tailored to individual PDAC cases, depending on miR-21 status. Targeting miR-21 networks may facilitate the development and advancement of miR-based clinical applications alone or in combination with CDK4/6 inhibitors for improved treatment in PDAC.

EACR22-1137

Control of disseminated breast cancer cell dormancy in the bone marrow by TGFB2 and BMP4 signaling

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Introduction

Breast cancer-related deaths are due to metastases that arise years after treatment and primary tumor removal. This delay in the time to metastasis is due to the dormancy of disseminated tumor cells (DTCs) in secondary organs. The bone marrow (BM) is of major interest as DTCs in this site are associated to poor prognosis. TGFB2 was found to induce breast cancer cell (BCC) dormancy in the BM, but other pro-dormancy factors are present in this organ and several signals are integrated. Among them, the Bone Morphogenetic Proteins (BMPs) are a major component of the BM. BMP4 has been implicated in regulating stem cell quiescence in several organs and types of cancers. Nevertheless, the precise role

of BMP4 and its interplay with TGFB2 in BCC dormancy in the BM has not been assessed. We aim to understand the importance of coordinated BMP4 and TGFb2 signaling in regulating BCC dormancy in the BM.

Material and Methods

3D matrigel models, mammosphere assays and Fluorescent Cell Cycle Indicator (FUCCI) were used in a wide variety of human and mouse BCC lines. To characterize activated pathways and the heterogeneity of the dormant (G0) compartment, bulk and single cell RNAseq were performed. To validate the findings in situ, a 3D model of the human BM niche, where the expression of TGFB2 and BMP4 can be regulated, is being optimized.

Results and Discussions

Growth arrest was induced by TGFB2 and BMP4 in human and mice BCC lines in 3D assays (matrigel). Interestingly, when combined, TGFB2 and BMP4 displayed a stronger anti-proliferative effect compared to individual ligands, showing that a long-term growth arrest signal is induced. Use of the FUCCI reporter also confirms the increase in the proportion of cells in the G0 phase of the cell cycle, specifically in the TGFB2+BMP4 condition. No difference in cell death or senescence-associated phenotype was observed. Single-cell RNAseq analysis revealed the heterogeneity of the G0 compartment with a unique deep dormant cluster in the TGFB2+BMP4 condition, with a blended gene signature from TGFB2 or BMP4 alone treatments. Validation of these findings in a 3D human-like model of the BM niche is ongoing.

Conclusion

We conclude that co-signaling by both TGFB2 and BMP4 in the BM may allow BCC to coordinate a signal for a deep dormancy that neither factor can achieve alone. By providing a better understanding of BMP and TGFβ signaling our results could open new opportunities to treat patients by preventing cancer metastasis and relapse.

EACR22-0165

POSTER IN THE SPOTLIGHT

PICH in Burkitt's lymphoma development and maintenance: a potential therapeutic target

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Introduction

Plk1-interacting checkpoint helicase (PICH) is involved in the resolution of ultrafine anaphase DNA bridges and, therefore, is important to safeguard chromosome segregation and stability. Recently, an important role of PICH in cancer has been proposed. It is overexpressed in a wide spectrum of human tumors and high PICH expression levels correlate with a worse clinical outcome. In addition, PICH is required for RAS-induced malignant transformation *in vitro* and seems to be important to sustain the proliferation of cancer cells. PICH is an important component of cell mitosis, but its role in tumor development and progression remains unclear.

Material and Methods

In order to address the actual relevance of PICH in cancer we have used novel PICH-deficient mouse models in combination with a transgenic cancer mouse model. Since PICH

is predominantly expressed in spleen and thymus, we have utilized the E μ -Myc mouse model which develops Burkitt's lymphoma (BL).

Results and Discussions

PICH deficiency delayed the appearance of *c-Myc* induced lymphomas in *Pich* heterozygous females. Using an inducible knock-out mouse model in an E μ -Myc transgenic background, we have found that *Pich* depletion in adult mice increased mice survival. However, some *Pich*-deficient mice still developed tumors. Further analysis of tumors showed PICH expression suggesting that PICH is essential to form this type of tumor. Importantly, *Pich* depletion in healthy adult mice did not cause any obvious phenotypic alteration or toxic effect. In human lymphoma cells, PICH depletion by shRNA resulted in cells with reduced proliferative potential, increased apoptosis and reduced survival. Altogether our results show that PICH is involved in lymphoma development in mice and that the proliferation capacity of the lymphoma tumors is diminished upon PICH depletion. BL is the most frequent childhood non-Hodgkin's lymphoma in Europe. The current treatments for BL have limited effectiveness and are mainly based on a combination of the antibody treatment rituximab and intense chemotherapy. These regimens cause short and long-term side effects in patients, especially in children. Therefore, it is necessary to find new and safer strategies to treat BL.

Conclusion

In this context, we consider PICH as a target for novel anti-cancer therapeutic strategies with potential benefits for BL patients.

EACR22-0232

DSTYK-dependent mitophagy impairs immunotherapy response in non-small cell lung cancer

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Introduction

Despite recent advances in prevention, screening and therapeutic strategies, lung cancer remains the leading cause of cancer-related death worldwide and poses a major challenge for public health policy in developed countries. Although considerable improvement in survival has been achieved in the last decade, a significant proportion of NSCLC patients are still not benefiting from these strategies

Material and Methods

Clinical relevance of *DSTYK* alteration in lung cancer was analyzed using The Cancer Genome Atlas (TCGA) public data. *DSTYK* expression and location was assessed in human and murine lung cancer cell lines. Expression profiling of a *DSTYK* network was performed by RNA sequencing. Genetic inhibition *in vitro* and *in vivo* using xenograft and syngeneic models was done to test *DSTYK* role as a molecular target and study its molecular mechanism. A cohort of immunotherapy (IT) treated patients was used to analyze the relevance of *DSTYK* alteration in IT response.

Results and Discussions

Here, we identify DSTYK, a dual serine/threonine and tyrosine non-receptor protein kinase, as a novel actionable target frequently altered in non-small cell lung cancer (NSCLC) subtypes through mutations and copy-number alterations that associate with a lower overall survival (OS) and poorer progression-free survival (PFS) in multiple patient cohorts. Abrogation of DSTYK in murine and human lung cancer experimental systems prevents mTOR-dependent cytoprotective autophagy and induces accumulation of autophagosomes. In addition, we show that DSTYK inhibition contributes to dysfunctional autophagy through impaired lysosomal biogenesis and maturation and induced ROS accumulation through reduced mitophagy. Finally, using immune competent models, we demonstrate that inhibition of DSTYK sensitizes immune-resistant lung tumors to anti-PD1 treatment.

Conclusion

In summary, through a comprehensive functional and mechanistic dissection, we have uncovered DSTYK as new therapeutic target in lung cancer. Prioritization of this novel target for drug development and clinical testing may expand the percentage of NSCLC patients benefiting from immune-based treatments.

EACR22-0338

COMBINED INHIBITION OF E2F AND TYMIDYLATE SYNTHASE IS HIGHLY EFFICIENT IN BOOSTING METASTATIC PROSTATE CANCER CELL APOPTOSIS

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Introduction

A hallmark of metastatic prostate cancer (mPCa) is the aberrant E2F target gene expression, which could account for the tumor's resistance against chemotherapy. 5-fluorouracil (5-FU) inhibits Thymidylate Synthase (TS), a key enzyme for *de novo* synthesis of nucleotides, resulting in replication stress. Although 5-FU induces apoptosis of prostate cancer cells, mPC cells show resistance to the treatment. We speculate that mPC relies on the salvage pathway for nucleotide synthesis to overcome the nucleotide pool reduction and subsequent replication stress imposed by 5-FU. TK1 and DCK, two rate-limiting enzymes of the salvage pathway, are known to be regulated by E2F factors, suggesting that E2F activation could account for the resistance to 5-FU, through the regulation of TK1 and DCK. The goal of this study was to determine the impact of E2F activity in the resistance of mPC to 5-FU and to determine whether a combined inhibition of E2F and TS would be deleterious for mPC cells.

Material and Methods

Gene expression and response to 5-FU treatment were analyzed in the mPC cell lines PC3 and DU145. To determine the impact of E2F in the resistance to 5-FU, E2F activity was prevented by using siRNAs or E2F inhibitor HLM-006474.

Results and Discussions

We show that 5-FU upregulates *TK1* and *DCK* expression in PC3 and DU145 cells. Furthermore, silencing of *E2F1* and *E2F2* induces replication stress, prevents 5-FU-induced upregulation of *TK1* and *DCK*, and potentiates 5-FU-induced apoptosis in PC3 and DU145 cells. Knockdown of *TK1* and *DCK* recapitulates this effect. By contrast, exogenous supply of nucleosides increases the viability of *E2F1*- and *E2F2*-depleted cells treated with 5-FU. Interestingly, combination of the E2F inhibitor HLM with 5-FU substantially reduces viability and colony forming capacity of PC3 cells and inhibits PC3 *in vivo* xenograft growth in nude mice.

Conclusion

Collectively, our data stress the relevance of E2F1 and E2F2 as key mediators of chemoresistance to 5-FU and point to the E2F-TK1/DCK regulatory axis as a therapeutic target in mPC.

EACR22-0448

Induction of heparanase 2 (Hpa2) expression by stress is mediated by ATF3

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Introduction

The activity of heparanase, an enzyme that cuts heparan sulfate (HS) side chains of heparan sulfate proteoglycans, is strongly implicated in tumor angiogenesis and metastasis attributed to the remodeling of the subepithelial and subendothelial basement membranes. In many cases, heparanase induction correlated with increased tumor metastasis, vascular density, and shorter survival rate post-operation. Heparanase 2 (Hpa2) is a close homolog of heparanase that lacks HS-degrading activity and functions as an endogenous inhibitor of heparanase. As a result, Hpa2 appears to attenuate tumor growth but mechanisms that regulate Hpa2 expression and determine the ratio between heparanase and Hpa2 are largely unknown.

Material and Methods

Applying, reporter gene assay, site-directed mutagenesis, Crispr/cas9 gene editing, in vitro translation, and electro-mobility shift assay, we have characterized stress response sequences in the Hpa2 promoter and identified activating-transcription-factor 3 (ATF3) as the transcription factor that mediates Hpa2 induction by stress.

Results and Discussions

Here, we focus on the Hpa2 promoter and its sensitivity to stress conditions. We report that while endoplasmic reticulum (ER) stress and hypoxia, each alone, resulted in a modest increase in Hpa2 expression, combining ER stress and hypoxia yielded a profound, over 40-fold, increase in Hpa2 expression. A prominent induction of Hpa2 expression was also found in cells exposed to heat shock, proteotoxic stress, lysosomal stress, and chemotherapy (cisplatin), strongly implying that Hpa2 is tightly regulated by conditions of stress.

Conclusion

Collectively, we revealed, for the first time, a molecular mechanism that underlies Hpa2 gene regulation and function. Furthermore, we demonstrate that induction of Hpa2 and ATF3 by conditions of stress that often accompany the rapid expansion of tumors is translated to improved survival of cancer patients.

EACR22-0652

Autophagy inhibition sensitises glioblastoma cells to Src family kinase inhibitors Si306 and its prodrug

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Introduction

Glioblastoma (GBM) is among the most frequent and aggressive brain tumors characterized by infiltrating nature, high proliferation, and resistance to chemotherapy and radiation. GBM exhibit high expression of Src tyrosine kinase which regulates proliferation, survival, and invasiveness of tumor cells, making Src a potential target for GBM therapy. Numerous Src family kinase inhibitors (SFKI) were reported to induce autophagy, thus protecting cells from undergoing cell death. However, inhibition of autophagy was shown to sensitize cells to SFKI in several cancer types.

Material and Methods

Human GBM cell line U87 and its multidrug-resistant (MDR) counterpart U87-TxR were transfected with RFP-LC3, an autophagy marker. The ability of two SFKIs, pyrazolo[3,4-d]pyrimidines Si306 and its prodrug pro-si306, to induce autophagy in RFP-LC3-transfected GBM cells was evaluated by flow cytometry and fluorescent microscopy. Cell viability was assessed by MTT assay. The autophagy induction and autophagic flux were evaluated by Acridine orange assay, immunocytochemistry and immunoblotting. Cell proliferation rate was analyzed by CFSE assay. Cell death was detected by Annexin/Propidium Iodide assay. PARP-1 cleavage was assessed by immunoblotting.

Results and Discussions

SFKI treatment resulted in degradation of RFP-LC3 after 3 h treatment as well as in formation of RFP-LC3 puncta in GBM cells demonstrating autophagy induction. The effect of SFKIs on autophagy induction persisted after 48 h, as demonstrated by autophagy markers LC3 and p62. Inhibition of autophagy by Bafilomycin A1 sensitized both U87 and U87-TxR cells to Si306 and its pro-drug after 48 h. The anti-proliferative effect of Si306 and pro-Si306 was additionally increased after autophagy inhibition by Bafilomycin A1. Furthermore, while single SFKI treatments did not cause significant cell death, combination treatments with autophagy inhibitor induced necrosis in U87 and U87-TxR cells after 48 h. Detection of necrotic PARP-1 fragment further confirmed necrotic cell death.

Conclusion

Taken together, these data suggest that autophagy induced by Si306 and pro-Si306 has a protective role in GBM cells, and that autophagy modulation may be used to enhance the anticancer effects of SFKIs. In addition, as the ability of the SFKIs to induce autophagy was not diminished by the presence of the MDR phenotype makes these compounds promising for treatment of MDR cancers.

EACR22-0666

PML is required for chemotherapeutic response in AML

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Introduction

Acute myeloid leukemia (AML) is characterized by the clonal expansion and differentiation arrest of myeloid progenitors in the bone marrow. Only few AML patients are cured, due to relapse occurring in more than half of them. Our laboratory focuses on the Promyelocytic Leukemia protein (PML). PML polymerizes to form nuclear bodies (NBs), particularly upon oxidative stress. Biochemically, PML NBs recruit many partner proteins within their core, and these will undergo post-translational modifications, in particular SUMOylation. Functionally, PML NBs are involved in fundamental pathways such as metabolism, senescence, response to oxidative stress or apoptosis. PML was discovered in the context of acute promyelocytic leukemia (PML/RARA translocation), in which arsenic and retinoic acid cure patients in a PML-dependent manner. We questioned whether PML plays a general role in AML response to chemotherapy.

Material and Methods

Mll/Enl oncogene was retrovirally transduced into *Pml*^{+/+} or *Pml*^{-/-} mouse haematopoietic progenitors, and engrafted these cells in syngenic mice that rapidly developed leukemia. We treated leukemic mice with doxorubicin and cytarabine, the classic 7+3 regimen used in AML patients.

Results and Discussions

We observed that mice bearing *Pml*^{-/-} leukemia exhibited a very poor response to the treatment compared to *Pml*^{+/+} ones. Accordingly, mice bearing *Pml*^{+/+} *Mll/Enl* leukemia survived significantly longer after treatment than those bearing *Pml*^{-/-} *Mll/Enl* AMLs. These results strongly support a general role of PML to induce AML cells death upon chemotherapy. When we explored the downstream mechanisms through which PML might be involved in chemotherapy response. Importantly, P53 and its targets appear to be similarly activated in both genotypes. PML is also involved in metabolism and mitochondrial biogenesis. In Actinomycin D-treated AMLs, we reported that chemotherapy unexpectedly targets the mitochondria, yielding Reactive Oxygen Species production and activating a PML senescence/apoptotic checkpoint. We are currently exploring whether a similar mechanism could explain the striking differences that we have observed.

Conclusion

Our results demonstrate the key importance of PML in chemotherapy response in AML. Taken the frequent downregulation of PML expression in advanced solid tumors, PML targeting could be of broader significance in cancer.

EACR22-0742

TRPV channels activation induces cell death in Human Pancreatic Ductal Adenocarcinoma cell lines

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Introduction

Pancreatic Ductal Adenocarcinoma is one of the most aggressive and lethal malignancy. The main therapeutic strategy is surgical resection followed by adjuvant chemotherapy, but only 10-20-% of patients have a resectable form, while if it is metastatic is not applicable. So new therapeutic approaches are necessary. Several studies demonstrated the importance of Transient Receptor Potential Vanilloid (TRPV) channels in physiological and pathological

processes such as cell proliferation, apoptosis, differentiation, suggesting that they could be potential anticancer targets. Herein, we evaluated the involvement of TRPV channels in Overall Survival (OS) and Recurrence Free Survival (RFS) in PDAC patients. Then, using two human PDAC cell lines, PANC-1 and MIAPaCa-2, it was evaluated the expression of TRPV1, TRPV2, TRPV3 and TRPV4 channels, and the anticancer activity of a TRPV ligand, alone and in combination with gemcitabine and paclitaxel, two chemotherapeutic drugs used in PDAC therapy.

Material and Methods

Firstly, using data from Pan-cancer database, OS and RFS associated to TRPV channels expression in PDAC patients were calculated. Then, TRPV channels gene and protein expression was evaluated in PANC-1 and MIAPaCa-2 using western blot analysis and RT-PCR. TRPV ligand anticancer effect was studied evaluating the cytotoxicity and cell death, alone and in combination with gemcitabine and paclitaxel. In addition, modulation of PDAC cell death pathways in TRPV stimulated cells was evaluated by RT-PCR profile array.

Results and Discussions

Database data showed a significant correlation between OS and RFS, and TRPV1, TRPV3 and TRPV4 expression. The expression profile of TRPV channels in PDAC cell lines was determined. Activation of TRPV channels induced cytotoxicity and apoptotic cell death. The combination of the TRPV ligand with chemotherapeutic drugs increased their efficacy. Moreover, TRPV activation led to a modulation of cell death pathways.

Conclusion

These preliminary data evidenced as TRPV channels could be potential targets for PDAC therapy. Moreover, the activation of these channels could be an adjuvant in PDAC chemotherapy.

EACR22-0994

Autophagy-related Gene-5 (ATG5) is essential for the anticancer effect of Picropodophyllin on colorectal cancer cells

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Introduction

Insulin-like growth factor-1 (IGF-1) and its receptor (IGF-1R) are overexpressed in cancer cells and were found to link obesity to different types of cancer. but their effects on cell migration and induction of metastasis were not previously evaluated. IGF-1R plays a crucial role in colorectal cancer (CRC) cell proliferation and migration. Accordingly, IGF1-antagonists, e.g., Picropodophyllin (PPP) was investigated as a potential anticancer therapy. Autophagy is a crucial process in CRC cell metabolism, proliferation and stress response, with a contextual role that depends on the cancer cell metabolic reprogramming and the oxygen and nutrient status of the tumour microenvironment.

Material and Methods

In this study, CRISPR-Cas9 using ribonucleoprotein was performed to knock out the HCT116 (CMS4) CRC cell line for autophagy related gene-5 (ATG5) and ATG7. In addition, we investigated the migration of different CRC cell lines, the HT29 (CMS3) and the metastatic SKCO-1 cell lines (CMS3-predicted) and LoVo (CMS1). The expression of IGFR1 was tested in all cell lines using WB. Both IGF-1 (10nM) and its inhibitor, PPP (10uM) were tested on the cell lines. The scratch (wound healing) assay was performed and area of separation was quantified (using Image J software).

Results and Discussions

In HCT116, HT29 and SKCo-1 (highly expressing IGF-1R), IGF-1 treatment increased cell migration, whereas hypoxia suppressed the migration of cells (both treated and untreated with IGF-1). PPP suppressed the migration of cells under both hypoxic and non-hypoxic conditions. Knocking out of ATG5 abolished the effect of hypoxia on cell migration, and abolished the effect of PPP under non-hypoxic condition. However, PPP is still effective in ATG5 KO cells under hypoxic condition. Knocking out ATG7 reversed the effect of hypoxia on cell migration, but it did not affect the action of PPP on the tested cell lines.

Conclusion

In presence of intact autophagy machinery, hypoxic cells may maintain its migration capacity. However, knocking out ATG5 abolishes the effect of hypoxia-induced migration suppression. Such an effect is not demonstrated with ATG7 knocking out, instead it is reversed (favoring cell migration). PPP effect on cell migration depends on ATG5, but not ATG7. Due to the complex role of autophagy, it should be precisely modulated when selected as adjunctive treatment modality for CRC.

EACR22-1186

Salinomycin-induced ferroptosis requires mTOR pathway

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Introduction

Ferroptosis is a newly form of non-apoptotic cell death, defined by the iron-dependent accumulation of lipid-based reactive oxygen species (ROS). Ferroptosis constitutes a promising anti-cancer therapeutic strategy, since it specifically targets aggressive cancer stem cells (CSCs). CSCs are a small population of self-renewing and tumor-initiating cancer cells resistant to conventional therapy, and though to be responsible for metastasis.

Salinomycin (Sal), a promising lysosomal iron-targeting molecule (or its derivative AM5) targets efficiently CSCs by ferroptosis. Due to their higher level of autophagy and iron, CSCs are more sensitive to Sal-induced ferroptosis compared to their non-CSC counterpart. Still, ferroptosis molecular mechanisms remain to be clarified.

Recently, emerging evidence has supported the connection between ferroptosis, GSH-xCT axis and mTOR pathway. The xCT/GSH axis is known to inhibit ferroptosis as a ROS reducing-mechanism. Here we report that mTOR pathway, regulates Sal-induced ferroptosis.

Material and Methods

For this study, we used a well established stem-like model: human mammary epithelial HMLER CD24^{low}/CD44^{high} cells. Chemical inhibitors and silencing RNA were used to inhibit mTOR and xCT. Cell death, intracellular iron, ROS and amino-acid level were measured using various probes by flow-cytometry. Protein levels were analysis by western blot.

Ultimately, a metabolomic study was realized, and we are working on a metabolic flux analysis to confirm our data.

Results and Discussions

As a result, we showed that **inhibition of mTOR pathway** (mainly through mTORC1) **suppresses Sal-induced ferroptosis**. Mechanistically, it acts on iron homeostasis and subsequent ROS production.

Furthermore, GSH-xCT axis seems to be surprisingly involved in this protection. We are currently modulating xCT and its partners, to address their impact on ferroptosis. Lastly, we showed that Sal itself activated several mTOR substrates.

Taken together, our data suggest a new mechanism of Sal requiring mTOR pathway (and GSH-xCT axis).

Conclusion

My project is to determine by which mechanism(s) mTOR could regulate Sal-induced ferroptosis, leading us to a better understanding of ferroptosis execution. Eventually, in cancer combined therapy strategy, the association of Sal and mTOR inhibitor would cause opposite effects, thus it is crucial to find out the mechanisms that lead to its resistance.

EACR22-1222

Ru(III)-based compounds to treat triple negative breast cancer: focus on cell death pathways and metastatic potential.

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Introduction

According to World Health Organization, breast cancer (BC) is the most common cause of cancer death in women. The triple negative breast cancer (TNBC) phenotype is the most aggressive and has to date limited treatment options. Therefore, the discovery of novel therapeutical approaches is crucial. Our research group is focusing on a newly synthesized Ru(III) complex – named AziRu – incorporated in different nucleolipid nanostructures to improve its stability and in vivo delivery.

Material and Methods

HoThyRu/DOTAP anticancer activity was measured with proliferation assay. Cell death pathways triggers were investigated using fluorescence microscopy kits and validated through Western Blot analysis. Migration capability was assessed at optimized condition by Wound Healing Assay while invasion capability measured by the use of matrix coated transwell. Moreover, MDA-MB231 xenograft mice models were set up to investigate in vivo antiproliferative activity.

Results and Discussions

Among our compounds, HoThyRu/DOTAP was the most effective against TNBC in vitro model represented by MDA-MB-231 cells. Since HoThyRu/DOTAP demonstrated significant antiproliferative activity against TNBC, we next investigated its capability to trigger multiple cell death pathways *i.e.*, apoptosis and autophagy. Fluorescent microscopy showed early apoptosis and autophagy activation already after 12h of HoThyRu/DOTAP treatments. The evaluation of the main proteins engaged in the regulation of cell death pathways after 48 and 72h of treatment suggested our Ru(III) nanosystem as capable to sustain these processes

over time. Moreover, we focused on the metastatic potential of TNBC demonstrating that HoThyRu/DOTAP can inhibit migration and invasion of MDA-MB-231 cells, as proved by different functional assays and by the modulation of principal EMT related proteins. Finally, we showed the efficacy of HoThyRu/DOTAP *in vivo* by using a xenograft model of MDA-MB-231.

Conclusion

The induction of different cell death pathways by multi-target Ru-based formulations and the reduction of metastatic potential could represent a valid and appealing chemotherapeutic option for breast cancer treatment.

EACR22-0151

An axonal guidance molecule as a new promising therapeutic target in Glioblastoma

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Introduction

DX, a recently discovered axon guidance protein, is known to be crucial for proper development of the nervous system. While its function during embryogenesis has been intensively studied, its role in tumorigenesis has not been studied yet. DX has been found to regulate Netrin-1 signaling, especially during brain development, through binding to Netrin-1 ligand and DCC and UNC5B receptors. Given that Netrin-1 is frequently overexpressed in a large set of human tumors, we have been interested in investigating the role of DX in Netrin-1-expressing tumors.

Material and Methods

Down-regulation of DX in GBM cells was performed using small interfering RNA strategy. 48 h after transfection, cell death was analyzed by caspase-3 activity assay using the Fluorimetric Assay Kit (Gentaur Biovision, Brussel, Belgium), quantification of DNA fragmentation using the TUNEL method (Roche) and incorporation of propidium iodide was assessed by IncuCyte™ ZOOM (Essen BioScience). Gene expression was analyzed by Western Blot or quantitative reverse transcription-PCR (LightCycler, Roche).

Results and Discussions

A bioinformatical analysis first revealed that DX is overexpressed in several human cancers, especially in highly aggressive glioblastoma multiforme (GBM) and low-grade glioma (LGG). Given that treatment options for GBM are limited, it is of interest to find new molecular targets in order to improve therapeutic strategies of this highly aggressive tumor. Our preliminary analysis showed that DX expression in GBM patients is correlated with a poor prognosis. *In vitro* studies using GBM cell lines revealed that silencing of DX expression triggers apoptotic cell death, which is abolished in an UNC5B-deficient cell line model. Moreover, the direct interaction between DX and UNC5B observed by ELISA and co-immunoprecipitation suggests that DX survival effect involves mainly UNC5B receptor. Importantly, we observed that the downregulation of DX expression potentiates the efficiency of the commonly used chemotherapeutic agent temozolomide and irradiation *in vitro*.

Conclusion

These results lead us to define DX as a new therapeutic target for GBM treatment. It remains now important to i) confirm the survival role of DX in the GBM human pathology, ii) further investigate the molecular relationship between Netrin-1 signaling members and DX, iii) elaborate a “proof of concept “ strategy validating that interference with DX constrains GBM tumor progression *in vitro* and in preclinical models.

EACR22-0277

Counter-regulation of thymosin beta-4 expression and primary cilium formation by nuclear factor (erythroid-derived 2)-like 2 for HeLa cervical cancer cell survival

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Introduction

Antenna-like primary cilium(PC) on the surface of cell membrane is observed in quiescent cells and proliferating cells. Reactive oxygen species (ROS) produced by SD increase antioxidant gene transcription via nuclear factor (erythroid-derived 2)-like 2(Nrf2). Thymosin beta-4(TB4) is a novel regulator for PC formation. Here, we investigated whether cancer cell survival by SD could be associated with PC formation via Nrf2-regulated TB4 expression.

Material and Methods

We used RNA interference of TB4, immunofluorescence staining to observe PC, Nrf2-knockdown cells, and pCDNA-Nrf2 plasmids. We also used RT-PCR, Gaussia luciferase assay, and western blotting, MTT assay to measure cell viability and flow cytometry to measure ROS.

Results and Discussions

SD increased PC formation and TB4 transcription. CC viability was further decreased by treatment with ciliobrevin A to inhibit PC formation or TB4-siRNA. SD increased ROS, including H₂O₂. N-acetylcysteine inhibited ROS production following H₂O₂ treatment or SD, which also decreased PC formation and TB4 transcription. Meanwhile, H₂O₂ increased PC formation, which was attenuated in response to TB4 siRNA. Treatment with H₂O₂ increased Nrf2 expression, antioxidant responsive element activity, and PC formation, which were inhibited by the Nrf2 inhibitor clobetasol propionate. Nrf2 knockdown via expression of Tet-On shNrf2 enhanced ROS production, leading to increased PC formation and decreased TB4 expression; these effects were counteracted by Nrf2 overexpression.

Conclusion

Our data demonstrate that Nrf2 counter-regulates TB expression and PC formation for CC survival under conditions of SD, suggesting cervical CC survival could be upregulated by PC formation via Nrf2 activation and TB4 expression.

EACR22-0285

mTOR inhibition overcomes Venetoclax resistance in pediatric T-cell lymphoblastic leukemia through induction of the Integrated Stress Response.

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Introduction

Approximately 20% of T-cell acute lymphoblastic leukemia (T-ALL) patients do not respond to current therapy, and their clinical outcome is dismal. Improved therapeutic strategies for T-ALL patients remain an unmet clinical need. Venetoclax is a specific B-cell lymphoma-2 (BCL-2) inhibitor showing activity in hematological malignancies such as acute myeloid leukemia (AML). However, T-ALL cells are resistant to Venetoclax due to upregulated expression of other anti-apoptotic members of the BCL-2 family. We recently showed that inhibition of mTOR, often deregulated in T-ALL, increases Reactive Oxygen Species (ROS) levels in the cells. Since previous studies have shown that a sustained increase in ROS can trigger the pro-apoptotic branch of the Integrated Stress Response (ISR), we tested whether mTOR inhibition could synergize with Venetoclax in inducing apoptosis in T-ALL cells.

Material and Methods

Experiments were carried out in T-ALL cell lines, patient-derived xenograft (PDX) cells, and primary tumor cells isolated from T-ALL patients. The cells were treated with Venetoclax as single agent or in combination with the mTOR inhibitor Everolimus. Cell death was measured using Annexin/PI staining and flow cytometry. Gene expression was analyzed by quantitative RT-PCR and immunoblotting.

Results and Discussions

The combination of Everolimus and Venetoclax induced significant cell death in T-ALL cell lines, PDX cells, and primary samples from T-ALL patients. Treatment with Everolimus activated the ISR, as demonstrated by the induction of EIF2 α phosphorylation and increased levels of the mRNA coding for DNA damage-inducible transcript 3 (DDIT3), a marker of the ISR. Pre-treatment of T-ALL cell lines and PDX-derived cells with ISRIB (an inhibitor of ISR) blunted cell death induced by Everolimus alone or in association with Venetoclax.

Conclusion

Our results demonstrate that treatments that increase ROS levels combined with BCL-2 inhibitors unleash a strong apoptotic response in T-ALL cells through induction of the ISR. Further experiments are ongoing to validate the efficacy of the proposed therapeutic approach in a T-ALL xenograft model.

EACR22-0811

Gossypol selectively induces necroptosis in squamous cell carcinoma cells

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Introduction

Cutaneous squamous cell carcinoma, the second most common form of skin cancer, is still rising. Especially the treatment of the invasive form still represents a big challenge. BH3 mimetics have been developed to induce Bcl-2 dependent apoptosis. In this study, we investigated the effect of gossypol (GP), a polyphenolic aldehyde derived from cotton seed, on cutaneous squamous cell carcinoma. As these tumor cells show an elevation of anti-

apoptotic Bcl-2 proteins, GP could be a potential treatment option. It is important not only to address tumor cells in context of the efficacy of an anti-cancer drug, but also to focus on healthy cells in order to elaborate well-tolerated doses for prevention of undesired side effects.

Material and Methods

Cutaneous squamous cell carcinoma (SCL-1) cells and normal human epidermal keratinocytes (NHEK) were treated with different concentrations of GP and cell viability was assessed. Z-VAD and Nec1 were used as apoptosis and necroptosis inhibitors, respectively. Fluorescent antibodies against RIP3 and pRIP3 were used to quantify RIP3 phosphorylation and Mitotracker green and TMRM staining were used to evaluate mitochondrial membrane potential and morphology. Also, the oxygen consumption was measured, and mass spectroscopy was used to evaluate the effect of GP on mitochondrial protein expression. Western Blots were performed using antibodies directed against p62 and LC3 I/II.

Results and Discussions

GP lowered cell viability in SCL-1 at concentrations showing no toxic effects on NHEK. Interestingly, induction of necroptosis was found instead of the postulated apoptotic pathway. Furthermore, an elevation of autophagy markers like LC3-II or p62 was detected indicating an autophagic flux. In addition, GP lowered the mitochondrial membrane potential, increased mitochondrial fragmentation and had a negative impact on respiratory parameters (ATP, spare respiratory capacity). These findings are supported by proteomics data showing predominant effects on mitochondrial proteins. Interestingly, the GP induced effects on mitochondria were mainly detected in tumor cells with little effect on NHEK. This suggests that GP qualitatively attacks mitochondria but is much more potent in tumor cells assuming that the metabolic state of the cells is an important target of GP.

Conclusion

The selective toxicity as well as the strong mitochondrial impact on SCL-1 at a concentration having no negative impact on NHEK indicate a possible therapeutic window for the treatment of cutaneous squamous cell carcinoma.

EACR22-0817

Effect of a combinational treatment of nanoceria and chloroquine on triple negative breast cancer cells (TNBC): an in-vitro approach

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Introduction

Standard treatment of triple negative breast cancer (TNBC) consists of chemotherapy and comes with severe side effects. We follow a new therapeutical strategy recently described as “old weapons for new battles”. Here, a combination of cerium oxide nanoparticles (CNP), that have been shown by our group to selectively kill many cancer cell types without affecting healthy cells, with the “old drug” chloroquine (CQ) was tested. CQ, an inhibitor of autophagy is/was being tried in cancer therapy as single drug, albeit showing enormous side effects in-vitro and in-vivo. Ideally, the CQ concentration can be lowered by adding CNP to diminish toxic effects on non-cancerous cells.

Material and Methods

MDA-MB-468 triple negative breast cancer cells and MCF-12A non-tumorigenic epithelial breast cells were either mock-treated, incubated with CNP or co-treated with CNP and

various concentrations of CQ. To analyze the migration capacity, confluent cells were divided into two sections by scratching of a defined area of cells and then the ongoing distribution of cells was monitored via microscopy (*Image J wound healing tool plugin*). Alternatively, transwells were used that contain a special membrane including pores, and the cells are attracted by a conditioned medium below the membrane. Migrated cells can be stained and counted. To determine the mitochondrial ROS levels of both cell lines, MitoSox Red staining was used.

Results and Discussions

CNP lowered MDA-MB-468 cell viability, whereas MCF-12A were not affected. In combination with CQ, TNBC viability was further decreased, establishing 5 μ M of CQ/300 μ M CNP as an ideal concentration with a similar toxic effect as a 3-fold higher CQ treatment alone. The combination prevented cell migration in a similar manner as CQ alone. Both CNP and CQ are redox-active substances, and we have shown before that CNP lower the viability of various cancer cell types by a significant increase in the ROS level. The application of MitoSOX proves that the basal mitochondrial ROS level in the TNBC is already higher than in the non-cancerous cells, which might be one of the reasons for the observed results and is worth further studies.

Conclusion

Further improvement of chemotherapeutics of TNBC is still very much needed, and CNP has been shown to be selectively toxic for tumor cells. Together with the “old drug” CQ both are a promising tool to selectively kill breast cancer cells and to lower TNBC migration while giving the opportunity to lower the CQ concentration and the involved side effects.

EACR22-0877

The effects of cannabidiol via TRPV2 channel in chronic myeloid leukemia cells and its combination with imatinib

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Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative disorder characterized by accumulation of immature cells in bone marrow and peripheral blood. Although successful results were obtained with tyrosine kinase inhibitors, several patients show resistance. For this reason, the identification of new strategies and therapeutic biomarkers represent an attractive goal. The role of Transient Receptor Potential (TRP) ion channels as possible drug targets has been elucidated in different types of cancer. Indeed, TRP channels play a relevant role in ion homeostasis and trafficking, and are associated with tumour progression, metastasis, proliferation and chemoresistance. Among natural compounds known to activate TRPs, cannabidiol (CBD) displays anti-cancer properties alone or in combinations with conventional chemotherapies.

Material and Methods

The expression of TRPV channels were evaluated in three CML cell lines in blast crisis K562 (ACC 10 DSMZ[®]), KU812 (ACC 378 DSMZ[®]), MOLM6 (ACC 611 DSMZ[®]) through RT-PCR, western blot, cytofluorimetric analysis and confocal microscopy. The effects of CBD treatment, via TRPV2, were assessed by cell viability, cytotoxic assay, cell growth assay and cell cycle assay. The stimulation of mitophagy and the expression of differentiation markers was evaluated by western blot, FACS analysis and confocal microscopy. The involvement of TRPV2 was studied by gene silencing.

Results and Discussions

We demonstrated that CBD, through TRPV2, inhibits cell proliferation and cell cycle in CML cells, promoting mitochondria dysfunction and mitophagy as shown by mitochondrial mass reduction and up-regulation of several mitophagy markers. These effects were associated with changes in the expression of octamer-binding transcription factor 4 and PU.1 markers regulated during cellular differentiation. Interestingly, a synergistic effect by combining CBD with the standard drug imatinib was found and imatinib-resistant cells remain susceptible to CBD effects. Therefore, the targeting of TRPV2 by using CBD, through the activation of mitophagy and the reduction in stemness, could be a promising strategy to enhance conventional therapy and improve the prognosis of CML patients.

Conclusion

Autophagy and mitophagy are important processes responsible for cell fate in cancer. Our results highlight the involvement of TRPV2 channel in the modulation of stemness and chemoresistance suggesting that it could represent a novel pharmacological target for the CML therapy.

EACR22-1011

TOP ABSTRACT

Paradoxical activation of oncogenic signaling pathways as a cancer treatment strategy

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Introduction

Most of the current clinical and experimental cancer therapy is based on targeted inhibition of mitogenic signaling pathways, attempting to restrain the viability of cancer cells. Unfortunately, such approach has proven to be relatively ineffective for treating most advanced cancers due to mechanisms that rewire signaling pathways, allowing cancer cell survival and proliferation in the presence of drug. On the other hand, it is becoming increasingly clear that too much oncogenic activity can be as lethal to cancer cells as the inhibition of oncogenic pathways. We hypothesized that a “paradoxical” deliberate further activation of already activated signaling pathways could be combined with the inhibition of stress response pathways that help cancer cells survive in the presence of hyperactive oncogenic signaling.

Material and Methods

Using SW-480 colorectal cancer cells as a model, we performed a stress-focused drug screen and a genome-wide CRISPR screen to identify synthetic lethal drug combinations with the Protein Phosphatase 2A (PP2A) inhibitor LB-100. These findings were validated across a panel of additional six colorectal cancer cells (DiFi, DLD1, HCT-116, HT-29, LoVo, and RKO). Time course flow cytometry and DNA combing were used to address DNA replication dynamics. Live cell microscopy was used to analyze mitotic errors.

Results and Discussions

We show that LB-100 leads to further activation of oncogenic signaling pathways in *KRAS*- and *BRAF*-mutant cancers, associated with strong increases in DNA damage, proteotoxic and mitotic stress. Compound screen and CRISPR synthetic lethality screen converge to identify inhibition of the mitotic entry gatekeeper kinase WEE1 as synthetic lethal with LB-100. These findings were validated across a panel of colorectal cancer models with diverse genetic backgrounds. Mechanistically, LB-100 induces strong replication stress, which, when combined with sublethal doses of WEE1 inhibitor, leads to replication collapse and aberrant entry into mitosis followed by cell death. Remarkably, CRISPR-based LB-100 resistance screens identify downregulation of MAPK and WNT signaling as major mechanisms of resistance.

Conclusion

These data show that the combination of LB-100 and WEE1 inhibition is lethal for a panel of colorectal cancer cells. Moreover, loss of oncogenic signaling is apparently a major escape route for treatments that are based on paradoxical activation of oncogenic signaling, supporting the prospect of “tumor-suppressive” acquired resistance to this type of therapy.

EACR22-0008

Validation of the Sos family of RasGEFs as potential therapy targets for *KRAS*^{G12D}-driven lung adenocarcinoma

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Introduction

Lung adenocarcinoma (LA) is a leading cause of cancer-related deaths in the world. About 25% of LA are *KRAS*-driven, most affecting *KRAS* codon 12. Specific inhibitors of *KRAS*^{G12C} and *KRAS*^{G12D} have been recently developed, but restricted therapeutic options are available for patients bearing other RAS pathway-related mutations. Sos family of GEFs act as universal RAS activators regulating RAS/MAPK signaling pathway and encompasses two, ubiquitously expressed, homologous members, Sos1 and Sos2. We developed an inducible KO mouse strain to bypass the embryonic lethality of Sos1-null mutations, allowing to evaluate the functional significance of Sos1/2 during adulthood. Here, we aimed at evaluating the effects of Sos1/2 disruption on development and/or progression of *KRAS*-driven LA tumors as well as on tumor microenvironment. Ascertaining the exact functional contributions of this family of RASGEF proteins may provide a basis for their use as diagnostic markers or therapy targets for human LA.

Material and Methods

We crossed our Sos1/2 KO model with a transgenic mouse model of *KRAS*^{G12D}-driven LA to characterize the effect(s) of Sos1/2 depletion in LA. Tumor initiation and promotion was followed by using microCT. Tumor size, number of tumors and the malignancy of the tumors were determined by histology. Immunohistochemical and flow cytometry studies were used to evaluate the effect of Sos-depletion in cancer-associated fibroblasts and macrophage response. The impact of Sos-dependent modulation on tumor microenvironment was measured by orthotopic *KRAS*^{G12D}-mutant cells transplantation.

Results and Discussions

Our results show that *Sos1/2* deficiency in *KRAS*^{G12D} mutants significantly increased mice survival and delayed tumor initiation and progression. In particular, tumor size, the number of surface tumors as well as the grade of tumor pathology was significantly reduced in *Sos*-depleted mice when compared with *Sos*-wild type mice, being in close correlation with a reduction of cell proliferation and pERK levels into the tumor. Interestingly, *Sos* absence resulted in a modulation of tumor-induced macrophage response and cancer-associated fibroblasts activation.

Conclusion

Our data suggest an important functional contribution of *Sos1/2* not only in the development of *KRAS*-mutant tumor cells but also give clues for the use of the *Sos1/2* isoforms as potential therapy targets or markers of disease progression or drug resistance in these types of tumors.

EACR22-0014

Empty spiracles homeobox genes *EMX1* and *EMX2* act as tumor suppressors in sarcomagenesis by regulating the WNT pathway

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Introduction

The *EMX1* and *EMX2* genes are two transcription factors homeobox genes involved in cell proliferation, migration, and differentiation processes, during brain development and neural crest migration. They play a role in the specification of positional identity, the proliferation and differentiation of neural stem cells. In general, they act as transcription factors in early embryogenesis. The *EMX*'s potential as tumor suppressor genes has been suggested in some cancers.

Material and Methods

We performed bioinformatic, quantitative mRNA and protein expression analysis, cell models of sarcoma by ectopic expression of *EMX* and *WNT* genes. By cell biology methods we measured tumorigenesis and populations enriched on stem cell phenotypes. Also, murine knock out *Emx* genes and immunosuppressed mice models were used for *in vivo* validations.

Results and Discussions

EMX1/EMX2 act as tumor suppressors in sarcomas by repressing the activity of stem cell regulatory genes. *EMX* protein downregulation, induced the malignance and stemness of cells both *in vitro* and *in vivo*. In murine knock out *Emx* genes models, 3MC-induced sarcomas were more aggressive and infiltrative, had a greater capacity for tumor self-renewal, and higher stem cell genes expression than those in wild-type models. These results showing that *EMX* acted as stemness regulators were reproduced in different subtypes of sarcoma.

It has been suggested *EMX2* is an inhibitor of Wnt-1 and a negative correlation with the WNT pathway was preliminarily established in other cancers. We demonstrated canonical

WNT pathway is one of the mechanisms explaining the interactions of *EMX1/2* and stem cell genes in sarcoma. The WNT-*EMX1/2* relationship was validated *in silico* with sarcoma patient datasets, *in vitro* in primary derived sarcoma cell lines, and *in vivo*, and *EMX* expression was found to negatively regulate the WNT pathway. In addition, the constitutive activation of the WNT pathway reverts to a more aggressive phenotype with stem cell properties and stemness gene transcription increased even in the presence of *EMX* overexpression.

Conclusion

In conclusion, our data establish the relationship among the WNT pathway, stem cell genes and the *EMX* genes, because *EMX* repress WNT signalling and activation of WNT pathway bypass *EMX*-dependent stemness repression and induces sarcomagenesis. Additionally, *EMX* genes negatively regulate cancer stem cells, acting as tumor suppressors in sarcoma, suggesting the relevance of the WNT/b-catenin/stemness axis as a therapeutic target in sarcoma.

EACR22-0048

The ideal candidate for inclusion in combination therapies targeting MYC-driven cancers

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Introduction

Cancer is a disease that affects both adults and children. Improved detection and prevention have an important role to play in improving this situation, but new and better treatments are urgently required.

Material and Methods

One such strategy is to target the transcription factors encoded by the MYC family of proto-oncogenes (MYC, MYCN and MYCL), which are among the most frequently deregulated in human cancers (>50% of cancers), including AML. MYC family deregulation exerts profound effects on the biology of tumour cells and is often associated with relapse and therapy resistant disease. Thus, this family of oncoproteins represent very attractive therapeutic targets but, as transcription factors lacking a catalytic active site, direct inhibition of their activities has proven to be very challenging and has not yet been accomplished. Promising approaches include targeting MYC family expression and identifying critical elements of pathways that have been reprogrammed by MYC family members and whose inhibition or removal cause the cells to die (so-called synthetic lethality). Synthetic lethality may arise due to a number of factors including impairment of pathways critical to survival of the cancer cell or the removal of positive feedback loops that promote expression of driver oncoproteins.

Results and Discussions

This project sought to establish whether targeted destruction of MYC protein combined with inhibition of the checkpoint kinases CHK1 and WEE1 exerted synergistic effects against the HL60 AML cell line. Dose-response analysis of the MYC-targeting drug Verlindamycin, the CHK1 inhibitor AZD-7762 and the WEE1 inhibitor Adavosertib demonstrated that all three inhibited HL60 cell growth at micromolar or nanomolar concentrations.

Conclusion

Functional analysis of Verlindamycin showed that 72 hours following initiation of treatment, cells had begun to undergo apoptosis. Combinatorial treatment presented the effect of degrading MYC and inhibiting its translation in combination.

EACR22-0050

The Hippo signaling co-activators YAP and TAZ suppress invadopodia formation and matrix degradation in multiple cancer cell lines

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Introduction

Metastatic cancer cells are known to possess adhesive and mechanosensitive, actin-rich structures called invadopodia, which degrade the extracellular matrix (ECM) and, thereby, facilitate tumor cell invasion. Multiple studies have highlighted the role of invadopodia, yet its molecular mechanisms in cancer metastasis remain elusive. The Hippo pathway co-regulators, YAP and TAZ, are well-known mechanosensing molecules that respond to chemical and physical signals from the ECM. Yet, their specific involvement in invadopodia formation is not clear. A better understanding of the specific roles of the Hippo pathway in invadopodia formation and the consequent matrix degradation will be instrumental for designing and applying novel and better-targeted therapies against metastatic cancer invasion. YAP and TAZ are commonly believed to promote cancer. Unexpectedly, we now show that YAP and TAZ can act as highly potent negative regulators of invadopodia formation and function in multiple cancer cell lines by downregulating key invadopodia components, including Tks5 and MMP14.

Material and Methods

We utilized various strategies to modulate YAP/TAZ signaling in multiple human cancer cell lines, including the knockdown of these regulators, their overexpression, or the use of an inhibitor. We then tested the effect on invadopodia formation and matrix degradation by quantitative microscopy and the cellular levels of major invadopodia components.

Results and Discussions

This study revealed that knockdown of YAP and TAZ, and treatment with the inhibitor verteporfin, greatly enhanced gelatin degradation and invadopodia formation on the tested cancer cell lines. Conversely, overexpression of these proteins suppressed invadopodia-mediated matrix degradation. Proteomic profiling analysis of MDA-MB-231 cells, following co-knockdown of YAP and TAZ, revealed a significant differential increase in the levels of seven invadopodia-associated proteins, including the crucial invadopodia components Tks5 and MMP14.

Conclusion

Overall, the results indicate that YAP and TAZ act as effective negative regulators of invadopodia formation and function by suppressing key invadopodia-associated proteins offering novel potential approaches for anti-metastatic therapies.

EACR22-0108

In situ detection of KRAS mutations in the context of pancreatic cancer subtypes

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is known for its aggressive biology and lethality. Transcriptomics have identified two major subtypes, termed 'classical' and 'basal-like'. *GATA6* is elevated in 'classical' PDAC and a valuable marker to distinguish both subtypes. Interestingly, aggressive PDACs have been linked to higher *KRAS* levels, mutant *KRAS* being the driver mutation in PDAC. Here, we assess a novel technique to successfully detect *in situ* *KRAS*^{WT} and *KRAS*^{G12D} on human tumours and co-visualize *GATA6* as a discriminating marker for PDAC subtypes.

Material and Methods

Samples were obtained from patients undergoing pancreatic surgery UZ Brussels, Belgium. Genotyping was done with NGS. BaseScope validation was performed in human PDAC cell lines with known *KRAS* genotype determined by sanger sequencing (Su86.86, HPAF-II, BxPC3; all from ATCC). BaseScope™ is used to detect the *KRAS* at RNA level while simultaneously detecting tumor (subtype) biomarkers at RNA or protein level, using RNAscope™ and immunofluorescence respectively. Image analysis is performed with HALO (Indica™) software.

Results and Discussions

BaseScope™ assay was validated to reliably detect *KRAS*^{WT} and *KRAS*^{G12D} on human PDAC cell lines. Positive control probe (*PPIB*) was detected in 91% of cells and only 0,7% showed false positive signal for the negative control probe (*DAPB*). In the homozygous mutant cell line Su86.86 only 5% of the cells was positive with the wild type probe while 78% of cells stained with the mutant probe.

Dot quantification on sections from *KRAS*^{G12D} mutant PDACs indicate a negative correlation (N=4; R=-0.7) between *KRAS* and *GATA6* expression, i.e high *GATA6*-expressing samples generally have a lower amount of both *KRAS*^{WT} and *KRAS*^{G12D} (respectively 0.15±0.04 and 0.03±0.01 dots/cell; t-test+Welch correction; N=7) , compared to low *GATA6*-expressing showing increased levels of *KRAS*^{WT} and *KRAS*^{G12D} cells (respectively 0.25±0.04 and 0.17±0.1 dots/cell; t-test+Welch correction; N=3), suggesting a more basal-like subtype. Interestingly, both phenotypes could be detected within one patient, demonstrating spatial heterogeneity.

Conclusion

Conventional transcriptomic profiling destroys tissue architecture hampering the ability to study histological and molecular heterogeneity. Here, we report the feasibility to successfully perform *in situ* detection of *KRAS*^{WT} and *KRAS*^{G12D} on human PDAC. Spatial subtyping that incorporates the spatial detection of mutations will provide critical insights into tumour heterogeneity and plasticity.

EACR22-0184

PTCH1 C-terminal domain mutations enhance colon cancer tumorigenesis

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Introduction

The canonical Hedgehog (Hh) signalling pathway, driving activation of SMO and GLI1, has been associated with tumorigenesis and progression of different cancer types; however, its role in colon cancer remains controversial. The C-terminal domain (CTD) of the Hh receptor PTCH1 has SMO- and GLI-independent “non-canonical” functions that might contribute to its tumour suppressor function. Here, we report the identification of PTCH1 CTD mutations in cancer and their role in tumorigenesis.

Material and Methods

Based on mutation calls from TCGA COAD and Genomics England GeCIP databases, we generated two independent PTCH1 CTD mutated clones (C9 and C15) in metastatic SW620 cells using CRISPR/Cas9 and a PTCH1 WT isogenic clone (SRC). The oncogenic role of the mutations was investigated using cell proliferation assays, anchorage-dependent and -independent colony formation, xenograft growth in athymic nude mice, growth factor signalling, and RNA-seq. Data were confirmed by qPCR and western blotting. Lentiviral shRNA delivery was used to silence overexpressed candidate genes.

Results and Discussions

Bioinformatic analysis revealed 3 hotspot mutations in the CTD of PTCH1 in ~20% right-side colon cancer cases: S1203(fs) and R1308(fs) and Y1316(fs), resulting in premature truncations of the CTD. The C9 and C15 clones engineered to carry a CTD truncation, acquired a spindle-like shape, increased *in vitro* cell proliferation by ~241 % and ~178%, anchorage-independent growth and migration by ~8- and ~4-fold compared to parental PTCH1 WT cells. Xenograft studies using C9 cells confirmed they formed 183% larger tumours than SCR cells *in vivo*. RNA-seq analysis showed that C9 and C15 cells have increased EGFR-MAPK, PI3K-AKT and TGF β signalling. MEK and PI3K inhibitors reduced proliferation of PTCH1 CTD mutant cells while SMO inhibition had no effect, ruling out the involvement of canonical Hh signalling. Surprisingly, GLI1 levels were upregulated in C9 and C15 cells in a SMO-independent manner. GLI inhibition with GANT61 abolished cell proliferation and colony formation, highlighting its pivotal role in the more aggressive phenotype of these cells. Treatment with GANT61 or shGli1 reduced ERK and AKT phosphorylation and *AREG*, *EREG* and *EGFR* expression. Complementary studies showed that both clones displayed an enhanced autophagic flux, reduced apoptosis and metabolic reprogramming.

Conclusion

Our study highlights the role of PTCH1 CTD mutations in cancer progression through dysregulation of non-canonical Hh signalling.

EACR22-0280

Mimicking PGC1 α tumor suppressive activity in prostate cancer.

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Introduction

Prostate cancer (PCa) therapies show favourable clinical responses, but a percentage of patients will still develop metastatic cancer that cannot be predicted nor efficiently treated. In this context, we identified the transcriptional co-regulator PGC1a as a prognostic factor in PCa with tumor and metastasis suppressive activity, although the molecular mechanism that could guide us towards effective therapies is still unknown.

Material and Methods

Our project integrates computational analysis of PCa patient databases and *in vitro* functional assays.

Results and Discussions

As a first strategy to infer the biological pathways associated with differential PGC1a expression, we performed GSEA on 5 PCa cohorts with clinical and transcriptional data. This computational approach pointed out that those patients with lower PGC1a expression have a molecular fingerprint enriched in MYC and E2F target genes. Furthermore, PGC1a and MYC mRNA expression was inversely correlated in PCa patients, as well as in PCa cell lines with inducible PGC1a expression, suggesting that PGC1a expression could modulate MYC transcriptional program. Indeed, RNAseq analysis of our *in vitro* PCa model with differential expression of the tumor suppressor showed that PGC1a re-expression is associated with a profound repression of MYC and E2F target genes. Interestingly, this repression is preceded by activation of Interferon (IFN) Type I pathway at the transcriptional and signalling level. We then hypothesised that loss of PGC1a in PCa patients drives non-canonical transcriptional reprogramming that increases MYC expression and its targets, thereby sustaining the acquisition of PCa aggressiveness. Thus, we challenged to inhibit MYC activity by expressing Omomyc in aggressive PCa cells and tried to phenocopy PGC1a activity. Our data show that expression of Omomyc in PC3 cells induces a strong reduction in 2D proliferation, cell cycle arrest and invasive growth, associated with altered expression of MYC canonical targets. Interestingly, chronic Omomyc expression in PC3 cells induces the activation of the IFN response pathway both at transcriptional and protein level.

Conclusion

MYC inhibition could be new and feasible therapeutic approach to treat and blunt PGC1a low-tumors. Our strategy could boost both intrinsic and extrinsic tumor suppression by activating the IFN pathway. Conceptually, these ideas support the implementation of precision medicine strategies based on PGC1a, but also open a new window of therapeutic opportunities for aggressive PCa.

EACR22-0282

The p53 target gene Zmat3 drives tumour development in cooperation with the critical effectors of p53-mediated apoptosis and cell-cycle arrest, puma and p21

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Introduction

The p53 transcription factor is a key barrier to cancer progression. Although for p53 to suppress cancer transcriptional activation has been shown to be essential, the role of specific targets remains elusive.

Material and Methods

We used *in vivo* shRNA and CRISPR library screens in mice to identify p53 transcriptional target genes that are critical for its tumour suppression activity.

Results and Discussions

ZMAT3, a double-stranded RNA binding zinc finger protein, was identified as a hit in those screens and we have demonstrated that in mouse *Kras*^{G12D} - driven lung cancer model and *Em-Myc* - driven lymphomas ZMAT3 unlike loss of p53, had minor impact on the rate of tumour development or severity of malignant disease. Thus, we hypothesised that additional co-occurring mutations in *Puma* and *p21*, the essential mediators of p53-driven cell death and cell cycle arrest (respectively), are required to resolve Zmat3 tumour suppressive activity and examine this by generating *Puma*^{-/-}*Zmat3*^{-/-}, *p21*^{-/-}*Zmat3*^{-/-} and *Puma*^{-/-}*p21*^{-/-}*Zmat3*^{-/-} deficient mice. Notably, we found that *Puma*^{-/-}*p21*^{-/-}*Zmat3*^{-/-} mice spontaneously developed malignancy at a high frequency in the first year of life, which indicate that several p53-regulated processes must fail simultaneously for tumours to develop in addition to ZMAT3 loss.

Conclusion

Our results demonstrate that p53 is a highly potent tumour suppressor because it can activate several tumour suppressive processes that cooperate and overlap to provide effective safeguard against cancer development.

EACR22-0290

Acute promyelocytic leukemia can be cured by a PML-driven cell-autonomous mechanism, independently from immune control

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Introduction

PML/RARA fusion protein expression leads to acute promyelocytic leukemia (APL) through the disruption of nuclear structures called PML nuclear bodies. These domains modulate stress response, particularly senescence, through the recruitment and post-translational modifications of a variety of partner proteins. All-Trans Retinoic Acid (ATRA) and Arsenic TriOxide (ATO), the current standard of care, cure the vast majority of patients, and both PML and PML/RARA have been identified as the direct targets of the treatment. However, the precise role of PML in APL cure still remains unclear. Previous work has failed to identify the actual functions of PML required for treatment response, including in innate immunity. Moreover, multiple studies have revealed the role of the immune system for therapy response and cure in cancer. Yet, any role of immunity in APL cure remains largely unexplored.

Material and Methods

We generated a variety of PML/RARA expressing mice in backgrounds where mutations in *Pml* abolish specific functions of PML (nuclear localization, partner recruitment or nuclear bodies formation). Then, APL blasts were transplanted in syngenic immunocompetent receivers to assess APL response to ATRA+ATO treatment. Transcriptomic analysis of treatment response was performed at different time points in these *Pml*-mutant APLs. Finally, APL cells were transplanted in immunodeficient NSG mice and these mice were treated to assess the role of the immune system in APL cure.

Results and Discussions

We found that Pml delays APL onset and aggressiveness in either untreated and treated acute transplant recipients. Critically, APLs defective for PML nuclear bodies formation or partner recruitment initially responded to ATRA+ATO treatment, but quickly relapsed, while mice transplanted with *Pml*^{+/+} APL could be cured. *Lilrb4* and *Tnfrsf14*, two immune checkpoint genes, were strongly deregulated in a Pml-dependent manner, but we failed to demonstrate interactions between APL blasts and immune cells upon treatment. Accordingly, highly immunodeficient mice transplanted with APL could still be cured with the ATRA+ATO combination.

Conclusion

Our work demonstrates that PML nuclear body formation and partner recruitment are required for APL cure and that the later can be achieved without immune system involvement. These findings emphasize that a potent targeted therapy can cure cancer in an autonomous, immune system independent, manner.

EACR22-0336

The Role of c-Myb in Tumorigenesis

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Introduction

Transcription factor expression and/or activity is altered in various cancer types. Due to the elaborate function of these proteins, many different processes, both pro- and anti-tumorigenic, can be dysregulated. c-Myb, a critical transcription factor in hematopoiesis and cell lineage commitment, has been described as a proto-oncogene in leukemia. In addition, recent studies have linked c-Myb overexpression and mutations to several solid cancers, including breast and colon cancer. However, contradicting reports on whether c-Myb overexpression correlates with a good or bad prognosis for cancer patients have emerged. In this study we aim to elucidate the exact role of c-Myb in tumorigenesis.

Material and Methods

We established a doxycycline (DOX) inducible system for c-Myb in cancer cell lines, which allows us to regulate c-Myb expression during tumorigenesis. The influence of c-Myb overexpression on tumor growth was analyzed both *in vitro* and *in vivo*. For our *in vivo* studies, tumor-bearing mice were given control or DOX containing food; and tumorigenesis was studied using histology, cytokine arrays and flow cytometry based analysis.

Results and Discussions

Our *in vivo* colon cancer model shows a significant reduction in tumor growth upon c-Myb overexpression. Since no changes in tumor cell proliferation or apoptosis were observed both *in vitro* and *in vivo*, we analyzed the tumor microenvironment in more detail. While no major changes were found in angiogenesis, cytokine arrays showed clear differences between control and DOX treated mice. In addition, we observed significant changes in different myeloid cell populations and activation state of CD8⁺ T cells within the tumor microenvironment.

Conclusion

This study shows that c-Myb in colon cancer cells can act as a tumor suppressor, by altering different immune cell populations and the cytokine profile within the tumor microenvironment.

Deciphering the role of the E3 ubiquitin ligase SMURF2 in molecular pathways of cancer

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Introduction

Accumulating evidence shows that activities of SMURF2, a HECT-type E3 ubiquitin ligase and suggested tumor suppressor, are linked to the regulation of core cellular processes pertinent to cancer onset, progression and therapeutic response. These processes are in the focus of the investigation in our laboratory.

Material and Methods

Different approaches ranging from genetics through molecular biology and biochemistry to animal pathophysiology and human sample analyses were employed.

Results and Discussions

SMURF2 was found to operate as a potent tumor suppressor in both murine and human cells. Its genetic inactivation changed cell identity and motility, replication rate and capacity to maintain the untransformed phenotype and, substantially, to repair DNA damage. As a consequence, the ability of *SMURF2*-depleted cells to preserve genome integrity was compromised, propelling the generation of diverse numerical and structural chromosomal alterations. Mechanistically, we showed that depletion of *SMURF2* leads to alterations in the epigenetic states, affecting chromatin compaction, DNA damage response and gene expression to promote uncontrolled cell growth and oncogenic phenotypes through the dysregulated RNF20/H2Bub1K120 and EZH2/H3x3MeK27 axes (Blank M et al., *Nat Med* 2012; Manikoth Ayyathan et al., *Oncogene* 2020). SMURF2 was also found to operate as an essential regulator of DNA topoisomerase II α (Topo II α), protecting this vital enzyme from proteasomal degradation and preventing the formation of pathological anaphase bridges, one of the major causes of chromosomal translocations (Emanuelli et al., *Cancer Res* 2017). Furthermore, our studies linked the distorted expression of SMURF2 to cancer progression, and suggested that alterations in SMURF2 subcellular localization could impede its tumor suppressor function by upregulating SMURF2 interactions with 14-3-3 proteins (Emanuelli et al., *Cancers* 2019). We have also found that SMURF2 controls the protein abundance of nuclear lamins, *i.e.* lamin A and its mutant form progerin, whose altered expression is associated with Hutchinson-Gilford progeria syndrome (HGPS) and may be related to cancer (Borroni et al., *Aging Cell* 2018). Additionally, our another study uncovered SMURF2 as an essential regulator of poly(ADP-ribose) polymerase-1 (PARP1), governing its activity, interactions and functions (Ilić et al., *FASEB J* 2021).

Conclusion

These findings point to SMURF2 as a pleiotropic cellular factor playing in carcinogenesis multiple roles through distinct molecular mechanisms.

The role of PAPP in tumorigenesis and its implication in programming cell death pathway.

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Introduction

The *PAPPA* (Pappalysin-1 or pregnancy-associated plasma protein-A) gene is located at 9q33.1 and encode a glycoprotein, which belongs to the superfamily of zinc-dependent metalloproteases (metzincins). *PAPPA* is involved in the insulin-growth factor (IGF) pathway by cleavage insulin-like growth factor binding proteins (IGFBPs), mainly IGFBP-4. Some studies associated the increase of IGFBP-4's proteolysis and *PAPPA* expression with the appearance of mammary tumors in pregnant transgenic mice. However, other studies suggested that *PAPPA* is epigenetically silenced in precursor lesions of breast cancer and the downregulation of *PAPPA* generates more invasiveness. Therefore, the role of *PAPPA* is controversial in breast cancer.

Apoptosis is a programmed cell death pathway that is responsible for maintenance of organismal homeostasis. The balance between cell division and cell death is broken in diseases such as cancer. However, this cell death mechanism could be helpful to research new markers and drugs targeting aspects of apoptosis. In this study, we focus in the implication of *PAPPA* in tumorigenesis and its possible repercussion in the promotion of apoptosis in breast cancer.

Material and Methods

PAPPA was overexpressed in different breast cancer cell lines and tumorigenesis was analysed using different functional assays: growth curves, clonability assays and tumorspheres assays. We have also analysed several stem cell markers by RT-qPCR. Additionally, we measured apoptosis through FACS by using Annexin V and propidium iodide stainings as well as analysing several apoptosis markers by western blot.

Results and Discussions

The overexpression of *PAPPA* caused a decrease in the proliferation of the cells and in the number of clones formed in the three breast cancer cell lines. In addition, the overexpression of *PAPPA* decreases some stemness properties of cells such as the formation of holoclones and mammospheres. Moreover, apoptosis seems to be increased when *PAPPA* is upregulated.

Conclusion

PAPPA overexpression decrease some tumorigenic and stemness properties of the cells and this effect may be due to the induction of apoptosis.

EACR22-0506

Tonic T cell receptor signaling is leukemogenic while its strong stimulation suppresses leukemia development

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Introduction

T cell acute lymphoblastic leukemia (T-ALL) is an aggressive T cell precursor malignancy in which T cell receptor (TCR) expression is common. Although mutations in TCR components are rare in T-ALL, TCR stimulation in T-ALL cells triggers an apoptotic program reminiscent of thymocyte negative selection, suggesting variations in TCR signaling intensity might have specific functions in leukemogenesis, similar to what is seen in normal thymocytes. Here, we aimed to determine the role of TCR activation in leukemogenesis in a TCR-driven transgenic model.

Material and Methods

We used mice expressing MHC class II-restricted transgenic TCR specific for the HY male antigen (Marilyn), in which female thymocytes receive weak TCR signals upon recognition of self-antigens presented by MHC (self-pMHC), are positively selected and differentiate into mature T cells, while male thymocytes receive strong HY-dependent TCR signals and die through negative selection. We also used OT-I mice, which express a transgenic TCR with high affinity for MHC class I-presented ovalbumin SIINFEKL peptide (OVAp) and lower affinity for mutant OVAp.

Results and Discussions

Aged Marilyn females, but not males, developed T-ALL characterized by infiltration of non-lymphoid organs and *Notch1* mutations, TCR expression, immature phenotype, and intermediate CD5 levels (similar to double-positive thymocytes), suggesting basal TCR stimulation (tonic signaling) fostered T-ALL development.

Strong *in vitro* TCR activation of Marilyn T-ALL cells resulted in increased CD5 and CD69 expression (activation marker) and cell death. Female Marilyn T-ALL cells expanded and generated disease in infused syngeneic females (HY-negative) but weakly or not at all in males (HY-positive). Additionally, cognate antigen (DBY peptide) treatment in Marilyn T-ALL infused females resulted in increased survival compared to PBS-treated counterparts, indicating TCR stimulation by agonist antigen hampered leukemogenesis. To further investigate the anti-leukemic effects of TCR stimulation by agonist antigen, we bred OT-I mice into the ETV6-JAK2 fusion transgenic T-ALL model. OVAp treatment resulted in increased apoptosis in mice infused with ETV6-JAK2;OT-I T-ALL and also induced apoptosis *in vitro*. Interestingly, the SIINFEKL (T4) and SIIGFEKL (G4) lower affinity OVAp, induced apoptosis less efficiently.

Conclusion

Together, these data indicate tonic TCR stimulation levels promote T-ALL development, while stronger agonist-induced TCR stimulation progressively suppresses T-ALL development.

EACR22-0560

RNF144B contributes to p53-dependent tumor suppression by preserving genomic stability

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Introduction

Mutation in the TP53 tumor suppressor is a key event in cancer development. 40 years of investigation haven't been enough to understand how p53 suppress tumor growth. Cell cycle arrest, senescence and apoptosis have long been considered essential effectors of p53-mediated tumor suppression; however, several studies have shown that combined genetic inactivation of these processes were dispensable for tumor suppression. Other under-

appreciated p53 targets must mediate its potent tumor suppressive function. Recently, an *in vivo* screen revealed that several DNA repair genes activated by p53 were critical tumor suppressors, as their knockdown (KD) caused the acceleration of MYC-driven lymphoma. RNF144B, an E3 ubiquitin ligase, arose as one of the most potent hits on the screen. Here, I aim to understand how RNF144B contributes to p53 tumor suppression.

Material and Methods

I have used mouse and human p53-proficient cancer and healthy cell lines for my experiments. I have performed several *in vitro* and *in vivo* assays to evaluate the cellular and molecular mechanisms of RNF144B in tumor suppression. Finally, I have performed a proteomic analysis to unravel putative targets of RNF144B's E3 ubiquitin ligase action.

Results and Discussions

It has been described that RNF144B has a potent tumor suppressor role in lymphoma, however it is unknown if this effect is conserved in other cellular contexts. With that in mind, I have generated different RNF144B deficient cell lines and performed tumor growth *in vivo* studies. RNF144B Knockdown (KD) was able to increase tumor growth to a similar extent than knocking down p53 itself in both MEF subcutaneous and orthotopic lung cancer models. Remarkably, RNF144B KD MEFs presented with important polyploidies, which could indicate RNF144B's involvement in chromosomal stability. Our proteomic analysis supported this hypothesis, as it suggests that RNF144B is involved in regulating microtubule binding proteins which, if affected, could give rise to an aberrant mitotic process. Finally, upon gamma irradiation, RNF144B KD MEFs exhibited an aberrant DNA repair response, similar to p53 KD cells, further consolidating RNF144B's involvement in genomic stability.

Conclusion

Taken together, our work shows that RNF144B is a p53-activated gene that contributes to tumor suppression not only in lymphoma but also in other cellular contexts. Our results indicate that RNF144B is involved in preserving genomic stability and DNA damage response regulation.

EACR22-0622

CEACAM6 is a positive regulator of gallbladder cancer aggressiveness

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Introduction

Gallbladder cancer (GBC) comprises the most common biliary tract cancer (BTC). It is a rare disease often manifesting as aggressive malignancy due to its asymptomatic character at early stage. Most GBC are unresectable and their therapy options are limited to adjuvant therapy with gemcitabine and cisplatin. Finding targeted therapies and understanding molecular drivers in GBC are still urgently needed. This study combines proteomic analysis of GBC, characterization of potential oncogenes, and identification of potential biomarkers for GBC by sequencing technology to obtain a deeper insight on GBC oncogenesis.

Material and Methods

Formalin-Fixed Paraffin-Embedded (FFPE) tissues of 5 GBC and 5 healthy gallbladder samples were provided by the tissue Bank of the National Center for Tumor Diseases (NCT, Heidelberg, Germany). Herein, quantitative mass-spectrometric analysis was performed using the 10plex™ Tandem Mass Tag (TMT) system and an Orbitrap Fusion™ Lumos™ mass spectrometer. Deregulated proteins detected by mass spectrometry were analysed and oncogenic protein candidates were evaluated. Transient siRNA knockdown and stable inducible cell lines were established to characterize protein candidates in GBC cell lines. RNAseq data were analysed to unravel the significant pathways perturbed in GBC. Furthermore, in vitro experiments were conducted to investigate relevant biological functions of the candidates.

Results and Discussions

In total 4,827 proteins were detected of which 676 proteins were significantly downregulated and 1,090 proteins were upregulated (adjusted $p \leq 0.05$). Carcinoembryonic Antigen-related Cell Adhesion Molecule 6 (CEACAM6) presented as one of the strongest upregulated and most significant proteins among 1,090 upregulated proteins in GBC sample (fold-change= 5.54, $p \leq 0.01$). RNAseq analysis of CEACAM6 overexpressing cells confirmed significant pathways related to GBC aggressiveness. Correspondingly, in vitro experiments revealed CEACAM6's role in decreasing cell adhesion, promoting migration, and supporting invasion of GBC cells. Conversely, CEACAM6 knockdown abolished these phenotypes of GBC aggressiveness. Therefore, inhibition of CEACAM6 may be an effective therapeutic option in GBC.

Conclusion

This study reveals that CEACAM6 plays a pivotal role in supporting GBC aggressiveness by increasing migration and invasion while decreasing cell adhesion.

EACR22-0644

A TBX3 oncogenic signalling axis important in breast cancer

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Introduction

Breast cancer is the leading cause of cancer-related deaths in women globally with 58% occurring in low-middle-income countries. There is therefore a need to identify more effective breast cancer therapies. The transcription factor TBX3 is critical for breast development and when expressed in postnatal mammary tissues it contributes to breast cancer. Importantly, TBX3 has been validated as a novel therapeutic target and identifying breast cancer oncogenic factors that co-operate with TBX3 may represent important anti-cancer drug targets. The aims of this project are therefore to identify and characterise (1) signalling molecules that upregulate TBX3 and (2) protein partners that cooperate with TBX3 to promote breast cancer.

Material and Methods

To determine if c-Myc regulates TBX3 expression, luciferase reporter and qRT-PCR assays were performed. Nucleolin and Hsc70 were identified and validated as TBX3 co-factors by affinity purification coupled with mass spectrometry, immunoprecipitation assays and confocal microscopy/co-localisation analysis. The functional significance of (1) TBX3-Hsc70 was investigated by siHsc70, MG132 (proteasomal inhibitor) and western blotting and (2) TBX3-nucleolin by RNAi/overexpression in rescue experiments coupled with scratch motility

assays. The effect of the nucleolin targeting aptamer, AS1411, on TBX3/nucleolin levels, subcellular localisation, ability to regulate target genes and as a therapy for breast cancer was tested in western blotting, immunofluorescence, qRT-PCR and MTT assays. All experiments were carried out in two breast cancer cell lines.

Results and Discussions

We show that c-Myc upregulates TBX3 transcriptionally. Hsc70 enhances TBX3 stability and nucleolin cooperates with TBX3 to promote breast cancer cell migration. The nucleolin targeting aptamer, AS1411, inhibits breast cancer cell viability and migration with no effect on normal breast epithelial cells and mislocalizes TBX3 and nucleolin to the cytoplasm blocking their ability to activate the breast cancer oncogene, ID1.

Conclusion

c-Myc/TBX3/Hsc70/nucleolin is an important oncogenic pathway in breast cancer and AS1411 disrupts this pathway making it a promising therapeutic agent for treating breast cancer.

EACR22-0688

Loss of 14-3-3 σ leads to decreased tumor formation and sensitivity to 5 fluorouracil due to an increase in the nuclear localization of YY1

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Introduction

14-3-3 proteins are small, dimeric, acidic proteins that regulate multiple cellular signalling pathways by binding to their ligands in phosphorylation dependent or independent manner. Among the seven mammalian isoforms, 14-3-3 σ is expressed only in epithelial cells and exclusively forms homodimers. 14-3-3 σ functions as an oncogene in colon cancer and as a tumor suppressor in breast cancer. Our previous work has demonstrated that 14-3-3 σ -/- cells showed the induction of EMT. However, the loss of 14-3-3 σ leads to decreased tumor formation in immunocompromised mice and sensitivity to γ -radiation and 5-fluorouracil.

Material and Methods

Clonogenic survival assays were performed by treating cells with γ -radiation or 5FU. Immunofluorescence assays were performed and data was represented as average nuclear intensity. RT-PCR was performed and fold change in gene expression was plotted. GST pulldown assays were performed followed by Western blotting. RNA seq data was analysed using TCGA database in samples with high 14-3-3 σ and low YY1 levels. YY1 ChIP data was analysed using ENCODE database.

Results and Discussions

To determine the mechanisms underlying the decreased tumor formation and increased sensitivity to 5FU in 14-3-3 σ -/- cells, we observed that the nuclear localisation of YY1 was

significantly increased. Ectopic expression of 14-3-3 σ in 14-3-3 σ ^{-/-} cells leads to increase in resistance to 5FU and a decrease in nuclear localisation of YY1. GST pulldown assays suggested that 14-3-3 σ interacts with YY1 indicating 14-3-3 σ might regulate the subcellular localisation and thus transcriptional function of YY1. Further RNA seq data and pathway analysis suggested that UPR pathway was enriched in 14-3-3 σ positive and YY1 negative category. Analysis of YY1 ChIP data using ENCODE database from HCT116 cells demonstrated that enrichment of YY1 was less on the promoters of UPR genes. We observed that UPR genes are downregulated both at mRNA and protein levels in 14-3-3 σ ^{-/-} cells and its levels are increased in 14-3-3 σ overexpressing cells.

Conclusion

The phenotypes observed in 14-3-3 σ ^{-/-} cells suggest that 14-3-3 σ is required for tumor progression and resistance in HCT116 cells. Our results have identified a potential mechanism by which 14-3-3 σ can contribute to therapy resistance by restricting YY1 to the cytoplasm and preventing repression of the UPR pathway. Further, disruption of the 14-3-3 σ YY1 complex could be a novel therapeutic strategy which will allow sensitization of colorectal cancers to chemotherapy.

EACR22-0756

A characterization of the most common RNF43 mutations in gastric cancer in vitro and in vivo

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Introduction

The E3 ubiquitin ligase RING finger protein 43 (RNF43), is a tumor suppressor that negatively regulates the WNT/ β -catenin signaling pathway by ubiquitinating Frizzled receptors and sequestering TCF4 to the nuclear envelope. RNF43 is mutated in multiple types of gastrointestinal tumors. The most recurrent mutation is the G659Vfs*41 mutation with a frequency of ~5-8% in stomach, colon and pancreatic cancer. Although being the most frequent mutation found, its effects on RNF43 functionality and cellular responses are controversially debated.

Material and Methods

We selected the most common *RNF43* mutation found in gastric cancer patients according to TCGA and COSMIC database, G659fs, and introduced it in gastric cancer cells. Mutations also found in gastric cancer patients such as R117Pfs, L120Hfs, D196Mfs, and C312* were also introduced by CRISPR/Cas9 gene editing for comparison. Cell proliferation, DDR activation and cell viability upon γ -radiation was analyzed in gastric cancer cell bearing mutated *RNF43*. In addition, we generated a mouse model carrying the *RNF43* G659Vfs*41 mutation to examine its effects *in vivo*.

Results and Discussions

Mutations in *RNF43* and in particular the G659Vfs*41 mutation conferred gastric cancer cells resistance to γ -radiation. Interestingly, we observed altered morphology of the stomach of mice carrying this mutation. In addition, gastric organoids from mutant mice showed higher growth compared to organoids from wild type mice.

Conclusion

Collectively, our findings indicate that mutations in *RNF43* confer gastric cells resistance to DNA damage induced by radiotherapy. In addition, we successfully generated a mouse model to study the effect of *RNF43* mutation G659Vfs*41 in gastric carcinogenesis and therapy response.

EACR22-0812

E-cadherin variants associated with distinct clinical phenotypes induce distinct motile and adhesive profiles

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Introduction

Germline mutations of E-cadherin (*CDH1* gene) contribute to hereditary diffuse gastric cancer (HDGC) and congenital malformations such as oral facial clefts (OFC). However, the molecular mechanisms through which E-cadherin loss-of-function leads to distinct clinical outcomes remain unknown. We propose that E-cadherin mediated disorders result from abnormal interactions between E-cadherin mutant cells with the extracellular matrix (ECM), and consequently from aberrant integrin activation, affecting the direction and coordination of epithelial migration. Thus, our main goal is to identify the key players and critical signaling events that are responsible for abnormal lip/palate closure in E-cadherin germline mutation carriers.

Material and Methods

To investigate the migratory behavior promoted by OFC or HDGC variants, we performed a comprehensive characterization of cell motility by exploring gap closure migration assays coupled with time-lapse microscopy and bioimaging analysis. For subsequent identification of molecules that control aberrant cell motile behavior, we used a high-throughput ECM array. Specifically, cells transfected with vectors encoding the wild-type E-cadherin or variants found in OFC and HDGC contexts were assayed in this platform, and the number of cells attached to each ECM spot was quantified as a direct measure of cell-matrix adhesive ability.

Results and Discussions

We verified that OFC and HDGC variants yield distinct motile profiles. Cells expressing OFC variants migrate faster and in a direct way, as verified by low travelled distances and poor diffusion rates. In contrast, HDGC variants show a high cell displacement and low velocity during wound closure. Regarding ECM adhesion, OFC mutants also present greater attachment abilities, when compared with wild-type cells. Remarkably, cells expressing OFC variants adhere to a wider variety of matrices than those expressing HDGC variants. This data further suggests that OFC variants lead to increased cell plasticity and high responsiveness to different microenvironments.

Conclusion

Herein, we demonstrated that E-cadherin variants associated with distinct clinical outcomes induce altered cell migratory phenotypes and ECM adhesion preferences, implicating that aberrant integrin activation may underlie *CDH1* pleiotropy.

EACR22-0822

Production and evaluation of HMGB1 and HMGB2 knockouts in SKOV3 ovarian cancer cell line

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Introduction

Ovarian cancer is one of the most common cancer types among women in the world leading for an estimated 239,000 new cases and 152,000 deaths worldwide each year. Almost 70 % of ovarian cancer diagnosis occur when the cancer has already spread in the abdominal cavity and this make the 5-year survival rate drops from 45% to 25% when diagnosed in advanced stages of the cancer progression.

Several studies investigated the association of high mobility group box (HMGB) proteins with cancer due to their importance and the variability functions inside and outside the cells. HMGB1 and HMGB2 proteins are the most abundant members among HMGB family and they contribute to a variety of hallmarks of cancer such as instability in the genome, increased in the mutations rates, tumor promoted inflammation, growth repressors insensibility, cellular energetics deregulation, immune destruction evasion, metastasis and angiogenesis stimulation.

Material and Methods

In the present work, the production of HMGB1 and HMGB2 knockouts in SKOV3 ovarian cancer cell line will be done using CRISPR/Cas9 technology to analyze the effect on the transcriptional regulation of several oncogenic biomarkers related to the cancer onset and progression.

Results and Discussions

We got two candidates (5D2 and 3F1) that are HMGB1 knockout. The RT-qPCR results for HMGB1 knockout candidate (5D2) showed significant down-regulation of several genes related with the synthesis and remodulation of rRNA such as UBTF and RSS1 (p-value < 0.05), while the other HMGB1 knockout candidate (3F1); RSS1 gene was found to be significantly down-regulated (p-value < 0.05). Additionally, we got two candidates (10F2 and 3E2) that are HMGB2 knockout. The RT-qPCR results for HMGB2 knockout candidate (10F2) showed significant down-regulation of several transcriptional regulators genes such as HAKAI, PARP1 and SP1 (p-value < 0.05), while the other HMGB2 knockout candidate (3E2); HAKAI and KRR1 genes were found to be significantly down-regulated (p-value < 0.05).

Conclusion

Several genes related with the synthesis and remodulation of rRNA such as UBTF and RSS1 were significantly down-regulated in HMGB1 knockout candidate (5D2), while RSS1 gene was found to be significantly down-regulated in HMGB1 knockout candidate (3F1). On the other hand, several transcriptional regulators genes such as HAKAI, PARP1 and SP1 were significantly down-regulated in HMGB2 knockout candidate (10F2), while HAKAI and KRR1 genes were found to be significantly down-regulated in HMGB2 knockout candidate (3E2).

EACR22-0850

A gene dosage-dependent effect unveils NBS1 as both a haploinsufficient tumor suppressor and an essential gene for SHH-medulloblastoma

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Introduction

Introduction: Inherited or somatic mutations in *MRE11*, *RAD50* and *NBN* genes increase incidence of tumors, including MB. On the other side, *MRE11*, *RAD50* and *NBS1* protein components of the MRN complex are often overexpressed and sometimes essential in cancer.

In order to solve the apparent conundrum about the oncosuppressive or oncopromoting role of the MRN complex, we explored the functions of *NBS1* in a MB prone animal model.

Material and Methods

We generated and analyzed mono- or biallelic deletion of the *Nbn* gene in the context of the *SmoA1* transgenic mouse, a SHH-dependent MB prone animal model. We used normal and tumor tissue from these animal models, primary cerebellar granule cell progenitors (GCPs) from genetically modified animals, and *NBS1*-depleted primary MB cells, to uncover the effects of *NBS1*-depletion by RNA-Seq, by biochemical characterization of the SHH-pathway and the DNA damage response (DDR) as well as on the growth and clonogenic properties of GCPs.

Results and Discussions

We found that monoallelic *Nbn* deletion increases *SmoA1*-dependent MB incidence. In addition to a defective DDR, *Nbn*^{+/-} GCPs show increased clonogenicity compared to *Nbn*^{+/+} GCPs, dependent on an enhanced Notch signaling. In contrast, full *Nbn*^{KO} impairs MB development both in the *SmoA1* mice and in a SHH-driven tumor allograft.

Conclusion

Our study indicates that *Nbn* is haploinsufficient for SHH-MB development while full *Nbn*^{KO} is epistatic on SHH-driven MB development, thus revealing a gene dosage-dependent effect of *Nbn* inactivation on SHH-MB development.

EACR22-0892

Spry1 as a potential new hypoxia modulator in cutaneous melanoma

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Introduction

Hypoxia has been recognized as one of the crucial features of solid tumors. It is mediated by the activation of the transcription factor hypoxia-inducible factor 1 α (HIF-1 α), which affects and regulates the expression of several genes involved in metabolism, angiogenesis as well as other biological mechanisms. Our recent findings indicated that the knock-out of the MAPK modulator Spry1 (Spry1^{KO}) reduced the epithelial-mesenchymal transition (EMT) potential of BRAF^{V600}-mutant cutaneous melanoma (CM) cells. Since hypoxia has been reported to participate to EMT in order to trigger BRAF^{V600}-mutant CM metastatic progression, the present study was design to exploit whether Spry1 contributes to hypoxia in CM.

Material and Methods

SPRY1 gene was knocked-out using the CRISPR-strategy in BRAF wild-type (wt) and mutant CM cell lines. By using *in vitro* and *in vivo* models, the effects of Spry1^{KO} on HIF-1 α -related genes were investigated through RNA-sequencing, quantitative real-time PCR, Western blot, and immunofluorescence analyses. The Luminex platform was used to analyze cytokines secretion, whereas the angiogenic potential was assessed through *in vitro* tube formation assays.

Results and Discussions

Reduced HIF-1 α nuclear expression and mRNA down-regulation of HIF-1 α -related genes were observed in all Spry1^{KO} clones, regardless of their BRAF mutation status. Of interest, the nuclear expression of the hypoxia-related long non-coding RNA miR210HG was strongly reduced, suggesting that it may function as a key hypoxia regulator in CM. E-cadherin expression, which is usually down-modulated during EMT, was restored in Spry1^{KO} xenografts, whereas lower expression and secretion of MMP1, MMP2, MMP3 and MMP8 were measured in BRAF wt and mutant Spry1^{KO} compared to their parental CM cell lines. Finally, vessels formation and CD31 expression were impaired in all Spry1^{KO} clones, thus indicating a substantial reduction of the tumor-associated angiogenic potential.

Conclusion

Taken together, these data suggest that Spry1 may function as a key regulator of hypoxia in CM, regardless of BRAF mutation status and that the targeting of Spry1 may represent a promising therapy strategy to counteract the hypoxic signaling and progression of CM.

EACR22-0920

Allele-specific knockdown of β -catenin reveals new targets associated with poor prognosis in liver cancer

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Introduction

β -catenin is a well-known effector of the Wnt pathway and a key player in cadherin-mediated cell adhesion. Oncogenic mutations of β -catenin are highly frequent in liver primary tumors. Those mutations are mostly heterozygous allowing the co-expression of wild-type (WT) and mutated β -catenins in tumor cells. We investigated the interplay between WT and mutated β -catenins in liver tumor cells, and searched for new actors of the β -catenin pathway.

Material and Methods

We used the HepG2 cell line that has the specificity to carry two different β -catenin alleles: one encoding wild-type (WT) β -catenin and the other encoding a constitutively active mutated β -catenin. This specific setting allowed us to design a RNAi strategy to specifically deplete WT and/or mutated β -catenin. The impact was characterized using transcriptomic and functional analyses. We compared our data with transcriptomic data from mice that develop liver tumors upon activation of β -catenin in hepatocytes (APC^{KO} and β -catenin ^{Δ exon3} mice) and from human HB specimens. We further analyzed samples by immunohistochemistry.

Results and Discussions

Allele-specific knockdown of β -catenin in HepG2 cells allowed uncoupling of the two functions of β -catenin in the same cellular background: nuclear/transcriptional activity, mainly mediated by the mutated β -catenin, and membrane/structural activity, mediated by the degradable WT β -catenin. Our results showed that WT and mutated β -catenin play an antagonist role in hepatocyte differentiation, attested by alteration of hepatocyte markers expression and bile canaliculi formation. Searching for new targets of β -catenin acting on hepatocyte differentiation, we focused on Fascin-1. We found that Fascin-1 expression alters liver tumor cell plasticity. Using mouse models, we found that Fascin-1 is highly expressed in mutated β -catenin-induced undifferentiated tumors. Finally, we found that Fascin-1 is a specific marker of the embryonal component in poor-prognosis human HBs.

Conclusion

Combining allele-specific knockdown and transcriptional analysis, we identified β -catenin targets whose expression is antagonistically altered by WT and mutated β -catenin and associated with hepatocyte differentiation status. We highlighted Fascin-1 as a new player in the modulation of cancer cell plasticity associated to β -catenin pathway alteration in the liver and as a new potential target in HB.

EACR22-0930

The c-Myc/TBX3/nucleolin signaling axis promotes the transformation of sarcoma-initiating cells

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Introduction

Sarcomas are aggressive cancers of mesenchymal origin whose clinical management is severely challenged. This is in part due to a lack of understanding of the molecular mechanisms underpinning the transformation of human mesenchymal stromal/stem cells (hMSCs), the sarcoma-initiating cells. T-box transcription factor 3 (TBX3) is upregulated by the oncogenic c-Myc in several sarcoma subtypes where it interacts and cooperates with

nucleolin (NCL) to promote proliferation and migration. While there is evidence that c-Myc can initiate the transformation of hMSCs into sarcomas, whether TBX3 and NCL may be involved are not known. The current study addresses this through investigating whether (1) the c-Myc/TBX3/NCL axis contributes to hMSC proliferation and migration, (2) the overexpression of TBX3 in hMSCs promotes key hallmarks of cancer and if the overexpression of NCL can enhance these abilities.

Material and Methods

Whether c-Myc regulates *TBX3* in hMSCs was examined using siRNA, qRT-PCR, luciferase reporter and chromatin-immunoprecipitation assays. Immunoprecipitation assays and co-localisation analysis were used to assess whether TBX3 and NCL interact in hMSCs. TBX3 and TBX3+NCL overexpressing hMSCs were generated using lentiviral gene transfer and the impact of TBX3 and TBX3+NCL overexpression was investigated in 2D- and 3D-cell culture models. These included colony formation unit assays, senescence-associated β -galactosidase staining, growth curves, calcein AM staining, 2D-scratch motility assays, 3D-collagen invasion assays and global gene expression profiling by microarray analysis and gene set enrichment analysis.

Results and Discussions

TBX3 is transcriptionally activated by c-Myc at a conserved E-box motif in hMSCs and *TBX3* interacts and cooperates with NCL to promote hMSC proliferation and migration. Overexpression of *TBX3* in hMSCs promoted bypass of senescence, proliferation, migration and invasion which corresponded with a decrease in levels of senescence markers and an increase in levels of cell cycle progression, migration and invasion markers. Transcriptomic analysis revealed that genes upregulated upon *TBX3* overexpression overlapped with c-Myc targets, were involved in cell cycle progression, and were associated with sarcomagenesis. Furthermore, NCL enhanced the ability of *TBX3* to promote 3D-hMSC proliferation and invasion.

Conclusion

The c-Myc/TBX3/NCL oncogenic molecular pathway may be a key mechanism that transforms hMSCs into sarcomas.

EACR22-0954

Structural and functional analysis of Intrinsically disordered DNA binding domain of BRCA1 and its clinically reported missense mutations

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Introduction

BRCA1 consists of an N-terminal RING domain, a central DNA binding region, and two tandem BRCT repeats at its C-terminus. Several studies have been performed to classify the pathogenicity of variants of uncertain significance (VUS). However, categorizing the pathogenicity of mutations from a larger cohort of patients is still a challenging task. Therefore, the mechanism by which these variants cause the disease remains undeciphered.

Material and Methods

Biophysical characterization of protein: Purified proteins were assessed for their biophysical characteristics with the help of Circular Dichroism spectroscopy for alterations in their folding patterns.

Small Angle X-ray Scattering (SAXS): The radius of gyration, $D_{\max}$ and intrinsically disordered properties were determined at varying protein concentrations by Guinier approximation, Kratky plot and pairwise distribution function. The data were processed using the ATSAS suite package.

X-ray crystallography: BRCA1 BRCT crystals were grown at 22°C using the hanging drop vapour diffusion method.

EMSA: Samples were electrophoresed on 1% agarose gel at 100V and 4°C.

Structure prediction and molecular dynamics simulation: Model generated using ROBETTA was simulated for 100ns and compared for its disorderiness.

Results and Discussions

Far-UV CD spectra display a secondary structure alteration in BRCA1 Ser282Leu, Thr1691Arg and Gly1801Asp. The addition of 2,2,2-trifluoroethanol (TFE) led to the gain in alpha-helicity of the central region and therefore, less susceptibility towards proteolysis. Further, conformational analyses by SAXS demonstrates an extended, less compact structure of the BRCA1 central region. The ensemble optimization method shows that the wild-type protein exists in multiple conformations with a Rflex value of 51% while the Ser282Leu mutant shows higher flexibility with a Rflex value of 59%. Reduced DNA binding ability was observed for the Ser282Leu mutant as compared to the wild-type protein.

Conclusion

We have characterized the BRCA1 central region, its behaviour in solution, and elucidated how its structural plasticity and heterogeneity is a prerequisite for the proper functioning of the protein. Induced folding in presence of TFE revealed a protease-resistant fragment within the central region of BRCA1. This shows that the protein has an inherent propensity to form α -helices in presence of its binding partner. Intrinsically disordered behaviour of the protein as depicted from SAXS were in corroboration with the Molecular Dynamics Simulation.

EACR22-1230

Regulatory role of miR-449a as a potential biomarker in Head and Neck Carcinoma

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Introduction

Head and neck squamous carcinoma (HNSCCs) and laryngeal squamous cell carcinoma (LSCC) are the most common histological subtype characterized by aggressive and invasive phenotypes of head and neck carcinoma (HNC). miRNA expression and mechanisms of action on biological pathways appear strictly tissue and cellular context-specific, especially in the histological patients' samples, pointing out their potential exploitation as early non-invasive biomarkers. Moreover, miRNAs, are involved in maintaining homeostasis and exerting post-transcriptional gene expression regulation.

Material and Methods

We applied a customized in silico prediction analysis to identified miR-449a target genes. To investigate biological functions, we evaluated miR-449a expression levels in four HNSCC cell lines by qRT-PCR. We selected two HNSCC cell lines (HNO-210 and FaDu). Using lentiviral stable miR-449a transfection for in vitro studies we evaluated miRNA biological function (proliferation, invasion and migration). We performed qRT-PCR analysis to evaluate genes differentially expressed in our cellular models and Western Blot analysis to identify modulation of proteins involved in specific molecular pathways.

Results and Discussions

Functional studies showed significant effects. Using TaqMan Gene Expression Assay we detected differential expression of key genes upon miRNA: up regulation of AKT, STAT3, MAPK involved in EMT inducing transcription factors and down regulation of PTEN and KRAS. These data are additional supported investigated the effects of miR-449a on EMT, IL6/JAK/STAT and PTEN/PI3K/AKT signaling pathways modulation in TGF- β and IL-6 stimulated condition.

Conclusion

We provide evidence of the role of miR-449a regulation of EMT, IL6/JAK/STAT and PTEN/PI3K/AKT in head and neck carcinoma in vitro model.

EACR22-1268

Identifying potential therapeutic targets for cancer prevention

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Introduction

Most efforts done in cancer research are focused on finding treatments to eliminate the presence of cancerous cells already present in our tissues. However, studies focused on developing cancer prevention strategies are scarce. Up to now, cancer prevention strategies are mostly focused on the promotion of healthy habits and early diagnosis screens. Hence, there is not any clinical indication for cancer chemoprophylaxis in the general population, and the most appropriate molecular targets for cancer prevention are not defined. Thus, it would be extremely useful to promote the development of potential cancer prevention strategies. Remarkably, the majority of hereditary cancer syndromes are caused by mutations in genes involved in the DNA damage response (DDR), indicating the relevance of this pathway to prevent cancer.

Material and Methods

This study is based on a CRISPR-activation system, which uses a catalytically dead Cas9 (dCas9) protein fused to a transcriptional activator domain that can be directed to any gene promoter using specific guide RNAs (gRNAs). We generated a gRNA library targeting DDR-related tumor suppressor genes (TSGs) and we carried out a screening in order to identify those that confer protection against DNA damaging agents.

Results and Discussions

We have identified several hits that seem to confer extra protection from DNA damage when increasing their endogenous expression in human cells. Once validated, the most relevant TSGs identified will be investigated *in vivo* by developing genetically modified mice with activation of those TSGs.

Conclusion

The identified TSGs whose overexpression does not affect cell viability or proliferation and leads to a better protection against certain types of DNA damage will be defined as potential therapeutic targets for cancer prevention.

EACR22-0019

RNA-binding protein IGF2BP1 promotes neuroblastoma metastasis in extracellular vesicle-dependent manner

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Introduction

Neuroblastoma (NB) being a highly metastatic cancer, is one of the leading causes of cancer-related mortalities in pediatric patients. More than 50% of NB cases exhibit 17q21-ter partial gain, and it is independently associated with poor survival, thus indicating the clinical importance of genes at this locus in NB. One such gene at 17q21 locus is *IGF2BP1* (Insulin-like growth factor 2 mRNA-binding protein 1), which is found to be upregulated in patients with metastatic NBs, and is associated with significantly reduced survival. Previous studies have shown its oncogenic potential in multiple malignancies, however, its importance in NB is poorly understood. This study was designed to uncover the role and mechanisms of IGF2BP1 in NB metastasis with an overarching objective to establish IGF2BP1 as a potential therapeutic target for treatment of metastatic NBs.

Material and Methods

We developed a novel highly metastatic transplantable mouse NB cell line, and utilized it along with other established syngeneic NB mouse models to understand the role of IGF2BP1 in NB progression. IGF2BP1-modulated NB cells were injected intravenously, and the development of metastatic tumors was monitored using *in vivo* bioluminescence imaging. Mouse survival was analyzed by Kaplan-Meier method. Pro-metastatic function of extracellular vesicles (EVs) and EV-dependent role of IGF2BP1 were accessed by studying the effects of NB cell-derived EVs on the growth of experimental metastasis, and pre-metastatic niche (PMN) formation.

Results and Discussions

Down-regulation of IGF2BP1 in highly metastatic NB cells resulted in significant reduction of their metastatic potential, and prolonged survival of mice in the model of experimental metastasis. Conversely, overexpression of IGF2BP1 increased the metastatic ability of the less aggressive NB cell line and significantly reduced survival in mice, thus implicating the regulatory role of IGF2BP1 in NB metastasis. NB cell-derived EVs promoted the development of experimental metastasis in mice, and increased the expression of PMN markers (CD45⁺ cells, Fibronectin) in the liver. Importantly, EVs derived from IGF2BP1 down-regulated cells failed to accelerate the growth of experimental metastasis, and failed to induce PMN formation in mouse liver.

Conclusion

Our findings demonstrate the significance of IGF2BP1 in NB metastasis, the importance of EVs in NB progression, and the pro-metastatic function of IGF2BP1 in an EV-mediated fashion. Overall, we establish IGF2BP1 as a promoter of NB metastasis.

EACR22-0135

Generation and Characterisation of a Murine Prostate Cancer Organoid Biobank for Personalised Medicine

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Introduction

Although prostate cancer (PCa) has good survival rates upon early detection, it was the second most frequently diagnosed cancer type in 2021. Due to the growth and ageing of the population, it is expected that the PCa burden will rise to 2.3 million new cases by 2040. The most common therapy approach, androgen ablation, mostly results in castration resistant tumours and a switch to alternative tumour pathways, hindering the efficient treatment of this disease. Due to the high genomic heterogeneity of PCa there is still a lack of reliable biomarkers and representative preclinical models to investigate the mechanisms involved in its initiation and progression. Here, we developed a biobank of mouse PCa derived organoids, which harbour distinct genetic mutations, in order to study the effects of defined mutations on tumour progression and therapy response.

Material and Methods

Organoids were derived from healthy or malignant murine prostate tissue. The PCa mouse models used were either based on the Cre-mediated knock out of the tumour suppressor *Pten* alone, or in combination with *p53*, or the transcription factor *Stat3*. Using a lentiviral tamoxifen-inducible Cre vector, the same mutations were induced in wildtype (WT) organoids carrying respective specific flox sites *in vitro*. After confirmation of the knockout, the changes in phenotype, proliferation, and marker expression were analysed.

Results and Discussions

The knockout of *Pten* alone, or in combination with *p53* or *Stat3*, in the WT organoids lead to transformation of cells reflected by a higher proliferation rate and the activation of cancer specific pathways, including the PI3K/Akt pathway. The morphology of the *in vitro* transformed organoids recapitulated the phenotype of the tumour organoids derived from PCa mouse models. In the next step, the gene expression, proliferation, and drug response of these organoids (generated from tumours or through *in vitro* deletion) will be compared to the *in vivo* tumours of the corresponding transgenic models. With this, we expect to validate our model system for the assessment of tumour specific pathways and drug response.

Conclusion

Due to the lack of suitable patient-derived PCa organoid models, which have been difficult to generate from primary PCa tumours, we propose that our murine organoids will be a useful tool to study tumour biology and drug response of genetically defined PCa tumours. Importantly, these organoid systems will help to greatly reduce the number of animal models used for drug testing and cancer research.

EACR22-0137

Cdc42 regulates Neuroblastoma progression affecting undifferentiated progenitor cells

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Introduction

Neuroblastoma (NB) tumors arise in the embryonic neural crest due to an uncontrolled behavior of sympathetic nervous system progenitors, giving rise to aggressive tumors. NB intratumoral heterogeneity has emerged as a defining characteristic that can explain its aggressive behavior. Recent single cell expression analyses in NB tumors have described different cell profiles that could be responsible of NB origin, such as neuroblasts or progenitor cells resembling neural crest stem cells. In our laboratory, we have described the existence of a progenitor population, with high expression of the CD44 marker and metastatic characteristics which are enriched in malignant NB. Genomic analyses on NB tumors have identified Rho GTPase signaling as one of the most frequently mutated in aggressive NB, including Rho GTPases GEFs (activators), GAPs (inhibitors) and other regulatory factors. This family of proteins regulates many cellular processes like cell proliferation or differentiation, and have a role in cancer progression. Particularly, the Rho GTPase Cdc42 have an essential role in controlling morphology and differentiation in neuronal-like cells. CdGAP is one of the most known Cdc42 GAPs, specially enriched in neural crest progenitor population. Their deregulation could be having an impact on aggressive NB

Material and Methods

Expression studies were performed using NB patients cohorts. NB cell lines stably expressing Cdc42 and silencing CdGAP were generated. *In vitro* functional assays and *in vivo* xenografts studies were carried out using NB cell lines and patient derived cells

Results and Discussions

Expression analyses show deregulation of Rho GTPases signaling as a hallmark of NB progression, correlating with clinical parameters, especially related with Cdc42 signaling. Functional assays performed indicate that Cdc42 re-expression is detrimental to tumor progression, affecting cell proliferation and differentiation. CdGAP upregulation was found in CD44High cells, apparently controlling Cdc42 action. Silencing CdGAP or activating Cdc42 decreases stem cell maintenance in NB progenitors by affecting factors involved in neural crest cells specification, like Sox9

Conclusion

Rho GTPase signaling is altered in high-risk NB cases, indicating their possible role in aggressive NB. In particular, Cdc42 plays a role in NB cell proliferation and differentiation. CdGAP has been found to be specifically upregulated in progenitor NB cells, being a possible culprit for Cdc42 deregulation in this malignant cell population.

EACR22-0181

Loss of RET promotes mesenchymal identity in neuroblastoma cells

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Introduction

The Anaplastic Lymphoma Kinase (ALK) and REarranged during Transfection (RET) receptor tyrosine kinases (RTKs) are expressed in both the developing neural crest and in

the pediatric cancer neuroblastoma. Previous work has identified the RET as a downstream target of ALK activity in NB models. Here we have investigated ALK and RET in neuroblastoma, with the aim of better understanding their respective contributions.

Material and Methods

Using neuroblastoma cell lines and immunoblotting assay, we study how ALK activity regulates RET phosphorylation and expression. Using CRISPR/Cas9 we generate RET knock out neuroblastoma cell lines and performed a multi-omics approach, combining RNA-Seq and proteomics to characterize the effect of deleting RET in a neuroblastoma context.

Results and Discussions

We show that ALK activation in response to ALKAL2 ligand results in the rapid phosphorylation of RET in NB cells, providing additional insight into the contribution of RET to the ALK driven gene signature in NB. Gene expression analysis of *RET* KO NB cells identified a reprogramming of NB cells to a mesenchymal (MES) phenotype that was characterized by increased migration and upregulation of the AXL and MET RTKs as well as integrins and extracellular matrix components. Strikingly, the upregulation of AXL in the absence of RET reflects the development timeline observed in the neural crest as progenitor cells undergo differentiation during embryonic development.

Conclusion

Together, these findings suggest that a MES phenotype is promoted in mesenchymal NB cells in the absence of RET, reflective of a less differentiated developmental status.

EACR22-0289

Inhibition of protein disulfide isomerases PDIA1 and AGR2 are potential novel treatments for pancreatic cancer.

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Introduction

As a lethal condition with poor prognosis, pancreatic cancer treatment needs to move away from conventional cytotoxic therapies and towards the development of novel therapies through the discovery of precise molecular targets. A potential target is protein disulfide isomerase (PDI); a group of enzymes recognised for their roles in disulfide bond formation within the endoplasmic reticulum (ER). PDI acts to refold misfolded proteins and alleviate ER stress, a characteristic commonly recognised in cancer, as part of the unfolded protein response (UPR). This demonstrates the pro-protective roles of PDI and highlights PDI as a potential therapeutic target for cancer treatment. Here we examine the impact of inhibition of PDI in pancreatic cancer cell proliferation and the downstream effects this has on ER stress.

Material and Methods

The siRNA knockdown of PDIA1 and AGR2 was confirmed via western blot and a PDI enzyme activity assay in three pancreatic cancer cell lines: Panc-1, Mia-PaCa-2 and AsPC-1. PDI activity was also measured after treatment with PDI inhibitors: PACMA-31 and CCF642. The effect of PDI knockdown and PDI inhibitor treatment on the expression of ER stress markers; BIP, ATF4 and ATF6 was also measured via western blot. Proliferation assays (BRdU incorporation and CyQuant assays), apoptosis (Caspase 3/7 assay), and cell viability (ATP assay) were also performed following PDI knockdown and inhibitor treatments.

Results and Discussions

Expression and enzymatic activity of PDIA1 and AGR2 were significantly knocked down after 48hrs siRNA treatment. On average, both AGR2 and PDIA1 knockdowns resulted in an 80% inhibition of PDI activity, with AGR2 knockdown in ASPC-1 showing the greatest inhibition at 91%. Cell proliferation was also inhibited with an average of 63% inhibition. An 82% inhibition of PDI activity and a 78% inhibition of cell proliferation was shown at 100µM Pacma-31 treatment and 10µM CCF642 treatment across all cell lines. PDI knockdown and inhibitor treatment also affected the expression of ER stress markers. These data demonstrate the pro-protective nature of PDIA1 and AGR2 in pancreatic cancer.

Conclusion

Targeted inhibition of PDI family members PDIA1 and AGR2 in pancreatic cancer may be a novel therapeutic strategy. Further work will investigate how PDI inhibition impacts the UPR through potential apoptotic pathways to examine the effects of ER stress, and whether this also translates in animal models of pancreatic cancer.

EACR22-0573

Genetic inactivation of transcriptional repressor ATF7IP: functional analysis in lung cancer

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Introduction

Lung cancer (LC) is the cause of over 1.3 million deaths annually, and is still the most lethal type of cancer worldwide. The endeavors to identify the gene alteration-profile underlying LC development have established that recurrent alterations known to be important in the development of this disease include the genetic activation of oncogenes and the inactivation of tumor suppressor genes. Understanding some aspects of LC genetics has been paramount to the implementation of new therapeutics such as tyrosine kinase inhibitors. Further characterization of LC genetics can therefore improve the diagnosis and treatment of patients.

A recent work from our laboratory has identified *ATF7IP* as a recurrently inactivated gene in LC. ATF7IP is a transcriptional regulator that can promote or inhibit transcription depending on its binding partners. Its best known role is to bind to and promote SETDB1's activity. SETDB1, a histone methyl transferase, catalyzes the deposition of H3K9me3, promoting chromatin compaction, gene silencing and heterochromatin formation. ATF7IP also directly interacts with MBD1, a methyl-CpG binding protein, providing a platform to couple DNA methylation and H3K9me3 deposition. Because ATF7IP's role in cancer development is unknown, we aim to characterize its contribution to oncogenesis.

Material and Methods

A panel composed of both ATF7IP-mutant and ATF7IP-proficient LC cell lines has been selected. The latter have been chosen to approximately match the histopathological type of the mutant cell lines. ATF7IP, SETDB1, MBD1 and H3K9me3 levels have been compared between both groups of the panel using Western Blot. ATF7IP expression is to be restored in the mutant cell lines in a doxycycline-dependent manner using a Tet-On Advanced expression system.

Results and Discussions

Our results show that ATF7IP expression is highly variable between LC cell lines and that it strongly and positively correlates with SETDB1 levels. ATF7IP-mutant cell lines generally

present lower H3K9me3 deposition when compared to their wild-type counterparts, although the correlation is not as strong as with SETDB1. These results are in line with the previously published literature. The data on the characterization of ATF7IP restitution in LC cells will be presented.

Conclusion

ATF7IP is a strong tumor suppressor candidate, although its role in oncogenesis is still unknown. The restoration of its expression in cell lines that naturally lack ATF7IP expression will provide the necessary context to understand this gene's role in cancer.

EACR22-0649

CCDC6, a novel partner of the transcription factor MNT and its cellular effects

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Introduction

MAX network transcriptional repressor (MNT) is a protein from the MYC/MAX/MXD network, which controls MYC oncoprotein activity and other cell functions and is deregulated in different tumors. Coiled-coil domain containing protein 6 (CCDC6) was identified as the CCDC6-RET fusion oncoprotein in human thyroid papillary carcinoma. CCDC6 might play a key role as a tumor suppressor gene and participates in DNA damage response leading to apoptosis under DNA damage. Recently, our group has focused on new biological functions of MNT and MNT partners different from MAX. In this study, we investigated MNT-CCDC6 interaction and its effects in cellular processes, such as cell proliferation or DNA damage response.

Material and Methods

MNT interacting proteins were determined by MNT immunoprecipitation (IP) followed by mass spectrometry in rat pheochromocytoma cells deficient in MAX (URMT) and a derivative cell line with a Zn²⁺-inducible MAX allele (URMax34). The domains involved in MNT-CCDC6 interaction were determined by co-IP with deletion mutants in HEK293T. The localization of the complex was determined by cellular fractionation assays followed by IP in HeLa. Two cellular models were used to evaluate the biological consequences of CCDC6-MNT interaction: HAP1 cell line (from chronic myelogenous leukemia) and HAP1 MNT-KO (MNT knockout by CRISPR-Cas9); as well as HeLa pLKO and HeLa shCCDC6 (stably CCDC6 silenced). Cell proliferation was analyzed after cisplatin and etoposide treatment to determine the effect of CCDC6 and MNT loss on DNA damage response.

Results and Discussions

A protein that reproducibly appeared as interactor with MNT even in MAX-deficient cells was CCDC6. We confirmed MNT-CCDC6 interaction in HEK293T, UR61 and HeLa cells. The interaction mainly occurs in the cytoplasm and requires the C-terminal of CCDC6 and LZ domain of MNT. HAP1 MNT-KO cells proliferate faster than the control, suggesting that MNT limits cell proliferation. Wound healing experiments show a role of MNT in cellular migration. MNT and CCDC6 knockdown increase anti-apoptotic BCL-XL levels in HAP1 cell line, suggesting an effect of these proteins on apoptosis. Moreover, CCDC6 loss increased the resistance of HeLa cells to cisplatin-induced DNA damage. We are investigating whether the CCDC6 interaction is involved in the MNT phenotypes.

Conclusion

We have discovered a MNT-CCDC6 interaction, which opens a new path to the understanding of MNT's wide functions in cell biology and its effects as a modulator of MYC-mediated oncogenic activity.

EACR22-0655

The Role of Cyclin C in BCR-ABL Induced Leukemia

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Introduction

Cyclin C regulates transcription by interaction with cyclin-dependent kinase (CDK) 8 or 19. The role of cyclin C in tumorigenesis is highly context-dependent. Oncogenic activity of cyclin C-CDK8/19 has been reported in several cancer types and we have recently shown that CDK8 acts as tumor promoter in acute B-lymphoblastic leukemia (B-ALL). In contrast, heterozygous loss of the CCNC locus has been reported in cases of T-ALL where cyclin C functions as tumor suppressor. This project aims to explore the role of cyclin C in B-ALL.

Material and Methods

We have generated transgenic mice lacking cyclin C in the hematopoietic system by crossing *Ccnc*^{fl/fl} to *VavCre* mice. Oncogenic transformation of bone marrow from these mice using the BCR-ABL^{p185+} oncogene allows *in vitro* characterization of cyclin C-deficient leukemic cells. Intravenous injection of these cells into immunodeficient NSG mice facilitates the study of leukemia progression *in vivo*.

Results and Discussions

Conditional deletion of *Ccnc* in the hematopoietic system of transgenic mice is well tolerated. We now report that cyclin C plays a key role in the establishment of murine BCR-ABL induced leukemia. Cyclin C is crucial for the transformation and immortalization of primary bone marrow cells with the BCR-ABL^{p185} oncogene. To explore the effects of cyclin C for *in vivo* cancer growth we established cyclin C deficient BCR-ABL^{p185+} cell lines and injected them into NSG mice. In contrast to control cells, cyclin C deficient BCR-ABL^{p185+} cells failed to elicit leukemia.

Cyclin C is not only crucial for the leukemic transformation and immortalization of primary cells, but also proved to be indispensable for *in vivo* leukemia progression.

Conclusion

Our data show that cyclin C plays a pivotal role in the establishment and maintenance of BCR-ABL1+ leukemia.

EACR22-0735

CCDC80 as a Novel PAX8-Regulated Tumor Suppressor in High-Grade Serous Ovarian Cancer

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Introduction

High grade serous ovarian cancer (HGSC), the most prevalent and aggressive subtype of epithelial ovarian cancer, often originates from transformation of fallopian tube secretory epithelial cells (FTSEC). One of the hallmarks of fallopian tube lineage in HGSC is the nearly ubiquitous expression of the transcription factor and fallopian tube lineage marker PAX8. PAX8 is considered a master regulator of transcription in fallopian tube development and in HGSC, and plays an essential oncogenic role in HGSC. PAX8 is known to play a major role in survival, proliferation and migration of HGSC cells. However, little is known about PAX8 target genes in this disease. Therefore, our aim was to identify novel mediators of the oncogenic role of PAX8 in HGSC and to discover the involved molecular mechanism.

Material and Methods

To address our aim, we analyzed four genome-wide PAX8 transcriptomic and binding location datasets. Our analysis revealed several candidate PAX8 regulated genes in multiple analysis, and some of them were experimentally validated to be PAX8 regulated in multiple HGSC cell lines using real-time quantitative PCR. Additionally, we utilized gene knockdown and overexpression approaches and performed *in vitro* assays including cell proliferation, apoptosis, migration, and colony formation in several HGSC and immortalized benign FTSEC cell lines to test the functionality of the potential selected PAX8 target genes.

Results and Discussions

The analysis and its validation highlighted CCDC80 as a robust negative transcriptional target gene of PAX8 in multiple HGSC cell lines. Our results show that CCDC80, which is known as a nuclear and/or extracellular protein, is localized to the nuclei of HGSC cells, where it inhibits cell migration and cell autonomous growth. CCDC80 silencing in immortalized benign FTSEC expressing high CCDC80 levels enhanced their migration and ability to grow from a single cell, highlighting CCDC80 as a novel tumor suppressor protein in HGSC.

Conclusion

Our results suggest that CCDC80 is a novel PAX8 regulated tumor suppressor gene in HGSC, and that the negative regulation of CCDC80 by PAX8, can drive transformation. To explore the molecular mechanism of the tumor suppressive role of CCDC80 we plan to explore CCDC80 dependent changes in proteomic profile and CCDC80 dependent signaling pathways.

EACR22-0815

MYCN mRNA is a post-transcriptional regulator of RNA transcripts via microRNAs in High-Risk Neuroblastoma

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Introduction

MicroRNAs (miRNAs) are small RNA sequences which downregulate gene expression through targeting complementary sequences in mRNA 3'-untranslated region (UTR), known

as miRNA response elements (MRE). miRNAs can regulate multiple mRNAs; whereas a single mRNA transcript can be regulated by multiple miRNAs. Competition for shared miRNAs between protein-coding and noncoding transcripts, known as “competing endogenous RNA” (ceRNA), represents a mechanism of gene regulation. ceRNAs act as a miRNA sponge or decoy for common miRNAs. By altering the pool of miRNAs, ceRNAs affect expression levels of other mRNAs.

In NB, the amplification of the *MYCN* oncogene (MNA) is associated with high-risk disease and its 3'-UTR contains highly conserved MRE, suggesting strong functional relevance. *MYCN* mRNA can thus function as a sponge that regulates other mRNAs via shared miRNAs, acting as ceRNA. In MNA, the increase of mRNA abundance may affect ceRNA networks and consequently alter the transcriptional output of key genes.

Material and Methods

We generated and analyzed both genome-wide miRNA and gene expression microarrays from the same 20 NB tumors. *MYCN* status was characterized using qPCR or/and SNP arrays. Bioinformatic tools and publicly available databases were used to examine mRNA/miRNA interactions. NB tumors, patient-derived xenografts (PDXs) and cell lines were used to validate the ceRNA network by qPCR, luciferase assays and *MYCN* transfection in non-amplified samples.

Results and Discussions

Unsupervised analysis of miRNA microarray data revealed differential profiles among NB clinical risk groups. These miRNA expression profiles were found to be significantly associated to MNA status. We developed and applied an R-based bioinformatic tool that performs correlation expression analyses between miRNA and RNA transcripts. The analysis identified a potential ceRNA network associated with MNA. After data filtering, the most promising *MYCN* targets and their associated miRNAs were validated in NB cell lines, in a set of independent NB tumors and PDX. The results enabled us to identify a ceRNA network that affect biological pathways that contribute to the aggressive phenotype of the disease.

Conclusion

High levels of *MYCN* mRNA act as a miRNA sponge deregulating the expression of several ceRNAs that compete for common miRNAs. These findings could provide a potential functional explanation of how genes with no previously known direct interactions are potentially interlinked and identify interactions between relevant signaling pathways.

EACR22-0961

Evaluation of microRNA 622 as biomarker and therapeutic target of Triple-Negative Breast cancer cells

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Introduction

Triple negative breast cancer (TNBC) is an aggressive tumor characterized by the absence of the estrogen and progesterone hormone receptors and the absence of epidermal growth

factor receptor (HER2). New biomarkers and molecular targets for this tumor are needed. Altered levels of specific microRNAs (miRNAs) in body fluids are associated with cancer progression and could represent novel non-invasive biomarkers for diagnosis and prognosis of breast cancer (BC) patients. miRNAs based therapy is a promising novel specific approach for cancer treatment. Here, we focused on miR-622.

Material and Methods

q-RT-PCR was performed to investigate the expression of miR-622 in human cancer tissues, plasma, BC cell lines and in mouse model. Stable and transient transfections were performed to investigate the role of miR-622 in the TNBC phenotype. *In silico* analysis, expression studies, migration assay, and vascular mimicry experiments were performed to better understand the molecular mechanisms underlying the role of this miRNA in the TNBC cell line and in mouse model-bearing TNBC.

Results and Discussions

Recently, we have demonstrated that circulating level of miR-622 was significantly decreased in the plasma collected from BC patients respect to plasma of healthy controls and its down-regulation was associated with TNBC and luminal A subtypes. The data was confirmed also in BC tissues. miR-622 forced expression decreased the motility phenotype of BC cells via the down-regulation of NIAK1 serine/threonine kinase. Transient transfection of mimic specific for miR-622 in TNBC cell line modulates the epithelial to mesenchymal transition markers and the vessel loop formation. Preliminary data show that the preclinical BALB/C mouse model of TNBC recapitulates the results obtained *in vitro*.

Conclusion

Our overall results highlighted the potential role of miR-622 as promising biomarker and as a therapeutic target for TNBC.

EACR22-0989

RAS and BRAF mutations in colorectal cancer drive CD73 expression associated with oxaliplatin resistance and an immunosuppressed phenotype.

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Introduction

CD73, encoded by *NT5E* catalyzes the purinergic end reaction from AMP to adenosine (Ado) and is overexpressed in most cancer types. Extracellular Ado mediates immunosuppression. In addition to its enzymatic function, CD73 is a signal and adhesive molecule that can regulate proliferation, migration, invasion and angiogenesis. The aim of this work was to study the role of CD73 in colorectal cancer (CRC).

Material and Methods

CRC cell lines: HT29, Lovo, DiFi, HCT116, SW48-wt (KRAS wt), SW48-G12V (KRAS mutant) and HTOXA (HT29-derived, resistant to oxaliplatin). *In vitro*, CD73 protein expression and activity was measured by western blot (D7F9A, Cell Signaling) and 5'-Nucleotidase (CD73) Activity Assay Kit (Abcam), respectively. Ado-related gene expression

data was obtained from GSE7609. CD73 protein levels were analyzed by IHC in TMAs from 119 patient tumor samples treated with first-line oxaliplatin- (N=76) or irinotecan-based schedules (N=35). *RAS/BRAF* mutation status was available in 82 samples. RNA-seq data was obtained from the TCGA project (456 COAD, 166 READ). Biological and clinical data was downloaded from cBioPortal. Consensus molecular subtypes (CMS) data was obtained from the Colorectal Cancer Subtyping Consortium.

Results and Discussions

CD73 protein was present in all *RAS/BRAF* mutant and absent in DiFi and SW48 (*RAS/BRAF* wt) cell lines. CD73 protein levels correlated with CD73 activity in SW48-wt (0 mU/mg) and G12V cells (47.97mU/mg). 60.6% of samples stained positive for CD73 and had a higher proportion of *RAS/BRAF* mutations than negative samples (67.5 vs 32.5% p-value=0.04). A higher proportion of CD73-negative tumors responded to oxaliplatin-based treatment compared to positive ones (92.3 vs 48.3% p-value=0.007). No differences were observed in irinotecan-treated patients. HTOXA cells had higher levels of *NT5E*, *ENTPD2*, *ADA*, *ADK*, *ADORA2B*, *CDA*, *UCK1*, *UPP1*, *XDH* and *SLC29A1* Ado-related genes, than HT29. *In silico* studies confirmed a positive association of *NT5E* with *RAS/BRAF* mutations. CMS1 and 3, displayed higher levels of *NT5E* (p-value<0.001). CIBERSORTx analysis revealed an increase in T $\gamma\delta$ and NK cells and a decrease in T CD4+ memory activated cells correlated with *NT5E* expression (p-value <0.05).

Conclusion

Our *in vitro*, clinical and *in silico* data suggests a link between CD73, *RAS/BRAF* mutations, resistance to oxaliplatin and an immunosuppressed profile. CRC patients with *RAS/BRAF* mutant-tumors may benefit from combinations of CD73 inhibitor and oxaliplatin with or without immunotherapy.

EACR22-1015

Mapping the proximal interactome of the RAD51 paralogs: disclosing novel functionality and clinically relevant partners

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Introduction

Replicative stress has emerged as a major source of genomic instability, which can lead to cancer development. In this context, repair of DNA double strand breaks by homologous recombination (HR) is crucial to preserve genomic integrity as well as to protect and rescue stalled DNA replication forks. The mammalian RAD51 paralogs (RAD51B, RAD51C, RAD51D, XRCC2, and XRCC3) have been shown to play crucial functions in both processes. Additionally, germline mutations in the different RAD51 paralogs favor the development of hereditary breast and ovarian cancer (HBOC), highlighting the

antitumorigenic role of the HR pathway. However, it remains largely unclear how these factors promote genome stability.

Material and Methods

To gain better insight into their contribution in the maintenance of genome integrity, we mapped the proximal interactome of the five human RAD51 paralogs using the BioID approach. Stable inducible HEK293 Flp-In T-Rex cell lines expressing RAD51 paralog genes fused to biotin ligase (BirA) enzyme were generated and processed for mass spectrometry analysis. Preys were identified by SAINT analysis and functionally validated using publicly available CRISPR-based chemogenomic screens. The RNA expression of a subset of proximal interactors of the RAD51 paralogs was subsequently probed in a cohort of breast cancer patients (n=2976) using kmplot tool to identify those components with potential clinical relevance.

Results and Discussions

Our proximal mapping of the different RAD51 paralogs identified several members of important functional cellular complexes like the PTIP-DNA damage complex (e.g. NBN), the spliceosome (e.g. SNRNP200) and the eIF3 complex (e.g. eIF3A). Interestingly, CRISPR-mediated targeting of these factors modulated the response of RPE1-hTERT cells to different chemotherapeutic agents, including cisplatin and Olaparib. Importantly, among these factors we identified a subset with RNA expression levels correlating with a poorer outcome for patients affected by breast cancer.

Conclusion

Here we present the first proximal mapping of the RAD51 paralogs, thereby identifying previously unrelated molecular complexes as functional partners and unraveling clinically relevant proximal interactors in the pathobiology of breast cancer.

EACR22-1095

Investigating the roles of YAP and TAZ in human cutaneous squamous cell carcinoma progression

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Introduction

Non-melanoma skin cancers (NMSCs) are amongst the most frequently diagnosed human cancers. Cutaneous squamous cell carcinoma (cSCC) accounts for the majority of NMSC deaths. Studies demonstrate a requirement of the transcriptional co-regulator proteins YAP and TAZ (WWTR1) for cSCC initiation and progression in murine epidermis. Many past studies on YAP/TAZ focus on either one of the paralogues or had assumed similar functions between them. However, emerging evidence demonstrates that YAP and TAZ have distinct roles with independent transcriptional partners and programs. We have therefore assessed distinct and non-redundant roles of YAP and TAZ in a human cSCC cell culture model.

Material and Methods

Our human cSCC model consists of a panel of isogenic human epidermal cell lines representing different stages of neoplastic progression (MET1, MET2, MET4), which were derived from the clinical progression of a primary human cSCC through to distant metastasis in a single immunosuppressed individual. We generated sublines of MET1, MET2 and MET4 expressing YAP-/TAZ-targeting inducible shRNAs to study the functions of YAP and TAZ

using variety of 2D and 3D cell culture assays, immunofluorescence microscopy, western blotting, and published microarray data for bioinformatics analysis.

Results and Discussions

We observed variable requirement of YAP and TAZ for cSCC proliferation in 2D and 3D culture conditions: MET1 (cSCC in-situ) was strongly dependent on YAP, MET2 (invasive, poorly differentiated recurrence of MET1) was dependent on both YAP and TAZ, whereas MET4 (lymph node metastasis of MET1/MET2) was not dependent on either paralogue. Surprisingly, we observed no obvious correlation between sensitivity to YAP/TAZ depletion and YAP/TAZ expression. In addition, YAP/TAZ nuclear/cytoplasmic ratio (often used as a proxy for YAP/TAZ activity) was highest in MET4 cells, despite their independence on YAP/TAZ.

Conclusion

Our findings suggest that YAP is the predominant paralogue in cSCC but cooperates with TAZ during cSCC invasion; losing dependency on YAP/TAZ after metastasis. Altogether, our findings strongly argue against the use of YAP/TAZ expression and nuclear/cytoplasmic ratio as predictors for YAP/TAZ activity in cSCC. As analysis of YAP/TAZ target gene expression provides a more robust and quantifiable way to assess YAP/TAZ activity than measuring its expression level, subcellular localisation or phosphorylation, our future work will aim to identify a cSCC-specific YAP/TAZ transcriptional signature.

EACR22-1189

The Effect of Atorvastatin on Oncogenic miRNAs in Hematological Malignancies: A Central Study

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Introduction

Targeted therapies were shown to increase survival rates in Hematological Malignancies (HM) patients who still face major challenges such as the relapse of a more aggressive form of the disease, metastasis, and drug resistance. The efficacy of statins as anti-cancer drugs has been demonstrated in several malignancies but poorly investigated in HM. By studying its effect on oncogenic miRNAs, we investigated the effect of statin therapy on HM patients. The data was used to identify enriched pathways that were altered due to statin treatment.

Material and Methods

A panel of 95 plasma circulating miRNAs involved in tumorigenesis, apoptosis, and differentiation were relatively quantified using qPCR for blood samples obtained from 12 HM patients, 4 with Chronic Myeloid Leukemia (CML), 4 with Non-Hodgkin Lymphoma (NHL), and 4 with Essential thrombocythemia. miRNA expression levels were measured pre- and post-administration of a 6 week atorvastatin treatment. Significantly differentially expressed miRNAs were those with a fold change > 2 or < 0.5 using the Livak method with a two-sided P value < 0.05. To further understand the underlying mechanism of statin differentially regulated miRNA, GO and KEGG pathway enrichment analyses were conducted for identified target genes using the DAVID 6.8 bioinformatics tool.

Results and Discussions

The data showed no significant fold change differences in miRNA among the different malignancies. Out of 95, 14 miRNAs exhibited significant fold changes post-treatment with statins including miR-198, miR-29a+b+c, miR-204, miR-222, miR-224, miR-155, miR-128b,

miR-296, miR-199a+b, miR-194, miR-125a, miR-200a, and the let-7-family that were upregulated and miR-150 that was downregulated post statin treatment. Higher mir-222, mir-194, mir-128b and mir-199b expression were significantly associated with better overall survival using the Cancer Genomic Atlas leukemia and lymphoma patient cohorts. Enrichment analysis identified the PI3k-Akt pathway as well as other pathways involved in epithelial-mesenchymal transition.

Conclusion

Up to our knowledge, this is the first study to investigate the effect of statin on miRNAs in HM patients. Atorvastatin upregulated several tumor suppressor genes involved in mediating better prognosis. The data can be used to enhance personalized treatments for patients with hematological malignancies by helping to predict the different pathways that may be targeted and therefore result in better survival outcomes in these patients.

EACR22-1141

Identifying targetable cancer-related deubiquitinases through a CRISPR/Cas9 dropout screen

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Introduction

Ubiquitination is a central mechanism in the regulation of many cellular processes. A protein's ubiquitination state is governed by the E1, E2, and E3 ubiquitin ligases which add ubiquitin, and the deubiquitinases (DUBs) which remove it. Processes that cancers are highly dependent on, such as protein degradation, angiogenesis, and the cell cycle, rely on working ubiquitination machinery. This makes targeting DUBs a viable strategy in cancer treatment. Most DUBs are cysteine proteases which are known to be druggable. To date, there are several DUB inhibitors that have been shown effective in various in vitro and in vivo cancer models. In this study, we seek to identify DUBs that are essential for cancer cell survival and are thus potential targets in cancer treatment.

Material and Methods

We performed a CRISPR/Cas9 dropout screen for all known cysteine DUBs, integral proteasome subunits, and proteasome interacting proteins using a guide library incorporating random sequence labels in the colorectal cancer cell line HCT116 and isogenic mutants for key cancer-related genes (*FBXW7*, *KRAS*, *PIK3CA*, *TP53*). Essential DUBs in the parental cells were identified through MaGeCK. For the essential DUBs, we obtained the associated survival data from the TCGA database. Differential dependence on DUBs for the different mutants was measured using UMI lineage dropout analysis.

Results and Discussions

We found that only a small fraction (10 of 87) of the cysteine DUBs are essential for cancer cell survival suggesting limited dependence on any single DUB or redundancy in DUB function. Among the essential DUBs, USP39, USP36, and USP14 are associated with decreased survival for several cancer types. Dependency on DUBs is highly similar among the different studied mutants with very few exceptions. Notably, HCT116 TP53(R248W/-), which exhibits reduced p53 and p21 expression, is significantly less dependent on USP7 compared to parental cells.

Conclusion

In general, colorectal cancer cells have limited dependence on cysteine DUBs. Mutations in the studied cancer-related genes do not affect DUB dependency to a large extent. Whether the findings extend to other cancer types has yet to be investigated. Most importantly, we have identified several DUBs that are of potential therapeutic or prognostic significance in cancer.

EACR22-0040

Pharmacological blockade of platelet-CysLT1 receptor as a novel target to prevent breast cancer progression and metastasis to bone

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Introduction

Platelets are well known to contribute to cancer progression and metastasis. However, few platelet-specific targets have been exploited therapeutically given the paramount function of platelets in hemostasis. In order to define new anti-metastasis therapies targeting the pathological tumor cell-platelet interactions without abrogating normal platelet functions, our lab developed a high throughput assay that showed that pharmacological blockade of cysteinyl leukotriene receptor 1 (CysLT1R) with zarfirlukast, a drug already widely used in asthma, dose-dependently inhibited the mitogenic action of human platelets on human MDA-B02 breast cancer cells. Here, we investigate the role of cysteinyl leukotrienes (CysLT) in modulating platelet protumoral functions and bone metastasis.

Material and Methods

Human washed platelets from healthy volunteers were incubated with MDA-B02 human breast cancer cells and the effect of the CysLT1R antagonist zarfirlukast was tested on platelet-induced cancer cell survival, migration and invasion using *in vitro* assays, as well as on tumor cell induced platelet aggregation (TCIPA) using live-cell imaging and confocal microscopy. The anti-metastasis effect of CysLT1R blockade was evaluated by injecting osteotropic MDA-B02 breast cancer cells into the tail artery of nude mice that received a daily treatment with zarfirlukast. After 7 days bone marrow cells were collected and placed in culture to quantified tumor cells that colonized bone.

Results and Discussions

Our MDA-B02 breast cancer cell line produces CysLTs as showed by ELISA and LC MS/MS but do not express CysLT1R whereas platelets do, enabling us to evaluate the effect of CysLT specifically on platelet biological responses. We found that CysLT1R blockade with zarfirlukast 1) inhibited platelet-induced MDA-B02 cancer cell survival, migration and invasion *in vitro*; 2) decreased platelet adhesion on cancer cells and TCIPA; 3) reduced by 90% the number of tumor cells that colonized bone in our *in vivo* model of bone metastasis

Conclusion

We found that breast cancer cell-derived cysteinyl leukotrienes promoted platelets protumoral actions and that CysLT1R blockade with zarfirlukast prevented breast cancer cell metastasis to bone. Our results suggest that platelet-CysLT1R is a potential therapeutic target for bone metastasis in breast cancer.

Survival and colonisation axis of metastasised cells in the secondary tissue: to target or not to target?

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Introduction

We have previously shown that plasticity ratio (PR; mesenchymal to epithelial markers) plays a critical role in successful metastasis across multiple stages. Here we highlight the importance of PR during survival in the secondary tissue followed by subsequent colonisation and how one can manipulate it for drug discovery.

Material and Methods

Wild type HT29 (PR 0.22) and engineered HT29#8C5 (PR 0.96) was initially used as a baseline to understand the functional differences between them during survival and colonisation. Cells with higher PR showed higher expression of PD-L1 and lesser susceptibility to apoptosis. Their ability to crosstalk with the secondary microenvironment was more evident from the higher exosome uptake capability. Metabolically, they had low dependence on lactate and glutamate but showed elevated autophagy and ROS generation. High PR cells had more cells in the G0 phase and lesser cells in the M phase than HT29.

Results and Discussions

Treating the 8C5 cells with retinoic acid (RA), a molecule that promotes MET, we could successfully decrease the PR along with a concomitant increase in chemosensitivity and a significant transition of cells from G0 to M phase. RA treated 8C5 cells formed more colonies in the clonogenic assay and had lesser invasive prowess. Interestingly, when we treated Colo205, a wild type metastatic colon cancer cell line (PR 1) with RA, there was again a measurable change in PR and we saw an increase in colony formation. Treatment of Colo205 with a chemotherapeutic agent also generated cells with lower PR and increased colony formation ability by promoting a G0 to S transition, suggesting the shift from M to E can be inadvertent and, therefore, a dual-edged sword.

We next tested the hypothesis of maintaining cells in the high PR axis by treating 8C5 cells with AGN193109. The compound abrogated RA mediated PR change and reduced colony formation in the clonogenic assay by preventing the shift of cells from the G to M phase. Currently, we are testing this hypothesis on primary patient tumors, comparing the two strategies of MET promotion and M-form maintenance.

Conclusion

Metastasis success depends on dissemination, survival and subsequent colonisation of the disseminated cells, and all efforts targeting the dissemination step has failed, suggesting there are more downstream rate-limiting steps. Our work identifies the colonisation axis as critical and suggests that targeting it in the right way might delay metastasis in clinical settings.

Statins affect cancer cell plasticity with distinct consequences for tumor progression and metastasis

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Introduction

Cancer progression is the result of the tumor cells' ability to toggle dynamically between cell states, termed plasticity. Multiple factors can impact cancer cell plasticity, one of them being (targeted) drugs. Statins are among the most commonly prescribed drugs with around every fourth person above the age of 40 on statin medication. Therefore, it is of utmost clinical importance to understand the effect of statins on cancer cell plasticity, and its consequences to not only cancer patients but also patients that are on statins.

Material and Methods

We performed an *in vivo* screen to identify FDA-approved compounds that can impact cancer cell plasticity. For this, we adapted a high-throughput screening platform based on DNA barcoding that allows to interrogate hundreds of compounds *in vivo* irrespective of their biochemical binding properties and modes of action. In addition, we utilized comprehensive STRING network analysis of transcriptome, proteome and phosphoproteome data combined with multiple mechanistic *in vitro* and functional *in vivo* transplantation models of several human cell lines and primary PDX-derived cells.

Results and Discussions

We demonstrate that cholesterol-pathway inhibition induces a mesenchymal cell shift in cancer cells, a feature that is until now solely associated with increased metastasis. Yet, while statins on the one hand increase metastatic seeding ability - the early steps of the metastatic cascade - they reduce cellular plasticity, trap cancer cells in a mesenchymal cell state and therefore inhibit mesenchymal to epithelial transition and tumor- as well as metastasis- formation *in vivo*.

Conclusion

Taken together, we provide a thorough mechanistic overview of the consequences of statin use for each step of cancer development, progression, and metastasis. Our findings emphasize that targeted therapies can have opposing effects on primary tumor and metastases formation in comparison to tumor progression, underscoring the importance of proper timing of anti-cancer therapies and the distinct inhibition of plasticity can have on different stages of tumor development and progression.

EACR22-0124

Tissue interface dependent induction of an invasive phenotype of migrating breast cancer cells.

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For the formation of metastases, invasive tumour cells escape the primary tumour and travel to a distant part of the body. The escaping cancer cells are challenged by various tissue microenvironments with distinct composition and mechanical properties, and steep microenvironmental changes at tissue interfaces. In the case of breast cancer invasion, microenvironmental stiffness and extracellular matrix density decrease during migration from the primary tumour to the adjacent healthy tissue (M.-Q. Gao et al., 2010).

Material and Methods

In a previous study it was shown that three-dimensional collagen-I matrices can be sequentially reconstituted, to mimic such tissue interfaces of tumour microenvironments, whereby the matrix properties can be adjusted via reconstitution conditions including collagen-I concentration and pH value (J. Sapudom et al., 2016; L. Kalbitzer et al., 2018). This setup allowed the investigation of the influence of local differences in the topological and mechanical properties of the extracellular matrix on migrating cancer cells *in vitro*. The first observation with this approach was the appearance of an altered migratory phenotype of highly invasive MDA-MB-231 breast cancer cells after transmigration, suggesting a direct influence of tissue interfaces on the tumour cellular behaviour (J. Sapudom et al., 2016).

Results and Discussions

We now reveal insights in underlying mechanisms by studying mechanotransductional signalling, DNA damage and gene expression. A peak incidence of DNA damage was identified directly at the matrix interface, possibly causing the observed induction of genes involved in a more aggressive phenotype, including a higher cell proliferation and resistance against chemotherapeutics. Ongoing studies using other invasive breast cancer cell lines verify the general principle of matrix interface induced DNA damage and phenotype changes during transmigration of tumour cells.

Conclusion

In sum, our findings suggest that distinct tissue interfaces may have an impact on breast cancer cells triggering highly invasive phenotypes as a possible result of mechano-transductional activity and DNA damage during breast cancer progression and metastasis.

EACR22-0132

Modelling of tumor cell plasticity and therapy resistance in colorectal cancer

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Introduction

Metastases formation and acquired drug resistance represent a major issue for cancer treatment. Both mechanisms are associated with tumor cell plasticity, which is linked to epigenomic perturbations. During metastasis, cancer cells dissociate from the primary tumor and invade into secondary tissues. This is enabled by epithelial mesenchymal plasticity (EMP), a process where cancer cells acquire mesenchymal features and at the same time, maintain key epithelial characteristics. In this intermediate EMP state, cancer cells also show a high drug resistance. So far, mainly 2D *in vitro* cell culture models were used to study EMP. However, these simplified models only barely reflect the complex multistep process of EMP *in vivo*. Here we have developed a 3D patient-derived colorectal cancer (CRC) organoid model, to induce EMP-like states, as a system to test phenotypic plasticity and therapy response in CRC.

Material and Methods

EMP was induced in CRC organoids carrying *APC* and *KRAS* mutations, through treatment with TGF- β . Characteristic morphological changes were detected/visualized by IF-microscopy and alterations in gene expression of stem cell and EMP marker genes were analyzed by RT-qPCR.

Results and Discussions

The treatment of organoids with TGF- β induced an outgrowth of a subpopulation of cells from 3D to 2D, which was associated with morphological changes. In addition, gene and protein expression of EMP associated transcription factors, such as *SNAI2*, and EMP markers including fibronectin (mesenchymal marker), was increased. Interestingly, TGF- β treatment also induced elevated expression of the epithelial marker E-cadherin and downregulated stem cell marker genes, such as *LGR5*, which might indicate increased differentiation of cells in this model.

Conclusion

TGF- β treatment of CRC patient-derived organoids induces an EMP-like phenotype, which was characterized by upregulation of both mesenchymal and epithelial marker genes. Through RNA-sequencing we will get a deeper insight into the underlying mechanisms of TGF- β induced plasticity in our 3D organoid model. As a next step we will analyze the sensitivity of our organoids to standard chemotherapy, such as 5-FU or oxaliplatin.

EACR22-0186

Tumor microenvironment remodeling by cancer cells-released tracks on type I collagen

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Introduction

Metastasis is the leading cause of cancer-related deaths. During this process, tumor cells acquire invasive and migratory capacities in order to invade the surrounding tissues. To achieve this, the tumor microenvironment is modified to facilitate cancer cells proliferation and dissemination. Multiple mechanisms are involved in this evolution, including cell-cell communications through the tumor microenvironment and the extracellular matrix modifications. Indeed, extracellular vesicles such as exosomes or migrasomes are already known to induce protumor features such as migration, promoting tumor development and metastasis formation.

Material and Methods

To characterize those collagen-associated tracks we used invasive and non invasive breast cancer cell lines, MDA-MB-231 and MCF-7 respectively.

We used different methods to investigate their structure and functions such as correlative and superresolution microscopy, mass spectrometry and RNA sequencing.

Results and Discussions

Indeed, we characterized these tracks, their structure as well as their composition in terms of proteins and nucleic acids (miRNA), and could show that they are different from classical extracellular vesicles known so far. These tracks are characterized by a discoidin domain receptor 1 (DDR1) staining in the extracellular space. Moreover, these tracks are very stable structures and can be internalized by neighboring cells. After internalization, they modify the differentiation status of cells able to internalize these tracks, promoting epithelial–mesenchymal *transition* and cancer cell proliferation, invasion and also matrix degradation.

Conclusion

These data suggest that these collagen-associated tracks have a role in cell-cell communication and participate in the remodeling of the tumor microenvironment. Even if their function and in vivo relevance needs to be fully elucidated, these tracks seem to be a new player in the tumor invasion process and could provide a better understanding underlying this process.

EACR22-0194

Overexpression of CXCL8/CXCR2 ligand/receptor system indicates melanoma metastasis formation into the liver

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Introduction

Organ specific metastasis formation involves cancer cell interactions with the host microenvironment. Endothelial cells covering blood vessels in different organs express altered chemoattractants and adhesion molecules supporting the homing of cancer cells. Melanoma has a fully comprehensive metastatic potential, however, this organ specificity is one of the crucial unanswered questions in cancer research. Our aim was to investigate receptor/ligand associations which could contribute to the increased invasive potential in melanoma cell lines after co-culturing with hepatic endothelial cells.

Material and Methods

To measure the effect of co-culturing with hepatic endothelial cells on the invasive potential of melanoma cell lines, Matrigel Invasion Chambers were used. After the incubation, invasive melanoma cells were recovered as well as non-invasive cells were selected for further analyses. Real-time qRT-PCR were performed to determine the expression of cytokine and chemokine receptors, while the expression of chemokine ligands in the endothelial cells was examined using Proteome Profiler Array.

Results and Discussions

According to our results, WM983A, WM1361 and WM3248 melanoma cell lines showed significantly increased invasive potential after co-culturing compared to the control cells. TNFRSF10D receptor was down-regulated in the selected invasive cells compared to the non-invasive ones in these three cell lines, however, in cell lines with stable invasion potential, the expression level of TNFRSF10D receptor was constantly low. Additionally, altered expressions of other genes (NGFR, CCR1, IL1R1, IL17A, TNFRSF12A, TNFRSF21, and TNFRSF10A) were characteristic in all of the selected invasive cells compared to the non-invasive ones. After co-culturing (with WM278, WM793B and WM3248 melanoma cell lines), hepatic endothelial cells were also recovered, and we found increased CXCL8 and CXCL1 chemokine expression in the endothelial cells. Interestingly, both compartments of the CXCL8/CXCR2 ligand/receptor system were overexpressed as the result of co-culturing

hepatic endothelial cells with WM3248 cell line, which was associated with increased invasive potential.

Conclusion

Our findings suggest that several cytokine and chemokine receptors play a role in organ specific invasion, and the CXCL8/CXCR2 ligand/receptor system possibly contribute to the invasion targeting the liver and have significant role in the organ specific metastasis formation.

EACR22-0220

An exploration of the role of exercise in modulating breast cancer progression in vitro: a systematic review and meta-analysis

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Introduction

Breast cancer is the most prevalent cancer in women worldwide. In the United Kingdom, approximately 5% of all breast cancers are already metastatic at the time of diagnosis. An abundance of literature shows that exercise can have beneficial effects on the outcome and prognosis of breast cancer patients, yet the molecular mechanisms remain poorly understood. There are several in vitro models that aim to recapitulate the response of breast cancer to exercise in vivo; this systematic review and meta-analysis summarises the existing literature and provides evidence that exercise significantly affects breast cancer progression in vitro.

Material and Methods

The following search terms were used to conduct a systematic literature search using a collection of databases (last search performed May 2020): “in vitro,” “exercise,” and “breast cancer.” The literature was independently reviewed, and data were extracted from included publications. Only studies that investigated the effects of exercise on breast cancer in vitro were included. Risk of bias was assessed and standardised mean differences (SMD) were calculated to determine pooled effect sizes.

Results and Discussions

Six hundred sixty-six papers were identified using the search terms. After duplicates were removed and 578 papers were excluded according to selected criteria, 10 relevant papers remained to be subsequently included in the quantitative analysis. The meta-analysis presented herein demonstrates a statistically significant reduction on breast cancer cell viability, proliferation, tumourigenic potential (SMD: 1.76, $P = 0.004$, SMD: 2.85, $P = 0.003$, and SMD: 3.15, $P = 0.0008$, respectively), in response to various identified exercise interventions. A clear direction of effect was found with exercise on breast cancer cell migration in vitro, however this effect was not statistically significant (SMD: 0.62, $P = 0.317$).

Conclusion

To our knowledge, this is the first meta-analysis and systematic review investigating and summarising literature on exercise and breast cancer in vitro, highlighting models used and priority areas for future research focus. The results of this meta-analysis support the need for further research comprising 3-D models that are more physiologically relevant, allowing researchers to expose breast cancer cells to different types and levels of physical activity in vitro. This will further the understanding of the molecular underpinnings of the role of physical activity in breast cancer progression.

FASN as a novel Hakai interacting protein: impact on intestinal inflammation and cancer

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Introduction

Important evidences pointed out the link between the epithelial plasticity and inflammation at early stages, in pre-neoplastic inflammatory processes, such as in Colitis or Chron's disease. E3 ubiquitin-ligases are important for the cellular protein homeostasis and their deregulation is implicated in several disease, including cancer. The E3 ubiquitin-ligase Hakai is an oncogene and plays a key role in EMT and tumour progression. Hakai interacts with E-cadherin at cell-cell contacts mediating its ubiquitination and degradation. Hakai is overexpressed in colon cancer, however, the implication in colitis-associated cancer (CAC) is unknown. In this work, we aim to determine the possible role of Hakai in intestinal inflammation.

Material and Methods

Several mice models of colitis and associated cancer were used including AOM-DSS mouse model, an Acute Colitis mouse model and an IL10 deficient mouse model. We performed Hakai immunohistochemistry assays in whole large intestine samples from these models and quantified the staining intensity of Hakai and other markers. Interactome analysis of Hakai was done and effect on selected proteins was studied by plasmid and siRNA transfection, western-blotting, qRT-PCR, immunoprecipitation, immunofluorescence and ubiquitination assays. Lipid accumulation was assayed by oil red staining.

Results and Discussions

Our results identified Fatty Acid Synthase (FASN) as a novel Hakai-interacting protein. Hakai induces FASN ubiquitination and degradation via lysosome, thus regulating FASN-mediated lipid accumulation. Quantification of the images obtained from Hakai immunohistochemistry's shows a low level expression of Hakai in the inflamed epithelial tissues of mice treated with AOM/DSS. An inverse expression of FASN expression with Hakai expression was detected in inflammatory AOM/DSS mice model. These results are confirmed in the other two animal models.

Conclusion

Hakai could play an important role in pre-neoplastic inflammatory process in the intestine and in tumour development. Further studies are needed to deepen into the role of Hakai in IBD in mice models and human biopsies in order to better understand the regulatory mechanism and pathways that may influence intestinal homeostasis and its breakdown in IBD.

Ovarian cancer dissemination relies on PDGFR β and S1PR1 for tumorsphere formation and proliferation

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Introduction

Epithelial ovarian cancer (EOC) is the fifth-leading cause of cancer death among women and the most lethal gynaecological malignancy due to its high incidence in western countries. The transcoelomic mode of metastasis is the most frequently observed in high-grade serous ovarian carcinoma (HGSOC), which is the most aggressive one. During the transcoelomic mechanism of spread, shed cells from the primary tumor promote the production of malignant ascites and aggregate as multi-cellular clusters or tumorspheres, so as to escape from anoikis cell death and survive until they find a new surface to adhere and disseminate.

Material and Methods

The present study is based on tumorspheres isolated from HGSOC ascitic liquid patient samples and on a three-dimensional SKOV3 ovarian cancer cell culture model that we call spheroids. Performed *in vitro* assays include cell culture associated techniques as well as RNA extraction and Real-Time PCR, Western Blot and immunofluorescence. *In vivo* studies in immunodeficient female mice have consisted of orthotopic injection of ovarian cancer cells and intraperitoneal injection of ovarian cancer spheroids.

Results and Discussions

Stable silencing of PDGFR β impairs ovarian cancer spheroid formation as well as these spheroids' disseminative capacity *in vivo*. Pharmacological inhibition of PDGFR family also diminishes ovarian cancer spheroid formation, which is too observed in tumorspheres from HGSOC patients. At the molecular level, this inhibition is accompanied by lower expression of CD44 and Fibronectin, although spheroid proliferation is irrespective of PDGFR β expression. Nevertheless, S1PR1 is the one in charge of directing spheroid cell proliferation through activation of ERK, increasing ovarian cancer spheroid formation as well as their disseminative capacity *in vivo*. Pharmacological inhibition of MEK impairs ovarian cancer spheroid proliferation, which is also observed in tumorspheres from HGSOC patients.

Conclusion

We propose PDGFR β to promote ovarian cancer cell aggregation and S1PR1 to direct proliferation of the cells within tumorspheres. These findings shed some light on two key molecular mechanisms involved in the HGSOC transcoelomic dissemination process, providing potential targets so as to hamper ovarian cancer progression.

Polarised contractile jiggling drives the collective amoeboid migration of cell clusters

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Introduction

Cell migration is essential to most living organisms and deregulated in numerous diseases, including cancer. Tumour cells hijack the three modes of migration that are known to date. Single cells migrate using two distinct mechanisms, either a traction-dependent mesenchymal motility or a contractility-driven propulsive amoeboid locomotion. The migration of cohesive groups of cells, also called collective migration, has only been described as a focal adhesion- and traction-dependent process, where the driving forces are mostly exerted at the front by leader cells. Whether collectives can also migrate without adhering to their surrounding environment has been suggested by the observations of fixed and live primary cancer specimens, but has never been directly addressed.

Material and Methods

We used a broad range of biological material, including primary cancer specimen from several type of cancers, patient derived xenografts and cell lines in custom microfluidic devices coated with the anti-adhesive polymer polyethylene glycol

Results and Discussions

Here, we prove the existence of a new mode of collective migration that we named collective amoeboid migration. Cell clusters confined into non-adhesive microfabricated channels migrate in a rounded morphology, independently of the formation of focal adhesions or protruding leader cells. They coordinate and behave like a giant super cell to mobilise their contractility towards the back. Optogenetics showed that this polarised contractility of the actomyosin cortex powers migration. Yet, unlike all other known modes of cell migration, particle image velocimetry revealed that the clusters are propelled in absence of persistent retrograde flows of cells or actin. Instead, using theoretical modelling, we show this migration relies on a physical mechanism of polarised jiggling in which fluctuating contractions within the clusters triggers self-propulsion.

Conclusion

Thus, we identified an undescribed mechanism of collective migration shared by various cancer types based on asymmetric fluctuating deformations that yield directed cluster motion in non-adhesive environments. This mechanism is consistent with the migration of engineered robots and could be extended to understand the movement of other biological systems. Since collective amoeboid migration was identified from tumour explants, it could participate in their metastatic spread by enabling cancer cells to cross a wide spectrum of environment for which they may not express the matching repertoire of adhesion receptors.

Extracellular vesicle-mediated crosstalk between pancreatic cancer and Schwann cells

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Introduction

Neural invasion (NI) involves the growth and invasion of cancer cells around and into nerves, a process that has been known as a hallmark of pancreatic adenocarcinoma (PDAC) due to its extremely high incidence rate (>90%). NI has been reported to occur at the early stage of PDAC development and glial cells of peripheral nerves, i.e. Schwann cells (SCs) have been shown to be attracted to cancer cells before the onset of cancer invasion. However, the mechanisms leading to NI have not been completely understood. We hypothesized that PDAC-derived extracellular vesicles (EVs) mediate the migration of SCs towards cancer and the reciprocal interactions between cancer and SCs eventually lead to NI.

Material and Methods

A 3D coculture assay with SCs confronted to cancer/normal epithelial cells was first conducted to evaluate the chemoattraction affinity of SCs towards cancer cells. To investigate the role of EVs in mediating migration of SCs, EVs were isolated from cancer cell lines and tissue explant model by differential ultracentrifugation and characterised according to the MISEV2018 guidelines. A novel 3D migration assay incorporating SCs and EVs in a Matrigel drop was employed to evaluate the migratory ability of SCs in response to EVs, which was further confirmed in the presence of EV uptake inhibitors. Protein alterations in SCs treated with tissue-derived EVs was evaluated by mass spectrometry.

Results and Discussions

Targeted migration of human SCs (hSCs) was observed towards PDAC cell lines, but not to the normal cells. It was further confirmed that murine SCs (mSCs) exhibited higher migratory ability when confronted to murine neuroinvasive PDAC cells than to non-neuroinvasive PDAC cells. The 3D migration assay demonstrated significantly stronger migratory ability of hSCs/mSCs after EV treatment from human PDAC/murine neuroinvasive cancer cells than that of the normal/non-neuroinvasive cancer cells, which could be reversed by heparin. At a translational aspect, the increased migration of hSCs could also be observed after treatment of tumor tissue-derived EVs from PDAC patients with neural invasion (Pn1) compared to those diagnosed with no neural invasion (Pn0) pathologically. Proteomic analyses indicated that hSCs treated with tumor tissue-EVs displayed significant changes in the expression levels of cytoskeleton and phosphoproteins.

Conclusion

PDAC-derived EVs may be responsible for the migration of SCs towards PDAC cells. This may underscore the mechanisms underlying the SC carcinotropism in PDAC-associated NI.

EACR22-0654

An intertwined endothelin-1/YAP/ZEB1 circuit controls the metastatic traits of high-grade serous ovarian cancer

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Introduction

Understanding the complex transcriptional network governing epithelial-to-mesenchymal transition (EMT) and metastatic growth in high-grade serous ovarian carcinoma (HG-SOC) is

paramount to develop new strategies for the management of this lethal disease. An intriguing scenario portrays the capability of the endothelin-1 (ET-1)/endothelin A receptor (ET_AR) axis to activate different transcriptional circuits orchestrating EMT, including the core EMT factor ZEB1. Because it has been recently reported that YAP promotes gene transcription upon ET_AR activation, in this study we evaluate the possible YAP/ZEB1 signaling cooperation in mediating the ET-1-triggered phenotypic plasticity and metastatic traits in HG-SOC.

Material and Methods

Mechanistic studies, including luciferase reporter gene, co-immunoprecipitation, in situ proximity ligation (PLA), and chromatin immunoprecipitation assays, were performed in patient-derived HG-SOC cells and cell lines. Functional assays were carried out *in vitro* and in HG-SOC xenografts by using an ET-1 receptors (ET-1R) antagonist. Survival analyses were conducted in publicly available databases of OC patients.

Results and Discussions

Here we provide evidence that in HG-SOC ET_AR activation can engender a multi-factorial transcriptional program by promoting the YAP/ZEB1 nuclear accumulation and their engagement in a DNA-binding platform with the oncogenic factor AP-1. This lead to the aberrant activation of common target genes regulating EMT, including *EDN1* (ET-1) and ZEB1, thereby creating a persistent ET-1R signaling activity. Given the reported ability of the ET-1R-induced Integrin-linked kinase (ILK) to promote EMT and cell invasive behaviour in OC, we document the involvement of this kinase in favouring the ZEB1/YAP alliance by mediating the ET-1-guided ZEB1 expression and YAP activation. Of therapeutic interest, the FDA approved ET-1R antagonist macitentan, breaking up the ILK/YAP/AP-1/ZEB1 circuit, inhibits EMT and cell plasticity *in vitro* and curbs metastasis formation in HG-SOC xenografts. Of translational relevance, high gene expression of ET_AR/ILK/YAP/AP-1/ZEB1 is a strong predictor of poor clinical outcome in serous OC patients.

Conclusion

This study highlights novel mechanistic insights of EMT and metastatic progression in HG-SOC uncovering the cooperation of ZEB1 and YAP signaling downstream of ET-1R/ILK pathway, and suggests that blocking this molecular network using an ET-1R antagonist should be a promising treatment option for this disease.

EACR22-0680

Glycosphingolipids are mediators of cancer cell plasticity through independent signalling pathways.

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Introduction

The reversible epithelial-to-mesenchymal transition (EMT/MET) is a pre-requisite for ovarian cancer cells to disseminate and invade organs of the peritoneal cavity. Our recent data suggest that globoside glycosphingolipids (GSLs) define and functionally modulate epithelial cancer cell plasticity. We hypothesized that the dynamic GSL expression also defines mesenchymal-like cancer cells and may functionally contribute to MET.

Material and Methods

Ovarian cancer tissue samples were investigated for expression of E-cadherin and GSL expression using immunochemistry in tissue microarrays (135 patients) and MALDI-MSI (9 patients), respectively. Various transcriptomic data were assessed to study the sialyltransferase ST8SIA1 with EMT marker expression. To characterise its functionality, we homozygously deleted ST8SIA1 in ovarian cancer cells using CRISPR-Cas9 and further validated the mutants by capillary gel electrophoresis coupled to laser-induced fluorescence detection (xCGE-LIF), flow cytometry, various *in vitro* and *in vivo* assays. Signalling pathways associated with GSL depletion were dissected by an additional (phospho-) proteomic analysis.

Results and Discussions

The epithelial marker E-cadherin was expressed in all analysed tissue samples, supporting the hypothesis that ovarian cancer remains epithelial. We identified 19 GSL lipofoms using MALDI-MSI and associated globoside and ganglioside structures to tumour and stroma, respectively. Sialic acid-rich gangliosides and related genes appeared as potential mesenchymal markers suggested by further bioinformatic analysis. The genetic deletion of ST8SIA1 reduced ganglioside GT3 but increased the expression of globosides associated with epithelial markers indicating that the cells underwent MET. The gain of an epithelial phenotype in Δ ST8SIA1 cells was further supported by reduced invasion and migration ($p < 0.05$) accompanied by enhanced spheroid formation ($p < 0.05$) and tumour growth in tumour xenografts. Phosphoproteomics identified in total 11.342 phosphorylation sites with 84 up- and 223 down-regulated in Δ ST8SIA1. Phosphorylation of peptides such as Erk^{Y202/T204} was reduced in Δ ST8SIA1 which is usually associated with MET while mTOR^{S2448} phosphorylation increased. Finally, we noticed that calcium-associated pathways were co-activated with mesenchymal-like cancer cells.

Conclusion

Here, we provide further evidence that GSLs play an important role not only in EMT but also actively contributing to the reverse transition into epithelial cells.

EACR22-0704

A NOVEL TRANSCRIPTIONAL LINK BETWEEN EPITHELIAL MESENCHYMAL TRANSITIONS AND IMMUNE INTERACTIONS IN BREAST CANCER

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Introduction

The interaction between cancer cells and the surrounding tumor microenvironment (TME) regulates cancer progression and metastasis. One aspect of this interplay is the crosstalk between tumors and the immune system. Tumors activate mechanisms leading to immune evasion, by which the ability of NK and T cells to kill cancer cells is suppressed. One of the mechanisms causing immune evasion is the generation of extracellular adenosine that is facilitated through an ecto-5'-nucleotidase, CD73 which converts extracellular adenosine monophosphate (AMP) into adenosine, thereby creating an immune suppressive milieu. Mechanisms regulating CD73 expression in cancer cells are largely unknown.

Material and Methods

Cancer cells can alter their interactions with the TME through epithelial-mesenchymal transition (EMT). EMT involves a shift in the balance between epithelial and mesenchymal transcriptional regulators. In this study, we have investigated the role of a pro-epithelial transcription factor, Grainyhead-like 2 (GRHL2), which has been reported to suppress EMT

while supporting survival and growth in breast cancer cells. ChIP-seq analysis in three human luminal breast cancer cell lines revealed genome-wide GRHL2 binding sites. In addition, Bru-seq analysis of a conditional CRISPR/Cas9 GRHL2 knockout (KO) model in MCF7 cells, revealed dynamic transcriptional alterations upon GRHL2 loss. We integrated ChIP and Bru-seq data and identified *NT5E*, the gene encoding CD73.

Results and Discussions

In a large panel of human breast cancer cell lines, RNAseq analysis showed that *GRHL2* levels were inversely correlated to *NT5E* with a marked decrease of *GRHL2* versus an increase of *NT5E* in basal B. Immunohistochemistry in human breast cancer patients confirmed the inverse relationship in most, but not all samples. Despite the negative impact of GRHL2 on CD73 expression and the role of CD73 in creating an immune-suppressive TME, immune infiltration was enhanced in tumors expressing low or no GRHL2 levels.

qPCR and Western blot analysis confirmed the emergence of CD73 upon GRHL2 loss in MCF7. The increased CD73 levels in GRHL2 KO cells were accompanied by enhanced adenosine levels. Our current work addresses the impact of GRHL2 deletion on the recruitment of naïve CD8 T cells isolated from human blood to breast cancer cells.

Conclusion

Taken together, our results reveal a novel role for GRHL2 in the regulation of CD73 expression and extracellular adenosine production and address the effect on breast cancer-immune cell interactions.

EACR22-0724

Epigenetic and mechanical functions of WDR5 and H3K4 methylation during the 3D migration of acute lymphoblastic leukemia cells.

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Introduction

Acute lymphoblastic leukemia (ALL) is the most common pediatric cancer and infiltration of leukemic cells is critical for disease progression and relapse. In spite of the canonical functions of epigenetics in transcription and DNA homeostasis, its contribution to the nuclear deformability of migrating leukemic cells remains unclear.

Material and Methods

We used ALL cell lines and primary samples from patients to study their response and migration in 3D environments and how they extravasate in a mouse xenograft model. We also performed, biophysical, transcriptional and ChIP-sequencing analyses to determine the mechanism by which WDR5 controls ALL progression.

Results and Discussions

We observed that WDR5 is critical for ALL cell invasion into 3D collagen matrices. Notably, blocking or silencing WDR5 also decreased the extravasation of ALL cells in an in vivo model. We identified that 3D constrained conditions promoted H3K4 methylation in ALL cells that altered the global chromatin configuration and transcriptional changes related to cell cycle and DNA replication. WDR5 inhibition did not impair cell adhesion or cell response to chemokines; but we demonstrated that 3D conditions affected the deformation and biophysical behavior of the nucleus in ALL cells.

Conclusion

We conclude that confined conditions have a fundamental role in ALL cell biology and provide novel molecular and biophysical mechanisms used by leukemia cells to disseminate.

EACR22-0814

Filamin A is critical regulator of the extrusive abilities mediated by E-cadherin dysfunction

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Introduction

E-cadherin loss of function is frequently associated with increased invasion, a hallmark of cancer cells. In Hereditary Diffuse Gastric Cancer (HDGC), a highly invasive cancer syndrome with a lethal outcome, E-cadherin mutations are causative events. In this syndrome, abnormal E-cadherin cells spread along and below wild-type cells, representing the first step of invasion and the result from a switch from apical to basal extrusion. However, the molecular and cellular mechanisms through which epithelial cells with E-cadherin dysfunction invade instead of being eliminated remain undetermined. We hypothesize that at early stages, Ecadherin mutant cells are sensed by wild-type epithelia suffering changes in cytoskeletal organization, cell shape and intracellular signaling, ultimately determining their ability to extrude basally.

Material and Methods

To investigate the extrusive signature induced by E-cadherin dysfunction, we have stably transfected AGS gastric cells with the wild-type protein and a panel of E-cadherin mutants affecting distinct domains of the protein. In particular, we have established a monolayer system where labelled mutant cells were mixed with non-labelled wild-type counterparts to monitor mutant cell fate within a normal epithelium. Structural organization, protrusion formation and dissemination of wild-type and mutant cells were also assessed in a 3D culture model.

Results and Discussions

We verified that cells expressing the juxtamembrane mutant R749W lead to an increase in basal extrusion phenotype, when compared with mutants affecting the extracellular and the intracellular domains. Moreover, R749W mutant cells form cellular aggregates with higher growth ratios and remarkable protrusive abilities. Interestingly, we found that the R749W mutant impacts the activity of Filamin A, with consequences at leading edge structures and cell movement throughout the extracellular matrix. The implication of Filamin A in cell

extrusive potential was further confirmed by its specific inhibition in wild-type cells that resulted in increased migration capacity and basal position of nuclei.

Conclusion

Our results suggest the existence of a molecular signature associated to basal cell extrusion, which involves cytoskeletal remodelling and can be explored for the development of new therapeutic strategies to impair the aggressive behaviour of invasive carcinomas.

EACR22-0864

Contribution of the epithelial-to-mesenchymal transition transcription factor PRRX1 to melanoma progression and plasticity

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Introduction

Melanoma is a very aggressive skin cancer, known for its high degree of plasticity and heterogeneity that confers high metastatic capabilities and resistance to therapies. Increasing evidences indicate that melanoma progression is not only regulated by mutation-driven mechanisms but also by non-genetic mechanisms that play an important role in melanoma phenotypic plasticity. The reactivation of the expression of neural crest genes in melanoma is associated to progression and resistance to therapies. Among those, epithelial-to-mesenchymal transcription factors (EMT-TFs) have been proposed to regulate reversible switches between phenotypic states driving melanoma progression. However the role of Prrx1, an important EMT inducer also expressed during neural crest development, has not been investigated in detail.

Material and Methods

We have generated a clinically relevant melanoma mouse model that cannot reactivate Prrx1 expression in melanocytes to address the contribution of this EMT-TF to melanoma initiation, progression and responses to therapies.

Results and Discussions

We have found that Prrx1 is expressed in melanoma and that Prrx1 down-regulation induces cell cycle retention and reduces cell proliferation and cancer stem-like properties in melanoma cells.

Conclusion

We have detected an effect of Prrx1 on proliferation in melanoma cell lines and their stem cell abilities, but we are still characterizing the effect of this transcription factor *in vivo* to melanoma initiation, progression and responses to therapies.

EACR22-0910

Uncovering the drivers of metastatic Prostate Cancer

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Introduction

Prostate Cancer is the most common cancer in men and despite its high rate of cure is the third cause of death by cancer in males in Europe. With the advent of technology, we have become aware of the relevance of depicting the driver molecular pathways in cancer disease, which spearheaded the need to develop precision medicine. The intensive research over the past years have allowed us to identify the key genetic drivers of Prostate Cancer pathogenesis. Yet, the epidemiology and the key drivers of metastatic Prostate Cancer, which account for most of its morbidity, remain to be elucidated.

Material and Methods

Retrospective analysis of cancer progression stages is a powerful tool to infer alterations that are selected in tumor cells. By comparing the gene expression patterns of metastatic lesions with those present in the primary tumor and normal tissue from multiple patient cohorts, we have identified a set of genes that are differentially expressed specifically in metastatic Prostate Cancer cells.

Results and Discussions

Transcription factor 19 (TCF19) is one of those candidates that is up-regulated in metastatic patient cohorts and which shows prognostic potential. TCF19 was initially discovered as a growth-regulated cDNA. It harbors a FH domain and PHD domain through which binds H3K4me3. Recent studies have postulated that TCF19 might have a role in driving cancer aggressiveness in multiple types of cancer. However, its real contribution to metastasis, in particular in the context of Prostate Cancer, and the molecular mechanisms underlying this process have not been uncovered yet.

Conclusion

We will present our latest results on the biological consequences of the perturbation of TCF19 in Prostate Cancer, and on the depiction of its role as a driver of cancer aggressiveness.

EACR22-0916

The epithelial, hybrid and mesenchymal status of lung cancer cells dictates the success in metastasis development through differential interaction with natural killer cells

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Introduction

Epithelial to mesenchymal transition is essential for cancer dissemination but its role in overt metastasis establishment remains an open issue. Cancer cells that retain both mesenchymal

and epithelial properties, the so-called hybrid phenotype, might possess the highest potential to seed metastasis.

Material and Methods

According to the ratio between expression of epithelial CDH1 and mesenchymal SNAI2 genes, we classified 22 NSCLC cell lines as epithelial (E), hybrid (H), mesenchymal (M). The disseminating/metastatic properties of NSCLC cell lines representative of each phenotype were tested *in vivo* by subcutaneous and tail-vein injections in SCID mice. Interaction with NK cells (NKs) was assessed *in vitro* in co-culture experiments and by the use of NKs blockade antibody *in vivo*. Primary tumors (n=78), classified as E, H and M according to IHC staining for CDH1 and SNAI2, were analyzed for the NKs marker (NKp46) by IHC. Prognostic value of NKs content was assessed.

Results and Discussions

In vivo experiments showed that H-cells had lower ability to disseminate to murine lungs compared to M-cells. However, compared to E- or M-cells, H-cells possessed an increased ability to generate overt lung metastases in an experimental metastasis assay. The increased metastatic ability of H-cells was correlated with an expansion of cancer stem cells (CSC) subset and reduced NKs infiltration to the metastatic lungs. The expression of IL-8, known to recruit NKs, was decreased in H-cells and, coherently, they were less prone to chemoattract NKs compared to E and M-cells in vitro migration assay. By contrast, tumorigenic activity of M cells is affected by NKs. Indeed, *in vivo* inhibition of NKs by specific antibody massively increased metastasis formation and expansion of CSC in mice injected i.v. with M-cells. Patients with H-NSCLC have a poorer OS than E and M (p=0,02, HR=2,3). IHC analysis of primary NSCLC confirmed that NKs content was significantly reduced in H-tumors compared to E-tumors. Low content of NKs in E-tumors predicts poor long-term survival (p=0,014 HR 12,2).

Conclusion

Hybrid phenotype correlates with the highest metastatic potential and poor prognosis, related to efficient cancer cell seeding, increased content of CSC and ability to avoid NKs recruitment. Conversely, fully M-cells show limited metastatic potential, possibly due the control exerted by NKs on their CSC subsets. In E-NSCLC, NKs content can predict patients' long-term survival.

EACR22-0964

Loss of Rnd3/RhoE favors entosis in hepatocellular carcinoma cells

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Introduction

Entosis is a form of cell-in-cell structures (CICs), it is defined by the invasion of one or more viable cells into another of the same type. The formation of entotic cells is extensively studied *in vitro* after matrix detachment or under glucose-starvated condition. The entotic cell engulfment is controlled by the epithelial adherens junctions and the proteins of RhoA/ROCK pathway. The new clinico-histopathological studies suggest that entosis might be associated with an aggressive phenotype and poor prognosis in many cancers. However, entosis has never been studied in hepatocellular carcinoma (HCC).

Material and Methods

Using various approaches, the aim of our study was to characterize the entosis mechanism in HCC. To do so, we searched for entosis inducers in HCC cell lines, we characterized the different stages of the process and we addressed the molecular regulators of this phenomenon.

Results and Discussions

Our results demonstrate an increase in the number of entotic events in HCC cells when cultured in suspension state and after nutrient-deprivation. We further used electron, confocal and time-lapse microscopy to describe the stages and the fate of entotic cells. Moreover, we found that entosis is induced after Rnd3 silencing in HCC cells. Rnd3 protein, belonging to the Rho GTPase family, is a negative regulator of RhoA/ROCK pathway. We demonstrated that entosis induced by Rnd3 inhibition is dependent on the presence of RhoA and the protein kinase ROCK. We further developed a global approach in order to identify specific markers of entotic cells. A proteomic analysis on the dissected HCC entotic cells allowed us to identify new markers of entotic cells. These markers are currently used to help the detection of entotic cells in human HCC tissues.

Conclusion

In conclusion, we found that HCC cells are able to perform entosis. The relationship between the expression of Rnd3 and the entosis mechanism is crucial, as our published data identified Rnd3 as a tumor suppressor in HCC. Altogether, these data suggest the involvement of entosis in liver tumor progression and highlight a new perspective for the entosis analysis in medicine research as a novel therapeutic target.

EACR22-1100

The impact of the clodronate-based ATP analogues on the skin cancer cells

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Introduction

The extracellular adenosine triphosphate (eATP) plays a crucial role in extracellular signalling in cancer microenvironment. Certain reports have shown its involvement in regulation of metastatic related processes, like cell migration rate, invasiveness and epithelial-mesenchymal transition [1]. To study ATP-dependent pathways, the series of ATP analogues were designed and tested as substrates, inhibitors and cofactors of enzymes, as well as agonists or antagonists of receptors. Clodronate-based ATP analogue (AppCCl₂p) was found to be an inhibitor of certain intracellular enzymes, such as PDGFRα, JAKs, FGFR2 and ANT [2]. In current studies, the class of clodronate-based ATP analogues were synthesized and tested for their properties as extracellular signalling molecules, which may modulate ATP-dependent processes in cancer cells.

Material and Methods

The series of clodronate ATP analogues, including thio-modified counterparts, were synthesized and tested for their cytotoxicity and ability to induce purinergic signalling pathways. Obtained compounds were also characterized for their impact on the migration rate of tested cells. The activity of ATP analogues was tested in the cancer squamous carcinoma (A431) cells, as well as the non-cancerous human keratinocytes. It should be noted, that for alpha-thio-modified counterparts, all studies were performed using diastereomerically pure compounds.

Results and Discussions

Performed studies have indicated significant impact of the one of the clodronate-based ATP analogues on the migration rate of squamous carcinoma cells. The alpha-thio-beta-gamma-CCl₂-ATP was shown to decrease the migration rate of cells in scratch assay. Interestingly, only one of the diastereomers showed such properties, whereas the effect of the other diastereomer was quite opposite. Simultaneously, none of the tested compounds induced strong toxic effect against human skin non-cancerous cells.

Conclusion

The clodronate-based ATP analogues seems to be a good candidate for the future research of the metastatic properties of cancer cells. Obtained data suggest one of the clodronate-based ATP analogues as a compound with anti-migration properties in human squamous carcinoma cells.

Acknowledgment: This project was financially supported by the grant 2017/26/D/ST5/01046 from National Science Centre in Poland.

References: [1] Zhang et al. Cancer Sci. 2017;108:1318-1327, [2] Malwal et al. J. Am. Chem. Soc. 2018, 140, 7568–757

EACR22-1154

Extracellular HSP90 chaperone complexes promote breast cancer progression

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Introduction

Cancer cells adapt to the proteotoxic stresses at which they are subjected by upregulating chaperone proteins expression and by becoming addicted to them. Chaperones, including Heat shock protein 90 (HSP90) and several of its co-chaperones, can also be actively released by cancer cells in order to provide important signals for the tumor microenvironment and to facilitate cancer progression. While the role of extracellular HSP90 (eHSP90) as a promoter of cancer progression is well established, the involvement of its extracellular co-chaperones remains poorly investigated.

Material and Methods

Protein–protein interactions were investigated performing immunoprecipitations, gel filtration chromatography and pull-down assays.

Downregulation of Morgana and HSP90 was obtained by shRNA lentiviral transduction in different breast cancer cell lines. The ability of cancer cells to migrate was evaluated through wound healing assay, in presence or not of different inhibitors (small molecules or antibodies) and/or recombinant proteins.

Results and Discussions

We recently found that, when released by breast cancer cells, eHSP90 interacts with the secreted co-chaperone Morgana promoting cancer cell migration through the binding to cell surface receptors, such as LRP1, TLR2 and TLR4. Our data indicates that eHSP90 interacts with other secreted co-chaperones and is capable of forming high molecular weight complexes in the extracellular milieu of breast cancer cells but not in the one of non-tumorigenic cell lines. The results that we obtained suggest a role for these aberrant eHSP90 complexes in promoting cancer cells migration.

Conclusion

Our work indicates the presence of an intricate chaperone network in the extracellular space of breast cancer cells that promotes cancer progression. A deeper understanding of the eHSP90 interactome could lead to the discovery of new potential targets for drug intervention in cancer.

EACR22-1156

Determination of a molecular signature to predict organ-specific metastasis using HCC model

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Introduction

More than 90% of cancer-related deaths are caused by cancer metastasis to vital organs. Metastatic cells are capable of detaching from the primary site, organizing the target microenvironment for its growth benefits, entering circulation, and adopting a new environment. Thus, it is crucial to understand metastasis biology to predict organ-specific metastasis and develop an effective management plan for cancer. Previously, we determined that HGF/c-Met signaling promotes liver colonization of hepatocellular carcinoma (HCC) cells in the liver. Thus, in this project, we aimed to determine the “mechanism of c-Met mediated liver colocalization of cancer cells” and “factors found in liver microenvironment regulating c-Met signaling”.

Material and Methods

First, we cloned fluorescent-labeled c-Met overexpressing HCC cells. Since HCC microenvironment is rich in collagen, matrigel, HGF, fatty acids, high glucose/fructose and insulin, we used these supplements in a variety of combinations to mimic the liver microenvironment in 3D-in vitro cells culture systems. We performed qPCR and western blot analysis to determine the molecular profile of cells grown in 2D and 3D conditions. Then, we used the zebrafish model to develop fatty liver and test the metastatic capability of cells.

Results and Discussions

We showed that c-Met overexpressing cells are highly invasive and metastatic in vitro and in vivo models, respectively. HGF or high glucose found in the microenvironment increased aggressiveness of the cells. We successfully delivered fatty acid into 3D cell culture and created a fatty liver in zebrafish using different combinations of palmitic acids, oleic acids, fructose, glucose and insulin. Molecular profiling studies showed that c-Met/caveolin-1 signaling pathway that regulates aggressive phenotype is highly affected by components found in the liver microenvironment

Conclusion

Our results provide information on the molecular profile of the liver microenvironment working in synergism with c-Met signalling in the orchestration of liver-specific metastasis. These findings suggest that c-Met might be used as a predictive biomarker to predict liver-specific metastasis.

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EACR22-1212

Mimicking liver microenvironment employing three-dimensional in vitro and in vivo zebrafish models

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common and aggressive cancers worldwide. It has an increasing incidence that is reflected by the rise in the incidence of non-alcoholic fatty liver diseases (NAFLD), especially in Western World. The prognosis remains dismal due to the difficulties in early diagnosis and the high level of metastasis, recurrence and therapy resistance. Thus, it is urgent to underpin the molecular mechanism of HCC. To do this, we aimed to develop 3D-in vitro cell culture methods and in vivo zebrafish model that are mimicking HCC microenvironment.

Material and Methods

We first cloned green fluorescent-labeled HCC cells. To mimic HCC specific microenvironments we used different ratios and combinations of supplements such as insulin, glucose, palmitic acid, and oleic acids to treat cells both in 2D and 3D cell culture systems. We used Nile red fluorescent dye to stain lipids. After Nile red staining, we used Fluorescent Microscopy to observe free fatty acid (FFA) accumulations in the cells. We performed MTT to analyze viability of cells to find optimum concentrations and variations of supplements. We used an in vivo zebrafish model to induce fatty liver using different combinations and ratios of supplements. We also analyzed frequently used model organisms to identify the most closely resemble gene expression patterns in NAFLD using bioinformatics tools.

Results and Discussions

Nile red staining results and viability test analysis demonstrated that optimum accumulation of FFA was managed at ratio 2 oleic acid: 1 palmitic acid in the presence of glucose (25mM) and insulin (100nM) in 2D system. When we embedded green fluorescent-labeled cells in collagen gel and treated with optimized concentration and ratio of FFA+glucose+insulin, we observed that cells formed branching-tubules and accumulated lipids compared to control groups. We also successfully delivered FFA to zebrafish liver and created fatty liver in vivo zebrafish model. The maximum delivery of FFA was managed when glucose was added to mediums. Importantly, when we delivered green-tagged cells to zebrafish larvae we could observe green cells in Nile red stained fatty liver. Our results showed that certain zebrafish models might be more appropriate to study angiogenesis in the NAFLD model.

Conclusion

This study might provide insight into designing new experimental systems using 3D systems and zebrafish model to mimic HCC microenvironment and analyze molecular mechanisms of HCC. This project was funded by TUBITAK(119S698).

Overexpression of α E-catenin/CTNNA1 supports cell migration via physical binding of CEP55 and activation of AKT/EMT signaling in hepatocarcinogenesis

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Introduction

Hepatocytes are highly polarized cells, which contribute to liver homeostasis via cell-cell contact. Interestingly, changes of junctional structures are frequently observed in hepatocellular carcinoma (HCC). However, how dysfunctional cellular interaction contributes to tumor formation and progression is poorly understood. α E-catenin (CTNNA1) belongs to the catenin family, which connects the cadherin- β -catenin complex to the actin cytoskeleton. Previous studies illustrated tumor-suppressive functions of CTNNA1 in many tumor types such as gastric and bladder cancer. However, if and how CTNNA1 contributes to liver cancer is unclear.

Material and Methods

Expression of CTNNA1 was investigated in human HCC expression data (e.g., The Cancer Genome Atlas) and confirmed by immunohistochemistry (tissue microarrays consisting of 744 samples). CTNNA1 binding partners were identified using BioID labeling followed by mass spectrometry, co-immunoprecipitation and proximity ligation assay. Functional (e.g., viability and migration assays) and mechanistic analyses (e.g., chromatin immunoprecipitation) were performed after RNA interference-mediated inhibition in liver cancer cell lines.

Results and Discussions

In contrast to other tumor types, CTNNA1 was significantly overexpressed in HCCs compared to normal/adjacent liver tissues with highest cytoplasmic expression in poorly differentiated tumors. Elevated CTNNA1 levels statistically correlated with poor clinical outcome of HCC patients. In vitro, CTNNA1 depletion decreased HCC cell viability, proliferation and migration but had no effect on cell apoptosis. Proteomics revealed known interaction partners for CTNNA1 (e.g., the baso-lateral polarity factor Scribble) and several new binding candidates such as CEP55 and PDLIM7. Transcription factors of the TEAD family and FoxM1 as well as the transcriptional co-activator YAP controlled CEP55 expression. CTNNA1 and CEP55 silencing reduced HCC cell migration rather than cell viability and proliferation. Furthermore, CEP55 knockdown suppressed epithelial-mesenchymal transition (EMT), which was illustrated by a downregulation of the genes CDH2, SNAI1, TWIST, VIM, ZEB1 and ZEB2 and reduced AKT pathway activity.

Conclusion

Overexpression of CTNNA1 supports cell migration via its physical interaction with CEP55 in hepatocarcinogenesis. Since CEP55 is regulated by known liver oncogenes, it serves as mechanistic link between these FOXM1/TEAD/YAP and AKT/EMT in hepatocarcinogenesis.

Phenotypes and function of small cell lung cancer circulating cell clusters

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Introduction

Small cell lung cancer (SCLC) is a highly metastatic, recalcitrant malignancy. Recently described molecular subtypes are based on predominant neuroendocrine (NE) or non-neuroendocrine (NNE) transcription factor expression. However, growing evidence points to intra-tumoural heterogeneity and co-operation between NE and NNE cells to facilitate invasion and metastasis. Circulating tumour cell (CTC) clusters were detected in blood samples from patients with SCLC correlating with worse outcomes. How CTCs cluster and which phenotypes comprise CTC clusters is unclear.

Material and Methods

An imaging workflow was developed to identify NE and NNE cells, singly and in clusters in blood from mice with subcutaneously implanted tumours comprising different ratios of NE and NNE cells. RNA seq data for SCLC cell lines, models derived from patients' CTCs (termed CDX) and patient tumours was used to predict unique cellular characteristics of these subpopulations. Predictions were validated using a novel *in-vitro* approach allowing comparison of single vs clustered cells.

Results and Discussions

NE SCLC cells were enriched in the blood of our mouse models and a significant proportion were within CTC-clusters. RNAseq data revealed expression of epithelial and cell–cell junction related genes were enriched in NE cells, and this was validated at the protein level. NE SCLC cells required cell–cell contacts to provide anti-apoptotic signalling without which apoptosis occurred via the intrinsic pathway. Further characterisation of signalling pathways involved in CTC cluster mediated NE cell survival is ongoing. Studies of intracardiac injection of single cells versus clusters have commenced to validate cell survival benefits of clustering in-vivo and how this impacts metastasis.

Conclusion

This study demonstrates that NE SCLC cells exploit their epithelial behaviours to facilitate clustering and survival in the blood stream, potentially supporting metastasis. Loss of cell-cell contacts provokes apoptosis and further research is ongoing to identify targetable nodes of this signalling pathway.

EACR22-0255

Impact of chromosomal instability and cGAS/STING activation on metastasis in cancer cells

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Introduction

A distinctive feature of cancer cells is chromosomal instability (CIN) which has been suggested to be associated with the ability to metastasize in many cancers. As metastasis is the leading cause of death in cancer, understanding whether and how CIN promotes metastasis is an important unmet need in cancer research. CIN results from errors in chromosome segregation during mitosis and can lead to the formation of micronuclei (MN). Their rupture leaks genomic DNA into the cytosol which is sensed by cGAS, an activator of STING. Based on conflicting *in vitro* data, we investigated the role of the cGAS/STING pathway as transducer of CIN and trigger of epithelial-to-mesenchymal transition (EMT) and metastasis through downstream activation of the non-canonical Nf-kB pathway.

Material and Methods

For our study, 12 tumor cell lines derived from metastases of pancreatic, colorectal and prostate cancer as well as from primary tumor sites of pancreatic cancer, respectively showing high, moderate and low metastatic potential in a large-scale xenograft model (Jin et al., Nature 2020), were selected. Copy number alterations were determined by flow cytometry and genome-wide SNP genotyping arrays. IF staining was performed to investigate chromosome mis-segregation, MN formation and cGAS signaling. The processing of NF- κ B2/p100, translocation of RelB to the nucleus and expression of epithelial (EpCAM, E-cadherin, claudin-4) versus mesenchymal (fibronectin, N-cadherin, vimentin) markers of EMT were examined by western blot analysis. All results were correlated to the levels of CIN and metastatic potential of the cell lines.

Results and Discussions

A significant correlation between higher metastatic potential and cGAS activation ($p < 0.05$) was only discernible for pancreatic primary tumor-derived cell lines but not for any of those derived from metastases. In addition, cell lines of primary pancreatic cancer with high cGAS activity exhibited a more mesenchymal character than those isolated from metastases. However, this correlation was independent of CIN as our cell line cohort showed almost equal fractions of aberrant chromosomes and unstable genomes. Moreover, the abundance of cGAS-positive MN did not correlate with non-canonical Nf- κ B signaling.

Conclusion

Our results do not confirm an overall correlation between CIN and the initiation of EMT and metastasis through downstream activation of the non-canonical Nf- κ B pathway via cGAS/STING signaling, suggesting that these effects might depend on tumor lineage and derivation site.

EACR22-0337

Targeting Adam17 in cancer associated fibroblasts restricts cancer cell migration

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Introduction

Cancer associated fibroblasts (CAF) within the tumour microenvironment (TME) promote cancer progression thus offering promising targets for more effective therapeutic intervention. Moreover, upregulation of a disintegrin and metalloproteases (ADAMs) have been reported in many cancers favouring their growth and invasion. ADAM17, which has been reported to be upregulated in cancer-associated fibroblasts (CAF), modulates more than 80 substrates, many of which are involved in cancer development and progression. Better understanding of the role of ADAM17 in CAF may identify novel mechanisms by which CAF contribute to cancer progression and identify new therapeutic targets.

Material and Methods

Primary normal oral fibroblasts (NOF) and oral cancer-derived CAF were challenged with the conditioned medium (C.M.) derived from H376 and H357 oral cancer cells. Proliferation was assessed via EdU incorporation assay and the expression of CAF markers analysed by western blot, qPCR assays and immunofluorescence. Adam17 protein and RNA levels and its proteolytic activity were assessed via western blot, qPCR and ELISA assays. NOF and CAF derived C.M. was used to treat H357 and H376 cells and proliferation, migration (transwell and wound healing assay), invasion assay (Matrigel coated transwell assay) assessed. EMT markers expression was analysed by western blot and qPCR.

Results and Discussions

Stimulation of NOF and CAF with cancer cell-derived C.M. induced upregulation of ADAM17 and CAF markers. ADAM17 silencing in NOF and CAF downregulated CAF markers and altered the composition of their secretome. Furthermore, the C.M. derived from ADAM17-deficient NOF and CAF decreased cancer cell migration and N-Cadherin levels. N-cadherin inhibition in cancer cells decreased their migration to a similar extent as that observed upon treatment with C.M. from ADAM17-deficient CAF.

Conclusion

Here, we discovered that cancer cells upregulate ADAM17 in neighbouring fibroblasts which in turn sheds factors promoting the migration of cancer cells at least in part by upregulating N-cadherin. Targeting of CAF-associated ADAM17 and cancer cell-associated N-cadherin hinders this crosstalk and hampers cancer cell migration. Collectively, this study identifies a novel ADAM17-N-cadherin axis contributing to pro-migratory CAF:cancer cell interactions.

EACR22-0493

Transcriptional networks governed by E2F factors control a proliferative to invasive switch

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Introduction

In order to disseminate and seed into distant organs, cancer cells become migratory and coordinate invasive, proliferative and genome stability maintenance programs. The ability of epithelial cells to engage into pro-invasive mesenchymal forms is governed by extensive changes in gene expression triggered by several transcription factors. Our group identified cell-cycle regulators E2F1 and E2F2 as key transcription factors necessary for the maintenance of differentiated phenotypes and preservation of genome stability. We now unveil a novel function for these factors whereby E2F1/2 activity negatively controls epithelial-mesenchymal transition-like (EMT-like) traits in colon cancer cells. How E2F factors regulate cell fate, and the impact of these novel functions of E2F1/2 in cancer cell biology remains to be resolved.

Material and Methods

Analysis of invasive features, DNA damage markers and proliferative capacity in colorectal cancer cell lines (HCT-116, DLD1, T84) was performed using quantitative-PCR, immunofluorescence, FACS analysis and transwell migration and invasion assays. E2F1/2 silencing was achieved with siRNA molecules and pharmacological inhibition. RNA-sequencing and Chromatin Immunoprecipitation (ChIP) analyses were applied to study E2F1/2-dependent transcriptional profiles that maintain the epithelial architecture and abrogate cell rewiring towards mesenchymal forms.

Results and Discussions

Here, we identify E2F1 and E2F2 transcription factors as regulators of the proliferative-to-invasive switch in colon cancer cells. E2F1^{low}/E2F2^{low} cells display a highly motile and invasive phenotype, while attenuating cell growth. Interestingly, increased DNA damage accompanies this malignant transdifferentiation to a mesenchymal-like state. While exogenous supply of nucleosides alleviated E2F1/2 deficiency-induced DNA damage, EMT-like phenotypes were not abrogated. Transcriptomic and ChIP analyses revealed that E2F1/2 factors repress the expression of the mesenchymal regulator *SNAI2* in colon

cancer cells upon binding to its promoter, together with genes involved in cell adhesion and migration.

Conclusion

Our study describes a novel mechanism mediating the shift from a proliferative to an invasive phenotype in colon cancer cells and reveals that while loss of E2F activity limits the growth of cancer cells and promotes genome instability, it concomitantly triggers a transcriptional program that favours invasive traits.

EACR22-0561

Biomimetic scaffolds as an *in vitro* tool to study breast cancer cell adaptation to organ-specific ECM in bone metastasis.

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Introduction

Bone is one of the most frequent sites of metastasis from breast cancer. The metastatic cascade is modulated not only by intrinsic features of tumor cells but also by the crosstalk with the microenvironment. In this context, the extracellular matrix (ECM) of both primary tumor and metastatic site plays a key role in orchestrating tumor progression. However, this study is hampered by the lack of *in vitro* models that recapitulate the organ-specific ECM.

Material and Methods

We used our previously characterized type IV collagen-based scaffold and a newly synthesized collagen-hydroxyapatite (HA) scaffold to mimic the ECM of primary tumor and bone site. We investigated how different physiochemical properties affects dynamics of cell growth, genetic and phenotypic features of three breast cancer cell lines: MDA-MB-231 (a triple negative breast cancer cell line), SCP2 (subclone of MDA-MB-231, *in vivo* selected with bone tropism) and MCF7 (luminal A). To evaluate the interactions between breast cancer and bone cells, the ability of tumor cells to induce osteoclasts differentiation from human monocytes has been evaluated by indirect co-culture. Moreover, we tested how ECM affects the pharmacological profile to bone targeted drugs in tumor cells and indirect co-culture.

Results and Discussions

Breast cancer cells cultured in the bone mimetic model showed a lower proliferation rate compared to when cultured in collagen matrix. By gene expression analysis, we showed that breast cancer cells cultured in 3D HA scaffold express at higher-level pro-inflammatory genes. Moreover, soluble factors secreted by breast cancer cells, cultured in 3D environment, can induce osteoclasts differentiation from their precursor cells. TNF- α , which has been found to be highly expressed by breast cancer cells cultured in 3D, could support this differentiation. Interestingly, different ECM seems to affect breast cancer sensitivity to bisphosphonate, which seems to be more effective in breast cancer cells cultured in 3D HA model.

Conclusion

We developed *in vitro* 3D organ-biomimetic models, which enable us to demonstrate that the mechanical structure and composition of the ECM affect breast cancer cells behavior in bone metastasis. These models will be used to dissect the multistep process of bone metastasis *in vitro*, to provide new insight on the role of microenvironment in stages difficult to model *in vivo*, as bone pre-metastatic niche and metastatic dormancy. This will enable us to identify novel targets to prevent or treat skeletal disease.

EACR22-0873

Pre-clinical models of small cell lung cancer brain metastasis enable molecular profiling of founder metastatic cells to identify potential vulnerabilities

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Introduction

Metastases are the predominant cause of cancer-related deaths, and Small Cell Lung Cancer (SCLC), an aggressive neuroendocrine (NE) tumour, is one of the most metastatic cancers (>80% patients present with metastatic disease). Incurable brain metastases are frequent and clinically challenging. Until now, lack of robust patient-derived preclinical models and a paucity of SCLC brain metastatic biopsies hampered molecular studies of SCLC brain metastasis. Our lab developed >60 CTC-derived patient explant models (CDX) in immunodeficient mice which are faithful models of the donor's disease. Using newly developed SCLC *in vivo* resection protocol, several of our subcutaneously implanted then resected CDX models routinely metastasise to the brain^{1,2}.

Material and Methods

We conducted a longitudinal *in vivo* study in brain-metastatic CDX3P, harvesting single CTCs and early founder brain metastatic cells. We adapted a FACS-based strategy using human CD147 antibody³⁻⁶ to purify CDX cells followed by single-cell RNA sequencing (scRNAseq).

Results and Discussions

CDX3P metastasises to the brain after 155-252 days. 58 single CTCs from 6 mice and 214 single brain metastatic cells from 2 mice were isolated and underwent scRNAseq. Initial bioinformatics analysis indicated that CTCs are more transcriptionally diverse than brain founder cells warranting deeper interrogation.

Conclusion

Our CDX models are robust tools to study mechanisms of tropism, founding and colonisation of metastasis to the brain. Bioinformatic analysis of this rich scRNAseq dataset is ongoing to characterise the molecular features underpinning transcriptional heterogeneity of these single CTCs and early brain metastasis founders. Our data will reveal shared transcriptional landscapes common to CTCs and brain metastatic founders to explore presence of brain-tropic CTC sub-clones and molecular features unique to brain metastasis founders. Future studies of established brain metastasis will explore programs for brain colonisation. Longer term function-testing of gene candidates of tropism, founding and colonisation will seek to identify precision therapies and liquid biopsies for early diagnosis to improve patients' quality of life and outcomes.

Tat-Cx43₂₆₆₋₂₈₃ reduces the viability of mouse lung cancer stem cells

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Introduction

Connexin43 (Cx43) is a gap junction forming protein related to cancer pathogenesis, having both pro-tumourigenic and anti-tumourigenic effects. Thus, Cx43 performs anti-tumourigenic effects in glioma cells by inhibiting the activity of Src oncoprotein. Based on this mechanism, a cell penetrating peptide was designed (Tat-Cx43₂₆₆₋₂₈₃), which preserves the capacity of inhibiting Src on different models of glioma. Src is involved in numerous signalling pathways related to different cancer types, including lung cancer. Src is particularly relevant in the stem cell subpopulation of lung cancer cells, involved in brain metastasis. Consequently, we asked whether the Src inhibitory peptide Tat-Cx43₂₆₆₋₂₈₃ could impair brain metastasis as shown in primary brain tumours. To do so, we first analysed the effect on lung cancer stem cells.

Material and Methods

Using the human lung cancer cell line, A549 (Non-Small Cell Lung Cancer) and the mouse lung cancer cell line LLC (Lewis Lung Carcinoma, also known as 3LL), we obtained stem cell subpopulations from differentiated adherent cells. With these subpopulations (adherent and non-adherent), we carried out cell survival studies and protein expression assays to assess the effect of Tat-Cx43₂₆₆₋₂₈₃.

Results and Discussions

Our results show that the levels of Src and its active form were higher in the non-adherent vs. adherent subpopulations of A549 and 3LL. The expression of other proteins such as Cx26, relevant on cancer stem cells, was higher in non-adherent vs. adherent 3LL subpopulations.

Tat-Cx43₂₆₆₋₂₈₃ did not significantly affect the cell growth and properties of A549 adherent and non-adherent cells. However, in 3LL cells a significant decrease in cell viability was found in the non-adherent subpopulation but not in adherent 3LL cells. A small increase on Cx43 expression was found on non-adherent 3LL cells with Tat-Cx43₂₆₆₋₂₈₃ treatment.

Conclusion

Tat-Cx43₂₆₆₋₂₈₃ is proving to have a significant antiproliferative effect in the non-adherent subpopulation of 3LL lung cancer cell line, as it does in glioma stem cells. These results aim us to explore further this effect, including lung cancer brain metastasis models to address Tat-Cx43₂₆₆₋₂₈₃ impact within the brain microenvironment, a favourable niche for the development of lung cancer metastasis

Regulation of tissue factor-dependent procoagulant properties by CD44: implication for metastasis of breast tumor cells

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Introduction

Epithelial-Mesenchymal Transitions (EMT) are well described to impact Circulating Tumour Cell (CTC) biology. Equipped with enhanced survival and invasive properties, EMT-shifted cells are suspected to be more prone to establish metastasis. Considering extensive literature linking coagulation to CTC protection within the circulation, our team demonstrated that EMT-shifted cancer cells express Tissue Factor (TF, F3, a membrane-associated activator of the coagulation cascade) and display enhanced coagulant properties that may contribute to their better survival and niching in experimental metastasis assays. Importantly, a CTC subpopulation expressing TF and vimentin in CTCs isolated from breast cancer patients was identified. On the other hand, microarray analysis evidenced that the stem cell marker CD44, also known as a fibrin (end product of the coagulation cascade) receptor, is overexpressed in EMT⁺ cells. The aim of this work is to study the implication of CD44 in the EMT/TF/coagulation/metastasis axis.

Material and Methods

We examined this CD44/TF/coagulation axis in breast cancer EMT⁺ (MDA-MB-231, Hs578T), EMT⁻ (MCF7, T47D) and EMT-inducible (EGF-treated MDA-MB-468, MDA-MB-468 harboring a doxycycline-inducible snail expression vector) cellular models. We used siRNA to inhibit CD44 expression and realized RT-qPCR, western blot, flow cytometry analyzes and promoter reporter assays to monitor regulations of our gene of interest. Furthermore, we realized *in vitro* clotting assays and TF activity assays to examine coagulant properties, and *in vivo* experimental metastasis assays to assess early metastatic potential.

Results and Discussions

We first confirmed that CD44 is induced during the EMT process concomitantly to TF. We then discovered that silencing CD44 in our EMT⁺ cellular models decrease TF expression, their pro-coagulant properties *in vitro* and their ability to accomplish early metastatic niching *in vivo*. Examining further the molecular mechanisms of TF regulation by CD44, we discovered that CD44 modulate TF transcription and we more particularly implicated Sp1 in the process.

Conclusion

Our results thus demonstrate a key role of CD44 in regulating TF expression in EMT⁺ cancer cells, and point to Sp1 as a transcriptional mediator of this regulation. This modulation of TF expression by CD44 in EMT⁺ cells led to a concomitant regulation of pro-coagulant properties, and impacts metastatic potential.

EACR22-0935

E-cadherin mutations induce remodeling of nucleus positioning and composition towards an invasive cancer program

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Introduction

Cell invasion is a critical step for tumor progression and dissemination into neighboring and distant tissues, which constitute the foremost cause of cancer mortality. Loss of the cell-cell adhesion molecule E-cadherin occurs in 70% of carcinomas and is widely described as an initiating event in the invasion process. Recently, data has emerged demonstrating that this milestone phenomenon is accompanied by an abnormal affair between cancer cells and the extracellular matrix (ECM). In fact, cancer cells display increased plasticity and a remarkable ability to overcome the fibrous structure of the ECM. Since the nucleus is the largest and stiffest organelle in the cell, we hypothesize that it represents a major determinant of cell reshaping and spreading capacity. Our main aim is thus to dissect the mechanisms through which the nucleus supports invasion of cancer cells.

Material and Methods

To determine alterations in nuclear composition and architecture that are distinctive of cells with E-cadherin impairment, we have used cell lines and *Drosophila* strains expressing either wild-type E-cadherin or the Y755C variant identified in Hereditary Diffuse Gastric Cancer (HDGC) patients. Nuclear morphological features and migratory rates were assessed by fluorescent markers coupled with confocal microscopy and advanced bioimaging techniques. The molecular profile of nuclei envelope from E-cadherin mutant and wild-type cells was subsequently investigated by implementation of a proteomic workflow, encompassing cellular fractionation and processing through high-resolution Mass Spectrometry (LC-MS).

Results and Discussions

Here, we have shown that the Y755C E-cadherin mutation induces evident changes in nuclear morphology, and an increased migration performance of border cells in *Drosophila*. In particular, detailed quantitative measurements revealed differences in nuclear area, mean intensity, perimeter, solidity and relative position to basal surface. Further, proteomic analysis of enriched nuclear fractions disclosed that the composition of the nuclear envelope from E-cadherin mutant cells is significantly different from that of the wild-type counterparts. Differentially abundant molecules include proteins relevant for spatial and structural integrity of the nucleus.

Conclusion

Overall, this work provide evidence that cells with E-cadherin loss of function activate a specific mechanotransduction program that imposes nuclear remodeling at physical and biochemical levels, endowing cells with a pro-invasive signature.

EACR22-0939

Unravelling New Strategies for Early Detection of Invasive Cancer Cells: a Focus on the Nuclear Architecture Dynamics

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Introduction

Cancer invasion is a hallmark of cancer progression and the leading cause of cancer mortality worldwide. However, the identification of invasive cancer cells at an early stage remains challenging. Therefore, there is an urgent need to develop novel biomarkers and innovative strategies to successfully tackle cancer invasion. The nucleus is a tightly regulated organelle and its dynamic and controlled architectural features are key to normal cell function. Indeed, alterations in nuclear morphology are observed in many diseases including cancer. Thus, in this study, we investigated fine-tuned nuclear patterns associated with invasion, taking into consideration the cell-extracellular matrix (ECM) interaction.

Material and Methods

In this study, we took advantage of an ECM Array platform (MicroMatrix 36, MicroStem) and a panel of invasive and non-invasive gastric cancer cells. A comprehensive analysis of nuclei architectural features was performed, including area, intensity, perimeter, eccentricity, circularity, solidity and entropy, among other parameters. For validation purposes, we also assessed cells transfected with wild type or mutant E-cadherin, which lead to distinct adhesion and invasion phenotypes.

Results and Discussions

Our results have shown that the invasive abilities of cancer cells are associated with unique nuclear modifications. Moreover, we verified that invasive cells adhere preferentially to specific ECM components. Interestingly, we could observe an association between nuclear phenotypes and increased attachment abilities, suggesting that the ECM may modulate nuclear morphometrics thus affecting the invasive behaviour of cancer cells. Of note, cell lines harbouring E-cadherin mutations corroborated the existence of a nuclear signature associated with invasive potential.

Conclusion

Overall, this study demonstrates that the use of nuclear architectural features could provide an efficient strategy to identify cells with invasive potential. Further investigation and integration of this approach with other cellular properties could have an impact in diagnosis, prognosis and therapeutic strategies.

EACR22-0959

Epithelial-mesenchymal transition induction in bladder cancer by TGF- β 1 treatment

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Introduction

Bladder cancer (BlCa) is the 10th most common malignancy worldwide. 5-25% of non-muscle invasive BlCa (NMIBC) cases progress to muscle-invasive BlCa (MIBC). Tumor microenvironment (TME) and epithelial-mesenchymal transition (EMT) have been described as key factors in tumor growth and progression. TME components release several soluble mediators, such as cytokines and proteases, capable of promoting epi(genetic) changes in malignant cells. Transforming growth factor β (TGF- β) is a cytokine that triggers TGF- β signaling, a crucial EMT-inducing pathway. During EMT, cellular trans-differentiation occurs, promoting a partial or complete loss of epithelial markers and a gain of mesenchymal markers. Thus, we aim to understand the relationship between TME and EMT in BlCa, to gather a better understanding of BlCa progression.

Material and Methods

Normal cell line, SV-HUC1 and BlCa cell lines, RT-112 and 5637, were treated with 20ng/mL TGF- β 1, for one week (0-7 days). Then, TGF- β 1 containing medium was replaced by normal medium, and cells were cultured for another week (7-14 days). RNA and protein were extracted to evaluate the possible alterations on EMT-related molecules, and to assess TGF β receptors and endogenous *TGF β 1* transcript levels.

Results and Discussions

Overall, in all treated cell lines, TGF- β 1 treatment promoted an EMT induction, both at transcript and protein levels. Here, a decrease of *CDH1*/E-cadherin and *CLDN1*/Claudins epithelial markers was observed, while an increase of *CDH2*/N-cadherin, *SNAI1*/Snail, *SNAI2*/Slug and *Vim*/Vimentin mesenchymal markers was assessed. Moreover, it was observed higher *TGF β RI* and *II* transcript levels for all the studied cell lines, as well as an increase of endogenous *TGF β 1* in SV-HUC1 and 5637 cells. Even a week after TGF- β 1 withdrawal (7-14 days), it was observed a global increase in all mesenchymal markers, similar to TGF- β 1 treatment (0-7 days), suggesting that EMT induction was maintained. *TGFB* receptors and *TGFB1* levels continued higher after TGF- β 1 withdrawal. Our findings suggest that TGF β -stimulated bladder cells are able to produce TGF- β 1 in an autocrine manner, and/or TGF- β downstream signaling pathways might become overactivated in a TGF- β 1 independent manner.

Conclusion

We showed that TGF- β 1 can induce EMT in bladder cancer cells, indicating a putative role in driving BlCa progression. Besides TGF- β 1, we are currently investigating novel BlCa TME players potentially involved in EMT induction.

EACR22-0979

Chronic hypoxia recapitulates metastatic ROS-resistant phenotype via MUC1-C

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Introduction

Hypoxia occurs in 90% of solid tumors and is associated with metastasis and mortality. We developed a hypoxia fate-mapping system that causes a Loxp-Cre permanent switch from DsRed to GFP expression under hypoxia. Our studies revealed that breast cancer cells that experienced intratumoral hypoxia (GFP+) were 5x more likely to form a metastatic lesion in the lung due to a reactive oxygen species (ROS)-resistant phenotype that promoted survival in the bloodstream. Hypoxia causes ROS, and both stressors activate a cell signaling response. We hypothesize that hypoxic cells experience high ROS levels intratumorally and acquire a resistant phenotype, which confers them a survival advantage in the bloodstream.

Material and Methods

To unravel the mechanism behind the GFP+ phenotype, we employed spatial transcriptomics on primary tumor sections or performed low-input RNA sequencing on CTCs isolated from a murine orthotopic model. Mechanistic studies were conducted in vitro under chronic hypoxia using gain or loss of function assays. A targeted Cre-CRISPR approach was developed to test the knockout of genes-of-interest only in cells that have been exposed to hypoxia.

Results and Discussions

Spatial transcriptomics revealed that hypoxic cells (GFP+) upregulate hypoxia- and NF- κ B-regulated transcriptional responses. The altered gene expression patterns are maintained even when GFP+ cells enter more oxygenated tumor regions or the oxygen-rich bloodstream, compared to DsRed+ cells. NF- κ B activation in GFP+ cells was confirmed in vivo using a NF- κ B-dependent mKate2 fluorescent reporter. RNA sequencing studies showed that cells exposed to chronic but not acute hypoxia are enriched for NF- κ B activation. MUC1-C was upregulated by both HIF-1 α and NF- κ B under these conditions. Abrogation of MUC1 reduced the levels of antioxidant enzymes, SOD1-3, resulting in higher ROS levels and apoptosis. Targeted CRISPR of MUC1 activated by Cre exclusively in GFP+ cells significantly increased ROS and caused reduction of GFP+ metastatic burden, which was recapitulated by MUC1-C therapeutic inhibition.

Conclusion

Intratumoral hypoxia upregulates HIF- and NF- κ B-transcriptional responses that are retained in CTCs to promote their survival against ROS in the bloodstream, which ultimately drive metastasis. HIF- and NF- κ B-regulated MUC1-C prevents ROS accumulation and apoptosis via SOD1-3 in hypoxia-exposed cells. Preliminary in vivo studies demonstrate that targeting MUC1-C reduces metastasis caused by cells exposed to intratumoral hypoxia.

EACR22-1023

E-cadherin (E-cad) and N-cadherin (N-Cad) expression assessment by double immunofluorescence (dIF) in prostate cancer (PCa) as a sign of epithelial-mesenchymal transition (EMT)

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Introduction

EMT is an important process in cancer metastasis and treatment resistance. "Cadherin switch" defined as down-regulation of E-cad and up-regulation of mesenchymal N-cad may

be considered as one of morphological features of EMT. Here cadherin expression in PCa was studied, as well as coexpression patterns seen, using dIF staining.

Material and Methods

A training cohort of 29 PCa samples obtained during radical prostatectomy was studied. 1 representative section per case was studied. Slides were stained with dIF technique using primary antibodies to E-cad (BioGenex, 1:100) and N-cad (ThermoFischer, 1:2000) and secondary goat antibodies labeled with AlexaFluor 488 and 555 dyes (ThermoFischer, 1:200), respectively. Staining was assessed semiquantitatively in the whole slide.

Results and Discussions

E-cad staining was generally homogenous in intensity in the same slide and reduced. On the contrary, N-cad staining exhibited heterogeneity in 16 (55.2%) of cases with separate groups of glands, glands or even cells within one gland exhibiting strong membranous staining while other cancer cells had weak or no staining. Both intensity and proportion of stained cells were assessed with calculating a weighed staining index (WSI). For E-cad median WSI was 30 (range 9-55), and for N-cad – 10 (1-50) due to much less percentage of PCa cells expressing N-cad (<10% in 15 (51.7%) cases). Moderate or strong N-cad staining in >1/3 of PCa cells was observed in 14 (48.3%) cases. However, no statistically significant correlations were found between WSI for E-cad and N-cad. Additionally, 65 high power fields (HPF, x40) from 9 cases with remarkable heterogeneity of N-cad staining with simultaneous presence of glands or cells with strong/moderate and weak/absent N-cad in the same HPF were assessed regarding staining intensity of E-cad in cells with different levels of N-cad. In 50 (76.9%) of them E-cad staining was the same, so we didn't observe significantly less E-cad in N-cad-expressing cells.

Conclusion

Neoexpression of N-cad may be a more potent marker of EMT in PCa than E-cad down-regulation as it was seen in only a small proportion of PCa cells. However, no significant associations were found between levels of N-cad and E-cad in the same cells, seen using direct coexpression assessments by dIF, as well as at the level of glands or cases, so further studies of mechanisms of both proteins regulation are needed.

EACR22-1093

Enhanced high-throughput stability of bioprinted breast cancer models for spheroid formation and migration with telocollagen

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Introduction

Type 1 collagen and Matrigel are gold standards for 3D cell culture and have recently been adapted for 3D bioprinting. However, there is still a knowledge gap regarding the scalability of natural matrices on bioprinted models for high-throughput spheroid formation and migration. We compared side by side the influence of type 1 collagen (TeloCol®-10) or Matrigel® on spheroid formation and migration of the breast cancer cell line MDA-MB-231, which is commonly used for research due to its spheroid forming and highly migratory capacity.

Material and Methods

We bioprinted droplets of the hydrogels type 1 collagen (TeloCol®-10) or Matrigel using a bioprinter with temperature-controlled syringe-pump printhead in 96-well plates. For the spheroid protocol, MDA-MB-231 mCherry tagged single cell solution was mixed with the

hydrogel at 6 mg/mL and 4×10^6 cells/mL, and bioprinted in 96-well plate as 1 μ L droplets. The migration assay entails a central core 1 μ L droplet embedded with cells (4×10^6 cells/mL, 6 mg/mL), covered by a cell-free outer 5 μ L droplet (4 mg/mL). The spheroid formation and migration were monitored every 3h, over 5-9 days, using the CELLCYTE X™ live cell imaging system.

Results and Discussions

The breast cancer cells grew consistently in TeloCol®-10, forming aggregates and spheroids after day 3, and maintained consistent growth until day 5. In Matrigel®, the cells aggregated into spheroids already by day 2, but from day 3 to 5, the droplets started to degrade at the edges, pushing the cell clusters to the center while leaving the cells growing on the well plastic. Interestingly, the red fluorescence from mCherry tagged cells was captured every 3 hours through the entire process showed that the cells in Matrigel® and TeloCol®-10 grew at the same rate. Similar results were obtained for the migration assay, where Matrigel® outer and inner droplets showed signs of degradation after day 3 and 7 respectively. TeloCol®-10 droplet-in-droplet kept the shape while the cells migrated from the inner droplet to the cell-free outer droplet. Overall, we were able to track the migrating cells in the TeloCol®-10 model for up to 9 days, while cells in the Matrigel® model had reached the border of the outer layer on day 7.

Conclusion

We demonstrated that TeloCol®-10, supported breast cancer cell spheroid formation and migration with higher stability over longer incubation times compared to Matrigel® under the same conditions, probably to the higher stiffness achieved by the telocollagen.

EACR22-1119

Paladin, a phosphatidylinositol 4,5-bisphosphate phosphatase overexpressed in colon cancer, is required for actin polymerization and liver metastasis dissemination.

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Introduction

Colorectal cancer is the fifth most diagnosed cancer worldwide and despite important improvement in both diagnosis and therapy, it remains the third cause of death by cancer. Most of colon cancer patients succumb to the development of distant metastasis and not to primary tumour growth. In this context, our laboratory has identified several potential biomarkers involved in the dissemination of colon cancer and correlated with a poor survival. In this work, we explore the function of paladin, a protein encoded by PALD1 (KIAA1274) gene, and never investigated in the cancer field. Paladin is an evolutionary conserved protein described as a phosphatidylinositol 4,5-bisphosphate phosphatase.

Material and Methods

Paladin abundance was investigated by western-blot in colon primary tumours and matched liver metastases. Biological function of Paladin was explored in HCT116, LS174T and LoVo cell lines. Liver-tropic HCT116 cells were selected in vivo after splenic injection in NOD-SCID mice. Tumour growth and metastatic potential of colon cancer cells was investigated by xenograft of PALD1 shRNA colon cancer cells in NOD-SCID mice. Cell migration was evaluated in vitro by wound-healing and Boyden chamber assays upon PALD1 silencing.

Phospho-proteomics analysis were used to highlight altered pathway upon PALD1 silencing. Highlighted pathway was validated by western-blot and co-immunoprecipitation experiments.

Results and Discussions

We discovered that paladin is required for cell migration and metastasis. We reported that paladin depletion altered the phospho-proteome within colon cancer cells. Thanks to immuno-precipitation experiments, we demonstrated that paladin was interacting with SSH-1, a phosphatase involved in colon cancer metastasis. Finally, we showed that paladin depletion in cancer cells altered the interaction between SSH-1 and actin.

Conclusion

Paladin is an under undervalued protein in oncology participating in actin cytoskeleton remodelling and necessary for an efficient cancer cell migration.

EACR22-1179

The crosstalk between melanoma cells in different phenotypic states and fibroblast activation protein- α expressing fibroblasts in the tumour microenvironment

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Introduction

Crosstalk between melanoma cells and melanoma-associated fibroblasts (MAF) in the microenvironment contributes to melanoma progression, metastasis and drug resistance. An improved understanding of the mechanisms underpinning the conversion of skin fibroblasts to MAF will enable more diverse ways of targeting this crosstalk. Given the heterogeneity of melanomas, we hypothesized that melanoma cells in different phenotypic states might influence the conversion of skin fibroblasts to MAF via different signalling pathways. MAF represent a heterogeneous population. One subset expresses fibroblast activation protein- α (FAP α) and exhibit myofibroblastic properties. We investigated: 1) factors secreted by melanocytic or dedifferentiated melanoma cells that drive skin fibroblasts to FAP α -expressing MAF and 2) the role of FAP α in MAF and on aggressive melanoma behaviour.

Material and Methods

Skin fibroblasts were treated with conditioned media derived from melanocytic or differentiated melanoma cells and MAF marker expression including FAP α and myofibroblast activity were assessed. MAF properties were analysed in FAP α overexpressing skin fibroblasts, FAP α -silenced MAFs and MAFs rendered enzymatically inactive for FAP α . Melanoma cells were cultured on top of live 3D fibroblast-derived ECM and their proliferation and migration were evaluated.

Results and Discussions

Results showed that TGF β 1 is the predominant factor secreted by dedifferentiated melanoma cells, which drives skin fibroblasts to FAP α -expressing MAFs, characterised by increased expression of FAP α and aligned ECM deposition. FAP α -silenced MAF decreased their expression of myofibroblastic markers and ECM remodelling capabilities. Conversely, overexpression of FAP α in skin fibroblasts, suggesting that MAF myofibroblastic activity is dependent on FAP α expression. Inhibition of FAP α enzymatic activity also decreased myofibroblastic activity in MAF and skin fibroblasts treated with TGF β 1, suggesting that the MAF phenotype is also dependent on FAP α enzymatic activity. Aligned ECM produced by FAP α -overexpressing skin fibroblasts or MAF decreased melanoma cell proliferation and

promoted their migration, compared to the disorganised ECM produced by empty vector control cells and FAP α -silenced MAFs.

Conclusion

These findings give novel insight into the crosstalk between melanoma cells in different phenotypic states and fibroblasts, which may lead to future treatment strategies that normalize the melanoma microenvironment.

EACR22-1181

Investigation of tumour cell lines motility and metastatic potential under hypoxic conditions

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Introduction

Hypoxic conditions and the resulting oxygen deprivation induce a number of changes in tumour cell behaviour that may contribute to tumour progression. Tumour hypoxia promotes neoangiogenesis and contributes to tumour cell resistance to radiotherapy and chemotherapy. Hypoxia inducible factor 1 (HIF-1) is a major regulatory protein of cellular responses to tissue hypoxia, regulating the expression of several molecules (transcription factors, enzymes, small G-proteins, adhesion molecules, membrane proteins) as transcription factors. HIF-1 α has been described to affect cell motility through the regulation of RhoA expression and activation, and we are investigating the role of the Rho family of small G-proteins (Rac1, cdc42 and RhoA) in metastasis. The aim of this study is to better understand how hypoxia affects the motility and metastatic potential of different tumour cell lines and which molecular events play a prominent role in these processes.

Material and Methods

We measured *in vitro* gene and protein expression of HIF-1 α , RhoA, Rac1 and CDC42 in proliferation and migration experiments under hypoxic and normoxic conditions. Following tail intravenous inoculation, we examined the *in vivo* metastatic ability of tumor cells and the expression of these genes in human clinical samples, looking for correlations with tumor progression.

Results and Discussions

The partial results obtained so far show a correlation between baseline motility and metastatic potential in the tumour cell lines studied, as well as between gene expression and clinical progression. In addition, we have also shown that a hypoxic environment enhances the migration and metastatic potential of cells with higher basal motility compared to less motile cells.

Conclusion

Consistent with our own observations described previously, the hypoxic environment induced cell-type-dependent changes in the levels and activation of small GTPases and resulted in variable migration and metastatic potential in different human tumor cell lines.

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EACR22-1237

Phylogenetic analysis of metastatic progression in thyroid cancer

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Introduction

Thyroid cancer (CT) is the most common cancer of the endocrine organs and is classified into: well-differentiated carcinomas, characterized by a mostly favourable prognosis and sensitive to standard treatments, and undifferentiated carcinomas (UTC), considered the most lethal and highly refractory endocrine neoplasia to current therapies. UTCs have an invasive and highly metastatic phenotype. The metastatic cascade models describe lymph node metastasis origin from cancer cells spread out directly from primary tumor that in turn seed distant metastasis. According this, lymphadenectomy was defined as an elective therapy to increase the progression free survival for TC patients. However, recent TC trials discredit the improvement in patient outcome after lymph nodes removal. We aim to unveil the origins of TC metastases to determine which specific subclone is able to colonize distant organs.

Material and Methods

Thyroid carcinogenesis model was previously generated starting from human embryonic stem cells which underwent to a thyroid differentiation protocol. At day 22 of differentiation, thyroid progenitor cells (TPCs) were engineered with the CRISPR CAS-9 technology to introduce mutation in BRAFV600E/TP53R248Q and NRASQ61R /TP53R248Q gene in order to recapitulate UTC, when injected in immune-compromise mice. Barcode-mediated clonal tracking technology allow a comprehensive clonal analysis of primary tumors and the corresponding metastasis. The Barcode library will be analyzed by Next-generation sequencing (NGS).

Results and Discussions

We permanent labelled D22 double-mutated TPCs using highly complex lentiviral barcode library to map primary tumor lineages in multiple metastatic sites. We generated thyroid orthotopic mouse model using labelled cancer cells. Primary tumors and metastases will harvest and will undergo to DNA extraction and NSG analysis. Parental engineered TSCs will also include in the analysis.

Conclusion

Given its impact on patient care, understanding the evolutionary history of metastases is critical in cancer treatment. Phylogenetic studies on the origin of metastases should dissect the metastatic events that determine the emergence of one or more subclones, which have a common or distinct origin. and we will unveil the distinct nature of clones prevailing in the several metastatic nodules.

EACR22-1239

2D and 3D-human vasculature models to study cancer cell interaction with endothelial

cells in metastasis formation

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Introduction

Interaction of cancer cells with endothelial cells (ECs), which line blood vessels, is the early and critical event in metastasis formation. Despite the contribution of animal models to cell-cell interaction and cancer studies, a human organ-specific vasculature in vitro model to study breast cancer organ tropism is needed.

Material and Methods

Here, we first set-up a 2D microfluidic model to study the interaction (firm adhesion and migration) between human patient-derived cancer cells and brain, lung and primary ECs under shear stress coupled to live-cell imaging. Then, we fabricated and developed a microfluidic 3D-chip featuring an in vivo-like cylindrical geometry resembling organ specific vessels, as the blood brain barriers, with brain ECs alone or in co-culture with pericytes in a 3D ECM matrix growing under rheological shear stress

Results and Discussions

We characterized the 3D cylindrical vessels formed by permeability and cell junction expression (VE-cadherin, ZO1 and PECAM1). Furthermore, we quantified the cell surface expression of cell adhesion molecules and integrins, known to mediate cell adhesion, in both cancer cells and organ specific vascular ECs, and their firm adhesion under shear stress.

Conclusion

These models may provide an important tool to unravel the molecular mechanism of cancer cell interaction with organo-specific vasculatures and new targets to prevent and/or reduce breast cancer metastasis.

EACR22-1257

Harnessing UTC tumorigenesis and evolution process via clonal tracking

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Introduction

Thyroid carcinoma (TC) is the most common malignancy of endocrine organs. Especially the anaplastic thyroid cancer (UTC) has an invasive and highly metastatic phenotype, representing a major subject of oncology research. Despite significant advances in the TC's hallmarks and biological behavior, the cell subpopulation hierarchy responsible for tumorigenesis, cancer promotion, and progression is still unclear.

Material and Methods

The TC model was generated starting from human embryonic stem cells, which underwent to specific differentiation protocol. On day 22 of differentiation, we obtained double-mutated BRAF/TP53 and NRAS/TP53 thyroid progenitors (TPCs) using CRISPR/CAS9 technology.

Barcode-mediated clonal tracking technology will allow a comprehensive clonal analysis of primary tumors heterogeneity. The Barcode library will be analyzed by Next-generation sequencing (NGS).

Results and Discussions

We established a new tumorigenic “genetic model” of UTC by the subcutaneous injection into immunocompromised mice of double-mutated TPCs. Then, to track the promotion and progression of UTC, we generated an intratumoral heterogeneity model by the injection of double-mutated TPCs permanently transduced with a barcode library. Then, a barcoded engrafted tumor has been consecutively transplanted to establish serial PDX passages. Immunohistochemistry analysis of P1 and P3-deritumorumor revealed conservation of UTC histologic features. DNA extracted from P1 and P3 tumor tissue was analyzed by digital PCR to confirm the presence of the specific mutation responsible for the transformed phenotype. P3-derived cells retain high migratory and invasion capacity compared to P1 derived cells together with high MMP-9 expression levels. P3 versus P1 cells are also characterized by a critical metabolic switch. Then, DNA extracted from the parental engineered TSCs, P1 and P3 derived tumors will be analyzed by NGS to track the Spatio-temporal diversity of clones and to determine which clones are responsible for the tumor promotion.

Conclusion

The generation of barcoded TPCs *in vitro* and the establishment of progressive PDX passages will allow us to identify the cellular subclones mainly involved in thyroid tumor initiation, promotion, and progression. The several PDX passages represent one step of tumor progression recapitulating the evolution and the heterogeneity of patient pathology.

EACR22-0592

Hydroxychloroquine might promote progeny cells generation by Therapy Induced Senescent Renal Cancer Cells in hypoxia

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Introduction

Therapy Induced Senescence (TIS) has been considered as a positive outcome of the therapy, as TIS cells are unable to proliferate. However, recent data have shown that TIS cells can produce progeny due to amitotic divisions. In consequence, it can lead to tumor relapse. Autophagy is the self-degrading process which occurs in the tumor hypoxic microenvironment where it promotes tumor cell survival. New evidence indicates that autophagy may play a crucial role in TIS cells survival. This study investigates whether autophagy inhibition could stop renal cancer TIS cells from escaping their non-proliferative state by reactivating their proliferative potential, or by selectively eliminating them.

Material and Methods

Human Renal Cancer cell line (RCC4) was cultured in normoxia (~19% O₂) and hypoxia (1% O₂). TIS in cells was induced by incubation with Vinblastine (VIN). Hydroxychloroquine (HCQ) was used as autophagy inhibitor in TIS and non-TIS cells. Incubation with HCQ lasted 24H. Metabolic activity was assessed with MTT and proliferation with BrdU ELISA test. Western Blotting was used for protein expression analysis, qPCR for gene expression analysis, flow cytometry for cell cycle distribution and polyploidy analysis, fluorescence microscopy for nuclei morphology analysis.

Results and Discussions

Our previous studies indicated that in TIS autophagy has been promoted. It led to conclusion that autophagy could be targeted in senolytic therapy. HCQ significantly decreased metabolic activity and proliferation of TIS and non-TIS cells. Treatment with HCQ decreased cell number, which corresponded with cell cycle blockade as well as accumulation of pro-apoptotic cells. Therefore, cytotoxic effect of autophagy inhibition was induced in both TIS and non-TIS cells. However, expression of molecular senescence markers in TIS cells, p21 and p53 was downregulated in hypoxia. Simultaneously, increased polyploidization was observed in hypoxia upon HCQ treatment as well as accumulation of cells with regular sized nuclei, suggesting progeny generation. Those observations correlated with disruption in autophagy-flux.

Conclusion

Temporal autophagy inhibition can promote cellular death of both TIS and non-TIS cells. Pharmacological inhibition of autophagy in TIS cells might also accelerate the process of progeny generation, especially in hypoxia suggesting an important role of autophagic flux in the TIS control. Research was funded from Sonata Bis 7 grant no. 2017/ 26/E/NZ3/00434 rewarded by the National Science Centre, Poland.

EACR22-0912

BH3 profiling of senescent cancer cells uncovers their resistance to apoptosis

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Introduction

Mammalian cells are continuously challenged by a myriad of endogenous and exogenous insults, which leads to cellular stress. The degree of inflicted damage determines if a cell undergoes apoptosis or survives in a non-proliferative state of cellular senescence. It has been shown that treatment with various anti-cancer agents can induce senescence in both normal as well as in cancer cells. These senescent cancer cells rely on anti-apoptotic signaling for their survival, which can be targeted by so-called 'senolytic agents'. Our group and others have shown that eliminating senescent cancer cells can potentially be an effective anti-cancer treatment strategy. ABT-263 is a broad acting senolytic that targets anti-apoptotic proteins BCL-2, BCL-xL and BCL-W. Our group has shown that ABT-263 has a senolytic effect in certain cancer cell lines, while not in others. This raised the question why certain senescent cancer cells undergo into apoptosis after treatment with ABT-263, while others remain refractory.

Material and Methods

We assessed the RNA and protein levels of pro- and anti-apoptotic proteins in a panel of 13 cell lines made senescent by aurora kinase A inhibitor alisertib and chemotherapeutic agent etoposide. In addition, we performed BH3 profiling, a technique that measures apoptotic priming (readiness to undergo into apoptosis) and identifies apoptotic dependencies. In this technique, mitochondrial outer membrane permeabilization is measured after exposure with synthetic peptides resembling the active domains of BH3 family proteins.

Results and Discussions

RNA and protein levels of the BCL-2 family proteins did not clarify the difference between ABT-263 sensitive and resistant cells. However, BH3 profiling showed that senescent ABT-263 sensitive cells highly depend on BCL-xL signaling, while ABT-263 resistant cells did not. Moreover, we found that ABT-263 sensitive cells were more primed to apoptosis compared to resistant cells. Intriguingly, this correlation was already apparent in the parental state.

Conclusion

These findings show that the parental state influences the senolytic efficacy of ABT-263 in the same cells when senescent. This suggests a possible predictive index for future senolytic therapy.

EACR22-0629

A lamin-based reporter system for screening of senescence in cancer

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Introduction

Cellular senescence is a highly regulated cell-cycle arrest important for embryonic development and aging, but can be induced prematurely for instance by mitochondrial dysfunction, activation of oncogenes or DNA damage. Therapy-induced senescence (TIS) is a promising anti-cancer strategy, but the search for TIS-inducing compounds is hampered by the difficulty of identifying senescent cancer cells. Hence, a senescence biomarker for cancer cells suitable for compound screening would be very valuable.

Material and Methods

We made reporter constructs with transgenic lamins, validated that their incorporation in cancer cells did not influence cell behavior, senescence-induction or endogenous lamin expression. We made various senescence reporter candidates and chose our senescence reporter based on that the transgenic lamins behaved similarly to the endogenous lamins during senescence-induction. We developed an imaging analysis program to quantify the fluorescent intensities of the transgenic lamin-fusion proteins and a data-analysis program which distinguishes senescent from non-senescent cells based on the changes in these intensities upon senescence-induction.

Results and Discussions

We developed a novel screening system for senescence in cancer, SenoQuant, consisting of a transgenic lamin-based reporter, imaging- and data-analysis program to distinguish between senescent and non-senescent cancer cells. We validated our screening system in MCF7 (breast), DU145 (prostate) and A549 (lung) cancer monolayers and spheroids. Further, we extended the reporter with an IL-6 promoter-element, which activation is indicative for Senescence-Associated Secretory Phenotype (SASP)-activation and show that we are able to identify senescent cells expressing SASP-factors.

Conclusion

We developed the SenoQuant system and demonstrated that it can distinguish senescent from non-senescent cells in *in vitro* cancer models and that the addition of the IL6 promoter-element may be useful to identify senescent cells expressing SASP-factors. Both features may be suitable for applications in longitudinal-monitoring of senescent cells relevant for industry or clinical settings to monitor treatment response.

The presence of a tumoral senescence signature negatively affects overall survival in non-small cell lung cancer.

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Introduction

Cure and long-term survival for non-small cell lung cancer (NSCLC) remains hard to achieve. Cellular senescence, an emerging hallmark of cancer, is considered as an endogenous tumor suppressor mechanism. However, senescent cancer cells can paradoxically affect the surrounding tumor microenvironment, ultimately leading to cancer relapse and metastasis. As such, the role of cellular senescence in cancer is highly controversial.

Material and Methods

In 155 formalin-fixed paraffin-embedded samples from surgically resected NSCLC patients with pathological (p) TNM stages I-IV (8th edition), cellular senescence was assessed using a combination of four immunohistochemical senescence markers, i.e., lipofuscin, p16^{INK4A}, p21^{WAF1/Cip1} and Ki67. A tumoral senescence signature (SS) was defined by the presence of high-level lipofuscin and high p16^{INK4A} and/or p21^{WAF1/Cip1} expression ($\geq 30\%$ NSCLC cells positive) in combination with low Ki67 expression ($< 30\%$ NSCLC cells positive), and was correlated to clinicopathological parameters and outcomes, including overall (OS) and disease-free survival (DFS).

Results and Discussions

A tumoral SS was present in 48 out of 155 NSCLC patients, but did not correlate to any clinicopathological parameter, except for p53 mutation status. In a rather heterogeneous patient cohort who had surgery with curative intent, i.e., excluding pTNM stage IV, Kaplan-Meier survival analysis showed a difference in median OS for patients with a tumoral SS ($n = 47$, 32%) compared to patients without a tumoral SS ($n = 100$, 68%) of 62 versus 88 months, with a clear trend for worse OS for patients with a SS approaching significance ($p = 0.061$). In a selected homogenous patient cohort who fulfilled the following criteria: (a) one type of histology, i.e. adenocarcinoma, (b) without known EGFR or KRAS mutation, (c) curative (R0) resection and (d) no neoadjuvant systemic therapy or radiotherapy, the median OS and DFS for patients with a tumoral SS ($n = 24$, 31.2%) compared to patients without a tumoral SS ($n = 53$, 68.8%) was 42 versus 109 months ($p = 0.007$) and 35 versus 61 months ($p = 0.16$), respectively. In multiple Cox PH model analysis correcting for age and pTNM stage I-III, a tumoral SS remained a significant prognostic factor for OS (HR: 1.96, $p = 0.028$).

Conclusion

The presence of a tumoral SS significantly affects OS in NSCLC adenocarcinoma. In this light, adjuvant senolytic therapy could be an interesting strategy for NSCLC patients harboring a tumoral SS, ultimately to improve survival of these patients.

EACR22-0977

Targeting USP7/RRM2 axis drives senescence and sensitizes melanoma cells to epigenetic drugs

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Introduction

A fundamental trait of melanoma is cell cycle dysregulation, leading to uncontrolled proliferation. High mitotic rate is associated with aggressive histologic features and targeting cell cycle regulators was proposed as a promising strategy in melanoma therapy. Deubiquitinating enzymes are key regulators of the ubiquitin-proteasome system and of the cell cycle, and their dysfunction lead to tumorigenesis. The DUB ubiquitin specific protease 7 (USP7) is up-regulated in several cancers, but its role in melanoma has never been deeply investigated.

Material and Methods

USP7 expression is investigated in melanoma patients tissues and its function in several melanoma patient-derived xenograft models (PDXs). Changes in the proteomic and transcriptomic profiles are investigated in USP7-silenced cells, identifying a new USP7 target assessed by deubiquitination and co-immunoprecipitation assays. The efficacy of USP7 inhibitor is tested *in vitro* and *in vivo*, also combination treatment is performed.

Results and Discussions

Our *in vivo* drop out screen in PDXs identifies USP7 as an essential gene in melanoma patients. We describe a new oncogenic role of USP7, whose downregulation induces cellular senescence, arresting melanoma growth *in vivo* and proliferation *in vitro* in all melanoma subtypes.

We provide a comprehensive understanding of targets and networks affected by USP7 depletion by performing a global transcriptomic and proteomic analysis and we show that RRM2 is a novel USP7 target and that its expression is regulated by USP7 during the S-phase of the cell cycle. The ectopic expression of RRM2 in USP7-depleted cells rescues the senescent phenotype.

Pharmacological inhibition of USP7 with P5091 phenocopies the shUSP7-induced senescent phenotype. We prove that the bifunctional HDACs/LSD1 inhibitor domatinostat has an additive antitumor effect, eliminating P5091-induced senescent cells, paving the way to a new therapeutic combination for melanoma patients.

Conclusion

Our research highlights the oncogenic role of USP7 in melanoma, providing a mechanistical landscape illustrating the shUSP7-senescent phenotype. We also establish a central role of USP7/RRM2 axis in melanoma maintenance.

Importantly, our work sheds light on alternative therapeutic option to melanoma patients increasing the efficacy of P5091, targeting the residual, senescent cells with domatinostat.

Regulation of TNBC by an alternate mRNA translation initiation and its potential role on breast cancer cell metabolism

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Introduction

Translational control and metabolic reprogramming are hallmarks of advanced cancers. Important genes involved in cancer progression express mRNAs that are selectively translated, including regulators of cancer cell metabolism.

Cancer cells acquire an altered metabolism, switching from oxidative phosphorylation (OXPHOS) to glycolytic phenotype (Warburg effect), to increase reliance on alternate metabolic pathways to support growth, proliferation, and metastasis. Triple-negative breast cancer (TNBC), one of the most aggressive and highly metastatic subtypes with the poorest outcome, is characterized by elevated glycolysis and low OXPHOS. Still, the exact mechanism for this metabolic switch is largely unknown.

Using a TNBC cell model it has been shown an alternate mechanism of cap-dependent but mTORC1/eIF4E independent mRNA translation by the eIF4G homolog, DAP5, which directly binds the cap-binding protein eIF3d. Our research indicates that this alternate translation initiation could be essential in regulating mRNAs involved in breast cancer metabolism.

Material and Methods

DAVID analysis from genome-wide transcriptomic and translomic studies, metabolomic assays including Glycolysis Assay [Extracellular Acidification], Oxidative Phosphorylation and Mitotracker Assay, as well as RT-PCR and western blot analysis were performed from well-characterized triple-negative aggressive breast cancer cell lines, 4T1 and MDA-MB-231, Non-silencing (Nsi) control and silencing DAP5 (shDAP5) samples.

Results and Discussions

Several mRNAs related to glucose metabolism decreased after silencing DAP5 and on the opposite, critical mRNAs associated with OXPHOS increased, independent of their steady-state mRNA abundance. Silencing DAP5 increased Oxidative Phosphorylation activity and decreased the glycolytic rate in TNBC breast cancer cells.

The first glycolytic enzyme, hexokinase 2 (HK2), was reduced after silencing of DAP5. Cancer cells express high levels of HK2 and this is, at least in part, responsible for the accelerated glucose flux. The E-cadherin transcriptional repressors Snail (SNAI1) which has traditionally been implicated in promoting EMT and to be correlated with aberrant glycolytic metabolism was also reduced.

Conclusion

Our data indicates that the alternate mRNA translation initiation mechanism by DAP5 of specific mRNAs correlated to glycolysis and OXPHOS could play an essential part in the

mechanism by which TNBC breast cancer cells orchestrate a metabolic switch required for malignant progression and metastasis.

EACR22-0128

ANTI-ANGIOGENIC THERAPY INDUCES RESISTANCE TO GLUCOSE DEPRIVATION IN OVARIAN CANCER XENOGRAPHS

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Introduction

Cancer is characterized by genetic and metabolic heterogeneity and can be considered as an evolving entity with sub-clonal populations, undergoing selection driven by microenvironmental pressures or external stimuli such as drug treatment. Anti-angiogenic therapy by targeting vascular endothelial growth factor (VEGF) was previously found to elicit rewiring of tumor metabolism, causing marked alterations in glucose, lactate and ATP levels in tumors. Putatively, anti-angiogenic therapy might select specific subpopulations of tumor cells endowed with distinctive capability in resisting to glucose starvation. Herein, we evaluated whether differences in sensitivity to glucose starvation existed at the clonal level in ovarian cancer cell lines and tumor xenografts. We assessed if this phenotype was modulated by anti-VEGF therapy and explored the metabolic basis of this phenomenon.

Material and Methods

We obtained clonal populations from both ovarian cancer cell lines and tumor xenografts and discerned glucose deprivation resistant (GDR) and glucose deprivation sensitive (GDS) clones based on their *in vitro* behaviour upon glucose deprivation. By genetic, transcriptomic and metabolic analysis, we characterized the two different populations of clones obtained upon glucose deprivation.

Results and Discussions

We observed that established ovarian cancer cell lines contained clonal populations which disclose strikingly different viability following *in vitro* glucose starvation. Genetic alterations were not likely to account for the marked difference in tolerance to glucose starvation observed in GDR versus GDS clones. *In vitro*, GDR clones appear to better survive glucose starvation by increasing pyruvate and lipids uptake and mitochondrial oxidative phosphorylation (OXPHOS), compared with GDS clones. Interestingly, ovarian cancer xenografts exhibited a significative enrichment in the amount of GDR clones following treatment with the anti-VEGF drug bevacizumab and acquired increased tumorigenic properties *in vivo*, associated with higher microvessel density.

Conclusion

We demonstrated a significant metabolic heterogeneity at the clonal level in ovarian cancer cell lines which was partially responsible for the onset of resistance to glucose deprivation after anti-angiogenic drug treatment. Specific features of these therapy-enriched clonal subpopulations could be potentially targeted for counteracting adaptive resistance to anti-angiogenic therapy in cancer.

EACR22-0162

Primed Mesenchymal Stem Cell- derived Extracellular Vesicles may Facilitate Breast Cancer Cell Recurrence Through Metabolomics

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Introduction

Breast cancer cells (BCC) favourably metastasise to the bone marrow niche, where they interact with resident mesenchymal stem cells (MSCs) and can enter dormancy, evading treatment. Recent evidence has postulated the role of MSC-derived extracellular vesicles (MSC-EVs) in the onset and reversal of this dormancy. Our previous studies demonstrated that differing effects of MSC-EVs on the BCC line, MCF7, were dependent on MSC state. Specifically, MSC-EVs vs primed MSC-EVs, derived from injury-stimulated MSCs, result in a more dormant or active MCF7 phenotype respectively. This study aimed to understand the mechanism caused by the two MSC-EV populations.

Material and Methods

The metabolic effect following MSC injury was examined via several key metabolic assays, including the JC-1 mitochondrial activity dye, fluorescent glucose analogue uptake and lactate secretion assays. A subsequent metabolomics screen of MSC-EV cargo was carried out. 3D MCF7 spheroid cultures, generated via the ultra-low attachment method, were treated with MSC-EVs and primed MSC-EVs. The metabolic response of these spheroids was then investigated using identical metabolic assays, in addition to a 3D Seahorse Mito Stress Test. Finally, the gene expression profiles of MSC-EV treated spheroids was assessed via RNA-seq analysis.

Results and Discussions

A carbon metabolism shift was identified upon MSC injury, which was further translated to the primed MSC-EV treatments, highlighting clear differences in respiratory metabolites between the two MSC-EV populations and potentially explaining their opposing effects on MCF7 activity. Further investigation demonstrated that treatments caused differing metabolic BCC profiles, resulting in a metabolic BCC shift. Finally, RNA-seq analysis of MSC-EV treated 3D MCF7 spheroid cultures highlighted the two treatments differential influence on mitochondrial and glucose import pathways, further supporting a metabolic mechanism regulating BCC dormancy and recurrence.

Conclusion

Ultimately, this work establishes a novel potential mechanism for BCC dormancy exit, via the effect of primed MSC-EVs on BCC activity through changes in carbon metabolism.

EACR22-0218

Modulation of OXPHOS and pharmacological response in ovarian cancer

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Introduction

Ovarian cancer is the most fatal of all female reproductive cancers. The standard of care is platinum-based chemotherapy; recently, its treatment was greatly transformed by the introduction of poly(ADP-ribose) polymerase (PARP) inhibitors, which exploit homologous recombination (HR) deficiencies to directly target cancer cells.

Metabolic adaptation has emerged as a hallmark of cancer and recent studies highlighted the importance of oxidative phosphorylation (OXPHOS) in ovarian cancer.

Our aim was to investigate if and how the modulation of OXPHOS influences the pharmacological response of ovarian cancer to standard of care treatments.

Material and Methods

The sensitivity to OXPHOS inhibitors and standard therapies (i.e. PARP inhibitors and chemotherapy) was evaluated *in vivo* on patient-derived ovarian cancer xenografts (OC-PDXs), orthotopically implanted in nude mice, and *in vitro* using murine and human ovarian cancer cell lines.

Results and Discussions

Chemotherapy: Cisplatin (DDP) treatment induced the emergence of a sub-population of cells from malignant ascites with higher mitochondrial content and reduced expression of glycolysis-related genes, suggesting a shift toward OXPHOS after chemotherapy. The combination of DDP with the OXPHOS inhibitor IACS-010759 (respiratory chain complex I inhibitor) significantly improved the survival of mice, indicating that the effect of DDP was enhanced by the impairment of the oxidative metabolism.

PARP inhibition: The HR status correlated with the OXPHOS activity in both OC-PDXs and ovarian cancer cell lines. Indeed, 78% HR-deficient (HR-D) OC-PDXs were characterized by a high OXPHOS activity compared to only 7% HR-proficient (HR-P) OC-PDXs. Moreover, HR-D was associated with a superior sensitivity not only to PARP inhibition, but also to OXPHOS inhibition both *in vivo* and *in vitro*.

Conclusion

OXPHOS modulation is a promising strategy to improve chemotherapy efficacy laying the ground for further studies on such combination.

HR-D OC-PDXs and ovarian cancer cell lines rely on oxidative metabolism for energy requirements and are very sensitive to OXPHOS inhibition. Further studies are needed to assess whether alterations of OXPHOS influence the response to PARP inhibition.

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EACR22-0222

HIGH GRADE SEROUS OVARIAN CARCINOMA PRESENTS DIFFERENT METABOLIC PROFILES DEPENDING ON ITS MUTATIONAL STATUS

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Introduction

High grade serous ovarian carcinoma (HGSOC) is the most diagnosed and lethal type of ovarian cancer. Half of HGSOC present mutations in BRCA 1, BRCA 2 or other genes related to the homologous recombination repair pathway (HR). In order to maintain their

homeostasis, HR deficient tumours present a higher dependence on other repairing mechanisms, such as those dependent of PARP activity.

Material and Methods

Mostly this work was performed with the ID8 cell line model, which is a murine HGSOC model, with a *Trp53* KO or a double KO for *Trp53* and *Brca2*. The data obtained was later validated with in-vivo studies using C57/BL-6 mice and patient samples

Results and Discussions

Our results show that to maintain PARP activated, tumoural cells have to change their metabolism to a more oxidative one, to provide high amounts of NAD⁺, which is PARP's substrate. HR-deficient tumours derived from patients presented a more oxidative profile, with higher respiration rates which correlates with higher activity of the PDH, and lower levels of lactate and glucose transporters. Also, a metabolomic analysis showed that our HR-deficient model present higher levels of Krebs cycle metabolites. Moreover, they showed a higher sensitivity to inhibitors of the respiratory chain such metformin or oligomycin. In addition, our in vivo studies show that HR-deficient tumours present lower tumour volume when treated with metformin.

Conclusion

This study shows a mechanistic link between two major cancer hallmarks, which suggests novel possibilities for specifically treating HR-deficient cancers with inhibitors of the respiratory chain

EACR22-0266

Loss of the serine/threonine kinase MLK4 promotes invasive capability and metabolic rewiring in pancreatic cancer

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Introduction

Pancreatic cancer (PC) is one of the most lethal types of cancer, with a poor prognosis that has changed little over the past few decades. The oncogenic KRAS mutation is the major event in PC, which acts as a molecular switch to activate various intracellular signaling pathways, inducing cell proliferation and metabolic rewiring. Unfortunately, there is only one clinically approved KRAS inhibitor, which selectively target the KRAS^{G12C} mutation. Therefore, the identification of critical molecular mechanisms underlying the pathogenesis of PC is necessary to design alternative therapeutic approaches. We focused our attention on MLK4, a poorly characterized serine/threonine kinase acting downstream of KRAS, which is known to contribute to tumorigenic processes in different cancer cell lines.

Material and Methods

We analyzed a cohort of PC patients to evaluate MLK4 expression in tumor samples. We used PC cell lines in which we knocked-out MLK4 and assessed proliferation rates and invasive capability *in vitro* and in xenograft models. Moreover, we performed metabolic

assays and Seahorse analysis to evaluate changes in cell metabolism and bioenergetics upon modulation of MLK4 expression.

Results and Discussions

Immunohistochemical analysis revealed that MLK4 levels are reduced in about 20% of pancreatic ductal adenocarcinoma samples, in line with expression data from TCGA, and are associated with poor prognosis. PC cell lines knocked-out for MLK4 showed reduced proliferation but increased invasive ability *in vitro* and in a zebrafish xenograft model. Metabolic and Seahorse analysis revealed a defective mitochondrial respiration and increased glycolytic rates in KO cells, which is associated with fragmented mitochondria and increased levels of ROS. Mechanistically, by confocal imaging and fractionation experiments we demonstrated for the first time the presence of MLK4 in the mitochondria, where it regulates ERK signaling and contribute to maintain mitochondrial homeostasis.

Conclusion

Taken together, our findings revealed an unexpected tumor suppressor role of MLK4 in PC. Loss of MLK4 increases the aggressiveness of PC cell lines and results in metabolic rewiring. The analysis of the molecular pathways regulated by MLK4 could pave the way to the development of new therapeutic strategies in the treatment of PC. Moreover, given the metabolic addictions of PC cells, the in-depth characterization of the metabolic alterations will open new insights in the molecular mechanisms underlying the metabolic adaptation of PC cells.

EACR22-0478

Varying Levels of Physiological Glucose Affect Metabolic Bioenergetic Profiles of Prostate Cancer Cells

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Introduction

The majority of cancer cells employ aerobic glycolysis described as the “Warburg Effect” rather than oxidative phosphorylation to fuel their increased growth and proliferation requirements despite being less efficient in ATP production. Unlike most cancers, Prostate cancer (PCa) cells appear to display distinct metabolic dependencies while progressing from an androgen-dependent (AD) state towards the more aggressive androgen-independent (AI) disease. We hypothesised that varying levels of physiological glucose could play a role in altering metabolic phenotypes of PCa cells and contribute to a more aggressive disease phenotype.

Material and Methods

We examined the metabolic bioenergetic profiles of a panel of PCa cell lines; PNT-1a, LNCaP, PC3 and DU145 representing non-malignant, AD and AI stages. The Seahorse XFe24 was employed to determine if cell lines utilize oxidative phosphorylation or glycolysis under varying glucose conditions: normal physiological, high-glucose and zero glucose. The metabolomics of the cell lysates and their supernatants were determined by liquid chromatography mass spectrometry (LCMS).

Results and Discussions

LNCaP cells representing early-stage AD disease favoured oxidative phosphorylation, varying glucose conditions did not alter the metabolic phenotype. In contrast, the more aggressive later-stage AI cell lines; PC3 and Du145 showed a high dependency on glycolysis under high-glucose and normal glucose. Under zero glucose, the AI cell lines favoured oxidative phosphorylation. AI cells also showed higher metabolic capacity and non-mitochondrial respiration in the presence of glucose. Whereas AD cells did not show an alteration in non-mitochondrial and maximal respiration across different glucose conditions.

Conclusion

Overall, results indicate that AI cell lines display more plasticity in their metabolic phenotype to support tumour growth and proliferation. These findings may have implications for the development of novel targeted therapeutics for AI disease aimed at specific metabolic phenotypes.

EACR22-0684

Human pheochromocytoma tumour cells show peculiar biophysical characteristics depending on their genetic profile

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Introduction

Pheochromocytomas and paragangliomas (PPGLs) are rare neuroendocrine tumours. PPGLs are genetically mutated in nearly 70% of cases, with germline or somatic mutations in one of the 20 susceptibility genes. The most frequently mutated genes are those encoding the 4 subunits of the succinate dehydrogenase (SDHA-D). PPGLs are mostly benign, but about 20% of patients develop metastases. Although the risk factors associated with metastatic evolution still remain poorly defined, SDHB mutations are considered to be among the strongest. One of the major obstacles to advancing knowledge on PPGLs was the lack of suitable human cell lines for in vitro studies.

Material and Methods

-

Results and Discussions

Here, we show metabolic and functional differences between the two recently developed human pheochromocytoma cell lines, the hPheo1 parental and its derivative knock-out for the SDHB subunit (hPheo1 SDHB KO). We confirmed the silencing of the SDHB subunit, by western blot, and the increase of intracellular succinate concentration, due to the SDH impairment in the hPheo1 SDHB KO, by mass spectrometry. Unsurprisingly, the intracellular ATP level in hPheo1 SDHB KO cells is significantly reduced compared to that of hPheo1 parental. Interestingly, in hPheo1 SDHB KO, the succinate specific cell-surface G-protein-coupled receptor (SUCNR1) is upregulated compared with hPheo1 parental, and this overexpression directly correlates with the concentration of succinate. Indeed, hPheo1 parental cells cultured in media supplemented with 1mM or 10 mM succinate for 72 h increase the expression of SUCNR1. Since chromaffin cells are neuroendocrine cells, we have also evaluated some electrophysiological parameters, such as the resting membrane potential (RMP), capacitance (C_m), and resistance (R_m) values. RMP values, estimated in

current clamp mode, are very depolarized in hPheo1 parental cells (-5.8 ± 6.1 mV), and significantly different to those recorded in hPheo1 parental cells (-12.9 ± 11.0 mV). Whilst C_m and R_m values result similar in both the cell types. Generally, the hPheo1 SDHB KO cells show reduced outward K^+ currents, compared with hPheo1 parental ones. However, only in hPheo1 SDHB KO cells we sometimes register large K^+ currents, suggesting an activation of K^+ ATP channels due to the lower intracellular levels of ATP.

Conclusion

In conclusion, we show for the first time that the SDH impairment in human chromaffin tumour cells not only induces metabolic and functional changes, but also alters some electrophysiological properties.

EACR22-0710

Estrogen receptor status correlates with fatty acid synthesis, choline metabolism and AMPK pathway activation in breast cancer

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Introduction

Current hormone-receptor based classification of breast cancer governs patient treatment options and long-term outcomes. Here, we examine the relationship between hormone-receptor status and alterations in metabolism, to better understand the potential for using metabolic targeted therapies in breast cancer.

Material and Methods

IHC staining (estrogen and progesterone receptors; ER and PR and fatty acid synthase/FASN) was performed on breast cancer MCF7 (ER+/PR+, n=8) and MDA-MB-231 (ER-/PR-, n=8) xenograft models, as well as patient-derived xenograft (PDX) models AB580 (ER+/PR-, n=13) and STG-139 (ER-/PR-, n=19) from a local biobank. Flash frozen tumour tissue was collected and subjected to *ex vivo* metabolic and genomic analyses. The findings in mouse models were verified using human tissue microarrays (TMAs).

Results and Discussions

FASN staining was lower in ER-/PR- MDA-MB-231 xenografts as compared to ER+/PR+ MCF7 xenografts ($p < 0.01$), in which FASN expression correlated positively with both ER and PR staining ($r = 0.75$ and 0.9 respectively). Similarly, in the breast cancer PDX models, which lacked PR and HER2, we observed a higher FASN staining in ER+ compared to ER- models ($p < 0.001$). Supporting these observations in mouse models, analysis of two independent breast cancer TMAs showed that FASN staining correlates positively with the expression of both ER and PR and that it is lowest in triple negative breast cancer.

To further assess the metabolic changes between our ER+ and ER- PDX tissues, we used magnetic resonance spectroscopy methods to analyse choline related metabolite concentrations. These studies revealed that choline, glycerophosphocholine (GPC) and total choline were significantly higher in ER- tumours as was the GPC/phosphocholine ratio ($p < 0.01$), in line with prior work. Our data suggests that differences in ER expression alone may contribute to this shift in choline metabolism.

To further understand the impact of hormone-receptor status on energy metabolism, we conducted differential gene expression analysis on our xenografts RNA-seq data, which revealed activation of AMPK pathway in ER-/PR- tumours.

Conclusion

Our data shows a strong relationship between FAS and PR/ER that can be observed in patient samples as well as in breast cancer PDX models and xenografts. Loss of ER expression influences choline and fatty acid metabolism and AMPK signalling. Further studies should focus on metabolic blockers targeting those pathways, which could be used in combination to aid existing treatment regimes.

EACR22-0716

Natural chalcone cardamonin specifically modulates cell viability and apoptosis in neuroblastoma cells.

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Introduction

Neuroblastoma is the most common extracranial malignant tumor with pediatric-onset and originates from the neural crest. Neural crest-derived cancers are typically more aggressive and have a higher tendency to metastasize. High-risk patients account for approx. 60 % of all neuroblastoma cases and require complex treatment regimens including surgery, chemo- and radiotherapy. Most chemotherapeutic agents lack specificity, which leads to severe toxic side effects and limited anti-cancer properties. The natural chalcone cardamonin (CD) was shown by our group to have promising potential in treatment of melanoma in context of a selective toxicity for those tumor cells, but is non-toxic on the studied normal (healthy) cells.

Material and Methods

The human neuroblastoma cell line SH-SY5Y and normal human dermal fibroblasts (NHDF), being a model for normal (healthy) cells and distributed throughout the whole human body, were treated with increasing concentrations of CD (1-15 μ M) for 24 to 96 h. Cell viability was studied via MTT and SRB Assay. Apoptosis was examined via Western Blot analysis, flow cytometry and immunofluorescence microscopy. In addition, mitochondrial superoxide was also studied via immunofluorescence.

Results and Discussions

CD significantly lowered SH-SY5Y cell viability in a dose and time dependent manner whereas NHDF cells were unaffected. A concentration of 10 μ M CD decreased the cell viability of neuroblastoma cells with high significance (< 50 %, p-value < 0.001) after a 24 h treatment. Moreover, PARP cleavage was significantly induced in neuroblastoma cells after CD treatment in a dose dependent manner (5 μ M CD, p-value: 0.001). For NHDF cells, no significant induction of PARP cleavage was observed.

Conclusion

Phytochemicals such as CD show good tumoricidal potential. This study promotes cell-type specific anti-tumor activity of CD. Cell viability and apoptosis was modulated in neuroblastoma cells whereas fibroblasts (healthy cells) were unaffected. Thus, CD could be a promising tool for anti-cancer treatment with lowered side effects and enhanced efficacy.

Elucidating the therapeutic potential of THC (Δ^9 -Tetrahydrocannabinol) in endometrial cancer

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Introduction

Endometrial cancer (EC) is the most frequent malignant tumor arising in the uterine corpus. We have previously reported that the expression of CNR1 is increased in EC in comparison to normal tissue, and that this increase is directly associated to tumor stage particularly in advanced stage III and IV EC tumors. CNR1 is a druggable heterotrimeric G-coupled protein-coupled receptor that is specifically activated by endocannabinoids, which are endogenous lipid signaling molecules that regulate multiple cellular functions such as cell proliferation, differentiation, migration, and survival. Taking this into account we decided to explore the biological role of CNR1 by exposing EC cells to Δ^9 -Tetrahydrocannabinol (THC), a CNR1 allosteric agonist prescribed to alleviate neuropathic pain in multiple sclerosis patients, spasticity, and others.

Material and Methods

HEC1A cells were treated with THC in a time and dose-dependent manner. Changes in lipid metabolism were inferred by analyzing gene expression by qRT-PCR, lipidomic analysis, transmission electron microscopy, Nile red staining and flow cytometry. Autophagy levels were assessed by using the mRFP-GFP tandem fluorescent-tagged LC3B construct (tfLC3) and western blot.

Results and Discussions

Treatment with THC reduces EC cell viability. Vacuole formation was observed under the microscope after THC treatment. Lipidomic analysis revealed an increase in triacylglycerol (TAG) in cell treated with THC, which was complemented with changes in the expression of lipid metabolic genes and lipid pools using a neutral lipid staining (BODIPY 493/503), Nile red staining and transmission electron microscopy (TEM). Furthermore, analysis by TEM revealed the presence of autophagic bodies surrounding lipid droplets, suggesting a close relationship between autophagy activation and lipid droplet formation. Finally, the therapeutic

potential of THC was studied using in vivo xenografts, where we observed that THC efficiently hampers tumor growth.

Conclusion

THC treatment compromises cell growth and modulates lipid metabolism and autophagy in vitro. The results observed in vivo warrant additional analysis in the future to further explore the potential benefit of THC for EC patients.

EACR22-0750

Identification of Novel Genes that Suppress Ras-mediated Glycolytic Hyperactivation in Tumor Cells Using Yeast as a Model Organism

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Introduction

Ras activation is the ignition key that stimulates glycolysis in tumor cells. Recently, an evolutionary conserved mechanism linking yeast fermentation to the activation of Ras and Warburg effect was reported. Namely, Ras in the *tps1Δ* yeast strain, with unrestricted hexokinase activity, mimics the activity of oncogenic Ras in cancer cells undergoing Warburg effect. Here, we investigated the link between the metabolic deviation and oncogenic potency using *tps1Δ* as a model system. We identified ALDOB as an endogenous inhibitor that interferes with this oncogenic pathway to hinder cancer progression under hyperglycemic condition.

Material and Methods

A human cDNA library was transformed into the *tps1Δ* yeast strain grown on glucose media, to discover new hits that inhibit active Ras in hyper glycolysis. Several genes that inhibit Ras pathway were identified via Sanger sequencing, but ALDOB/Aldolase B was further studied on a small-scale using survival assays, Annexin/PI for apoptosis assessment, Ras localization experiments and reactive oxygen species (ROS) measurement.

Results and Discussions

It was reported that the occurrence of mitochondrial dysfunction result in loss of respiratory activity, accumulation of ROS, appearance of apoptotic markers and switch from respiration to increased glycolytic activity. These were also features observed in a mammalian cell system expressing K-Ras (G12V) oncogene as well as in *tps1Δ* yeast cells. Based on these previous findings, our results revealed that **Aldolase B/ALDOB** was identified as a novel inhibitor of the Ras hyperactivation mechanism in *tps1Δ* yeast cells, reverting cells to ethanol production and normal completion of fermentation. This was further proven by the suppression of ROS and DNA damage, resistance to hydrogen peroxide and repression of apoptosis. In addition, ALDOB re-localized Ras from the mitochondria to the cytoplasm similar to the wild type cells. Our data hints to a possible role for ALDOB in regulating the Ras activity in mammalian cells. Thus, further investigations are being performed to identify the molecular mechanism of ALDOB in regulating glycolysis in mammalian cells.

Conclusion

In conclusion, ALDOB acts as a potential inhibitor of glycolysis as it corrected the defects of hyperactive Ras caused in *tps1Δ* yeast cells. These findings open avenues for understanding the metabolic cues of tumor cells that are activated or inactivated in response to glucose and pave the road for better treatment approaches of metabolic disease associated with cancer.

The p140Cap scaffold protein modulates the mevalonate pathway impairing breast cancer cells mitochondrial respiration

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Introduction

p130Cas associated protein (p140Cap), a multisite docking protein encoded by the SRCIN1 gene, is involved in the assembly of multi-protein complexes. It is mainly expressed in brain and epithelial-rich tissues such as mammary gland. p140Cap has been shown to explicate a tumor suppressor function in neuroblastoma and breast cancer (BC), where its expression correlates with a better prognosis. Indeed, it negatively impacts tumor features through several mechanisms, among all, by down-regulating Src-dependent integrin signaling or interfering with RAC-GTPases circuitries. However, its involvement in tumor metabolism remains largely unknown. Here we study the role of p140Cap in modulating mevalonate (MVA) pathway in BC cells. The MVA pathway is responsible for the biosynthesis of cholesterol, and non-sterol isoprenoids such as geranylgeranyl-diphosphate (GGPP), farnesyl-diphosphate (FFPP) and ubiquinone (UQ) and is often deregulated in several cancers.

Material and Methods

The *de novo* synthesis of cholesterol, GGPP and UQ was measured by radiolabeling cells with 1 μ Ci [3H] acetate for 24 h. The three different lipid extracts' radioactivity was measured by liquid scintillation. Cholesterol, GGPP and UQ synthesis was expressed as fmoles/10⁶ cells.

Mitochondrial cholesterol was measured both in mouse and human BC cells with p140Cap gain and loss of function, by a fluorimetric Cholesterol/Cholesteryl Ester Assay Kit Quantitation (Abcam), previous mitochondria isolation.

OCR analyses were performed at Seahorse and Oroboros accordingly to manufacturer's protocol.

Results and Discussions

We found that p140Cap expression correlates with an increased flux through the MVA pathway, and in particular an increased synthesis of cholesterol. We also observed that p140Cap overexpression modulates cholesterol distribution through the cell membranes, in particular, leading to cholesterol accumulation into mitochondria membranes. Oxidative phosphorylation (OXPHOS) occurs in mitochondria and leads to efficient generation of ATP. It is emerging as an active pathway in tumors and cancer stem cells. Interestingly, our data show that p140Cap high expression and the subsequent increase in mitochondrial cholesterol can impair mitochondrial respiration leading to a less aggressive cell phenotype.

Conclusion

Considering these promising preliminary data, we will further study and functional characterize this new role for p140Cap scaffold protein in impairing mitochondrial respiration, as it may affect breast cancer cells aggressiveness.

melanoma cells

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Introduction

Malignant melanoma is the deadliest form of skin cancer and treatment options especially against the metastasizing form are still challenging. The “old drug” doxorubicin (DOX) is an anthracycline derivative and an effective chemotherapeutic drug used for a wide range of cancer types. Its antitumor activity is based on interactions with genomic DNA, inhibition of topoisomerase II and generation of ROS. But DOX also shows diverse toxic effects with doxorubicin-induced cardiotoxicity (DIC) being the most problematic side effect. Cardamonin (CD) is a thiol-reactive natural chalcone found in *Alpinia* species and has anti-inflammatory, antineoplastic and antioxidant activity. Here, we studied the effect of a combinational therapy of DOX and CD.

Material and Methods

Human A375 melanoma cells, H9C2 rat cardiac myoblasts and normal human dermal fibroblasts (NHDF) were treated with increasing concentrations of DOX and CD alone or in combination. Cell viability, oxygen consumption and extracellular acidification were measured. Furthermore, Nrf2 nuclear translocation was analyzed via immunofluorescence staining and upregulation of heme oxygenase 1 (HO-1) was quantified via Western blot.

Results and Discussions

DOX alone led to a decrease in cell viability in all tested cell lines. On the contrary, treatment with CD alone caused a decrease in cell viability on tumor, but not healthy cells. Interestingly, a combinational treatment of CD and DOX resulted in a protective effect from DOX toxicity in normal cells while a high toxicity in A375 cells still existed. Both, DOX and CD alone time- and dose-dependently decreased maximal respiration in melanoma cells. The addition of CD resulted in a further decrease of the maximal respiration in melanoma cells compared to DOX alone. However, in cardiac myoblasts, CD protected the cells from DOX mediated downregulation of maximal respiration. CD initiated the translocation of Nrf2 to the nucleus and upregulated HO-1. As CD is thiol-reactive it might be able to react with thiol groups of Keap1 leading to a release of Nrf2. HO-1 is involved in heme degradation and has been shown to have antioxidant properties which might explain the protective effect seen in the combinational treatment in healthy cells.

Conclusion

A combinational approach of DOX and CD shows a protective effect on healthy cells while still showing a high toxicity in melanoma cells. A combination of CD and DOX might be a promising new therapeutical approach and might protect patients from DIC while still killing tumor cells.

EACR22-0784

The Effect of Inflammatory Cytokines on Steroidogenesis in Colorectal Cancer

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Introduction

Colorectal cancer (CRC) is globally the third most frequent cancer, with one of the highest rates of occurrence and mortality. Estrogens influence the incidence of and prognosis for CRC patients. Circulating estrogen sulfate (E₁S) is taken up by CRC via organic anion

transporting polypeptides (OATPs) where it is desulfated to estrone (E_1) by steroid sulfatase (STS). E_1 can be reduced by 17 β -hydroxysteroid dehydrogenases (HSD17Bs) to form biologically active oestradiol (E_2). We have previously shown that STS activity and HSD17Bs expression are altered in CRC to favour E_2 synthesis. Here, we hypothesised that inflammatory cytokines drive this change in E_2 synthesis pathways.

Material and Methods

The expression of estrogenic enzymes were measured via qRT-PCR in HCT116, Colo205, Lovo, and SW620 cells after treatment with inflammatory cytokines (TNF α , IL-6, IFN- γ) at various concentrations (2.5, 5, 7.5, 10, 20, and 40 ng/ml), and different incubation times (4-24-48 hours). Changes to STS activity in cells was measured through ^3H - E_1 S conversion to ^3H - E_2 . We also analysed The Cancer Genome Atlas (TCGA) human colon adenocarcinoma (COAD) RNA-Seq dataset (n=440) to generate Kaplan-Meier survival curves and correlation graphs comparing normal and cancerous colon tissue expression of TNF α , the HSD17Bs, OATPs, and STS.

Results and Discussions

Of all the inflammatory cytokines tested, only TNF α impacted on CRC estrogen metabolism. In HCT116 and Colo205 cells, TNF α (10, 20 and 40 ng/ml) treatment for 4- and 24-hours significantly increased STS activity, led to a decrease in HSD17B2 mRNA, and an increase in HSD17B7 and HSD17B12 expression. This suggests TNF α alters estrogen metabolism in CRC to favour E_2 synthesis. TCGA data analysis showed that patients with low HSD17B1 expression have a better survival outcome compared to patients with high HSD17B1. Also, CRC patients with high expression of HSD17B4, which favours E_2 to E_1 synthesis, have a better survival outcome compared to low expression. A significant correlation was seen between TNF α in CRC tissue and HSD17B4 ($p < 0.01$), HSD17B12 ($p < 0.05$), OATP1A2, OATP2B1, OATP3A1 and OATP1C1 ($p < 0.001$) expression, suggesting TNF α regulates these estrogenic pathways.

Conclusion

Increased E_2 in CRC is a coordinated action of estrogenic enzymes in response to TNF α . TNF α significantly increases STS activity in CRC cells and may increase the pathways for estrogen uptake through increased OATP expression. This suggests that TNF α is an important regulator of estrogen metabolism in CRC.

EACR22-1018

Cluster of Differentiation 109 influences cell growth in lung cancer cell line and identifies desmoglein-2 as potential interaction partner

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Introduction

Cell proliferation is of utmost importance for tumor initiation and development. Factors promoting these cell properties, might be considered therapeutic targets opening new

treatment options. Among other, cluster of differentiation 109 (CD109) is known to modulate TGF- β as well as JAK/STAT3 signaling, both of which are associated with tumor progression. Furthermore, CD109 is upregulated in many cancers such as lung cancer, glioblastoma and squamous cell carcinoma and its expression often associates with higher mortality rates compared to low-grade CD109.

Material and Methods

In our approach for a deeper understanding of the pathophysiological role of CD109 in lung cancer, we used a CRISPR-Cas9 model to knock out CD109 in H460 cells. These stem from a large cell cancer of the lung and we characterized them using migration and tumor spheroid assays. Moreover, we investigated the fluorophore-labeled extracellular vesicle (EV) distribution in mice with EVs loaded with or without CD109. Biochemical and mass spectrometry-based proteomics analyses helped to identify new possible interaction partners of CD109 at the cell surface.

Results and Discussions

We observed differences in cell growth and morphology when comparing CD109 deficient H460 cells with its parenteral cell line. Mass spectrometry analysis of immunoprecipitated CD109 from cell lysate identified desmoglein-2 (DSG-2) as a possible new cell surface interaction partner, which we confirmed in biochemical and microscopic experiments. Fluorescence imaging of CD109 expressing exosomes in mice showed a preferred distribution to DSG-2 expressing organs like heart, lung and liver. As a cell surface protein, important for cell-cell contact interaction CD109 could (i) stabilize DSG-2 at the cell surface and could (ii) target CD109 expressing exosomes/cells to DSG-2 expressing tissue. This is supported by metastases of CD109 expressing tumor cells to liver and lung, both with a high DSG-2 expression.

Conclusion

We show that CD109 influences cell growth, interacts with the important desmosomal protein DSG-2 endogenously in H460 cells and changes EV distribution after application into mice. These results broaden our understanding on the pathophysiological role of CD109 and indicate CD109 as a potential therapeutic target.

EACR22-1038

Metabolic changes of cancer cells under therapy: time-resolved fluorescent microscopy and pH-sensitive genetically encoded sensor for their monitoring

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Introduction

To improve the efficacy of chemotherapy, it is promising to search for methods enabling differentiation between responding and nonresponding tumors in the early stages of

treatment. Fluorescence imaging of the metabolic cofactors: reduced nicotinamide adenine dinucleotide (phosphate) NAD(P)H and oxidized Flavin adenine dinucleotide FAD, represents a powerful noninvasive instrument to monitor, in real time, the metabolic activities in living cells and tissues. The other metabolic parameter intracellular pH (pHi) is known to be involved into the “physiological” behavior of cancer cells, including response to the anticancer therapy. In this case genetically encoded fluorescent sensors are perspective instruments for obtaining information about pHi changes during treatment. The aim of this study was to investigate metabolic and pHi modifications under chemotherapeutic exposure.

Material and Methods

HeLa Kyoto cells and Hela Kyoto stably transduced with SypHer2 gene, (HeLa-SypHer2) investigation were used as cells monolayer or 3-D cultures.

Tumor xenografts were generated in nude mice by subcutaneous injection of Hela K or HeLa-SypHer2 cells.

Fluorescent imaging (NAD(P)H, FAD and pH-sensor SypHer2 fluorescence intensity) was performed using the fluorescence confocal laser-scanning microscope LSM 880 (Carl Zeiss, Germany) equipped with an FLIM module Simple Tau 152 TCSPC (Becker & Hickl GmbH, Germany).

The chemotherapeutic agents Cisplatin and Paclitaxel were used for investigation.

Results and Discussions

The metabolic shift to the oxidative phosphorylation from the glycolysis was demonstrated for both drugs in different tumor models. Analysis of the fluorescence of NAD(P)H and FAD in subpopulations with different responses revealed that the metabolic changes developed faster in the more responsive cells. Notably these changes pre-existed morphological changes. Also for both drugs the tendency to cytosolic acidification has been shown. The analysis of individual cells has revealed the differences in pHi dynamics between dying and surviving cells for both drugs.

Our present study, therefore, provides more insight into cancer cell response to cisplatin and demonstrates an involvement of pHi and energy metabolism in it. These findings contribute to an understanding of the mechanisms of chemotherapeutic drugs action and of the complex origin of drug resistance.

Conclusion

Our research demonstrate high sensitivity of FLIM-based metabolic imaging as an instrument for assesment of cancer cell response to therapy.

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EACR22-1206

The Role of The Extracellular Matrix Deposited Secreted Frizzled Protein 1 in Melanoma

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Introduction

Secreted Frizzled Protein 1 (SFRP1), is a protein deposited in the extracellular matrix (ECM) that belongs to the family of Secreted Frizzled Proteins (SFRPs). These ECM proteins are thought to act as Wnt antagonists, by either binding Wnt or Frizzled receptors. SFRPs are downregulated in some cancers, including breast and colon cancer, and were thought to have anti-tumorigenic effects. This because aberrant Wnt signaling was identified as a major actor in the initiation and development of certain cancers. However, our preliminary studies revealed that SFRP1 is overexpressed and correlates with poor patient outcomes in melanoma. Unlike most cancers that are often highly glycolytic, melanomas switch to oxidative phosphorylation (OXPHOS) during dissemination and in response to therapeutic stress. We hypothesize that SFRP1 driven ECM signaling is involved in melanoma metabolic reprogramming.

Material and Methods

Steady state RNA was isolated from xenograft tumors of C8161/A2058 melanoma cells that are proficient, deficient, or overexpressing SFRP1 and analyzed by RNAseq. These melanoma cells were cultured and analyzed by Seahorse & bioenergetic capacity was assessed. Steady-state Metabolome level was measured by Mass Spectrometry.

Results and Discussions

SFRP1 loss in C8161 cells decreased cell proliferation *in vitro*. Additionally, there was a reduction in subcutaneous tumor growth *in vivo*, which was partially rescued by SFRP1 re-expression. Conversely, subcutaneous injection of A2058 cells that overexpress SFRP1 in mice ensued an increase in orthotopic tumor growth. Hence, SFRP1 may exhibit tumor-promoting properties in melanoma. Also, our results show that reduced SFRP1 levels are paralleled by profound perturbations on cellular metabolism and bioenergetics. RNAseq revealed enrichment in genes involved in metabolic regulation and mRNA translation upon SFRP1 loss. SFRP1-deficient cells have an increased dependence on glycolysis compared to SFRP1-proficient cells. SFRP1 loss increased the activity of the mTORC2-AKT pathway, which acts as a major stimulating signaling pathway of glycolysis.

Conclusion

Our results support the idea that SFRP1 driven ECM signaling modulates metabolic reprogramming in melanoma to fuel cancer progression. By understanding the role of SFRP1 in melanoma, we hope to identify predictive response markers to SFRP1 inhibition therapy in patients. Moreover, the establishment of SFRP1's role in melanoma will shed better light on the molecular foundations of ECM signaling in cancer.

EACR22-1210

MiR423-5p/Malat-1 loop: a novel therapeutic approach to fight hepatocellular carcinoma.

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Introduction

DNA-RNA-Protein-Function is the founding of biology, but today we suppose that there is something more. Some RNAs, such as micro-RNAs (miRNAs) and long non-coding RNAs (lncRNAs), do not code for proteins but still exert powerful effects on cell biology and function of both normal and cancer cells. We recently found that miR423-5p is overexpressed in serum of hepatocellular cancer (HCC) patients responding to sorafenib, inducing autophagy in HCC cellular models. This could be due to the interaction with oncogenic lncRNA Malat-1, in silico miR423-5p predicted target.

Material and Methods

To explore the miRNA/lncRNA interaction we generated stable overexpressing clones of three different HCC Cell lines (HepG2, Hep3B and SNU387), using lentiviral transduction system. Functional assays were carried out to evaluate proliferation, migration, invasion capability and clonogenicity potential with different techniques. MiR423-5p and Malat-1 interaction was confirmed by RIP assay, then Mass Spectrometry (MS) proteomics and NGS expression analysis (lncRNA Seq, RNA Seq) contributed to further clarify the mechanisms. The most important genes together with miR423-5p and Malat-1 levels were also validated through RT-qPCR.

Results and Discussions

MiR423-5p overexpressing cell lines had reduced levels of Malat-1 and were struggling to proliferate, migrate and invade compared to parental cells, while Malat-1 overexpressing clones had opposite effects. MS experiments and other observations suggested a reduced level of mitochondrial proteins in cells transfected with miR423-5p pushing us to investigate the mitochondrial activity. Confocal microscopy evidenced miR423-5p-transfected cell lines to have a reduced number and smaller mitochondria compared to parental cells, while opposite effects were recorded in Malat-1 overexpressing cells. Interestingly, we found most of the mitochondrial related genes, directly correlated to cell energetic and metabolic activity, to be downregulated in miR423-5p-overexpressing clones and upregulated or unmodified in Malat-1 overexpressing clones. Finally, we performed transcriptome sequencing of lncRNAs and mRNAs, with interesting results.

Conclusion

The above findings demonstrate how strong is the impact of these two non-coding RNAs on cell phenotype and metabolism, offering an interesting starting point to be further investigated for novel precision medicine approaches possibly exploiting miR423-5p as a novel oncosuppressor.

EACR22-0073

Investigating melanoma cell stiffness using a novel scanning ion conductance microscopy system

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Introduction

The ability to differentiate cancer from non-cancer cells is an important step on the road towards improved cancer diagnosis, prognosis and treatment. Cancer progression and metastasis is correlated with changes in the cytoskeletal network and therefore alterations in

cell stiffness. It has previously been shown that softer cancer cells show higher metastatic and migratory abilities, suggesting that cell stiffness could be a biomarker for metastatic potential. However, other publications have shown opposing results, as is the case for melanoma. Following changes in cell stiffness can also provide information about drug treatment and its effectiveness. If cell stiffness plays such an important role in cancer spread, it may be an overlooked important effect of some anti-cancer drugs.

Here, we aim to be the first to assess the correlation between cell stiffness and metastatic potential of melanoma cell lines using novel scanning ion conductance microscopy (SICM). We also aim to evaluate changes in cell stiffness following the treatment of anti-cancer drugs.

Material and Methods

A detailed examination of the stiffness of single cells from three melanoma cell lines with increasing metastatic potential was carried out using SICM. SICM offers an advantage over other cell stiffness methods as single cells can be continuously scanned over several hours with no damage or interference with the cell surface. We used this continuous scanning method to assess cell stiffness changes in melanoma cells following treatment with anti-cancer drugs such as Taxol and Cisplatin.

Results and Discussions

We saw no statistically significant difference in cell stiffness between the melanoma cell lines but saw an increase in the mean stiffness in the highly metastatic cell line. This goes against the consensus that metastatic cells are softer to allow increased motility. This may be due to metastatic cells having increased plasticity. Treatment of melanoma cells with Taxol and Cisplatin saw an overall increase in cell stiffness following treatment. However, the work showed variability in the change of cell stiffness between cells, highlighting heterogeneity in the effect of anti-cancer drug treatment.

Conclusion

Our data highlights the variability in cell stiffness data available in the literature and that our work opposes the idea that softer cells can migrate and metastasise more easily. Our work also shows the importance of using cell stiffness as a tool in anti-cancer drug treatment and in assessing drug effectiveness.

EACR22-0075

A lysosomal lipid switch sensitizes to nutrient deprivation and mTOR targeting in pancreatic cancer

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive disease with limited therapeutic options. The unique metabolic features of PDAC unveil metabolic liabilities that can be therapeutically explored. The key player of metabolic adaptation to nutrient availability is the mTOR (mechanistic target of rapamycin) pathway. Targeting mTOR showed clinical benefit

in a minority of patients. Identification of critical regulators of mTOR signaling might be helpful to select patients that can benefit from mTOR inhibition.

Material and Methods

We measured the levels of PI3K-C2g protein in human resected PDAC samples and associated it with patient outcomes. We performed studies with *KRAS*^{G12D/+};*p53*^{R172H/+};*Pdx1-Cre* (KPC) mice and KPC *Pik3c2g*-null mice. We used CRISPR/Cas9 system to disrupt *PIK3C2G* gene in human PDAC cells. Functional studies were performed to determine the significance of loss of PI3K-C2g and the sensitivity of deficient cells to mTOR and glutaminase inhibition.

Results and Discussions

PI3K-C2g expression is reduced in about 30% of PDAC cases, associating with aggressive phenotype and increased phosphorylation of S6K. In KPC mice, loss of PI3K-C2g increases tumor development and progression, strongly reducing mice overall survival time. The ability of tumors with PI3K-C2g loss to grow faster correlates with hyperactivation of mTORC1 pathway dependent on Arf1/Asap1 axis and glutamine metabolism rewiring to support lipid synthesis. PI3K-C2g-KO tumors failed to adapt to metabolic stress induced by glutamine depletion, resulting in cell death.

Conclusion

Loss of PI3K-C2g activates mTOR signaling through Arf1/Asap1 axis and renders tumors vulnerable to RAD001 (mTOR inhibitor) and BPTES/CB-839 (glutaminase inhibitors). Therefore, our study identifies PI3K-C2g as a new mTOR inhibitor and indicates a novel therapeutic strategy of targeting the mTOR pathway and glutamine metabolism in PI3K-C2g deficient-tumors.

EACR22-0077

Hypoxia regulates acetylation/deacetylation of non-histone proteins through PLIN2 and SCD1 expression in cancer cells.

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Introduction

Tumor hypoxia induces a profound metabolic change in cancer cells and sustains cancer malignancy. Recently, we showed that tumor hypoxia increases fatty acid storage in PLIN2-positive lipid droplets (LD) and tumor reoxygenation increases lipid desaturation in cancer cells (Luis et al., 2021). Here we found that Stearoyl-CoA Desaturase-1 (SCD1) and PLIN2 are upregulated in cancer cells incubated under hypoxia.

Material and Methods

To explore whether targeting lipid storage in hypoxia can be exploited as a vulnerability in cancer cells, we deleted SCD1 and PLIN2 in MDA-MB231 and HT29 cells and explored their phenotypes under normoxic and hypoxic conditions in vitro.

Results and Discussions

PLIN2 depletion induces a drastic decrease in the number and the size of LD in cancer cells, reduced cancer cell migration and proliferation in vitro and reduced in vivo tumor growth. Depletion of SCD1 in cancer cells impacted the expression of PLIN2 and consequently LD

formation under hypoxia. The expression of FASN and ACC1 were decreased in PLIN2 depleted cells under hypoxia, whereas the expression of CPT1 was increased, suggesting a potential shift to fatty acid oxidation when LD are disrupted. To search for molecular mechanisms that might be regulated in cancer cells, because of Acetyl-CoA pool change in PLIN2 and SCD1 depleted cells, we performed a post-translational acetylome analysis and identified several non-histone nucleolar proteins, including SMC3, MEAF6, HNRNPK and TPR that were deacetylated by SCD1 and acetylated by PLIN2 at the same sites.

Nucleophosmin (NPM1), the key tumor suppressor protein, was the most strongly deacetylated protein in SCD1 expressing cells. CoIP analyses confirmed the role of SCD1 acts as a potential deacetylase-like protein of NPM1. Interestingly, deacetylation of NPM1 decreases its stabilization and potentially affects its tumor suppressor function. SCD1 expression was associated with increased cell migration and an increased deacetylation of vimentin and filamin-A, key intermediate filament proteins involved in actin binding and cell migration.

Conclusion

Overall, our data indicate that PLIN2 is involved in increased acetylation of nucleolar proteins as a cellular response to hypoxic stress, whereas SCD1 expression led to the deacetylation of tumor suppressor NPM1 and proteins involved in cell migration. Pro-tumorigenic function of SCD1 and stress response function of PLIN2 may be exploited as metabolic vulnerabilities to bypass cancer adaptation to hypoxic and metabolic stress.

EACR22-0217

p140Cap modulates cholesterol homeostasis and reduces Rac1 activity in lipid rafts of breast cancer cells

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Introduction

p130Cas-associated-protein (p140Cap), a multisite docking protein expressed in brain and epithelia, negatively controls cell migration, invasion and survival, down-regulating signalling pathways that control cytoskeleton remodelling, such as Src-dependent integrin signalling. p140Cap acts as an oncosuppressor in neuroblastoma and HER2-amplified breast cancer (BC), where a p140Cap-positive status is associated with a better prognosis. Aberrant cholesterol homeostasis is a metabolic hallmark in different tumours and affects membrane fluidity and cell migration. The mevalonate (MVA) pathway is the biosynthetic route for generating cholesterol, a crucial component of lipid rafts. Up to date, there is no information on the role of p140Cap in cancer cells metabolism.

Material and Methods

Gene Ontology (GO) analysis was performed on upregulated genes from p140Cap-overexpressing (p140oe) BC cells. To investigate the MVA pathway flux, we treated cells with radiolabeled acetate and measured the MVA end-products radioactivity. We evaluated HMGR mRNA and protein levels by qRT-PCR and immunoprecipitation. The activity of the transcription factor SREBP2 was analysed by luciferase assay. Cell migration was assessed via transwell migration assay. We analysed cholesterol levels using a Cholesterol Fluorimetric Assay kit and membrane fluidity through a pyrene-based kit. We enriched lipid rafts by sucrose gradient centrifugation and analysed signalling events by western blotting.

Results and Discussions

GO terms related to cholesterol metabolism are over-represented in a set of upregulated genes in p140oe MDAMB231 cells, indicating a putative role of p140Cap in BC cholesterol homeostasis. p140Cap promotes an increased synthesis of the MVA end-products and upregulates the rate-limiting enzyme of the pathway, HMGCR, both at transcriptional and post-translational levels. Transient expression of p140Cap in HEK293 cells enhances the activity of SREBP2, the master regulator of the MVA pathway. p140Cap determines an increased membrane cholesterol content, potentially contributing to reduced membrane fluidity and impaired BC cell migration ability. We found that p140Cap is present in lipid raft microdomains of MDAMB231 cells, where it negatively controls the activity of Rac1.

Conclusion

We hypothesise that p140Cap is involved in cholesterol homeostasis and curbs BC aggressiveness by inhibiting cell migration through reduced plasma membrane fluidity and hindering tumorigenic lipid raft-associated signalling events.

EACR22-0239

Metabolic stress effect on chemo-immunotherapy response in Non-Small-Cell Lung Cancer Model.

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Introduction

Lung cancer is one of the leading cause of cancer death worldwide, with an estimate incidence of about 2 million new cases per year. Among all lung cancers, non-small-cell lung cancer (NSCLC) is the most frequent (nearly 85%).

NSCLCs are frequently mutated in *STK11/LKB1* and co-mutation with *KRAS* is often reported. Considering the key role of *STK11/LKB1* in controlling cell metabolism, we hypothesized that NSCLC harboring mutations in this gene could be vulnerable to metabolic stresses. Different studies have highlighted how is possible to interfere with cancer metabolism using metformin and caloric restriction (CR). In this scenario, our work aimed at investigating the role of metabolic stress in determining the response to chemotherapy and immunotherapy in *LKB1* mutated NSCLC model.

Material and Methods

The cell lines with the genetic backgrounds of interest were generated from lung nodules of *KRAS*^{G12D}/*LKB1*^{wt} and *KRAS*^{G12D}/*LKB1*^{mut} transgenic mice. Once stabilized *in vitro*, the cell lines were inoculated intramuscularly in immunocompetent mice and treated with chemotherapy (cisplatin) or immunotherapy (anti-PD-1) alone or in combination with metformin and CR. The latter consisted of 36 hours of fasting every week for three weeks. Metformin was administered daily for the entire experiment, while cisplatin was given once a week and anti PD-1 twice a week, both for three weeks.

Results and Discussions

In our *in vivo* experiments, we compared the response of tumors characterized by the mutation in *KRAS*^{G12D} and the co-mutation *KRAS*^{G12D}/*LKB1*^{mut}. The results indicate that the addition of metformin and CR improves the activity of cisplatin in both tumors but a stronger effect was detected in the tumor presenting the deletion of *LKB1*, with a slowdown in tumor growth even beyond the end of the treatment. As expected immunotherapy alone is more effective in *KRAS*^{G12D} than in *KRAS*^{G12D}/*LKB1*^{mut} tumors even if the combination with metformin and CR enhances the effect in both tumors.

Conclusion

Our *in vivo* preliminary studies confirm our hypothesis that the addition of CR and metformin is able to improve the antitumor activity of cisplatin without increasing the treatment toxicity in tumors characterized by the co-mutations of *KRAS*^{G12D}/*LKB1*^{mut}. For immunotherapy, a slight improvement was observed in both models. Further investigations are ongoing to dissect the role of metabolic stress in addition to the combination of chemo and immunotherapy.

EACR22-0323

Uncovering cell interactions and metabolic vulnerabilities in the tumor microenvironment of breast cancer with Dual Ribosome Profiling (DualRP)

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Introduction

During cancer progression the tumor microenvironment (TME) acts as a pro-tumorigenic niche around malignant cells, where reprogramming of the metabolism occurs in stromal, cancer and immune cell types. In recent years targeting the metabolism of the TME has emerged as a promising strategy for cancer therapy. Nonetheless, current methods to study the molecular mechanisms behind metabolic remodeling in the TME expose the cells to severe stress and thereby introduce artefacts to the original gene expression patterns.

Material and Methods

In our research we apply Dual Ribosome Profiling (Dual-RP) to elucidate the cell interactions in the naïve TME of triple negative breast cancer (TNBC). In Dual-RP the ribosomes of two interacting cell types, are tagged with two distinct fluorophores, allowing cell type-specific isolation of ribosomes from a mixed population. Notably, Dual-RP not only provides gene expression data from two interacting cell populations, but also unravels potential metabolic vulnerabilities in combination with differential ribosome codon reading (Diricore) where ribosome stalling in specific codons is correlated to potential amino acids restrictions for cancer.

Results and Discussions

With the use of Dual-RP tailored models, we identified multiple signaling cascades affected in the TME of TNBC, and also under conditions of metabolic stress. In particular, the activation of ER-stress responses upon glucose deprivation is attenuated in both cancer cells and fibroblasts only when they are co-cultured. This rescue might be attributed to compensatory mechanisms involving the IFN-type-1 pathway which is substantially exacerbated in co-cultured cells. Furthermore, our findings *in vivo* also reflect the role of fibroblasts in supporting tumor growth and indicate limitation in alanine for breast cancer cells. In addition, we employed transgenic Ribo-Tag mice to characterize the immune response under check-point inhibition. Here the specific metabolic signatures of T cells and cancer cells were obtained in the context of their TME. Ultimately, we found vulnerabilities in

T cells for serine and aspartic acid, that were exploited to improve T cell-mediated cancer killing *ex vivo*.

Conclusion

Altogether we introduce a novel approach to study TME interactions *in vitro* and *in vivo*, and hope to shed light on the mechanisms driving metabolic reprogramming in TNBC and other types of cancer. Moreover, we can unravel the existence of amino acid limitations that hold the potential to benefit cancer therapeutic schemes.

EACR22-0407

Simultaneous blocking of glycolysis and oxidative phosphorylation causes cell death in Multiple Myeloma.

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Introduction

Multiple myeloma (MM) remains an incurable cancer despite advances in therapy. Therefore, the search for new targets is still essential to uncover potential treatment strategies. Metabolic changes, initiated by the hypoxic bone marrow environment, lead to both cancer cell survival and drug resistance. In this study, we aimed to evaluate the synergistic activity of monocarboxylate transporter (MCT) inhibition with metformin on MM viability and survival.

Material and Methods

Correlation between expression and survival was investigated using the Mulligan cohort. *In vitro*, multiple MM cell lines (OPM-2, LP-1, RPMI-8226, JJN3, ANBL-6) were used, as well as the stromal cell line HS-5. A tracer study, whereby RPMI-8226 cells were supplemented with ¹³C-glucose, was performed to measure glucose-to-lactate conversion in normoxia and hypoxia (1% O₂). MCT expression was measured on protein level by western blot. MCT1/4 was inhibited by syrosingopine, while metformin was used to inhibit complex I of the electron transport chain. Effects on viability were measured by CellTiter Glo, lactate concentration by Lactate Glo, and apoptosis by annexin V/7-AAD staining. Effects on glycolysis and oxidative phosphorylation were measured by the Seahorse Glycolytic Rate assay.

Results and Discussions

High MCT1 or MCT4 expression correlates with a significant lower overall survival in MM. Glucose-to-lactate conversion was increased in RPMI-8226 cells in hypoxia, confirming the Warburg effect in MM. JJN3 cells contain the highest expression of MCT1, while RPMI-8226 show the highest expression of MCT4 alongside the HS-5 cells. Both MCT1 and MCT4 expression are increased after 48h of hypoxia.

MCT1/MCT4 targeting by syrosingopine reduced viability in LP-1 and RPMI-8226, while apoptosis and intracellular lactate levels were increased. Metformin increased syrosingopine-mediated cell death in MM cell lines. Seahorse analysis showed that metformin decreased oxidative phosphorylation-mediated oxygen consumption rate and increased glycolysis-mediated extracellular acidification rate, while syrosingopine caused the opposite effect. Combination therapy reduced both oxygen consumption rate and extracellular acidification rate, blocking both crucial metabolic processes.

Conclusion

Combined targeting of oxidative phosphorylation by metformin and glycolysis by syrosingopine induces apoptosis in MM. This combination strategy could form a new approach to block MM survival and progression.

EACR22-0531

KRAS mutant lung cancer cells specific cell death, methuosis, associated with HBP pathway

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Introduction

Lung cancer is the leading cause of cancer related death in the worldwide, and has a poor prognosis. Oncogene KRAS mutation, which is the most frequent in non-small cell lung cancer (NSCLC), serves aggressiveness risk in tumor, and leads the metabolic reprogramming of cancer cells by promoting glucose, glutamine, and fatty acid absorption and glycolysis. In this study, KRAS mutant specific anticancer effect of MDM2 antagonist (MDM2-A) was investigated in an *in vitro* cell lines, and verified in an *in vivo* animal model using the NSCLC cells.

Material and Methods

KRAS mutant, and wildtype NSCLC cells were used *in vitro* cell analyses. Cell viability, proliferation, and death were measured by MTT, cell counting, colony analyses, and annexin V staining for FACS. Cell tracker dyes were used to investigate cell morphology, and tomography, and confocal microscope were used to examine cell images. RNA sequencing was performed to identify key target molecule or pathway, which was confirmed by qRT-PCR, western blotting, and metabolite analyses by UHPLC-MS/MS. Zebrafish and mouse xenograft model were used for *in vivo* analysis.

Results and Discussions

MDM2-A induced non-apoptotic cell death, methuosis, in KRAS mutant/p53 wildtype NSCLC cells, A549 and H460 specifically, but not in KRAS wildtype NSCLC cells HBP pathway, which is associated with glucose and glutamine uptake of KRAS mutant lung cancer cell metabolism, identified playing important role in the MDM-2A induced methuosis. Metabolite analysis confirmed HBP pathway was dysregulated by MDM-2A. [a3] GlcNAc recovered MDM2-A induced methuosis in KRAS mutant/p53 NSCLC cells, A549, and H460, which were verified by *in vivo* zebra fish, and mouse xenograft model. These effects were associated with the downregulation of KRAS, cMYC signaling pathway, and abnormal glucose metabolism.

Conclusion

Our results indicated the potential of MDM2-A in the treatment of KRAS mutant lung cancer cell, and provided methuosis associated mechanism.

EACR22-0575

The mTOR pathway regulates glucose-6-phosphate dehydrogenase expression and redox balance in T-cell acute lymphoblastic leukemia cells

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Introduction

Approximately 20% of patients with pediatric T-cell acute lymphoblastic leukemia (T-ALL) are incurable at present. T-ALL cells frequently present constitutive activation of the mechanistic target of rapamycin (mTOR) pathway, a characteristic that is associated with resistance to glucocorticoid (GC)-based chemotherapy. Building on our previous finding of altered redox balance in T-ALL cells, in the present study we investigated the links between the mTOR pathway and altered redox that could be exploited to kill T-ALL cells.

Material and Methods

GC-resistant T-ALL cell lines were treated with the mTORC1 inhibitor everolimus. Effects on gene expression were analyzed by RNAseq, quantitative RT-PCR and immunoblotting. Effects on reactive oxygen species (ROS) levels and cell death were assayed using fluorescent probes and flow cytometry. Results were verified in short-term cultures of freshly isolated T-ALL cells, patient-derived T-ALL xenografts (PDX) and *in vivo* in NOD/SCID mice inoculated with dexamethasone-resistant PDX.

Results and Discussions

In vitro studies showed that everolimus increased ROS levels, augmented lipid peroxidation, and activated the ROS-controlled transcription factor NRF2. These effects were accompanied by a decrease in the levels of NADPH and of glucose-6-phosphate dehydrogenase (G6PD), the rate-limiting enzyme of the pentose phosphate pathway (PPP), which produces NADPH needed by cellular ROS-scavenging enzymes. Everolimus induced mitochondrial inner membrane depolarization, release of cytochrome c into the cytoplasm, and dose-dependent apoptosis of T-ALL cells, but did not kill normal T-cells. siRNA-mediated knockdown of G6PD expression also produced an increase in ROS and cell death. On the other hand, forced expression of G6PD or pretreatment with the ROS scavenger N-acetyl cysteine interfered with everolimus-mediated induction of ROS and cell death. Importantly, the combination of everolimus and the GC dexamethasone had a synergistic effect on killing T-ALL cells. Studies of PDX-inoculated mice confirmed that everolimus could overcome dexamethasone resistance in conditions of high tumor burden that mimicked the clinical setting of acute leukemia.

Conclusion

These findings identify a link between mTOR pathway activation, G6PD expression, and ROS homeostasis in T-ALL cells and furnish mechanistic evidence to support the combination of glucocorticoids with mTOR inhibitors to treat GC-resistant T-ALL.

EACR22-0611

Uridine34 tRNA modifications and its involvement in prostate cancer (PCa)

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Introduction

Cancer cells typically show an increase in protein synthesis with specific subsets of pro-tumourigenic mRNAs being more efficiently translated. This increase in translation is often associated with more efficient translation initiation and elongation. tRNAs are RNA molecules that play a central role in protein synthesis by decoding mRNAs via direct base pairing between mRNA codons and tRNA anticodons. Modifications in the anticodon bases can affect the efficiency of decoding. We have recently reported that some key enzymes responsible for modifying uridine 34 (U34) in the anticodon of specific tRNAs to generate mcm5s2-U34 are overexpressed in some prostate and breast cancer cells. Synthesis of mcm5s2U34 requires three steps catalyzed by the elongator complex (ELP1-6), ALKBH8 and CTU1/2 in a sequential manner. Depletion of ELP3, the catalytic subunit of the elongator complex has been reported to diminish translational rates of mRNAs enriched in A-ending codons in different cancer types, including melanoma, intestine tumour and breast cancer.

Material and Methods

ELP3 knock-out prostate cancer cell lines (DU145, LNCaP) were generated using CRISPR-Cas9 approach. In these cell lines we evaluated the biological effects of ELP3 depletion through proliferation assay, colony formation assay and viability assay in 3D cell culture. Depletion of ELP3 activity was validated by nucleoside mass spectrometry. Translatome-wide effects of ELP3 depletion were assessed through polysome profiling followed by RNA-seq and protein mass-spectrometry. At single transcript level, the repercussion of ELP3 depletion was evaluated through fluorescence-based reporter system analyzed through FACS technology.

Results and Discussions

Here we show that CRISPR-KO of ELP3 impairs the proliferative potential of human PCa *in vitro* models, but it has no effect on untransformed prostate epithelial cell line (PNT1a). Importantly, ELP3 KO strongly impairs the clonogenic potential of both mouse-derived and human PCa cell lines. In human PCa cell lines, ELP3 depletion also causes a significant translational reshaping.

Conclusion

Our current efforts are focused on identifying and validating the transcripts and proteins most affected by ELP3 depletion in prostate cancer models. Our overall goal is to explore if inhibition of U34 modification pathway is a therapeutic vulnerability in various neoplasia, including prostate cancer.

EACR22-0681

Mesenchymal stromal cells achieve distinct effects on the growth of multiple myeloma cell and their resistant counterparts: significance of metabolic changes

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Introduction

Among the elderly, multiple myeloma (MM) remains an incurable, drug-resistant, hematological malignancy originating from the bone marrow (BM). The many constituents of the senescent BM niche contribute to myeloma genesis, neoplastic progression, survival and chemoresistance. Here, we investigate the role of BM mesenchymal stromal cells (MSCs) and therapeutics on MM cell proliferation, metabolism and drug response.

Material and Methods

MSCs were isolated from BM of patients undergoing hip arthroplasty (Ethical approval 187/18, 65 ± 9 years). Wild type (WT) MM cells (MM1.S) and their resistant counterparts (MM1.SR180Ixa) were analyzed after 72 h of co-culture with MSCs and/or treatment with bortezomib (BTZ). Gene expression analysis was examined by PCR. Proliferation, cell cycle progression and metabolic status of co-cultured cells were analyzed using flow cytometry, cellular assays and histochemical staining. Colony growth of MM cells was explored by a methylcellulose-based assay as an indicator of self-renewal potential.

Results and Discussions

We observed a decreasing effect of BTZ on Ki-67 expression in MM cells only on WT MM1.S cells, that seemed to be reverted in the presence of MSCs. MM1.SR180Ixa show a higher clonogenic potential than MM1.S, whose clonogenicity increased with the application of BTZ. The presence of MSCs stimulated an increased gene expression of ABC transporters in MM1.S cells treated with BTZ, whereas the effect was not observed when only BTZ was applied. We found that MSCs increased ATP production, preserved mitochondrial functionality and mass, while significantly decreased total cellular reactive oxygen species production in WT MM1.S cell treated with BTZ. BTZ did not affect the metabolic features of MM1.SR180Ixa cells. Since our preliminary results indicated that BTZ and MM can regulate senescent phenotype of MSCs in a different way, we assumed that molecular drivers of senescence actively participated in described effects of MSCs.

Conclusion

Our results indicate distinct growth, metabolism and BTZ response of the resistant MM compared to the WT MM cells, with additional intricacy brought by MSCs. To elucidate mechanistic background of MM persistence instructed by the senescent BM environment, our further research will clarify potential of senescence modulators to guide MSC-assisted self-renewal, metabolic re-configuration and drug resistance in MM cells.

EACR22-0723

The role of GAPDH in the selective toxicity of CNP in melanoma cells

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Introduction

Malignant melanoma is the most aggressive form of skin cancer, and once metastasized the prognosis is poor. A standard chemotherapy often results in severe side effects on normal healthy cells finally being difficult to tolerate for patients. Shown by us, cerium oxide nanoparticles (CNP) selectively kill A375 melanoma cells while not being cytotoxic with identical concentrations on non-cancerous cells. The redox-active CNP exhibit both

prooxidative as well as antioxidative properties, with the latter even being protective in combination with already established chemotherapeutics. CNP induce mitochondrial dysfunction in the studied melanoma cells via generation of H₂O₂, but that does not account for 100% of the toxicity. As cancer cells also rely on increased glycolysis as energy source (Warburg effect), we focused on CNP mediated thiol oxidation and identified GAPDH as a target protein.

Material and Methods

A375 melanoma cells were incubated with 300 µM of CNP for 4 and 24 hours and subsequently prepared for redox proteomics. The resulted data were verified using a 15 kDa DNA-maleimide conjugate (SulfoBiotics- Kit) which binds to free thiol groups of proteins and resulting in an electrophoretic shift. That shift can be monitored by immunoblotting: a higher shift represents less thiol oxidation of the desired protein and vice versa. For analysis of cellular lactate formation and GAPDH activity, commercially available kits were employed. MTT and SRB Assay were used for evaluating viability, and heptelidic acid (HA) was used as GAPDH inhibitor.

Results and Discussions

CNP significantly induced thiol oxidation of GAPDH. It has been shown before, that GAPDH activity is regulated via oxidation of a cysteine in the active center with a subsequent loss of activity. Upon CNP treatment, formation of cellular lactate and GAPDH activity were significantly lowered. The treatment of melanoma cells and melanocytes with the GAPDH inhibitor HA decreases viability only in the cancer cells but not in studied healthy cells, highlighting and proving the important role of glycolysis in cancer cells.

Conclusion

Further improvement of chemotherapeutics of skin cancer are still much needed, and CNP has been shown to be a promising therapeutical tool exerting a selective toxicity on tumor cells. Further understanding of its mode of action is necessary, and here we show for the first time that CNP can modulate glycolysis via oxidation and inhibition of GAPDH resulting in a loss of viability only for melanoma cells but not for melanocytes.

EACR22-0763

Aggressive characteristics of ligand binding domain activating mutations of ESR1 is mediated by urea cycle deregulation in breast cancer cells

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Introduction

Mutations in the ligand binding domain (LBD) of estrogen receptor α (ER) confer constitutive transcriptional activity and resistance to endocrine therapies in breast cancer patients. Accumulating clinical data suggest adverse outcome for patients harboring tumors expressing these mutations. Cancer cells modify the normal metabolic pathway enabling them to proliferate and thrive. The main goal of this study was to reveal metabolic properties of LBD-ER, and 537S-ER in particular, in increasing cell aggressiveness.

Material and Methods

Cells constitutively expressing physiologic levels of ER harboring activating LBD mutations were generated and characterized for viability, invasiveness and tumor formation *in vivo*. Gene expression profile was studied using microarray and RNAseq technologies. Metabolic properties of the cells were assessed using global metabolite screen and direct measurement of metabolic activity.

Results and Discussions

Cells expressing mutated ER showed increased proliferation, migration and *in vivo* tumorigenicity compared to cells expressing the WT-ER, even in the presence of estrogen. Expression of the 537S-ER was associated with upregulation of genes involved in invasion and metastases, as well as elevation of genes associated with tumor cell metabolism, such as the rate-limiting enzyme for arginine biosynthesis, ASS1, that was elevated in 537S-ER expressing cells. Indeed, analysis of metabolites indicated that 537S-ER activates the urea cycle, as evidenced by higher levels of urea in the 537S-ER cells compared to WT-ER. Inhibition of ASS1 inhibited colony formation and proliferation of 537S-ER cells in contrast to WT-ER. Accordingly, 537S-ER cells were more sensitive to arginine deprivation. Addition of citrulline was able to restore 537S-ER cells growth in arginine deprived media, however WT-ER cell growth was affected to a lesser extent.

Conclusion

Taken together, these data indicate an important role urea cycle and ASS1 have in mediating aggressive behavior of 537S-ER LBD mutant cells. This unique metabolic activity may serve as a potential vulnerability and aid in the development of novel treatment strategies to overcome endocrine resistance.

EACR22-0943

Metformin and simvastatin exert additive antitumor effects in glioblastomas via senescence state and telomere-lengthening: Clinical and translational evidence.

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Introduction

Glioblastoma (GBM) is one of the most devastating cancers due to its aggressive behavior and lack of therapies available, being its overall-survival from diagnosis ~14-months. Thus, identification of new therapeutic tools is urgently needed. Interestingly, metabolism-related drugs such as metformin (MF) and statins [e.g. simvastatin (SVT)] are emerging as efficient antitumor agents for several cancers. We evaluated the *in vivo/in vitro* effects of MF and/or SVT on key clinical/functional/molecular/signaling-parameters in GBM patients/cells and a preclinical mouse model.

Material and Methods

Human GBM cells [cell lines (U-87MG/U-118MG) and patient-derived cell-cultures] and mouse astrocytes progenitor cell-cultures were used to measure a set of key functional/molecular parameters and signaling-pathways in response to MF, SVT, and their

combination *in vitro*. Then, the response of MF, SVT, and their combination, treatment was analyzed *in vivo* using an exploratory/observational retrospective GBM patients cohort (n=61) and a U-87MG xenograft preclinical mouse model.

Results and Discussions

MF and SVT treatment exerted strong antitumor actions *in vitro* (i.e. proliferation/migration/tumorsphere-/colony-formation/VEGF-secretion inhibition, and apoptosis increase) in GBM cells. Notably, their combination additively modulated these parameters vs. individual treatments. These actions were mediated through the alteration of key oncogenic pathways (AKT/JAK-STAT/NFκB/TGFβ). An enrichment analysis uncovered a TGFβ-pathway activation and AKT-pathway inactivation in response to MF+SVT combination, which promoted a senescence-state linked to a senescence-associated secretory phenotype, telomere lengthening process, and the expression dysregulation of key splicing machinery components. The MF and SVT antitumor actions, but specially their combination, in GBM were confirmed *in vivo* in humans (i.e. MF+SVT combination treatment was associated with longer overall survival in GBM patients) and in a preclinical mouse model (i.e. intratumor administration of MF, SVT and specially MF+SVT, decreased growth/weight tumor vs. non-treated tumors).

Conclusion

We demonstrated the efficacy of MF, SVT, and, particularly, their combination, as a potential and useful therapeutic tool for the GBM management. Given the clinical safety of these drugs, these results offer a clinically relevant opportunity that could be tested for their use in humans with limited undesired side-effects.

EACR22-0983

Carboxylesterase 1 (CES1) is a promising druggable target in ovarian cancer

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Introduction

Tumour cells show an incredible metabolic plasticity. This flexibility is a key factor that allows cancer cells to cope with the low nutrient concentrations in the tumour microenvironment, thus sustaining their proliferation and metastatic spread. Recently, carboxylesterase 1 (CES1), has been identified in colorectal carcinoma (CRC) as an essential NF-κB-regulated lipase promoting cancer-cell survival and metabolic adaptation under condition of energy stress. Specifically, CES1 was able to promote the survival of CRC cells by increasing TAG breakdown to fuel fatty acid oxidation (FAO) and preventing their toxic build-up. Like CRC, ovarian carcinoma (OC) preferentially metastasizes to peritoneal cavity and infiltrates the omentum, a fat rich organ, and recent evidence pointed out the involvement of lipid metabolism in energy stress response. Therefore, we investigated whether CES1 could play an important role also in the metabolic adaptation of OC cells.

Material and Methods

Bioinformatic analysis of publicly available datasets of OC patients was conducted. Experimental validation exploited a panel of two primary and two metastatic OC cell lines that were analysed at the baseline for CES1 expression and bioenergetic parameters by qRT-PCR, Western blot (WB) and Seahorse XFe96. Changes in CES1 levels, metabolic

phenotype, autophagy flux and survival under energy stress conditions in presence or absence of specific CES1 inhibitor were evaluated by qRT-PCR, seahorse, WB, viability assay and FACS analysis.

Results and Discussions

We found that elevated CES1 expression correlates with worse prognosis in OC patients. Accordingly, we showed that CES1 was basally expressed only in the metastatic cell lines EFO21 and OV-56. Although primary cell lines OVCA-429 and ES2 were lacking basal CES1 expression, all OC lines expressed CES1 when cultured under metabolic stress conditions, suggesting that CES1 could be relevant for their adaptation to harsh metabolic environment. Indeed, pharmacological CES1 blockade by commercially available GR-148672X inhibitor impaired bioenergetic parameters and blocked autophagy flux, thus resulting in significant cell death of all the analysed OC lines. Notably, inhibition of CES1 signalling under energy stress conditions was able to also kill carboplatin-resistant OC cells.

Conclusion

These data underscore the clinical relevance of this enzyme in OC and suggest that CES1 could be a potential druggable target, especially in chemotherapy-refractory subsets.

EACR22-1035

PDK4 – a novel prognostic marker for prostate cancer

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Introduction

Prostate cancer (PCa), as the second most common cancer in men worldwide, is characterized by metabolism that lacks the Warburg effect, but prefers oxidative phosphorylation as the energy-generating pathway. The main regulators of this metabolic shift are the pyruvate dehydrogenase complex and pyruvate dehydrogenase kinase (PDK) 1-4. Since cancer metabolism and its key players are potential therapeutical targets, we aim to determine biomarkers responsible for the metabolic shifts in PCa.

Material and Methods

The human transcriptomic datasets TCGA PRAD and MSKCC were used to define the role of PDK4 on recurrence-free survival (RFS), differential gene expression, as well as pathway deregulations. Furthermore, tissue microarrays (TMA) of recurrent and non-recurrent PCa patients were stained for PDK4 via immunohistochemistry.

Results and Discussions

In a gene co-expression network analysis of the TCGA PRAD and MSKCC human transcriptomic datasets we discovered that PDK4 correlates with, and is a transcriptional target of STAT3, which we previously described as a tumor suppressor in PCa. Furthermore, low PDK4 expression is associated with decreased probability of RFS in primary and

metastatic PCa, whereby metastatic patients showed lower PDK4 expression than primary PCa patients. This was also reflected in the TMA analysis of the PCa patient cohort, where a low PDK4 score correlated with decreased probability of RFS and metastasis-free survival (MFS). Moreover, in a gene set variation analysis alterations in PDK4 expression are reflected by deregulations in major metabolic cancer pathways, most importantly TGF- β or PPAR signaling. In differentially expressed genes analysis the expression of transcription factors such as FOS, ATF3, SNAI2, or PPARG positively correlated with PDK4.

Conclusion

The correlation of PDK4 expression with RFS and MFS on RNA and protein level proposes PDK4 as a novel prognostic marker in PCa. Interestingly, PDK4 expression directly corresponds with the metabolic shifts taking place throughout progression from normal prostate tissue to primary and metastatic PCa. To further understand the regulatory network of PDK4 expression and determine therapeutical targets, we will validate these results in our *in vitro* and *in vivo* model systems.

EACR22-1059

Pharmacological targeting of myoferlin primes ferroptosis in pancreatic cancer cells.

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the seventh leading cause of cancer-related deaths worldwide and will become the second leading cause of cancer-related deaths by 2030. Myoferlin is a protein involved in membrane fusion and in membrane receptors recycling and is overexpressed in PDAC. We used an innovative small compound (WJ460) to target myoferlin in PDAC cell lines and reported the triggering of a mitophagy associated with an iron-dependent cell death, namely ferroptosis, considered as an alternative to apoptosis in cancer cells.

Material and Methods

1) Myoferlin was targeted thanks to specific siRNA or WJ460. 2) Tumour growth was evaluated by Panc-1 xenograft in 5 week-old NOD-SCID mice. 3) Cell proliferation was assessed thanks to Incucyte SX5 imaging. 4) Mitophagy and mitochondrial network integrity were assessed in lived cells using a Nikon A1R confocal microscope and transmission electron microscope. 5) Mitochondrial respiration was evaluated using Seahorse Xfp technology. 6) Intracellular iron concentration was assessed by fluorescence imaging using Incucyte SX5. 7) Ferroptosis induction was evaluated thanks to malondialdehyde production and rescued using Trolox as lipophilic antioxydant. 8) WJ460 and RSL3/Erastin synergy on cell growth was measured thanks to Chou-Talalay method.

Results and Discussions

We are able to demonstrate that myoferlin targeting using WJ460 mimicks gene silencing in different PDAC cell lines. Indeed, WJ460 treatment induces a mitochondrial fragmentation and a decrease of mitochondrial respiration. Besides, we report a significant increase of mitophagy in WJ460-treated cells inducing a decrease in mitochondrial ROS abundance. Aware of the importance of mitochondria in ferroptosis, we show that malondialdehyde, a main hallmark of ferroptosis, is increased upon WJ460 treatment. Furthermore, we show a significant increase of iron abundance in all PDAC cell lines under WJ460 treatment. Finally,

in order to bring clinical value to our data, we decided to combine WJ460 with ferroptosis inducers (RSL3 or erastin). We demonstrate that combination of WJ460 and RSL3/Erastin exhibited synergistic effects on PDAC cell proliferation.

Conclusion

Our findings shed light on the biological effects of WJ460, a myoferlin-interacting compound. We demonstrated that WJ460 cytotoxic effects were iron-dependant. Consequently, targeting myoferlin may pave the way for new therapeutic combination. We propose to combine myoferlin targeting and low concentration of ferroptotic inducers to eradicate PDAC cells.

EACR22-1079

Mitochondrial dynamics and OXPHOS participate in the resistance of glioblastoma multiforme cells to doxorubicin

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Introduction

Recent progress in the development of bio-accessible doxorubicin (DOX)-loaded liposomes opens perspectives for DOX application in glioblastoma multiforme (GBM) treatment. However, the ability of tumor cells to develop resistance to DOX has also been reported and ascribed to their oxidative phosphorylation (OXPHOS)-dependent adaptability to the chemotherapeutic stress. Accordingly, mitochondrial rearrangements, which regulate cellular bioenergetics, are crucial for cellular adaptation to microenvironmental stimuli. Taking these facts into account, we estimated the short-term metabolic GBM responses to DOX and the long-term consequences of DOX treatment for the metabolic profile of DOX-resistant populations.

Material and Methods

Human T98G GBM cells were subjected to pulse DOX (1 μ M; 48 hours) treatment followed by long-term (72 hours – 30 days) incubation after drug ablation. Rearrangements of mitochondrial architecture, redox status and mitochondrial membrane potential were visualized with fluorescence and transmission electron microscopy (TEM). Effects of DOX on the bioenergetic profile of GBM cells were examined with Seahorse® XFp approach, ATP/NADH analyses and by the estimation of glucose uptake efficiency (2-NBDG; ImageStream® X cytometry). Oligomycin A was applied to study the role of OXPHOS in DOX-resistance development.

Results and Discussions

DOX stimulation resulted in enhanced mitochondrial fusion in T98G cells, which was correlated with the gradual increase of OXPHOS intensity. This effect was accompanied by the gradual increase of ATP and total NAD/NADH content in the surviving cells. Moreover, the intensified mitochondrial fusion was almost completely abolished by OXPHOS inhibitor – Oligomycin A and this effect was followed by reduced survival rate of DOX-treated cells. These data confirm the interrelations between the metabolic flexibility of GBM cells and their resistance to DOX.

Conclusion

Collectively, we consider the mitochondrial plasticity as a self-protection mechanism that counteracts mitochondrial stress, thus protecting metabolic balance of DOX-treated GBM cells and providing the background for OXPHOS stimulation and drug-resistance. It can be

attributed to the selective expansion of GBM lineages characterized by enhanced metabolic flexibility or to the “*ad hoc*” cell adaptation to DOX-induced metabolic stress.

EACR22-1195

Acquired resistance to EGFR-targeted therapy in quadruple wild-type colorectal cancer: role of glycation stress

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Introduction

Colorectal cancer (CRC) is the third most common cancer diagnosed and the lack of effectiveness of current therapies leads to frequent relapses. Cetuximab (CTX) is the most used anti-EGFR targeted therapy in CRC. Genetic alterations, such as activating mutations, are commonly associated with a lack of response to CTX. Strikingly, patients with CRC diagnosed as “quadruple wild type” (4WT, non mutated KRAS, NRAS, BRAF and PIK3CA) also show a lack of response to this therapy. Reliance of cancer cells on glycolysis promotes tumorigenesis and malignant progression. Methylglyoxal (MG) is an unavoidable glycolytic by-product that glycates proteins, lipids, and DNA. Upon MG stress, cancer cells enhance their growth and metastatic potential.

Material and Methods

We used LIM1215 and DIFI human 4WT CRC cell lines after short/long-term challenge with CTX to assess MG stress. We measured glyoxalase 1 (GLO1), the major MG detoxifying enzyme, expression and activity. MG level was assessed using mass spectrometry and MG adducts were evaluated using specific antibodies. To explore further the potential link between MG stress and CTX resistance, we generated GLO1-depleted CRC stable clones using shRNAs. We aim to use patient-derived xenograft and organoids generated from 4WT CRC patients to demonstrate the occurrence of MG stress and the potential anti-cancer effects of MG scavengers.

Results and Discussions

Short-term CTX treatment allowed the selection of persistent cells that showed decreased Nrf2 transcription factor. As a consequence, these cells lost GLO1 expression and accumulated MG protein adducts. Conversely, long-term challenge with CTX generated resistant cells with elevated glycolytic rate and MG level when compared with parental cells. In good accordance, the sole induction of endogenous MG stress in GLO1-depleted LIM1215 and DIFI cells rendered them resistant to CTX. CTX efficacy in CRC is dependent upon a functional p38 pathway whose activation leads to apoptosis. Mechanistically, we will explore the possibility that MG inhibits p38 in favor of AKT activation thus triggering escape from apoptosis and therapy resistance.

Conclusion

Our data point to a novel connection between MG stress and acquired resistance to CTX in CRC. Ongoing analysis of MG stress level in organoid models will help evaluating the potential resensitizing effect of MG scavengers used alone or in combination with CTX in resistant CRC.

EACR22-1253

Palmitic acid enhances protein lipidation in oral cancer

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Introduction

Although glucose is considered the dominant metabolic substrate in rapidly proliferating tumors, dietary lipids have a critical role in cancer progression and metastasis. Western diets contain many lipids but not all are equally capable of promoting metastasis. To understand the mode of action of different dietary lipids on cancer progression, we studied the proteome and the lipo-proteome in oral cancer cells exposed to palmitic acid and oleic acid with known pro- or non-metastatic effects, respectively, according to our recent studies.

Material and Methods

We combined SILAC with lipid metabolic labeling using a palmitic acid analog (17-ODYA) and click-chemistry to assess the changes in the proteome and the lipo-proteome of human oral cancer cells growing with palmitic (PA) or oleic acid (OA). Furthermore, to better understand the effect of pro-metastatic lipids in vivo, GFP-bearing human oral cancer cells were orthotopically injected in immunodeficient NSG mice fed on a palm-oil or olive-oil enriched diet. Primary tumors were excised and tumor cells were FACS-sorted for proteome analysis. Proteomic assays were complemented with transcriptomic analysis.

Results and Discussions

In vitro stimulation of oral cancer cells with PA increased expression of CD36 and also enhanced the expression of proteins involved in lipid metabolism, cell adhesion, as well as pro-inflammatory and drug resistance processes; whereas proteins involved in cell cycle, RNA splicing and protein turnover showed reduced expression. In contrast, OA produced a completely opposite response at the proteome level. The proteomic rewiring elicited by PA strongly correlates with transcriptomic changes, indicating that metabolic response is mostly transcriptionally regulated. In-vivo proteomic and transcriptomic analysis of tumors from mice on a palm-oil or olive-oil enriched diet, partially reproduced the results observed in vivo. Interestingly, we found the family of palmitoyl-transferases enzymes among the mostly upregulated proteins preferentially in mice fed with a palm-oil enriched diet. This prompt us to study the changes in palmitoylome upon PA exposure, and found that PA increases the proportion of palmitoylated proteins, especially those involved in cell adhesion (desmosome/hemidesmosome).

Conclusion

PA and OA reshapes the proteome in opposite directions which correlates with their different metastatic potential. PA enhances palmitoylation in oral cancer cells.

EACR22-1263

Deciphering the role of ETFDH in neoplasia

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Introduction

Cancer cells undergo metabolic remodeling in order to support neoplastic growth and survive therapeutic insults. To adapt to constantly changing environments, cancer cells exert metabolic plasticity, which has a strong impact on therapeutic responses. However, the mechanisms that underpin metabolic plasticity of malignant cells remain obscure, thus limiting the efforts to exploit metabolic reprogramming of neoplasia in the clinic. To address this pivotal gap in knowledge, we performed database and literature searches to identify metabolic genes that are commonly suppressed in neoplasia. Electron transfer flavoprotein dehydrogenase (ETFDH) emerged as consistently downregulated across several cancer types (e.g. colon, liver cancer), while low ETFDH expression appears to coincide with disease progression and poor survival. ETFDH is a facultative component of the electron transport chain and is involved in the catabolism of multiple fuel sources including branched chain amino acids and fatty acids, which may contribute to metabolic plasticity. Notwithstanding these observations and the central role of ETFDH in metabolism, its function in cancer is entirely unknown.

Material and Methods

To study the impact of ETFDH loss on cancer, we performed CRISPR/Cas9 mediated gene editing to establish stable ETFDH knockout models across numerous cancer cell lines (HCT116, Nalm6, HepG2), along with models that rescue ETFDH expression. Bioenergetic and metabolomic analyses were performed to delineate changes on cancer metabolism. Western blotting was performed to study the role of ETFDH on mTOR signalling. Puromycilation assays were conducted to study changes on protein synthesis. In vivo orthotopic xenograft models were used to assess the impact of ETFDH loss on tumour growth.

Results and Discussions

ETFDH loss promoted cancer cell proliferation in vitro and increased tumour growth in vivo. Cells with the absence of ETFDH had higher accumulation of branched chain amino acids, which paralleled with increased mTOR signalling and higher rates of protein synthesis. ETFDH knockout cells also displayed increased mitochondrial bioenergetics. The dependency on OXPHOS and mTOR was highlighted by the increased sensitivity of ETFDH KO cells to inhibitors of the electron transport chain and mTOR inhibitors compared to controls.

Conclusion

This study highlights a potential role of ETFDH in contributing to the metabolic plasticity of cancer cells, which represents a novel metabolic vulnerability to target in the clinic.

EACR22-1275

POSTER IN THE SPOTLIGHT

Prostate cancer metastasis and post-translational modifications: deciphering the role of UFMylation

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Introduction

UFMylation is one of the most unknown of the numerous post-translational modifications found to date. These modifications may have the ability to alter the characteristic biological processes of their target proteins, including their stability and/or metabolism. However, to date, the role that UFMylation could have in the context of Prostate Cancer is unknown.

Material and Methods

In order to identify key pathways in the metastatic process, we made use of publically available Prostate Cancer databases using Cancertool. Furthermore, we developed molecular tools to genetically modify the UFMylation pathway, such as CRISPR/Cas9, shRNAs and overexpression of UFM1 or its specific ligases. Finally, to identify UFMyated proteins, we developed several stable cell lines expressing BioID and TurboID fusion proteins and analysed them via immunoblot, immunofluorescence, and biotin-affinity purification-based proteomics.

Results and Discussions

Through a bioinformatics approach, we found that UFMylation is altered in metastatic Prostate Cancer. On the one hand, we observed that the silencing of UFM1 leads to an increase in cell invasion without affecting cell proliferation. On the other hand, we identified relevant proteins in key metabolic pathways to be UFMyated.

Conclusion

The modulation of the UFMylation machinery during Prostate Cancer progression could unveil a role for protein metabolism in disease aggressiveness, by promoting the adaptation of the cancer cell signalling network to hostile microenvironments.

EACR22-0074

Innate immunity-related protein PGLYRP1 as a new biomarker for pancreatic cancer detection and cancer stem cells isolation

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a highly metastatic cancer with a 5-year survival rate of less than 10%. Neither chemotherapy nor immunotherapy have significantly

impacted these statistics primarily due to late detection of PDAC and the existence of highly chemoresistant “stem”-like cells known as cancer stem cells (CSCs). Our goal is to identify and validate new genes/proteins that could serve as new markers for CSC identification and PDAC early detection using the KPC mouse and human samples as preclinical models.

Material and Methods

Murine primary tumor cells were harvested from the KPC mouse model (*LSL-Kras^{G12D/+};LSL-Trp53^{R172H/+};Pdx-1-Cre mice*) and human primary cells were obtained from PDAC surgical tumors following published protocols. Murine cells were sorted and SurePrint G3 Mouse Gene Expression 8x60K arrays, followed by GSEA, were performed (using the Reactome Molecular Signature Database) to identify differentially regulated genes. Western Blot (WB), Flow Cytometry (FC), qPCR and Immunofluorescence (IFF) were performed following previously published protocols. ELISA assay was performed following the manufacturer's protocol.

Results and Discussions

Peptidoglycan Recognition Protein 1 (*Pglyrp1*) was found as one of the most upregulated genes in tumor-isolated murine CSCs VS. non-CSC populations. *Pglyrp1* is an innate immunity-related protein involved in antibacterial defence that is present in neutrophils and non-canonical monocytes; however, its presence in PDAC has never been addressed. *Pglyrp1* expression was assessed by qPCR, WB and FC in KPC and human primary cell lines, increasing in spheroids compared with 2D cultures. *Pglyrp1* distribution in tissues was determined by IFF, and while it was not present in healthy tissue, and mildly in acute inflamed tissue, it was expressed since the beginning of tumor formation, firstly restricted to PanINs and later present in adenocarcinoma tissue and metastases. Finally, we performed an ELISA to assess PGLYRP1 levels in sera from healthy donors and PDAC patients and found a significant difference between both groups, and across PDAC stages, increasing with tumor development and showing a parallelism with KPC mouse model IFF results.

Conclusion

These results highlight PGLYRP1 as a potential biomarker for both early disease detection and isolation of CSCs, in human and murine samples; however, further research will be needed to decipher the exact role of PGLYRP1 in PDAC.

EACR22-0152

STUDY OF T-TYPE CALCIUM CHANNEL EXPRESSION IN GLIOBLASTOMA CELLS UNDER STRESS

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Introduction

Glioblastoma multiforme (GBM) is a brain tumor of poor prognosis and few therapeutic options. T-type calcium channels (TTCC; namely Cav3.1, Cav3.2 and Cav3.3) play pivotal roles in GBM, Cav3.1/Cacna1g being the prevalent isoform and upregulated in

chemoresistant GBM. Glioma stem cells (GSC) are thought to originate the tumor and responsible for its therapeutic resistance, through their high adaptability versus different conditions/insults within the tumor microenvironment. Hence, here we have studied TTCC expression in GSCs compared to adherent conditions, and under the impact of hypoxia and irradiation.

Material and Methods

We used the U87-MG cell line and patient-derived primary cultures, grown as neurospheres (NS) or in adherent cultures (in presence of serum). NS cultures were exposed to “chronic” hypoxia (2% O₂ for 3 days) compared to normoxia (21% O₂). Patient-derived GSCs were submitted to fractionated radiotherapy at a dose of 2 Gy/day up to 8 Gy (4 consecutive days) to simulate clinical radiotherapy regimens. Western Blot and immunocytochemistry were performed to study autophagy or hypoxia markers. RT-PCR was used to quantitate TTCC expression.

Results and Discussions

Cav3.1 expression was maintained (U87MG cells) or reduced (patient-derived cultures) in GSCs compared to adherent cells. Exposure to a 2% O₂ atmosphere increased Cav3.1 channel transcripts and that of Cachd1 (an accessory subunit) in U87MG NS, while Cav3.2/Cacna1h was elevated in patient-derived GSCs. Cav3.1 overexpression was linked to increased autophagy in GSC. Thus, it is possible that Cav3.1 contributes to GSC survival in the hypoxic areas of the tumor. Furthermore, GSC cultures showed upregulation of Cav3.1 and Cav3.3/Cacna1i isoforms when subject to irradiation.

Conclusion

These results indicate that hypoxia and radiotherapy induce the expression of different TTCC isoforms in GSCs, confirming their adaptive roles and highlighting the functional differences between the three highly homologous Cav3 isoforms.

EACR22-0264

Isolation of a cell subpopulation enriched in cancer stem cell characteristics independently of surface marker expression

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Introduction

This project aims to study intratumoral cellular heterogeneity and treatment resistance in colorectal cancer (CRC) by unraveling cell subpopulations, including cancer stem cells (CSC). The objectives of this research project are: (1) to use the SdFFF technique to isolate cell subpopulations based on biophysical properties (label-free), (2) to phenotypically and functionally characterize the sorted cell subpopulations, (3) to analyze their response to chemotherapy, and (4) to compare the results obtained from early stage cell lines with those obtained from metastatic stage cell lines.

Material and Methods

Cell subpopulations were sorted from CRC cell lines and primary cultures by SdFFF. SdFFF-sorted cell subpopulations were then characterized phenotypically by analyzing the expressed CSC markers and functionally by studying their distribution in the cell cycle, their ability to form colonies/spheres and to form tumors in immunocompromised mice. Finally,

the response of these SdFFF-sorted subpopulations to conventional chemotherapies used in the treatment of CRC was analyzed in 2D and 3D culture models.

Results and Discussions

Three cell subpopulations were isolated by SdFFF based on their biophysical properties. Phenotypic and functional characterization identified a subpopulation composed of rather differentiated cells that express CSC markers more strongly but are poorly clonogenic/tumorigenic, and a subpopulation enriched in CSC characteristics: relative quiescent, clonogenic and able to initiate tumors in vivo. Results of chemosensitivity tests showed that the subpopulation enriched in CSC characteristics is more resistant to 5-FU as well as to 5-FU-based chemotherapies compared to the differentiated fraction. A difference in chemotherapy sensitivity between cell lines derived from primary tumors and those derived from CRC metastases has also been demonstrated.

Conclusion

Cell subpopulations sorted by SdFFF have distinct phenotypic and functional features. We identified two populations of interest: a subpopulation composed of differentiated cells, proliferating but poorly tumorigenic and sensitive to chemotherapies, and a subpopulation containing cells with CSC characteristics such as relative quiescence, clonogenicity, tumorigenicity and chemoresistance. Therefore, we highlighted a chemoresistant subpopulation enriched in CSC properties present in early and late stage cell lines and primary cultures, without using surface markers.

EACR22-0342

The atypical Wnt ligand, Norrin, undergoes importin-alpha dependent nuclear translocation

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Introduction

Secreted Norrin is a well characterized as a ligand that activates canonical Wnt signaling through binding to the Frizzled-4 (FZD4)/Low-density lipoprotein receptor-related protein 5 (LRP5) receptor complex to regulate vascular development and barrier function in the brain. Recently, we described a novel canonical Wnt-independent function of Norrin, where it stimulates GBM stem cell proliferation and self-renewal through Notch signaling.

Here, we sought to investigate the pathways underlying this Wnt-independent function of Norrin, including its function in mediating GBM stem cell growth.

Material and Methods

BioID proximity labelling screen was performed in Flp-In T-Rex (HEK293) cells to characterize the Norrin interactome. Norrin subcellular localization was investigated using cellular fractionation and immunocytochemistry. RNA interference was used to investigate the role of Importin-alpha in nuclear localization of Norrin and in Norrin-stimulated GBM growth. Primary Patient-derived GBM stem cells were isolated and expanded according to standard protocols.

Results and Discussions

Using the BioID technique, we identified 81 candidate Norrin-interacting proteins and strikingly, the majority of these hits corresponded to nuclear proteins. Therefore, we

characterized Norrin localization in cells transfected with wildtype and tagged Norrin. Western blot analysis revealed enrichment of Norrin in the nuclear fractions, and this was confirmed by immunocytochemistry. Notably, Importin-alpha, a key regulator of nuclear import in mammalian cells, was identified as a candidate Norrin interacting protein in the BioID screen. Pharmacologic inhibition of Importin-alpha reduced the accumulation of Norrin in the nucleus, and RNAi-mediated Importin-alpha knockdown resulted in a significant nuclear to cytoplasmic shift in Norrin localization. Functionally, blocking Importin-alpha in Norrin expressing cells increased paracrine activation of canonical Wnt signaling, suggesting a competitive interaction between Norrin nuclear import and secretion. Currently, we are examining whether Importin-alpha is required for the Wnt-independent effects of Norrin on the growth of primary patient-derived GBM stem cells.

Conclusion

Our results uncover a novel nuclear localization of Norrin, which could mediate Wnt-independent functions in stimulating GBM stem cells. Additionally, we identify Importin-alpha as the main regulator of this nuclear translocation.

EACR22-0354

Characterization of undifferentiated neuroblastoma tumor cells: contribution to metastasis

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Introduction

Neuroblastoma (NB) tumours are heterogeneous, therapy-resistant, metastatic tumours that originate from the neural crest of the sympathetic nervous system. Within the tumours, cellular heterogeneity has been observed, including at least two types of tumour cells with divergent gene expression profiles and phenotypes: undifferentiated stem-like mesenchymal cells (MES) and compromised adrenergic cells (ADRN). The undifferentiated cell population seems to exhibit stem cell properties, it is enriched in high-risk tumours, and has been suggested that they contribute to NB malignization by evading intensive multimodal therapy and being responsible for tumour relapses and metastasis formation. The formation of aggressive metastases takes place thanks to a multi-stage process in which cell migration and invasion are essential. Migratory and invasive cancer cells undergo dramatic molecular and cellular changes by reshaping their cell-cell and cell-matrix adhesion, as well as their cytoskeleton. Changes in cell shape are indicative of biochemical signals that can modulate cell phenotype and biological properties and are indicative of the behavior and evolution of the cells, influencing biological processes, such as proliferation, differentiation and stem cell fate.

The aim of this research is to study the specific adhesive, migratory and invasive properties of undifferentiated neuroblastoma cells (UNCs).

Material and Methods

Cultures are enriched in undifferentiated cells by growing in defined culture conditions as tumorspheres and FACS sorting from NB cell lines and patient-derived cells. Adhesion and spreading assay, cell morphology assay, migration analysis, membrane dynamics analysis, and matrix degradation assays were performed.

Results and Discussions

The results obtained show that UNCs have greater adhesion and spreading capacity than the rest of tumour cells. They also present defined, persistent morphological features. These characteristics are concordant with a UNCs greater cellular plasticity. In addition, UNCs migrate at a slower speed, but with greater directionality, exhibit increased blebs-mediated membrane actin dynamics, and degrade the extracellular matrix to a greater extent than the rest of the tumor cells. These results show that these UNCs are characterized by a particular migratory and invasive behaviour.

Conclusion

These preliminary results suggest that UNCs could show significant metastatic features and warrant further analysis of their contribution to aggressiveness in vivo.

EACR22-0396

CD73+/CD44+/CD90+ cells population in liver of Diethylnitrosamine (DEN)-treated male rats and therapeutic potentials of biologically synthesized silver nanoparticles.

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Introduction

The role of cancer stem cells (CSCs) is crucial in the initiation of a variety of cancers. CSCs are considered as pluripotent cells which replicate via asymmetric cell division and cause heterogeneity within the tumor tissue. Therefore, it is important to uncover the carcinogens that may cause cancer via increasing the CSCs population in a target tissue. The present study was designed to evaluate the population of CSCs like cells (CD73⁺, CD44⁺, and CD90⁺ cells) in the liver tissue of a diethylnitrosamine (DEN)-treated male rats and the possible therapeutic effects of biologically synthesized silver nanoparticles (AgNPs).

Material and Methods

Four groups; Control, DEN-treated, DEN+AgNPs, and AgNPs-treated were made. DEN (90mg/kg) and AgNPs (100mg/kg) were administered intraperitoneally and by oral gavage, respectively. After 5 weeks, animals were dissected and liver tissues were fixed in 10% buffered formalin and processed through the standard procedure of microtomy. Variations in morphological and histological features of liver-stained sections were observed under the microscope. CSCs population was analyzed by fluorescence-activated cell sorting (FACS) in the single-cell suspension of liver tissues and RT-qPCR was performed for relative mRNA expression of CD73, CD44, and CD90 genes.

Results and Discussions

Abnormalities in the cellular growth, disruption in the tissue architecture, enlargement of cells with mild pleomorphism, and expansion in the central vein of liver tissues were revealed by the histopathological studies of DEN-treated animals. However, a moderate inflammatory response was observed in the DEN+AgNPs treated group. The CD73⁺, CD44⁺, and CD90⁺ cells population were significantly increased in the DEN-treated group, however, the same cells population was decreased with AgNPs treatment. Elevated CD73⁺, CD44⁺, and CD90⁺ indicated that liver of DEN-treated animals contain the cells which are bearing the stem cell properties i.e., proliferation and self-renewal. Furthermore, the relative mRNA expression levels of CD73, CD44, and CD90 genes were significantly up-regulated in the DEN-treated group as compared to the control group. The expression of targeted genes was decreased in the DEN+AgNPs-treated group compared to the DEN-treated group.

Conclusion

Altogether, it was concluded that DEN may cause carcinogenesis via increasing the CSCs like cells population in liver tissues and AgNPs has the potentials to attenuate the hazardous effects of DEN.

EACR22-0470

Matrix Gla Protein: a new driver in ovarian cancer stem cells

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Introduction

Ovarian cancer (OC) is the most aggressive gynecological tumor, mainly due to the lack of efficient early detection methods and to peritoneal dissemination. Moreover, after surgical resection, in most cases tumor relapses and acquires chemoresistance.

This clinical evolution seems to be accounted for by a small population of ovarian cancer stem cells (OCSC), thus making them an attractive target for more efficacious treatments. However, this could only be achieved through the detailed characterization of the molecular and functional traits of this elusive cell subpopulation. Therefore, the identification of novel OCSC-associated targets and therapeutic approaches may offer alternative treatment options.

Material and Methods

To identify OCSC biomarkers, we compared the transcriptomic profile of primary, patient-derived OCSC versus their non-stem counterpart and we selected Matrix Gla Protein (MGP) as an interesting candidate upregulated in OCSC. Subsequent gain and loss-of-function studies were undertaken to investigate the role of MGP in OCSC biology

Results and Discussions

MGP is classically known as a calcium chelator, mainly associated with the inhibition of tissue calcification. However, MGP has also been found aberrantly expressed in different cancer types, yet its contribution to cancer progression and the underlying mechanisms remain elusive.

We found MGP to be specifically enriched in primary OCSC, and then obtained evidence that MGP mediates stemness-associated biological features including sphere formation and tumor initiation. Furthermore, MGP regulated the expression of several key genes involved in stemness and/or EMT.

We also found MGP to be upregulated in OC patients following chemotherapy, suggesting that the protein may be involved in OCSC-driven chemoresistance.

The molecular mechanisms underlying MGP-regulated OCSC pathophysiology were investigated by RNA sequencing experiments on MGP-manipulated OC cells, which unveiled genetic networks and pathways that are orchestrated by MGP to promote OCSC function.

Conclusion

The biological role of MGP in OC provides novel insight into the biology of OCSC, thus defining new pathways involved in OC stemness and paving the way to innovative therapeutic strategies for OC eradication.

EACR22-0542

Deciphering CML LSCs TKI-induced dormancy within a standardized 3D BM model

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Introduction

Despite the therapeutic progress in Chronic Myeloid Leukemia (CML) using Tyrosine Kinase Inhibitors(TKI) that target specifically BCR-ABL kinase,TKI treatment is not yet curative since many CML patients still retain leukemic stem cells(LSC) after treatment arrest.

Material and Methods

Human Bone Marrow(BM) is a complex microenvironment providing essential cues to resident cells. We developed a standardized 3D human BM-like model to decipher pathophysiological mechanisms that control stem cells(SC) and take place during hematological malignancies. For that, we used human mesenchymal stem cells(MSC), endothelial cells and CML LSCs ensuring a high reproducibility of the medullar niche . We generated a CD34⁺LSC model expressing Fucci system, for a dynamic analysis of cell cycle in response to different treatments. To expand our analysis we used different TKI such as Imatinib and Nilotinib but also Asciminib which is the first allosteric inhibitor of BCR-ABL1.The outcome could be analysed either by *in situ* imaging or flow cytometry of retrieved cells.

Results and Discussions

Previous single cell RNA-Seq analysis of BCR-ABL+ persistent cells showed a co-enrichment of BMP signalling, hematopoietic SC and quiescence signatures. In addition, TKI treatment of LSCs seems to foster their quiescence. However, a dual targeting of BMPR1B and Jak2/Stat3 efficiently impairs LSCs quiescence by enabling their differentiation. The 3D model allows a dynamic tracking of quiescent CML cells localization within the model and allows us to investigate how CML treatment remodels BM microenvironment and affects LSC quiescence. After adding LSCs to the 3D model, we identified an increased in BMP4 production, induced by TKI treatment. Current analyses involving multiplex IF and using specific markers for each compartment will localize quiescent LSCs within the system. Lastly, we revealed that non-TKI treatment were able to promote LSC dormancy in the 3D model.

Conclusion

We identified that BMP4/BMPR1b controls LSC dormancy through the Jak2/Stat3 signaling in their supportive niche.Importantly CML treatments are able to increase BMP4 production within the 3D BM-model. Surprisingly, TKIs and Asciminib, are increasing LSC dormancy suggesting that LSC persistence within the BM is not only related to BCR-ABL effect but also relies on the BMP-signaling. Hence, deciphering key elements that support LSC dormancy and ecosystem dynamic upon CML treatment will give better clues on CML treatment escape and new insights to prevent CML patients' relapses.

Common anti-cancer therapies induce somatic mutations in stem cells of healthy tissue

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Introduction

Comprehensive analyses of genome-wide mutation profiles has revealed that specific anti-cancer drugs, particularly 5-fluorouracil (5-FU) and platinum-based drugs, are highly mutagenic to cancer cells of treated patients. The systemic administration of these chemotherapies presents a potential genotoxic hazard to healthy tissues, however, the true mutational impact on normal cells of patients is not known. The mutational impact of anti-cancer therapies on healthy adult stem cells (ASCs) is of great interest as ASCs are long-lived to support tissue renewal and are considered the cells-of-origin in cancer and secondary malignancies.

Material and Methods

We have developed a genome-wide approach to identify all somatic mutations acquired *in vivo* in single healthy ASCs by combining clonal expansion of individual ASCs via organoid technology with WGS of the expanded clones. Here, we applied this approach to identify genome-wide somatic mutations in morphologically normal colon or liver ASCs derived from 14 colon cancer patients that received CapOx treatment. This commonly used first-line chemotherapy treatment regimen includes the 5-FU prodrug CApecitabine and the platinum-based OXaliplatin.

Results and Discussions

Our findings reveal that CapOx chemotherapy is mutagenic in colorectal ASCs and significantly increase the mutational burden in normal non-cancerous cells beyond typical age-related mutation accumulation. Unlike colon, liver ASCs do not acquire such somatic mutations resulting from systematic CapOx treatment. The CapOx mutagenic effect in colorectal ASCs is chemo-drug dependent. Platinum-based treatment induced 500-1000 mutations, equivalent to 10 to 20 years of normal aging, in each colon stem cell in all treated patients. In contrast, 5-FU treatment resulted in completely different mutation contributions, with considerable inter- and intra-individual variation. In fact, most ASCs showed a complete absence of 5-FU-induced mutations.

Conclusion

Thus, while 5-FU and platinum drugs effectively kill cancer cells, they also increase the somatic mutational burden in long-lived normal stem cells responsible for tissue renewal and thus pose a potential source for developing secondary cancers.

A genetically engineered mouse model to study oncofetal CRIPTO in aggressive lethal prostate cancer

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Introduction

Prostate cancer is the second leading cause of cancer-specific death and the fifth leading cause of death in men Worldwide. Early stages are treated with surgery and androgen-deprivation therapy, but cancer can become castration-resistant (CRPC). Human tumors express high levels of CRIPTO, a protein that has been shown to have multiple oncogenic effects both *in vitro* and *in vivo*. We hypothesize that CRIPTO could be involved in tumor initiation and late progression of PCa and we aim to study its oncogenic role in a newly generated mouse model where CRIPTO is knockout in different background genotypes mimicking tumor progression (NY, NPY, and NPKY) or overexpressed in organoids derived from the aforementioned mouse models genotypes.

Material and Methods

NPY (Nkx3.1^{CreERT2}; Pten^{flox/flox}; R26^{LSL-YFP/LSL-YFP}) and NPKY (Nkx3.1^{CreERT2}; Pten^{flox/flox}; Kras^{LSL-G12D/+}; R26^{LSL-YFP/LSL-YFP}) were crossed with conditional *Cripto* (Cr) knock out (CRIPTO^{flox/flox}) to generate respectively NPCrY and NPKCrY. *In vivo* experiments presented the following workflow: 8-weeks old mice were castrated and induced after 1 month from the castration with 5 daily injections of tamoxifen, after 2.5 weeks animals were treated with testosterone weekly for 10 weeks. Organoids were derived; briefly single cells were isolated from the prostate tumors and the YFP+/- population recovered by FACS sorting and cultured as organoids.

Results and Discussions

NPY animals mimic the first stage of the disease, developing high-grade prostatic intraepithelial neoplasia (PIN)/carcinoma lesions with local invasive epithelium, while NPKY animals develop the more advanced stage of the disease with invasive prostate adenocarcinoma with lung and liver metastasis. As expected, PIN lesions were detected in NPY animals and NPCrY. The dominant pattern present in NPKY and NPKCrY tissue is adenocarcinoma (100%) with a very high invasive phenotype, interestingly NPKCrY further presents histological alterations of the stroma.

GEMM organoids recapitulate molecular features of the tissue of origin, expressing both luminal and basal markers. The morphology varies between different genotypes, NPY, NPCrY and NPK organoids present a solid structure that is consistent with an oncogenic transformation. NY organoids instead, present a cystic morphology, which is consistent with low-grade PIN phenotypes.

Conclusion

NPKCrY advanced stages seem to present stromal alterations, this might suggest a role for *Cripto* not only restricted to the epithelium. Further analyses are needed.

EACR22-0640

The developmental regulator SOX1 exerts an oncogenic activity in gastric cancer

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Introduction

Gastric cancer (GC) is the fourth leading cause of cancer death worldwide, with an average 5-year survival rate of 32%, being of 6% for metastatic cases. *Helicobacter pylori* (*H. pylori*) infection is the major risk factor for GC development, particularly in individuals infected by highly virulent Cytotoxin-associated antigen A (CagA)-positive strains. SOX1 is a developmental transcription factor that plays an essential role in the regulation of neural stem cells. SOX1 has been shown to act as a tumor suppressor in different cancer types such as liver and breast cancer or nasopharyngeal carcinoma, whilst our group revealed that it exerts an oncogenic role in glioblastoma. Nevertheless, the role and impact of SOX1 in GC remained unknown and we aimed at answering that unresolved question.

Material and Methods

We explored the expression and clinical impact of SOX1 in GC patient samples. In parallel, we evaluated the role of SOX1 in the oncogenic action of *H. pylori*, in the regulation of gastric cancer stem cells (gCSCs) and in tumor progression *in vitro* and *in vivo*. In addition, we developed transcriptomic and bioinformatic analyses to unravel the molecular mechanism of SOX1, and we identified the main potential effector of SOX1 action in GC. Finally, we functionally validated the involvement of this effector identified in the pro-tumoral activity exerted by SOX1.

Results and Discussions

We reported SOX1 overexpression in GC and association with poor outcome. We observed SOX1 induction in GC cells infected by *H. pylori*, particularly in response to CagA+ strains. Of note, SOX1 knockdown experiments demonstrated that SOX1 is required for the oncogenic action of *H. pylori* in terms of induction of proliferation, nuclear β -catenin accumulation and stemness. Moreover, SOX1 knockdown impaired GC cell proliferation, gCSC self-renewal, and tumor initiation and growth *in vivo*. Transcriptomics revealed downregulation of E2F targets in SOX1-silenced GC cells, while bioinformatic analyses identified E2F1 among the genes most correlated with SOX1 in GC. We also found that E2F1 silencing phenocopied SOX1 silencing in GC cells, and we demonstrated that the ectopic restoration of SOX1 in E2F1-silenced cells rescued the pro-tumoral phenotype, indicating that SOX1 and E2F1 establish an oncogenic axis in GC.

Conclusion

Our clinical and functional findings reveal for the first time the relevance of SOX1 in GC, and postulate the targeting of the oncogenic axis established between SOX1 and E2F1 as a promising therapeutic strategy in GC.

EACR22-0914

Identification of novel sorting strategy to enrich for LSCs in CD34negative AMLs

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Introduction

Acute Myeloid Leukemia (AML) is one of the most common and aggressive adult leukemia characterized by a high rate of relapse due to therapy resistant persisting cells. Leukemia Stem Cells (LSCs) have been suggested as the origin of relapse and indeed, while current chemotherapies can eradicate proliferating blasts, they fail to completely eliminate this subpopulation. To specifically target LSCs, a better understanding of their identity and biology is essential. Until now, no markers able to uniquely distinguish between malignant and healthy hematopoietic stem cells have been characterized. Indeed, since the discovery of LSCs, they have been identified as CD34⁺CD38⁻ cells, a phenotype highly similar to normal Hematopoietic Stem Cells (HSC). However, the usage of CD34 as a positive marker to isolate LSCs is particularly challenging in NPM1^{mut} AML (circa 20% of total AML cases) that have very low or absent expression of CD34. Over the years, additional markers have been identified as aberrantly expressed in AML, but their application for the identification and isolation of LSC is limited due to the genetic and associated phenotypical heterogeneity of AML.

Material and Methods

Primary diagnostic AML samples were FACS-sorted using Lineage Markers, CD34, GPR56, NKG2DLigands. Sorted fractions were functionally tested in vivo and transcriptomic signatures were retrieved using RNAseq.

Results and Discussions

Using a genetically harmonized cohort of DNMT3A^{mut}, NPM1^{mut} primary diagnostic AML samples, we suggest a novel FACS sorting strategy using positive selection for GPR56 and negative selection for NKG2DLigands to successfully enrich for LSCs among CD34⁻ cells. Enrichment for functional LSCs was confirmed by primary and secondary transplantation into immunocompromised mice. Transcriptional analysis revealed that CD34⁻ and CD34⁺DNMT3A^{mut}, NPM1^{mut} LSCs are transcriptionally and functionally similar suggesting high plasticity and lack of a hierarchy. Finally, by integration of functional in vivo data and genetic analysis of the FACS-sorted stem enriched fractions, we demonstrated that CD34⁺ subpopulations do typically contain also normal and pre-leukemic HSCs. In contrast, CD34⁻ enriched stem cells are devoid of normal cells, including HSCs and thus, LSCs can be isolated from the CD34⁻ population with great purity.

Conclusion

In conclusion, we are suggesting a novel sorting strategy to enrich for LSCs with high purity, allowing more thorough molecular and phenotypical characterization of those therapy-resistant LSCs.

EACR22-0924

Dual inhibition of Sam68 and Rad51 as a synthetic lethal therapeutic strategy to target breast cancer stem cells

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Introduction

Despite the remarkable progress achieved in the treatment of localized disease, breast cancer (BC) is the most common malignancy and the second leading cause of cancer-related death in worldwide female population. Compelling evidence showed that a small subset of cells with stem-like properties, named cancer stem cells (CSCs), is spared by standard therapies and, thus, responsible for relapse and metastatic dissemination. CSC refractoriness is sustained by high expression of ABC transporters and antiapoptotic molecules, and a proficient DNA-damage repair machinery. Thus, the possibility to interfere with DNA repair pathways is a potential therapeutic strategy for BC treatment. Indeed, BCs harboring *BRCA* mutation or other dysfunctions compromising the homologous recombination (HR) repair machinery, a phenotype called BRCAness, benefit from the use of PARP inhibitors.

Material and Methods

Breast CSCs (BCSCs) were purified from serially transplanted patient-derived xenografts (PDX). This model heightened the frequency of stem-like cells and mirrored intratumor heterogeneity, representing a good model for drug testing.

Results and Discussions

Cells isolated from PDX, obtained from luminal and basal BC samples, displayed an enrichment in stem-like CD44^{high}/CD24^{low} subpopulation, characterized by high expression levels of Myc. A Chip-seq analysis on normal epithelial breast cells over-expressing Myc and BCSCs showed that Myc binds to and activates the promoter of Sam68, a multifunctional DNA/RNA binding protein involved in DNA damage response interacting with PARP1. Although Sam68 downregulation impairs PARP-induced PAR chain synthesis upon DNA-damage, chemotherapeutic agents did not reduce BCSC viability, due to Rad51 upregulation and increased HR activity. Interestingly, Rad51 inhibition by using small molecule or a CDK inhibitor in combination with Sam68 silencing hindered the *in vitro* and *in vivo* growth of BCSCs, displaying a “synthetic lethal” effect. This therapeutic strategy is also effective in BCs that relapse after endocrine therapy. Finally, the analysis of a large cohort of BC patients highlighted that a *MYC*^{high}, *SAM68*^{high} and *RAD51*^{high} signature is associated with worse relapse-free survival.

Conclusion

Taken together these data suggest that all molecular subtypes of BCs harbor a subpopulation of therapy-resistant stem-like cells, characterized by a *MYC/SAM68/RAD51* signature, and that the double targeting of Sam68-PARP1 axis and Rad51 impairs the expansion of aggressive BCSCs.

EACR22-1060

Targeting tumor cell dormancy as a new strategy to fight cancer

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Introduction

Tumor cell dormancy is a process whereby cancer cells enter a reversible cell cycle arrest or quiescence. This quiescent state allows cancer cells to survive chemotherapy treatments, leading to chemoresistance and relapse after an apparent remission of the disease. We developed a strategy that allows the identification and isolation of slow-cycling cancer cells (SCCCs) using the inducible expression of the histone 2B fused to the enhanced green fluorescent protein (H2BeGFP). By using gene expression microarrays we identified HIVEP2

as one of the highest overexpressed genes in SCCCs compared to rapid-cycling cancer cells (RCCCs).

Material and Methods

We created HIVEP2 knock-down (KD) and knock-out (KO) colorectal cancer cell lines using shRNA and CRISPR/Cas9 technology respectively. Then, we performed self-renewal capacity, H2BeGFP dilution, *in vitro* chemoresistance, and *in vivo* tumor growth experiments to check whether the modulation of the gene expression may alter the dormant status of the cells. For the overexpression, we are attempting two alternative options: introducing the cDNA exogenously or overexpressing the endogenous HIVEP2 by using the CRISPR/dCas9 activation technology. In addition, we have performed RNAseq from organoids derived from the models mentioned above.

Results and Discussions

Modulation of HIVEP2 expression showed its involvement with the self-renewal capacity of tumor cells, as well as resistance to chemotherapies both in RCCCs and SCCCs subpopulations. *In vivo* experiments with HIVEP2 KD tumor cells revealed an earlier tumor initiation capacity, a slight increase in tumor volume as well as a better response under irinotecan treatment. The forthcoming RNAseq results of our models will shed light on the mechanisms in which HIVEP2 is involved. This data will provide us with valuable information in order to elucidate its role in tumor cells focusing on tumor cell dormancy.

Conclusion

The discovery of HIVEP2 as a new potential key factor in tumor cell dormancy will help to understand the whole process and identify other key elements ruling this phenomenon. At the same time, a comprehensive understanding of the role of HIVEP2 can trigger a drug development process so one day it can reach the clinics and offer new therapeutic options for cancer patients with tumor relapse.

EACR22-1134

Sox4-mediated acinar cell plasticity in pancreatic regeneration and cancer initiation.

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Introduction

The pancreas is composed principally of secretory acinar cells. Upon acute inflammation of the pancreas, acinar cells reprogram and activate a molecular program enabling pancreas regeneration. However, somatic mutations in the Kras proto-oncogene prevents pancreas regeneration and induce neoplastic transformation of acinar cells, which develop into precursor lesions of pancreatic cancer (PanINs). We aim to unravel non-mutated genetic determinants of cancer initiation which are i) transcription factors, ii) de novo expressed upon tissue injury and iii) conserved in mouse and human acinar regeneration and transformation.

Material and Methods

We used the Ptf1aCreER mice to conditionally drive the expression of YFP, KrasG12D and delete Sox4 in adult pancreatic acinar cells. Tissue injury is induced by caerulein. Analysis

for reprogramming and transformation is, respectively, 2- and 21-days post-injury. Human primary exocrine explants were cultured in suspension and mimic human acute pancreatitis. Human pancreatic cancer samples were analysed. We used RNA in situ hybridisation, qRT-PCR and immunohistochemistry to unravel and validate our findings.

Results and Discussions

Sequencing data analysis defined Sox4 as significantly induced in acinar cells upon tissue injury in mouse and human. Conditional deletion of Sox4 in acinar cells upon induction of acute pancreatitis resulted in the upregulation of markers of neoplastic transformation (Junb, Krt19, Onecut2, Klf5), decrease of proliferation (ki67) and formation of well-defined lesions when compared to Sox4 wild type pancreata. GSEA defined a significant increase in Kras, Tgf-beta, Tnfa-via-NFkb and Il6-Jak-Stat3 hallmarks. Furthermore, pancreatic fibroblasts acquired a aSMA+ myofibroblast phenotype. However, refinement of the pancreas was not altered, and the pancreas reacquired normal histological and molecular features. Upon induction of cancer initiation by tissue injury and KrasG12D expression in acinar cells, deletion of Sox4 resulted in the absence of PanINs composed of mucin-producing and Dclk1+ tuft-like cells, but appearance of serous-like cysts. The stroma is less desmoplastic concomitant with a significant decrease in sonic hedgehog expression. We will combine single nucleus RNA sequencing and 3D tissue-clearing to get insights into intercellular crosstalk.

Conclusion

These findings will open avenues to restrain autonomous processes of cellular transformation and epithelial-derived signalling to alleviate desmoplasia.

EACR22-1198

Growth Factor Regulation of Castration-Tolerant Luminal Progenitors of the Mouse Prostate

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Introduction

Castration-resistant prostate cancer (CRPC) is a lethal disease. Once diagnosed, current therapeutic strategies do not improve overall survival more than a few months. The molecular and cellular mechanisms that drive cancer relapse remain poorly understood, which has prevented the development of efficient therapies for CRPC.

Our laboratory recently identified a population of castration-tolerant luminal progenitors that we called LSC^{med} according to their Lin⁻/Sca-1⁺/CD49f^{med} FACS profile. LSC^{med} cells are rare in WT mouse prostates, but are massively amplified in premalignant prostates driven by prolactin/STAT5 signaling (Pb-PRL mice), and even more in prostate cancer driven by loss of *Pten* tumor suppressor gene (*Pten*-null mice, a model of CRPC). This suggests that LSC^{med} cells contribute to pathological overgrowth of prostatic tissue. The goal of this study was to identify potential regulators of LSC^{med} progenitor ability.

Material and Methods

We first used *in silico* approaches. Comparative transcriptome analysis of LSC^{med}, luminal and basal cells FACS-enriched from WT mice was performed in combination with an

interactome study (CellPhone DB). Expression of the *in silico*-identified actors were confirmed by RT-qPCR. We then challenged the effects of these ligands on LSC^{med} cell progenitor and proliferative abilities using the organoid assay.

Results and Discussions

The *in silico* study highlighted expression of several genes associated with EGFR, IGF-1R and c-Met pathways and their potential interactions, respectively. These growth factor signaling pathways have been shown to regulate stemness and proliferation in various contexts including cancer. The expression of the EGFR, IGF-1R and c-Met in LSC^{med} cells sorted from WT, Pb-PRL and *Pten*-null mouse prostates was confirmed by RT-qPCR. As expected, EGFR signaling was mandatory for organoid formation by *Pten*-null LSC^{med} cells. Remarkably, our results show that IGF-1/IGF-1R and HGF/c-Met signaling pathways are able to rescue organoid formation and growth in the absence of EGF in culture medium, suggesting redundancy.

Conclusion

While EGFR-targeting therapies have been proposed to affect cancer stem cells, our ongoing experiments using natural ligands and pharmacological inhibitors suggest that alternative pathways may overcome EGFR signaling blockade during castration-resistant prostate cancer progression.

EACR22-1208

Establishment of an *in vitro* preclinical platform: patient-derived organoid models with lung and breast cancer.

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Introduction

Heterogeneity in cancer is regarded as one of the most substantial roadblock in the development of effective patient-specific therapies. Patients often present diverse tumor phenotypes that dynamically evolve throughout disease progression and clinical treatment. This heterogeneity within and across patients often explains widespread patient therapeutic responses. In this context, patient-derived organoids (PDOs), 3D structures composed of epithelial cells, are changing our understanding of cancer heterogeneity and its implications for personalized medicine. Our aim was to establish and characterize a PDO platform of lung and breast cancer as preclinical tool to research the molecular and cellular mechanisms underlying both tumors.

Material and Methods

Surgical resections were collected from patients diagnosed with lung or breast cancer. Organoid generation from patient material were divided in three main steps: 1) mechanical fragmentation of tissue pieces; 2) digestion of fragments into single-cell suspension; and 3) plating of cell preparations into matrigel domes to mimic the extracellular matrix. Organoids were passaged every 7–21 days based on confluency. Each newly organoid line was cryopreserved for further expansion.

Results and Discussions

We have successfully achieved an outgrowth efficiency of 70% for both normal PDOs obtained from tumor-adjacent epithelium and tumor PDOs from patients diagnosed with lung

cancer. In the case of breast cancer patients, the efficiency was slightly lower and was set around 50% of tissue samples.

Characterization of PDO cultures is essential to validate its predictive potential. To assess that PDOs represent the tumor of the patient, for each organoid line, expression levels of the main histological markers were evaluated and correlated with the tissue of origin. In parallel, we have performed immunofluorescence on paraffin-embedded organoid samples with routinely used cancer stem cell markers.

Conclusion

PDOs represent a superior preclinical system compared to previous models due to their inherent heterogeneity, long-term stability, applicability for high-throughput screens and enhanced capacity to capture tumor characteristics. Therefore, the implementation of a well-annotated patient-derived organoid biobank will be of great interest for drug discovery and personalized therapy. Understanding the sources and implications of tumor heterogeneity will undoubtedly improve our evolving definition of cancer and aid in the design of effective patient-specific treatment strategies.

EACR22-1224

The Effect of Ascorbic Acid Treatment on Osteosarcoma Stem Cells Grown in the presence and absence of FGF2

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Introduction

Osteosarcoma stem cell (Os-CSC) are mesenchymal origin stem cells derived from patient biopsies. The selection of stem cell population is achieved by spheroid growth in restrictive conditions through two generations. The essential component for their growth is the addition of fibroblast growth factor 2 (FGF2) that acts by blocking differentiation and promoting proliferation.

Material and Methods

The purpose of this study is to eradicate osteosarcoma stem cell population responsible for disease recurrence. According to previous *in vitro* studies, ascorbic acid (AA) can induce apoptosis of Os-CSC. However, the mechanism of action is still unclear. AA could induce differentiation of Os-CSC and collagen synthesis as well as it can interact with metabolism. As FGF2 is the differentiation blocker, we wanted to test the effect of AA with and without FGF2. Therefore, we performed MTT assay and calculated IC₅₀ values. Gene expression analysis for stemness and differentiation markers was also performed. Bioenergetic profiling of examined cell lines was performed using Seahorse XF Cell Energy Phenotype Test Kit. Metabolic changes were confirmed by Western analysis of GAPDH level.

Results and Discussions

The IC₅₀ value for HEK, U2OS and hMSC cells ranged from 15.26 mM to 25.63 mM AA, while the IC₅₀ value for Os-CSC ranged from 3.5 mM to 10.5 mM AA without FGF2 and 5.5-11 mM with FGF2. The presence of FGF2 blocked the collagen and alkaline phosphatase expression. However, the with AA restored AP expression. FGF2 did not affect basal metabolism of Os-CSC, while AA treatment increased it. Other cells showed slightly higher basal metabolic activity. Stressors increased OCAR while there was a slight increase ECAR in Os-CSC. Os-CSC have more quiescent metabolism, which is activated by treatment with

AA. The complete depletion of GAPDH expression in Os-CSC treated with AA in the absence of FGF2 was observed.

Conclusion

This study examined the effect of AA on Os-CSC isolated from tumour tissue samples and grown in the presence and absence of the FGF2. The presence of FGF2 makes Os-CSC less sensitive to AA treatment. FGF2 does interfere with collagen synthesis as well as metabolic phenotype so it is important to elucidate further the interference with AA.

EACR22-0011

Determination of oncosomes ability to transform cells: a new prognostic tool?

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Introduction

Colorectal Cancer (CRC) is the 4th leading cause of cancer-related death world-wide. Mortality has been steadily decreasing since the 1990s but relapse occurs within 2 years of curative resection and seems to be related to minimal residual disease. A major cause of these relapses is the presence of **Cancer Stem Cells** (CSC); resistant to conventional therapies but also hard to isolate and characterize. Cancer cells can communicate by extracellular vesicles (EV) that allow them to transfer aggressiveness. The EV content differs according to the cell of origin, therefore EV from CSC are probably more aggressive than those from differentiated cells. A new approach based on **electromagnetic signature** characterization has been optimized the last few years. This lab-on-chip consists in measuring the crossover frequency of individual cells by dielectrophoresis (DEP) technic in the ultra-high frequency (UHF) range. The lab-on-chip was previously used to analyze glioblastoma cells, we transferred the tool to CRC and tried to determine if a transfer of aggressiveness by EV could be detected by this way.

Material and Methods

Our models are two CRC cell lines from the same patient: SW480 and SW620. The cells were cultured in a conventional medium to obtain differentiated cells or in a define medium to obtain CSC enrichment. The cell types were characterized for the expression of stem-related genes by RT-qPCR and analyzed by the lab-on-chip. EV from cells were obtained by a multistep ultracentrifugation protocol and used to treat target cells to test the transfer of aggressiveness.

Results and Discussions

The DEP characterization of each population individually demonstrated a significant difference between the electromagnetic signatures of the different cell populations: CSC showed a lower crossover frequency that can be correlated with differential stemness markers. After treatment by EV from SW620-CSC on differentiated SW480 cells, electromagnetic signatures of cells were analyzed. It resulted in a decrease of the crossover frequency which tends to that of the SW480-CSC. This decrease is correlated with an overexpression of some stem-related genes.

Conclusion

Our lab-on-chip is enough sensitive to discriminate different cell types and can detect changes caused by a treatment with EV. Because the lab-on-chip uses unlabeled cells, the detection of CSC can be faster and easier than conventional techniques. This approach will

be useful on liquid biopsies to detect a potential tumor relapse and the care and support of patients.

EACR22-0197

Fatty acid synthase as a new therapeutic target for HER2 positive gastric cancer

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Introduction

Gastric cancer (GC) is the fourth leading cause of cancer-related death worldwide. HER2 overexpression/amplification is found in less than 20% of GC cases. Its remarkably heterogeneous intra-tumor expression coupled with the frequent co-existence of other alterations poses clinical challenges. Even if the humanized antibody trastuzumab (T) improved the clinical outcome of metastatic HER2+ GC, the high rate of therapy resistance sustains the need to refine the knowledge of HER2 biology. GC stem cells (GCSCs), a minor subset of neoplastic cells with stem-like features, are emerging as a key target to counteract tumor growth, aggressiveness and therapy resistance. The survival advantage imparted to CSCs by metabolic plasticity suggests that targeting crucial metabolic hubs, as the fatty acid synthase (FASN), the key enzyme involved in fatty acids (FAs) *de novo* biosynthesis, may be clinically relevant.

Material and Methods

The protein expression and functional role of FASN was examined by WB and FACS assays; its transcript was examined by qRT-PCR and GEP analyses in T-sensitive OE19 and N87 and T-resistant OE33 HER2+ GC cell lines, and in the patient-derived GTR0455 cells, cultured in adherent (2D) or gastrosphere promoting (3D) conditions. Molecular data were analyzed *in silico* in two public HER2+ GC datasets. The effectiveness of the specific FASN inhibitor TVB3166 to overcome anti-HER2 therapy resistance was tested *in vitro* in gastrospheres forming efficiency bioassays and *in vivo* in GTR0455-bearing mice.

Results and Discussions

To identify new biomarker(s) regulating HER2+ GCSC activities, we compared the transcriptome profiles of N87 and OE19 cells cultured in 2D vs 3D conditions finding a significant enrichment of FAs metabolism-related genes, including FASN, in 3D cell models. Consistently, its enrichment in HER2+ GC cases significantly correlated with a high stemness score and poor prognosis. We also observed that TVB3166 treatment significantly decreased the number of GCSCs in all tested GC cells. Additionally, our data evidenced that the pharmacological dual blockade of HER2 and FASN significantly decreased the capability to form gastrospheres vs monotherapy, and reduced the *in vivo* growth of GTR0455 tumors.

Conclusion

Overall, our findings sustain the possibility of combining FASN and HER2 inhibitors to improve the clinical outcomes of HER2+ advanced GC patients.

Dedifferentiated esophageal cells constitute a reservoir for esophageal pre-cancerous metaplasia

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Introduction

Esophageal adenocarcinoma (eAC) is a cancer with a poor prognosis which incidence has been multiplied by 4 in USA over the past 4 decades. Esophageal adenocarcinoma is the consequence of a multistep process in which chronic gastroesophageal reflux disease progressively induces esophagitis, Barrett esophagus (BE), dysplasia and finally eAC. While columnar metaplasia of the esophagus may show transitional, gastric or intestinal differentiation, BE is defined by the replacement of the squamous epithelium by an intestinal-like epithelium. Several studies have shown that cells from the squamo-columnar junction (SCJ) are competent to form BE-like metaplasia and eAC *in vivo* while esophageal cells are not. However, some columnar metaplasia and eAC can be surrounded by squamous cells, far from the SCJ, thus suggesting they may originate from squamous cells.

We recently showed that the hedgehog (HH) pathway is activated in the esophageal cells upon chronic acid reflux. When activated, the HH pathway triggers dedifferentiation of esophageal cells and the appearance of columnar cells *in vivo* but no sign of specialized columnar epithelium. Interestingly, recent multi-omics data suggest that human metaplasia and eAC may originate from a pool of undifferentiated cells. HH-dedifferentiated cells could thus be a reservoir for columnar metaplasia and/or eAC initiation.

Material and Methods

In this project, we analyzed publicly available copy number variation data in human eAC and developed a new transgenic mouse model to mimic gene amplification in the esophagus. We followed mutated esophageal cells by lineage tracing and characterized them by histology and RNA sequencing.

Results and Discussions

GATA4 is a transcription factor necessary and sufficient for columnar epithelial morphogenesis in the stomach. While it is absent from normal esophagus, we showed that it is overexpressed in human BE samples in 5 independent studies. Moreover, GATA4 is amplified in 13% of eAC from the *The Cancer Genome Atlas* database. Hence, GATA4 may be a good candidate to drive columnar metaplasia and/or eAC. Our data show that GATA4 ectopic expression in esophageal cells has virtually no phenotype. Nevertheless, when expressed in HH-dedifferentiated esophageal cells for 10 days, GATA4 induces a tissue reorganization and a transcriptomic profile that resemble the one from metaplastic cells although they show no sign of intestinalization.

Conclusion

Our data suggest that HH-dedifferentiated cells may constitute a reservoir for metaplasia in the esophagus.

Targeting of DNMT3B in the plasma cell cancer multiple myeloma disturbs cancer cell growth and clonogenicity and boosts the anti-tumor activity of the proteasome inhibitor bortezomib

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Introduction

Multiple myeloma (MM) is an incurable plasma cell cancer due to the development of drug resistance (DR). Epigenetic defects are well-known to play a major role in MM progression and relapse. However, the role of most epigenetic modifiers (epiplayers) in MM cell DR development remains unknown. Using RNASeq data from the MMRF CoMMpass study, we found that the epiplayer DNMT3B is significantly increased in the relapsed setting, suggesting a role in MM relapse. Here, we explored the role of DNMT3B in MM cell biology and drug response.

Material and Methods

Publicly available gene expression profiling data was used to analyse DNMT3B expression in MM cohorts. The DNMT3B inhibitor Nanaomycin A (NA) and inducible shRNA lentiviral vectors were used to specifically target DNMT3B. Viability and apoptosis were assessed using a CellTiter-Glo assay and AnnexinV/7AAD staining. Cell proliferation was measured by BrdU incorporation and cell cycle analysis, while the clonogenic capacity was evaluated by a colony formation assay.

Results and Discussions

DNMT3B mRNA levels increase during disease progression, correlating with a worse disease outcome in both newly diagnosed and relapsed patients, thus further indicating a role for DNMT3B in MM progression and DR. In line, NA treatment led to a significant and dose-dependent decrease in cell viability and proliferation and increase in apoptosis in the XG-2, XG-7 and AMO-1 human cell lines. We also validated the anti-MM activity of NA on primary human MM cells. Next, the effect of DNMT3B targeting on clonogenicity was evaluated. A significant and dose-dependent decrease in the number of colonies was observed when low doses of NA were added to AMO-1 (100 and 200 nM) and XG-2 (30 and 50 nM) cells on the day of plating, but not when added 7 days after plating. In contrast, treatment with a high dose (800 nM) significantly reduced the number of colonies at both timepoints, indicating that high doses of NA are cytotoxic whereas low doses impair MM cell proliferation and clonogenicity. The anti-clonogenic effect of DNMT3B targeting was also confirmed by DNMT3B knockdown in AMO-1 cells. Finally, combining NA (100 nM) with bortezomib (Bz, 4 nM) resulted in a significant decrease in colony formation compared to both single agents.

Conclusion

Together, our findings indicate that DNMT3B is a novel promising target to overcome or delay relapse in MM. In the near future, the anti-myeloma activity of DNMT3B targeting will be validated *in vivo* and the underlying mechanisms will be further determined.

EACR22-0387

Role of the thyroid hormone nuclear receptor TR α 1 in the plasticity of the colon cancer stem cells Diana FARHAT and Michelina PLATEROTI Université de Strasbourg, Inserm, IRFAC/UMR-S1113

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Introduction

Thyroid hormones (THs) act via the thyroid hormone nuclear receptors TRs and control the intestinal homeostasis through complementary Wnt, Notch and BMP signalling pathways. Previous studies suggested that TR α 1 perturbation could lead to the deregulation of these pathways and participates in tumour initiation and progression through an action on progenitors and on stem cells (SCs). Therefore, the aim of our project is to define in detail the cellular and molecular properties of the TR α 1-expressing cells by using the TR α 1-GFP mice, where the GFP reflects the expression of the TR α 1 protein in a WT or an Apc^{f/+} background.

Material and Methods

In order to map GFP/TR α 1 expression within the different cell populations, we are currently performing several approaches:

-A co-immunolabeling for GFP and proliferation (Ki67), differentiation (epithelial cell types) and SC/CSC markers (EPHB2, CD44).

-A double sorting for GFP and EphB2 (high and low populations) by using the TR α 1-GFP/Apc^{f/+} mice with or without T3 treatment to:

1) Analyze the stem potential of sorted populations from normal intestine by 3D organoid cultures and to 2) Analyze comparatively by single cell RNA-seq the SC/CSC or progenitors expressing TR α 1 in normal intestine or in cancers.

Results and Discussions

After performing the co-immunolabeling for GFP/TR α 1 and multiple stem cell and proliferation markers, we could demonstrate that TR α 1 is expressed in the progenitors and stem cells. Furthermore, we could validate the high stem potential of GFP/EphB2 high population in comparison with the other sorted populations with less TR α 1 expression. Recently, we performed sc-RNA-seq analysis in normal colon cells (bioinformatics ongoing) and started analyzing cell clusters in control and TH conditions.

Conclusion

Our results showed for the first time a new stem cell sub population characterized by its high TR α 1 expression. Furthermore, TR α 1 could increase the stemness and consequently the carcinogenesis.

EACR22-0465

Study of S100A9 as a new molecule for the diagnosis and treatment of advanced breast cancer

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Introduction

Breast cancer was the most diagnosed malignancy and the main cause of death among women in 2020. Although the early-stage disease is associated with a good prognosis, **metastatic advanced cases are considered incurable with current therapy options**. Therefore, there is an urgent need for a better understanding of metastatic breast cancer to find novel targets that could improve treatment.

The **calcium-binding protein S100A9** has been highlighted as a potential target for cancer treatment. Our group described that S100A9 is **overexpressed in HER2-positive tumors**. Moreover, S100A9 has been associated with a lower overall survival and treatment resistance. S100A9 is a soluble molecule that is secreted and can be detected in patients' blood. Although in breast cancer **the value of S100A9 as a biomarker is still premature**, S100A9 has been proposed as a biomarker in colon and prostate cancer. Our preliminary data evidence the **role of S100A9 in the acquisition of stem properties**. While its role in stemness is largely unknown, it is known that **S100A9 plays a key role in tumor immune microenvironment regulation** as it intervenes in the reprogramming of the tumor microenvironment into an immunosuppressive state.

Material and Methods

The non-tumorigenic human mammary epithelial cell line **MCF10A** and its overexpressing HER2 and S100A9 transformed cell lines (**MCF10A-HER2 and MCF10A-S100A9**) were used for the in vitro assays (flow cytometry, tumorsphere formation, western blot...). Moreover, **human plasma samples** of a cohort of breast cancer patients diagnosed in the University Hospital of Bellvitge were analysed using a S100A9 ELISA commercial kit.

Results and Discussions

Regarding S100A9 role as a biomarker, we have observed how **its levels in plasma were higher in the more aggressive breast cancer subtypes** and correlated with lower survival.

Concerning the role of S100A9 in stemness, we have seen how breast cancer lines with higher S100A9 intrinsic levels presented an **increase in tumorsphere formation and a higher CD24-, CD44+ and ALDH+ population** which are stem-like properties.

Finally, our research pointed out that S100A9 produced by tumoral cells could **modulate not only the innate immune response but also adaptative immunity**.

Conclusion

Our results point to **S100A9 as a potential key molecule in stemness, tumor immunomodulation and metastasis in breast cancer**. Thus, this project could have a great impact on increasing the knowledge on S100A9 role in breast cancer as a potential biomarker and treatment target.

EACR22-0487

Overexpression of adaptor protein Ruk/CIN85 in 4T1 breast cancer cells results in substantial dedifferentiation associated with attenuation of vitamin D3-dependent signaling

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Introduction

Vitamin D₃ is known to be involved in the control of differentiation of both normal and cancer cells. Our previous studies demonstrated that overexpression of adaptor protein Ruk/CIN85 in breast cancer cells resulted in potentiation of their malignant properties and cancer stem cells features. The aim of present study was to investigate the effect of Ruk/CIN85 overexpression on the expression of vitamin D₃ auto/paracrine system components and molecular markers of differentiation in breast cancer cells.

Material and Methods

Mouse breast adenocarcinoma 4T1 cells with stable overexpression of Ruk/CIN85 (RukUp) and control cells were used in this study. Gene expression was studied using RT²-PCR. Protein content was estimated using Western-blotting.

Results and Discussions

It was found that both mRNA expression and protein content (in the nuclear fraction) of vitamin D₃ receptor (*Vdr*) decreased considerably in RukUp cells. Additionally, *Rxr* (retinoid X receptor) expression decreased threefold compared to control. We demonstrated that overexpression of Ruk/CIN85 in 4T1 cells correlated with decreased expression of *Cyp27b1*, responsible for calcitriol synthesis, and increased expression of *Cyp24a1*, responsible for calcitriol degradation. Consequently, these data suggest that RukUp cells are characterized by decreased formation of both biologically active 1,25(OH)₂D₃ and its receptor, that may be potentially associated with decreased level of 4T1 cells differentiation. In order to prove this hypothesis, we analyzed expression of epithelial molecular markers. It was found that in RukUp cells mRNA expression of *Cdh1*, *Tjp1*, *Icam1*, *Fn1*, *Cfl1*, *Itgb1* was significantly decreased in comparison to control. Also, we found that various dedifferentiation molecular markers, such as *Cd44*, *Nanog*, *Klf4*, and *Id1* were overexpressed in RukUp cells.

Conclusion

Thus, overexpression of adaptor protein Ruk/CIN85 in breast cancer cells results in their dedifferentiation associated with diminished vitamin D₃ signaling.

EACR22-0557

A CD10-related pre-malignant stem-cell signature to identify high-risk patients in a broad range of solid cancers

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Introduction

Current molecular signatures have a limited predictive value often depending on the tumors they originate from. Here, we reveal a pre-neoplastic molecular signature identified from non-transformed CD10-expressing stem cells. The CD10 enzyme is overexpressed in several cancers and linked to stem cell features regardless of transformation.

Material and Methods

We used the MCF10A mammary stem cell line as well as models of early luminal transformation from long-term exposure of MCF10A cells to BMP2. By gain and loss of function, we tested the role of CD10 in stemness and transformation processes. A transcriptomic analysis identified a molecular signature upregulated in CD10⁺ MCF10A cells that was tested for its ability to predict tumor aggressiveness and response to treatment.

Results and Discussions

The “CD10 signature” is enriched in genes involved in sister chromatid segregation and asymmetric division and their expression is dependent on CD10. Remarkably, tissues

predisposed to transformation (naevus, BRCA mutated breast cells) already have an altered CD10 signature expression (CD10 score) compared to normal tissues. Using models of early luminal transformation, we showed that CD10 still marks cells with stem cells properties and that the CD10 score increases in early transformed cells. Breast tumors have a high CD10 score compared to normal breast and high CD10 scores correlate with poor survival, even in early stage. In addition, the CD10 score predicts sensitivity of breast cancer cell lines to drugs in use or in development. We then extended this analysis to other types of solid cancers. In various solid tumors, we observed an enrichment of the CD10 score in tumor tissues compared to paired healthy tissues. The increased risk gradient observed in a pan-cancer Cox model highlighted a correlation between CD10-score and survival. Interestingly, the CD10 score predicts survival at early stages of very aggressive cancers like pancreatic adenocarcinoma, lung adenocarcinoma or mesothelioma. In addition, the CD10 score is correlated with the sensitivity to drugs, including several PARP inhibitors.

Conclusion

In summary, we identified a novel molecular signature related to the CD10 function on stem cell and representative of pre-malignant cells. Altogether, our analyses demonstrate that the CD10-score is linked to cancer evolution and patient survival and may also contribute to identifying efficient therapies in patients in a broad range of cancers.

EACR22-0563

Targeting cathepsins B and X as new approach to improve cancer stem cells' directed therapy

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Introduction

Despite advances over the past years the effectiveness of cancer treatment is limited by development of resistance and cancer recurrence. Within heterogenous cell populations in tumors, drug resistance and tumor relapse emerge due to the presence of small population of cancer stem cells (CSCs) that are resistant to the most conventional antitumor therapies. Therefore, establishment of new drugs and therapeutic approaches that would, in addition to differentiated cells, be effective also towards CSCs is needed. In this study we evaluated cathepsins B and X as possible new targets to direct antitumor therapy towards CSCs. Lysosomal cysteine cathepsins B and X are well-established targets in different types of cancer and participate in various stages of development and progression of cancer including invasive tumor growth and metastasis formation. Cathepsins have been recognized as an important part of dynamic response in anticancer therapy that can be selectively regulated at multiple levels, including specific small molecular inhibitors.

Material and Methods

We isolated CSCs cell lines based on their ability to form tumorspheres and used western blot, ELISA, qPCR and enzyme kinetics activity to evaluate expression and activity of cathepsins B and X in CSCs. Next, selective, reversible small molecular inhibitors of cathepsins B and X were used to test the role of cathepsins B and X in cancer stem cells alone or in a combination with conventional chemotherapeutics.

Results and Discussions

We demonstrated that cathepsins B and X are present in higher levels in CSCs following tumorsphere formation compared to single adherent differentiated tumor cells. Cathepsins B and X could therefore serve as molecular targets able to enhance CSCs directed therapy.

Furthermore, our results reveal that inhibition of cathepsins B and X affects cells with CSC phenotype resulting in decreased expression of stemness markers and markers of mesenchymal cell phenotype. Cathepsins' inhibition increased differentiation of CSCs, thus improving the effectiveness of the conventional chemotherapy.

Conclusion

Taken together, our results define cathepsins B and X as suitable targets for CSCs directed antitumor therapy providing new a new approach that could serve to address main challenges in cancer treatment with the goal to improve effectiveness and overcome limitations of current antitumor therapy.

EACR22-0625

Identifying KRas mutant cell mechanisms to overcome cell competition and promote disease in pancreatic cancer initiation

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is among the deadliest human cancers (10% 5-year survival). The main factor is the late diagnosis of the disease; therefore, understanding early PDAC has great potential to develop new tools for cancer detection and prevention.

Activation of oncogenic KRAS is the first genetic event occurring, and it is associated with the appearance of pancreatic intraepithelial neoplasia (PanIN), driving the development of the disease. Additionally, alterations in other genes, such as TP53, are accumulated during tumour development.

Through cell competition genetically different cells compare 'relative cell fitness' establishing a loser-winner relationship in which 'loser' cells are eliminated. We demonstrated that when KRas is mutated in few cells within the pancreatic epithelium, they are removed from the tissue through cell competition; however, if cells are mutant for KRas and Tp53, they remain promoting PanINs and, eventually, PDAC.

Our aim is to understand how mutated p53 confers a competitive advantage to cells expressing KRAS mutations, allowing them to avoid cell elimination and remain in the tissue.

Material and Methods

We used the Pdx-1Cre^{ER};KRas^{G12D/+};TP53^{R172H/+};Rosa26^{LSL-RFP} mouse model to generate the genotypes KRas^{G12D/+}(KC) and KRas^{G12D/+};TP53^{R172H/+}(KPC). Adult mice were treated with low dose tamoxifen and tissue was collected 5 weeks post induction, when cell competition has occurred. We studied mutant cell dynamics through confocal imaging and RNA sequencing.

Results and Discussions

Our RNAseq data showed that both KC cells that remain in the tissue and KPC cells, dysregulate cell adhesion, cell migration, antigen presentation and differentiation transcriptional signatures; suggesting that KRas mutant cells de-regulate these pathways to avoid cell competition and elimination. However, some KC specific signatures could be found such as gastric/duodenal stem cell signatures, disruption of adherens junctions and downregulation of NFkB pathway, indicating that overcoming cell elimination also promotes some fate changes.

To link migratory signatures to a phenotype, we studied cell morphology and found that in both KC and KPC tissues two different populations can be identified. Our next objective is to identify if one of these populations is more likely to give rise to PanINs and tumors.

Conclusion

To overcome cell competition and elimination in the pancreatic epithelium KRas mutant cells downregulate cell adhesion molecules and acquire stem-like characteristics.

EACR22-0651

Extracellular vesicles from COPD patients modulate stem cell phenotype through HIF-1 α shuttle

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Introduction

Cigarette smoking is the main risk factor for the development of chronic obstructive pulmonary disease (COPD) or the onset of lung cancer. In COPD patients the lung microenvironment is characterized by inflammation and air flow reduction. It has been shown that the reduced air flow in COPD patients promotes the activation of HIF-1 α pathway that was associated to a reduction in lungs functionality and increased risk to develop lung cancer. The subset of CD133+ cancer stem cells (CSC) was demonstrated to be responsible for lung cancer initiation. Cells co-expressing CD133 and CXCR4 are defined as the subpopulation of metastasis initiating cells (MIC). Recently, the involvement of extracellular vesicles in the pathogenesis of lung diseases gained attention. The aim of this project is to evaluate the pro-tumorigenic role of EVs from COPD patients in the development of lung cancer, exerted through the modulation of CSC subsets phenotype.

Material and Methods

EVs were isolated by ultracentrifugation from plasma of heavy smoker volunteers with (COPD-EVs) or without (HS-EV) COPD enrolled in a LDCT screening trial. EV's characterization was performed accordingly to the MISEV guidelines using FC (Flow cytometry), NTA and TEM. The protumorigenic role of EVs was evaluated on transformed but non-tumorigenic Epithelial Cells (HBEC-KRAS^{V12high} cells) using FC, Real Time, 3D proliferation and migration assays.

Results and Discussions

The results showed that EVs were sized around 100 nm without significant differences between COPD and HS-EVs. These EVs possess the canonical EVs markers CD9, CD63 and CD81 and were internalized by recipient cells. First, we observed that COPD-EVs increased CD133+ and CD133/CXCR4+ cells compared to HS-EVs on epithelial cells. COPD-EVs were able to stimulate 3D growth of HBEC cells on VitroGel and their migratory ability. COPD-EVs promoted the Epithelial to Mesenchymal Transition in HBEC-KRAS^{V12high} cells by E-Cadherin down-modulation and SNAI2 upregulation. We showed that the effects in CSC modulation on epithelial cells were mediated by transfer of HIF-1 α hypoxic transcription fraction from COPD-EVs that is responsible for CXCR4 pathway up-regulation. Experiments using HIF-1 α inhibitor or CXCR4 silencing with EVs administration

clearly showed the role of hypoxia-induced CXCR4 pathway in the modulation of CD133 phenotype.

Conclusion

Our findings demonstrated that EVs from COPD patients are able to expand the CSC subsets in non-tumorigenic epithelial cells towards pro-tumorigenic/metastatic phenotype.

EACR22-0717

Establishment of treatment-naïve gastric cancer patient-derived organoids as models of drug screening

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Introduction

Gastric cancer (GC) is the 3rd leading cause of cancer-specific mortality. It is an aggressive cancer and despite great progress in treatment, the overall prognosis remains poor. Surgery remains the cornerstone treatment for GC. For patients who present with locally advanced disease peri-operative chemotherapy is the accepted treatment for this cancer. GC is a heterogeneous disease which makes it difficult to predict patient outcomes based only on the histologic and molecular classification. Consequently, there is a need for novel approaches to predict the efficacy of therapy for individuals. 3D organoid-based technology has shown tremendous potential for a wide range of basic research and translational applications. Patient-derived organoids (PDO) show great potential in drug discovery, screening and precision treatment.

Material and Methods

Fresh tissue specimens from 3 endoscopy/surgical gastric tumours were collected and processed within 24 hrs. Briefly, tissues were cut into small pieces, then mechanically dissociated, and the resulting single cells were plated in a 3D environment embedded in Matrigel as an extracellular matrix to form domes. The Matrigel: tissue cells were overlaid with specific feeding medium and cultured. Organoids and corresponding tissue specimens were characterized by transcriptomic and whole exome analysis. In addition, PDOs were used to assess drug responses to three different chemotherapeutic drugs.

Results and Discussions

Growth of organoids was typically detected after 5-6 days after plating. The 3D structures grew out from single cells or small cell clumps and eventually developed into dense spherical structures. Phenotypically, GC PDOs had cystic and hollow structures with a clear lumen. To evaluate GC PDOs as functional in vitro disease models, GC organoids were treated with increasing concentrations of 5-FU, oxaliplatin and taxotere for drug sensitivity screening. The dose-response curves and half-maximal inhibitory concentrations (IC₅₀) showed divergent response to each drug between PDOs. Furthermore, verification of PDOs ability to maintain similar characteristics as their matched original tumour was performed by GC markers, transcriptomics and whole exome sequencing.

Conclusion

Our results demonstrate the feasibility of developing GC PDOs, which have the potential for drug sensitivity screening and personalized medicine applications.

Novel key genes in the regulation of gastric cancer stem cell activity

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Introduction

Cancer stem cells (CSCs) are a distinct subpopulation of cancer cells within tumors, with unique properties of plasticity, unlimited self-renewal, multipotency and quiescent state that make them responsible for tumor origin and heterogeneity, therapy resistance, recurrence and metastasis.

In this context, our group, along with others, has demonstrated that SOX9, a developmental transcription factor that is a master regulator controlling super-enhancer dynamics in stem cell plasticity, regulates CSCs and plays a relevant oncogenic role in different tumor types including glioblastoma, pancreatic, colorectal, and gastric cancer (GC). However, the molecular network driven by SOX9 in the regulation of CSCs remains unknown. Thus, the aim of the present study is to identify these key genes, which represent promising potential therapeutic targets to overcome therapy resistance and cancer progression.

Material and Methods

To achieve our objective, we initially focused on GC and performed computational biology analyses to identify the top-genes most correlated with SOX9 in GC samples. We selected a group of candidate genes based on their clinical impact and completed loss-of-function studies *in vitro* and *in vivo* as well as transcriptomics to elucidate their role and mechanism in the regulation of CSCs and the processes linked to this population. In addition, we tested the antitumor effect of the pharmacological inhibition of one of the promising candidate genes *in vitro* and *in vivo*.

Results and Discussions

Among our initial panel of candidate genes, *DIAPH3*, *KIF11*, *HMGA1*, *FGFR4*, *EHF*, *ECT2*, *TPX2*, *KIAA1804* and *IL17RB* were downregulated in SOX9-silenced GC cells, whereas they were upregulated in critical aspects of GC, such as *Helicobacter pylori* activity and/or cisplatin resistance. Furthermore, we focused on the study of *DIAPH3* and *KIF11*, and found that their silencing in GC cells severely impaired cell proliferation and oncosphere formation, while induced cell senescence and apoptosis as well as reduced tumor initiation and growth *in vivo*. Of note, the pharmacological inhibition of *KIF11* severely reduced cell viability and oncosphere self-renewal *in vitro*, and exerted a drastic impairment on tumor growth *in vivo* when administered in GC xenografts.

Conclusion

In summary, our study demonstrates that *KIF11* and *DIAPH3* are critical regulators of gastric CSCs (gCSCs) and constitute promising targets in the disease. Indeed, our encouraging results obtained with a *KIF11*-selective-inhibitor brings us closer to the targeting of gCSCs.

Prospective isolation of cancer stem cells by Single Cell Trained Sorting in High Grade Serous Ovarian Cancer

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Introduction

The high mortality rate in High Grade Serous Ovarian Cancer (HGSOC) is mainly caused by frequent relapse and drug resistance, due to the presence of cancer stem cells (CSCs). Molecular markers have been used to isolate CSCs, but their precise identification has not yet been possible, even if instrumental for the development of novel therapeutic strategies. Here, we show a novel method, based on single cell-trained FACS sorting, for CSCs identification directly from fresh ascites of HGSOC patients that can be exploited to investigate CSCs features and to study new effective therapeutic strategies and molecular markers.

Material and Methods

We combined single cell Monoclonal Ovarian Cancer Spheroids (sMOCS), a 3D system for CSCs isolation from single cells, with GateID, a computational method that connects FACS parameters with phenotypic cellular features to perform marker-free purification of cell populations. Ascitic samples were processed and analyzed by FACS, recording all the available channels' indexes. Single cells were then sorted in 96-well-plate for the generation of sMOCS. FACS sorting index data were interpolated with sMOCS growth information to distinguish putative CSCs able to generate sMOCS from cells that do not grow. A Random Forest approach has been applied to identify the best FACS sorting parameters that distinguish putative CSCs. Then, sMOCS forming efficiency has been used to evaluate the ability of the selected gate in enriching for CSCs.

Results and Discussions

Variations between the two populations in FACS index data for all scatter and fluorescence channels were investigated: putative CSCs, able to generate sMOCS, presented specific values when compared to other cells, displaying that our strategy can identify unique features of CSCs. Then, we defined a first multi-dimensional gate able to enrich for our cells of interest. In particular, we obtained a higher sMOCS forming efficiency after selecting cells with our gate in 3 out of 4 samples. These preliminary results show the potential of our method in identifying CSCs using FACS indexes data, overcoming the issue of unspecific membrane markers.

Conclusion

The development of a method for the isolation of CSCs from fresh ascites of HGSOC patients can be exploited for the identification and characterization of CSCs, capturing inter- and intra-patients tumor heterogeneity, and use to study of effective therapeutic strategies, reducing time for analyses, and moving forward in the field of precision oncology.

EACR22-0987

Contribution of macrophages to the anti-tumour effects of peptides based on Connexin43 in mouse glioma models

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Introduction

Microglia and macrophages are part of the complex immune microenvironment of malignant gliomas. The peptide based on connexin43, TAT-Cx43266-283, is an inhibitor of Src that

exerts anti-tumour effects in different glioma models. Src is an important oncogene involved in several signaling pathways related to cancer, including immune activation. Therefore, we investigated the contribution of macrophages and microglia to the effect of TAT-Cx43266-283.

Material and Methods

In vivo and ex vivo glioma models in C57Bl6J immunocompetent mice, control vs TAT-Cx43266-283 treatment, were analyzed. For the in vivo model, m-cherry Gl261 glioma stem cells (Gl261-GSCs) were intracranially injected and macrophages and microglia were analyzed by immunohistochemistry at 7 or 15 days post-implantation. For the ex vivo model, Gl261-GSCs were implanted on organotypic adult brain slice cultures and visualized by fluorescence microscopy.

Results and Discussions

We found a higher percentage of MHCII-positive PU.1 cells (activated macrophages or microglia) within the tumours of TAT-Cx43266-283-treated animals when compared with the controls, at 7 days but not at 15 days post-implantation. To identify macrophages, we analyzed Ly6c expression in PU.1 cells. We found an increase in the percentage of Ly6c-positive PU.1 cells within the tumours of TAT-Cx43266-283-treated animals compared with the controls, at 7 days post-implantation, but not at 15 days. Since these results suggest that activated macrophages derived from infiltrating blood monocytes might play a role in the anti-tumour effects of TAT-Cx43266-283, we implanted GSCs in combination with monocytes and TAT-Cx43266-283 on brain slice cultures. Interestingly, the number of GSCs increased when implanted with monocytes for 7 days. However, the presence of monocytes and TAT-Cx43266-283 dramatically reduced the number of GSCs, suggesting that TAT-Cx43266-283 may regulate monocyte function. To confirm this proposal, we analysed the number of dying GSCs (caspase3-positive) engulfed by MHCII cells in the in vivo glioma model. Interestingly, we found an increase in phagocytosis of glioma cells after TAT-Cx43266-283 treatment at 7 days post-implantation.

Conclusion

All together our results suggest that the activation of macrophages in the glioma microenvironment contributes to the anti-tumor effects of TAT-Cx43266-283.

EACR22-1075

The combinatorial therapy by histone-modifying agents restores Naked levels and inhibits stemness in lung cancer stem cells

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Introduction

Cancer stem cells (CSCs) handle heterogeneity, multidrug resistance, and metastasis. Epigenetic mechanisms regulate several hallmarks of cancer, such as the abundant activation of Wnt/ β -catenin signaling (a pro-survival pathway) in CSCs. The low level of *Naked* (NKD2), an antagonist in Wnt/ β -catenin signaling, is related to aggressive tumor behavior such as Cisplatin resistance, invasion, and poor prognosis in lung cancer. The role of histone machinery in *Naked* expression remains unknown in lung cancer. Thus, we focused on if histone modifiers can restore *Naked* levels, modulate stemness and prevent invasion.

Material and Methods

CSC-enriched population (H1299/S) provided from the parental H1299 lung cancer cell line. Stemness verified by flow cytometry and western blotting. H1299/S cells are pretreated with histone demethylase inhibitor then exposed to histone deacetylase inhibitor (combinatorial therapy). Cell viability, NKD2 levels, Wnt activity, and EMT progression monitored by ATP assay, qRT-PCR, and western blotting. Alterations in histone modification profile assessed by ELISA and ChIP assays. The invasive ability is observed and compared by a 3D invasion assay.

Results and Discussions

Indeed, the low level of NKD2 was related to the aggressive character of H1299/S through Cisplatin resistance, Wnt activity, and mesenchymal phenotype compared to the parental counterpart. The combinatorial therapy exerted a significant and better anti-growth effect by itself in a dose and time-dependent manner, yet it couldn't potentiate the effect of cisplatin. However, cells exhibited reduced Wnt activity, EMT, and invasion properties by elevated expression of NKD2 restored via combinatorial treatment. The repressive histone mark level, H3K9me3, globally decreased in cells by therapy and slightly enriched in NKD2 promoter.

Conclusion

The future success of this combination approach in targeting CSCs may hold significant promise to prevent stemness and slow down migratory and invasive potential in lung cancer.

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EACR22-1211

Deciphering the transcriptomic regulation of tissue lipidomic distribution in colonocyte differentiation and colon cancer

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Introduction

The process of colonocyte proliferation and differentiation requires a fine and strict regulation along the crypt and its disruption leads to tumor initiation and cancer development. In previous publications, Matrix-assisted laser desorption/ionization – imaging mass spectrometry (MALDI-IMS) analysis showed how sensitive the membrane lipidome is to the pathophysiological state of the cell, changing concomitantly to the processes of differentiation and tumorigenesis.

Material and Methods

To further investigate the underlying regulatory mechanisms of this differential lipid phenotype, we used fluorescent-activated cell sorting (FACS) and healthy and tumor patient-derived biopsies to isolate several colonocyte subpopulations depending on the levels of EPHB2 staining. Next, we analyzed the lipidome and transcriptome of each subpopulation, containing approximately 15000 cells, by MALDI-IMS (n=5-8) and gene expression microarray (n=4), and we performed a multi-omic integrative analysis using a system biology approach.

Results and Discussions

Both -omics profiles highlighted the coordinated regulation of phospholipid species containing PUFA or MUFA fatty acids through the process of colonocyte differentiation. Thus, in healthy subpopulations, levels of PI species containing the PUFA arachidonic acid (AA; e.g. PI 38:4, PI 36:4) were higher in stem cell-like than in differentiated colonocytes, while this latter were more enriched in MUFA-containing PI, displaying a different composition of this bioactive hub. The gene expression profile presented an enrichment of eicosanoid metabolism in differentiated colonocytes concordant with the lipidomic changes. While in tumor stem cell-like colonocytes, the AA-containing PI levels were even higher, and gene expression was enriched in fatty acid biosynthesis, suggesting a different metabolic program. Application of weighted gene co-expression analysis enabled the identification of modules correlating with the levels of the PI species of interest in each subpopulation. Further network visualization and motif enrichment analysis facilitated the identification of metabolic hub genes (e.g. PLCD3, FABP1, and AKR1C3) and predicted transcription factors (e.g. E2F4, HNF4A) associated with a subpopulation and a pathological condition.

Conclusion

In this study, we described for the first time the transcriptomic networks involved in the regulation of specific membrane lipid molecular species phenotype corresponding to both healthy and tumor colonocyte differentiation states.

EACR22-1215

Elucidating glycosylation genes responsible for stemness capacity in triple-negative breast cancer by novel CRISPR library

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Introduction

Breast cancer is the 1st leading cause of cancer death in women. A few breast cancer patients experience metastasis and relapse. Within the tumour, a little subset of cells is poorly differentiated, giving them stem-cell properties and defined as cancer stem cells, which are known to be the responsible of migration, metastasis and recurrence. However, targeting specifically this population in breast cancer is challenging nowadays.

Differences between breast stem cells and breast cancer stem cells (BCSC) have been reported. Among others, posttranslational modifications in BCSC have been observed, specifically glycosylations, occurring preferentially in stemness markers.

We aim to elucidate the responsible(s) of these aberrant glycosylations through a novel CRISPR library designed by our laboratory, targeting specifically glycosylation genes in triple-negative breast cancer (TNBC). **Material and Methods**

TNBC MDA-MB-231 cell line was used. sgRNA guides of CRISPR library targeting glycosylation genes were designed according to Gene Ontology Consortium and cloned into a lentiCRISPR v2 vector. Screening was performed infecting GlycoCRISPR library with a representation of 300 times. Infected cells were plated in monolayer (2D) and in low attachment (3D) plates to form tumorspheres.

RT-qPCR was performed to observe changes in stemness sgRNA guides expression in tumorspheres generations.

Next Generation Sequencing of three experiments was analysed until obtaining a subset of potential hits.

Results and Discussions

GlycoCRISPR cells were maintained attached until 15 doubling times and then pelleted to check how CRISPR library affected them in 2D. Apparently, their phenotype did not change.

In tumorspheres, they were kept for 7 days and then passed until the 3rd generation. They were able to form tumorspheres along 3 generations, obtaining pellets from each generation.

Once DNA extraction and RT-qPCR were performed, results elucidated how stemness genes lost their expression throughout the 3D experiment, whereas this loss was not as much as in 2D. Therefore, we could confirm that tumorsphere approach worked.

Finally, subsets of hits have been obtained. Hence, the most relevant will be evaluated to be a good candidate for future research.

Conclusion

GlycoCRISPR library is a novel tool created to screen glycosylation genes, having the possibility to be used for further investigations in other groups. In addition, a subset of glycosylation genes has been obtained to be necessary for stemness condition in breast cancer cells.

Cancer Genomics

EACR22-0860

TOP ABSTRACT

Immune evasion impacts the distribution of mutations in driver genes across tumor types

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Introduction

Immune evasion is a cancer hallmark present across multiple tumor types. However, how immune evasion impacts the selective fitness of driver clones remains unknown. In recent years, the ratio of nonsynonymous to synonymous mutations (dN/dS) has been a widely used metric to detect selection in cancer genomes. Genes under negative selection are depleted of nonsynonymous mutations (dN/dS < 1) unlike genes under positive selection (dN/dS > 1). Here, we stratified cohorts based on the mutational status of escape genes and calculated dN/dS to find differentially selected genes that could be linked to the presence or absence of an active immune selective process. We hypothesized that immune-evasion expands the possible number of driver sites in the genome.

Material and Methods

We obtained 9896 individuals spanning 31 cancer types from The Cancer Genome Atlas (TCGA) project through the Genomics Data Commons (GDC) Portal. We used dNdScv and SSB-dN/dS, methods to estimate dN/dS in somatic data, to discover positively selected genes in our escaped and non-escaped cohorts. We then obtained six immune phenotypes from Thorsson et al. to further show that certain drivers are more favored in some microenvironmental context than others.

Results and Discussions

We found that across 31 cancer types, despite global dNdS values are larger than one for both cases, non-escaped cohort has stronger evidence of selection than its counterpart. We found that the relaxation of immune selection on multiple unknown driver sites leads to a diluting effect on dN/dS in common driver genes, and an increase on the number of predicted driver genes. In addition, we observed that escaped tumors have a larger proportion of aneuploidy events confirming that other genomic alterations can also lead to tumor-immune recognition.

Conclusion

We discovered new driver genes thanks to our stratification strategy. We also showed that there is a difference in the distribution of mutations in driver genes between escaped and non-escaped clones thus providing a source of heterogeneity that might lead to differential prognosis.

EACR22-0248

ORAL PRESENTATION

The ALT pathway generates telomere fusions that can be detected in the blood of cancer patients

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Introduction

Telomere fusions (TFs) can trigger the accumulation of diverse genomic rearrangements and the acquisition of oncogenic alterations leading to malignant transformation and resistance to chemotherapy. Despite their relevance in tumour evolution, our understanding of the patterns and consequences of TFs in human cancer remains limited.

Material and Methods

To detect TFs in sequencing data, we developed TelFusDetector. In brief, TelFusDetector identifies sequencing reads containing an enrichment of telomeric sequence repeats (TTAGGG and CCCTAA), considers their sequence orientation, base error rates and quality measures to identify TFs. We applied TelFusDetector to a total of 8,556 whole genome sequencing samples, including more than 30 cancer types, 306 cancer cell lines and healthy individuals used as controls.

Results and Discussions

Here, we have characterized the rates and spectrum of somatic TFs across >30 cancer types using whole-genome sequencing data. TFs are pervasive in human tumours with rates varying markedly across and within cancer types. In addition to end-to-end fusions, we find

novel patterns of TFs that we mechanistically link to the activity of the alternative lengthening of telomeres (ALT) pathway. We show that TFs can be detected in the blood of cancer patients, which enables cancer detection with high specificity and sensitivity even for early-stage tumours and cancer types for which early detection remains a high unmet clinical need, such as pancreatic cancer and brain tumours.

Conclusion

Overall, we report a novel genomic footprint that enables characterization of the telomere maintenance mechanism of tumours and liquid biopsy analysis, which has implications for early detection, prognosis, and treatment selection.

EACR22-0764

PROLINE DEHYDROGENASE AS NEW MARKER AND TARGET IN EGFR MUTANT NON-SMALL CELL LUNG CARCINOMA

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Introduction

Non-Small Cell Lung Cancer (NSCLC) is one of the most frequent types of tumor for incidence and the leading cause of cancer mortality. Identification of markers to better define diagnosis, prognosis and therapeutic options of NSCLC is needed. Proline dehydrogenase (PRODH), a mitochondrial flavoenzyme catalyzing the key step in proline degradation, is expressed in lung adenocarcinomas and expression appears to correlate with EGFR mutations. PRODH expression is also regulated by AMPK. Observing the synergism between metformin and EGFR-inhibitors, we propose that PRODH may be a mediator of metformin growth-inhibitory and proapoptotic effects, alone or in combination with tyrosine kinase inhibitors.

Material and Methods

The relation between EGFR mutations and PRODH expression was investigated by modulating EGFR or specific downstream signaling pathways, in particular JAK-STAT3, as we identified putative STAT3 binding sites in the *PRODH* promoter.

We developed two cellular models with *in vitro* acquired resistance to Osimertinib (EGFR inhibitor): we selected PC9 and H1975 cell lines, harbouring an in-frame deletion in exon 19 and T790M and L858R mutations of the *EGFR* gene, respectively.

Results and Discussions

Preliminary results show that PRODH expression is modulated upon STAT3 activation or transfection of constitutively active STAT3 construct. We will investigate if modulation occurs by direct promoter regulation. We hypothesize that STAT3 may be implicated in PRODH activation and that PRODH may be a new marker and therapeutic target.

In parallel, we investigated expression of PRODH in Osimertinib resistant cell lines (PC9-OR and H1975-OR) compared to corresponding parental cells: we detected increased level of PRODH in resistant cells, suggesting that it may represent an escape mechanism to EGFR

inhibition. Based on the molecular connection of PRODH with targets modulated by metformin, we tested the effect of metformin on PRODH levels and we show that metformin reduces PRODH protein levels *in vitro* in NSCLC cells. Also, considering the synergism of metformin with EGFR-inhibitors and the inter-dependence between EGFR and PRODH, we speculate that PRODH activation may be an EGFR resistance mechanism reversible by metformin also in OR-cells, thus suggesting a new combinational strategy.

Conclusion

We propose the involvement of PRODH as escape mechanism to EGFR and its role in the mechanism of antineoplastic activity of metformin.

EACR22-0208

Dissecting Early Molecular Events in Osteosarcomagenesis

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Introduction

Osteosarcoma (OS) is one of the most common malignant bone tumors in children and adults. This malignant tumor is highly aggressive, associated with poor clinical outcomes and metastasizes mainly to the lungs. Genomic instability is a hallmark of the tumor and marked by multiple random chromosomal aberrations with only a few recurring deletions and amplifications. Due to its rarity and biological heterogeneity, limited studies of its molecular basis exist and thus resulting in hindering the development of effective targeted therapies. Our lab recently demonstrated that WW domain-containing oxidoreductase (WWOX) is frequently inactivated in human OS and that WWOX restoration in WWOX-negative OS cells suppresses tumorigenicity. In addition, combined WWOX loss of function and that of p53 accelerates murine OS development and was shown to be a hallmark of most human OS cases. We aimed here to dissect early molecular changes of OS development to identify plausible druggable biomarkers.

Material and Methods

A double-knockout mouse model of *Wwox* and *Trp53* in osteoblast (using Osterix1-Cre) that expresses a tdTomato reporter was generated and used to track tdTomato-positive cells. RNA-sequencing of these young tdTomato cells in non-tumor bearing mice was performed to identify early molecular changes that might contribute to osteosarcomagenesis.

Results and Discussions

Using this mouse model, we detected early presence of tdTomato-positive cells in the bone marrow of non-OS-bearing mice (yBM). We found that these cells exhibit tumorigenic traits both *in vitro* and *in vivo*. Molecular and cellular characterization of these cells revealed their resemblance of OS cells. Interestingly, one of the main observed molecular pathways contributing to OS development was enriched Myc-targets, among which upregulation of MCM7 was validated both in yBM cells and OS tumors. Therapeutical validation both *in vitro* and *in vivo* indicated that MCM7 can be targeted using Simvastatin which resulted in attenuating OS tumor growth.

Conclusion

Our data reveals the presence of osteoblast exhibiting tumorigenic ability within the bone marrow of non-tumor bearing mice. Further molecular analysis of our model shall enable characterization of early molecular events of osteosarcomagenesis and facilitate development of targeted therapy for OS.

EACR22-0288

Recurrent splice site mutations promote oncogenesis in diffuse large B-cell lymphoma

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Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most frequent hematological malignancy in adults. Recently, large-scale studies have performed whole-exome sequencing of over 1700 DLBCL primary tumors to elucidate the molecular mechanisms and improve the molecular classification of the disease. However, we found that some of the largest studies to date ignored or underreported splice site mutations. Splice site mutations can cause major aberrations in gene products, impairing gene function. In this context, we aimed to identify and experimentally characterize previously underreported recurrent splice site mutations in DLBCL.

Material and Methods

We reanalyzed whole-exome sequencing data of >1700 DLBCLs from three external cohorts and implemented an in-house computational pipeline to identify somatic mutations at splice sites. We selected two of the most recurrent splice site mutations for functional validations: a splice donor mutation in intron 1 of *BCL7A*, and a splice donor mutation in intron 4 of *CD79B*. For *BCL7A*, we compared the functional effect of mutant and wild type proteins *in vitro* and *in vivo* and we used transcriptomics and proteomics to elucidate the molecular mechanism of the effect of the mutation. For *CD79B*, we tested the effects of the mutation

on surface B cell receptor (BCR) expression and on cellular signaling using flow cytometry and western blot.

Results and Discussions

We identified 29 genes that recurrently undergo splice site mutations in the three independent DLBCL cohorts. The mutations caused a wide range of alterations in messenger RNAs, including intron retention, usage of cryptic splice sites, exon skipping, intronic polyadenylation, and usage of alternative isoforms. Mutations in the splice donor site of intron 1 of *BCL7A* induced the usage of a cryptic splice donor site within exon 1, causing an in-frame deletion of 27 aa. This deletion prevented *BCL7A* from binding to the SWI/SNF chromatin remodeling complex and impaired its tumor suppressor role *in vitro* and *in vivo*. Mutations in the splice donor site of intron 4 of *CD79B* caused intron 4 retention, generating a truncated protein. Truncated *CD79B* increased surface BCR expression and oncogenic signaling via AKT and NF- κ B in DLBCL cell lines.

Conclusion

Splice site mutations are recurrent in DLBCL and they cause major alterations in key oncogenes, such as *CD79B*, and tumor suppressor genes, such as *BCL7A*.

EACR22-0476

Malignant Pleural Mesothelioma harbor early clonal fusions involving both tumor suppressor drivers and novel oncogenic alterations

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Introduction

MPM is a pleural tumour associated with asbestos exposure, with a long latency period (up to 40 years) between asbestos exposure and disease presentation. Our aim was to identify gene fusions that occurred early in the evolutionary history of the tumour.

Material and Methods

We conducted multiregional whole exome sequencing (WES) of 106 samples for 20 patients, as part of the MEDUSA study (Zhang et al., 2021). We identified gene fusion events, and filtered for tumour-specific events using the exome sequence from the matched blood. Gene fusions observed across all regional samples of a particular tumour were validated by Sanger sequencing of the breakpoint. Fusion transcripts were identified from tumour RNAseq and compared with gene fusions identified at the DNA level.

Results and Discussions

We found 24 truncal fusion events, three of which were novel (FMO9P-OR2W5, GBA3 and SP9). Tumour suppressor genes involved included BAP1, MTAP, and LRP1B, a potential oncogenic fusion CACNA1D-ERC2, and other truncal events such as in-frame PARD3B-NT5DC2 and out of frame STAB2-NT5DC2 fusions. Transcriptome sequencing confirmed a subset of gene fusions were transcribed.

Conclusion

Our findings suggest that gene fusions events occur early in mesothelioma evolution with marked heterogeneity across the cohort, as no recurrent truncal fusions event were found.

Some truncal fusion events involve genes previously shown to be mutated in MPM and are likely to be drivers of cancer progression

EACR22-0718

Variants in cis-regulatory elements of selected genes in laryngeal squamous cell carcinoma (LSCC)

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Introduction

Pathogenic variants in non-coding cis-regulatory elements in LSCC are not fully characterized. To fill this gap we used targeted NGS sequencing of the regulon defined as a region of 46 Mbp, overlapping proximal enhancers and promoters identified by the ENCODE and FANTOM consortia. We aimed at identification of variants with consequences on the expression of adjacent protein coding genes.

Material and Methods

LSCC (n=49) samples constituting of 7 LSCC cell lines and 42 tumors with paired blood samples were sequenced using the Illumina HiSeq 1500 (paired-end sequencing, 2x100 bp, mean coverage for tumor samples >100x, > 80x for blood). Libraries were prepared using the SeqCap EZ Choice XL Kit (Roche). Sequencing data were mapped to the genome using BWA, compared to the reference sequence using HaplotypeCaller (GATK) and somatic variants were identified using MuTect2 (GATK). We searched for gene regulatory regions with variants found in at least two samples with (I) variant allele frequencies (VAF) > 20%; (II) being variants of conserved nucleotides (phyloP30way Genome Browser track; conservation > 0.0). Variants were confirmed using pyrosequencing (PyroMark Q24) and studied using reporter assay for their effect on expression of the respective genes.

Results and Discussions

We identified 74 variants which correspond to 35 genes. Filtering for conservation and validation left 12 variants corresponding to 5 genes. We next lowered the VAF threshold to 10% for these genes and found 2 additional variants. Together, we identified 3 variants in *NFE2L2* (transcription factor (TF)), 2 in *PHC1* (Polycomb group, upregulated in LSCC), 4 in *SAP30* (histone deacetylase complex, upregulated in LSCC), 2 in *ZNF12* (TF, upregulated in LSCC) and 3 in *ZSCAN18* (zinc finger protein, downregulated in LSCC) regulatory regions. These genes have biological functions commonly impaired during malignant transformation.

Last, we performed reporter assay for the *PHC1* related chr12:008914173-C>G (hg38) variant which creates a TF binding motive **TGACGTCA** recognized by several TF including CREB and JUN abundant in LSCC. The variant results in up to 3 fold upregulation of *PHC1* in UTSCC29 and HEK cell lines. Functional analysis of the other variants is in progress.

Conclusion

We report the identification of variants in regulatory regions of potentially cancer related genes in LSCC and demonstrate a transcription enhancing effect of chr12:008914173-C>G on *PHC1*. Therefore, these variants may present a mechanism behind the deregulation of these genes in LSCC.

EACR22-0900

Identification of LINE-1 mobilizations and its clinical utility in cancer

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Introduction

About 17% of a human genome is composed by retrotransposons, repetitive DNA sequences that can mobilize along the genome. Although most retrotransposons are silent, a small subset of LINE-1 (L1) has been demonstrated to be active in some tumours using PanCancer data, representing an important source for somatic mutation that is not currently considered in cancer patient diagnosis. Sometimes, L1 transcription continues beyond 3' adjacent DNA unique sequences in a process called transduction, which can be used to identify active L1 elements. Based on this, our group has developed "RetroTest" to evaluate L1 activation in tumor biopsies and promote its translation to clinical practice.

Material and Methods

First, we have evaluated our method and measured L1 activation in a Head and Neck Squamous Cancer (HNSCC) dataset to characterize L1 activation and its possible clinical implications along tumor stages. Second, we have studied L1 activation as a possible source of new neoantigens that can favour immunotherapy response in lung (LC) and bladder cancer (BC).

For all the cohorts, we isolated DNA from FFPE primary tumor biopsies, performed targeted RetroTest libraries, sequenced them with Illumina, and analysed them with our bioinformatic pipeline.

Results and Discussions

For the first objective, L1 activity was evaluated in 97 HNSCC patients along the different stages. Our results reveal a really early L1-activation, even in the first stages of the tumor. For the second aim, L1 activation was measured in 10 BC and 30 LC patients before starting immunotherapy as first-line therapy. In BC patients, a statistical significance association was found, supporting that those patients with L1 activation reached immunotherapy response. In the case of LC, a positive trend was found although not statistically significant, suggesting that further samples should be addressed.

Conclusion

As a conclusion, our data supports L1 activation as an early HNSCC diagnosis biomarker and a possible role as a predictive response biomarker for immunotherapy in LC and BC.

Comprehensive transcriptomic analysis of intrahepatic and extrahepatic cholangiocarcinoma identifies distinct biological subclasses.

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Introduction

Cholangiocarcinoma (CC) is a rare and aggressive disease with limited therapeutic options resulting in dismal prognosis for most of the patients. Several previous studies with integrative molecular analyses and omics technologies aimed at characterizing the biology and the oncogenic landscape of CC primary tumors to identify the optimum therapies able to improve patients' survival. Still comprehensive transcriptomic analysis of intrahepatic (ICC) and extrahepatic cholangiocarcinomas (ECC) derived from different patient cohorts for obtaining biological insights and prognostic information is lacking.

Material and Methods

A total of 543 patients of primary tumor tissues profiled with RNAseq and microarray platforms were collected from 7 different publicly available datasets and used as training set to identify distinct biological CC subgroups. RNAseq raw sequencing were quality-checked using FastQC and aligned to reference genome (hg19) using STAR. Transcripts quantification was performed using the RSEM package for TPM calculation. Gene expression data from Affymetrix and Illumina microarrays were processed using R software. Empirical Bayes method was considered to adjust for batch effect. Unsupervised clustering analyses for biological characterization were performed using NbClust tool. Fast Unified random forest algorithm was considered as method to build a predictor on training set. For the validation set a single cohort of 131 patients with RNAseq data used. Quality was checked using FastQC and submitted to RSEM for TPM calculation.

Results and Discussions

After adjusting for batch effects associated to the different datasets, unsupervised clustering analysis identified 4 subgroups both for ICC and ECC cohorts. For each anatomical location gene set enrichment analysis showed pathways and specific CC signatures differentially enriched between all subgroups. The analysis of immune cell infiltration showed different immune profiles associated to each group, suggesting possible distinct immunoreactivity profiles. The developed class predictors for ECC and ICC are currently challenged on the validation set to evaluate the clinical outcome associated to each subgroup.

Conclusion

The comprehensive transcriptomic analysis of publicly available CC datasets allowed to identify and validate the presence of biologically distinct subgroups providing clinical insight into the disease. Such results represent the starting point for a complete molecular characterization of CC, including integration of genomics data.

Identification and somatic characterization of the germline PTEN promoter variant rs34149102 in a family with gastrointestinal and breast tumors.

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Introduction

Genetic variant located in non-coding region can affect processes that regulate protein expression, functionally contributing to human disease. Germline heterozygous mutations in the non-coding region of the *PTEN* gene have been previously identified in patients with PTEN hamartoma tumor syndrome (PHTS) diagnosed with breast, thyroid, and/or endometrial cancer. In PHTS patients with *PTEN* promoter mutations, protein analysis showed that these mutations were associated with aberrant PTEN expression, suggesting that alterations in *PTEN* promoter region can contribute to disease pathogenesis.

Material and Methods

Here, we examined an Italian family with a history of gastroesophageal junction (GEJ) and breast cancer. The index case showed major (macrocephaly, multiple oral papillomas on the tongue) and minor (lipomas) clinical criteria of PHTS. We performed a molecular analysis of major genes

(*APC*, *ATM*, *BARD1*, *BMPR1A*, *BRIP1*, *CDH1*, *CDK4*, *CDKN2A*, *CHEK2*, *EPCAM*, *MLH1*, *RE11A*, *MSH2*, *MSH6*, *MUTYH*, *NBN*, *PALB2*, *PMS2*, *PTEN*, *RAD50*, *RAD51C*, *RAD51D*, *S*, *MAD4*, *STK11*, *TP53*) involved in hereditary cancer predisposition syndromes. Moreover, the status of *PTEN* somatic alterations was investigated in GEJ adenocarcinoma of the index case.

Results and Discussions

The molecular analysis revealed the presence of a germline *PTEN* promoter variant (rs34149102 A allele) in the index case, which was also present in his mother and one maternal aunt with breast cancer. This variant was previously identified in patients with PHTS phenotype and was showed to be correlated with a reduction of the expression of full-length *PTEN* transcript. In order to investigate the putative functional role of the rs34149102 A allele variant, we investigated the status of PTEN alterations in GEJ adenocarcinoma tissue of the index case. We found that PTEN protein expression was absent in tumor tissue. Moreover, we identified the presence of a deletion involving one copy of *PTEN* gene, indicating the occurrence of somatically acquired inactivation of the second allele. Moreover, direct sequencing analysis of *PTEN* gene revealed the presence of rs34149102 A allele in tumor tissue of index case, suggesting that this variant may induce *PTEN* promoter dysregulation.

Conclusion

In conclusion, these results reveal the functional role of the PTEN promoter rs34149102 A allele variant in modulation of PTEN protein expression and highlight its contribution to hereditary cancer risk.

EACR22-1072

The deleterious accumulation of mutations is compensated by the effect of somatic copy-number amplifications

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Introduction

It is unclear how cancer genomes can tolerate large amounts of deleterious mutations. Common measures of mutation selection, such as the ratio between non-synonymous over synonymous mutations, indicates that the majority of protein-coding genes lack detectable signals of negative selection (99% of mutations are tolerated), and most mutations are potentially neutral events on cancer fitness. Conversely, evolutionary simulations, *in vitro* and *in vivo* proliferation studies and whole-genome CRISPR-KO screenings within tumor cell lines demonstrated the deleterious role of passenger mutations that lead to reduced *in vitro* proliferative fitness and slowed *in vivo* tumor growth. This raises the question of why cancers bear many mutations with such a weak selection if those are deleterious: our hypothesis is that selection against detrimental mutations is probably not efficient or partly hidden, but again, how can tumors solve this paradox?

Material and Methods

Using TCGA dataset, we performed an unbiased relationship analysis between amplification and diploid mutation frequencies with different conditions (haploinsufficient genes, cancer genes, high/low impact mutations, aggregation-causing mutations).

Results and Discussions

We demonstrated that copy-number amplifications frequently cover highly mutated regions to compensate for the deleterious impact of mutations by creating safe copies, thus protecting the genes. We showed that these compensatory events are highly influenced by gene and mutation properties. Indeed, mutations predicted to have high impact on protein structure and function, hence very deleterious, are not well tolerated and thus highly buffered by creating extra safe copies which preserve the functions of the genes within the gain. Supporting our hypothesis, mutations within LOF-intolerant genes showed to be more likely associated with extra copies when highly mutated, while this effect drops for LOF-tolerant genes. Beside LOF, aggregation-causing mutations demonstrated a strong loss of buffering, while non-aggregation-causing ones produced correlations as good as the ones considering the whole mutation subset or missense mutations. Moreover, over-representation analysis of unprotected gene sets contain unessential and cancer-unrelated functions; conversely, protected gene sets contain cellular essential functions and cancer-associated functions.

Conclusion

The deep understanding of these compensatory events may reveal new cancer vulnerabilities and might guide novel therapeutic intervention.

EACR22-1138

Systematic characterisation of chromatin modifiers in endometrial cancer

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Introduction

While chromatin remodelers are mutated in many cancer types, several lines of circumstantial evidence suggest they are particularly important in endometrial cancer (EC). *ARID1A* - a key component of the SWI/SNF remodelling complex, is one of the most frequently mutated EC drivers. Concurrent *ARID1A* and *ARID1B* loss is associated with dedifferentiated EC. Other chromatin remodelers, such as *CHD4* and *BCOR*, have been identified as drivers in EC. However, systematic analysis of alterations of chromatin modifiers in EC beyond the SWI/SNF complex is still lacking. We sought to address this and define the associated changes in chromatin architecture.

Material and Methods

We analysed whole genome sequence (WGS) data from the Genomics England 100,000 Genomes Project EC cohort (n=665) along with WES and RNA-seq from The Cancer Genome Atlas (TCGA) pan-cancer (n=10,295) and EC (UCEC and UCS, n=586) cohorts. We analysed chromatin accessibility profiling (ATAC-seq) data from the TCGA pan-cancer cohort (n=410) and a panel of EC cell lines (n=12).

Results and Discussions

We identified 15 chromatin modifiers as EC drivers in the GEL cohort. These include known EC drivers such as *ARID1A*, *ARID1B*, *ARID5B*, *CTCF* and additional pan-cancer drivers *CREBBP*, *SETD1B*, *SETDB1* and *SMARCA4* new to EC. *ARID1A* mutations are highly over-represented in EC (TCGA EC vs pan-cancer cohort, Fisher's Exact Test, Odds ratio = 10.53, p-value < 2.2e-16) with 40-53% of samples harbouring a mutation. The 1p36.11 region, which contains the *ARID1A* gene, is deleted in 66 samples in the GEL cohort.

BCOR, a member of the PRC1.1 complex, is mutated in 18% of all EC samples with a recurrent N1459S mutation in 9% of samples near-unique to EC. *CDH4*, a member of the NuRD complex, mutated in 20% of EC, has 2 missense hotspots in its functional domains. Conversely, alterations in *CDH3* and *HDAC2* consist in significant proportions of frameshift deletions. *CTCF* is mutated in half of the samples with mismatch repair deficiency subtype.

There are significant differences in the open chromatin regions in relation to SWI/SNF mutational status in EC and colorectal cancers, as well in EC derived cell lines.

Conclusion

Alterations in chromatin remodelers are highly recurrent in EC, with approximately two-thirds of cases harbouring mutations in at least one gene. The mutational status of chromatin modifiers correlates with altered open chromatin profiles. Investigating perturbations of chromatin architecture is critical for understanding differentiation and disease progression in EC.

EACR22-0046

Next-Generation Sequencing for detection and follow-up of NSCLC patients with rare EGFR exon 20 insertions

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Introduction

EGFR exon 20 insertions (ex20ins) have a frequency of ~2% in non-small cell lung cancer (NSCLC) and patients show poor responses to EGFR tyrosine Kinase inhibitors (TKIs). FDA-approved techniques (Cobas or Therascreen) only target <50% of ex20ins and next generation sequencing (NGS) can help to identify uncommon and new variants. Here, we present our experience with NGS in FFPE and liquid biopsy samples from patients with these uncommon mutations.

Material and Methods

FFPE biopsies from 447 NSCLC patients (p) arriving to our Hospitals from 2018 to 2021 were prospectively tested by NGS. Tumor DNA was purified using QIAamp DNA FFPE Tissue Kit (Qiagen) and submitted to NGS using a 30-gene GeneRead™ QIAact Custom Solid Tumor Panel (Genereader®, Qiagen). For blood monitorization, circulating free DNA (cfDNA) was automatically extracted from 4 ml of plasma using the Qiasymphony robot and QIAasymphony DSP Virus/Pathogen Midi Kit and tested by NGS.

Results and Discussions

Sixty-two of the 447 NSCLC patients (13.8%) included in the study harbored EGFR mutations in exons 18-21. Regarding ex20ins, they were detected in 7/447 p (1.6%), representing 11% (7/62) of the total population of EGFR-mut p. The majority of ex20ins p. were women 71% (5/7), non-smokers 86% (6/7) and had adenocarcinoma histology 100% (7/7). All ex20ins were located in the A767-C775 loop following the αC-helix, including p.A767_V769dup (n=2), p.S768_D770dup (n=1), p.V769_D770insGTV (n=1), p.N771delinsSH (n=1), p.N771_H773dup (n=1) and p.H773_V774insAH (n=1). Of them 5/7 (71%) would have been missed by Therascreen or Cobas. Some of the ex20ins p had additional co-mutations in TP53 (3/7) and MET (1/7). In addition, three of them showed concomitant amplification of EGFR and one co-amplification of CDK4 and MYC. Five of the 7 ex20ins were found in advanced stage patients, two of them received first line chemotherapy and two an EGFR TKI (afatinib or osimertinib), with short responses. A fifth p was included in a clinical trial of avimantanab combined with chemotherapy and had a partial response that is still ongoing after 6 months. Allelic fractions of the ex20ins in blood correlated with the clinical evolution of the disease.

Conclusion

Implementation of NGS in routine clinical practice allows identifying rare ex20ins in EGFR, including new variants, which would be missed by other methodologies. New therapeutic approaches are needed for ex20ins p, which respond poorly to current treatments.

EACR22-0271

SSP-ddPCR: A method to quantify rare somatic single nucleotide variants utilizing SuperSelective primers by droplet digital PCR

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Introduction

Somatic single-nucleotide variants (SNVs) occur every time a cell divides, appearing even in healthy tissues at low frequencies. These mutations may accumulate as “neutral” variants during aging, or eventually, promote the development of neoplasia or enhance metastases. Here, we present the SSP-ddPCR, a droplet digital PCR (ddPCR) based approach that utilizes tailored SuperSelective primers to accurately quantify and validate the proportion of rare SNVs.

Material and Methods

We selected five potentially pathogenic variants identified by whole-exome sequencing (WES) occurring at low variant allele frequencies (VAF) in at-risk colon healthy mucosa of patients diagnosed with colorectal cancer (CRC) or advanced adenoma (AAD). SuperSelective primers were designed and tested to precisely quantify the mutant sequence and confirm the observed WES VAF in these five variants and two additional APC SNVs detected in two CRC lesions.

Results and Discussions

Designed SuperSelective primers allowed specific quantification of SNVs present at low VAFs *in silico* and in clinical samples. SSP-ddPCR confirmed the presence of three out of five selected SNVs in normal colonic mucosa with allelic frequencies $\leq 5\%$.

Conclusion

These results indicate that SSP-ddPCR provides a rapid and feasible methodology to validate next-generation sequencing data and accurately quantify rare SNVs, and thus displays a potential tool for diagnosis and stratification of at-risk patients based on mutation profiling.

EACR22-0511

Sleeping beauty mutagenesis screens identify genes required for primary and metastatic breast cancer in cooperation with p53- versus p53:Rb-loss

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Introduction

Basal-like (BL) breast cancers (BC), most of which are also triple-negative (TN) tumors, are an aggressive subtype of BC affecting relatively young women. Despite their response to highly toxic chemotherapies, the metastatic forms are deadly. Therefore, there is an urgent need for developing more effective and less toxic therapies for metastatic BL tumors. The tumor suppressor genes *TP53* and *RB1* are frequently altered in BL tumors with their

concurrent alterations reported in approximately 40% of these tumors. Subset of BL/TN tumors with these alterations have poor prognosis. Alterations of *TP53* and *RB1* are undruggable; therefore, identification of oncogenic drivers that cooperate with their loss in TNBC may uncover novel therapeutic modalities.

Material and Methods

I employed conditional sleeping beauty (SB) mutagenesis screens to identify cooperating oncogenic drivers in primary mammary tumors and their matched lung metastases developed in *p53*- and *p53:Rb*-deficient mouse models, using a WAP-Cre deleter line to induce transposon mobilization. Genomic DNA from SB-induced tumors (primary and metastatic) were used to prepare SB insertion libraries which were then deep sequenced on Illumina platform to identify gene-centric common insertion sites.

Results and Discussions

SB mutagenesis accelerated tumorigenesis in mice with *p53* or *p53* plus *Rb* deficiencies relative to wild-type controls. This study identified known cancer genes such as *Met*, *Notch1*, or *PTEN* in both primary and metastatic tumors. Moreover, some of the genes that were identified by transposon insertions in *p53:Rb*-deficient metastatic tumors are involved in the Ras pathway (*Hras1* and *Sos2*) or cell motility/migration (*Rock2*, *Fam19a5*, and *Ntn1*). Enrichment of Ras pathway was also confirmed in BL/TN human tumors and cell lines by bioinformatic analysis. Finally, inhibition of ERK1/2 of Ras-Raf-MAPK pathway by ulixertinib (a potent ERK1/2 inhibitor) suppressed growth of BL/TN breast cancer cell lines with high Ras activity which also carry *TP53* and *RB1* alterations. Interestingly, this growth suppression was more pronounced in cells with stable *RB1* knocked-down relative to parental *RB1* wild-type cells.

Conclusion

These results suggest that genes involved in Ras pathway activation may promote metastatic *p53:Rb*-deficient TN breast cancer. Hence, therapeutic modalities such as ERK1/2 inhibitors may serve as potential therapeutic intervention against this metastatic TNBC subgroup.

EACR22-0569

Novel susceptibility genes in familial multinodular goiters

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Introduction

Multinodular goiter (MNG) is a common thyroid disease characterised as a benign, diffuse and nodular enlargement of the thyroid gland. The clustering of goiters within families and early age of onset in some families supports a genetic predisposition. Previous studies into the genetics of goiter development include linkage analysis of extensive pedigrees of families with a high incidence of goiters. Despite multiple candidate loci being identified, *DICER1*, a gene involved in microRNA biogenesis, is the only causative gene to

be identified within the candidate loci. Recently, *DGCR8*, another gene involved in miRNA processing, has been linked to familial MNG (fMNG). This study aimed to identify novel genes involved in predisposition to MNGs.

Material and Methods

Whole exome sequencing of blood-derived DNA collected from 32 individuals with MNG, 24 non-affected individuals from 9 families (2 branches of 1 family were treated as separate families). Variant prioritisation was performed according to 3 filtering workflows: 1) A per-family analysis; 2) An analysis looking at variants in genes included in a gene list and seen in at least 2 individuals per family; 3) An analysis investigating shared variants and/or genes in at least 2 families and in at least 2 individuals per family.

Results and Discussions

A *DICER1* truncating variant, c.418C>T, p.Gln140Ter was identified in Family 5. No alterations in other genes involved in miRNA biogenesis or variants shared by at least 2 individuals per family and seen in at least 2 of the 9 families were identified. Analysis of genes common in at least 2 of the 9 families and with variants present in at least 2 members per family yielded only 2 genes: *NCOR1* and *PIK3C2G*. Unique variants of uncertain significance (VUS) in *NCOR1* were identified in families 1 and 7. Moreover, a likely pathogenic variant and VUS in *PIK3C2G* were found in the 2 branches of Family 4, respectively. Further validations in the patients' neoplasms and in an additional validation set containing sporadic nodules and familial MNGs will be performed.

Conclusion

Germline pathogenic variants in *DICER1* were observed in 1 out of 9 families (11%) and no other putative driver gene of fMNGs or candidate genes in the linkage loci were identified in the other 8 families. Other potential candidate genes that require further investigation were identified on a per family-basis.

EACR22-0595

Chromosome engineering and CRISPR-Cas9 viability screening reveal increased metastatic capacity targetable by patient-specific synthetic lethality

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Introduction

Chromosomal aberrations are a frequent event in a majority of tumor entities and a hallmark of cancer. In hepatocellular carcinoma (HCC), chromosome 8p (chr8p) loss of heterozygosity (8pLOH) is overserved predominantly and is correlated with poor overall patient survival. However, no single chr8p tumor suppressor gene reveals strong pro-tumorigenic effects and can account for the increased mortality. Given the extent and complexity of 8pLOH, this suggests that co-suppression of multiple genes can concomitantly promote tumor growth.

Here, we use chromosomal engineering and CRISPR-Cas9 viability screens to investigate the effects of concurrent loss of multiple chr8p genes.

Material and Methods

Chromosomal deletions were introduced by a dual guide CRISPR-Cas9 approach. Single cell clones with 8pLOH were obtained by FACS sorting. PCR, Sanger Sequencing, fluorescence in situ hybridization (FISH) and RNA sequencing (RNAseq) were used to validate effective chr8p deletions. Functional effects were analyzed by viability, proliferation, migration and invasion assays. Gene dependencies were investigated by genome-wide CRISPR-Cas9 screens in 8pLOH clones and validated by RNAi mediated knockdown.

Results and Discussions

Chromosomal deletions were introduced into different HCC cell lines and stable single clones were obtained. PCR, FISH and Sequencing confirmed loss of heterozygosity in single cell clones. Genome-wide mRNA abundance was significantly altered and gene set enrichment revealed deregulations in migration and tumor microenvironment. Metastasizing potential of 8pLOH cells was further investigated in vitro and in vivo and goes in line with increased patient mortality. Subsequently, several metastasis suppressor genes were identified on chr8p in a reduced RNAi migration screen. Additionally, by performing a genome-wide knockout screen, we discovered and independently validated dependencies specific for 8pLOH patients with decreased survival. These include novel synthetic lethalties of DOCK1 and NUDT17 in Rho/Rac-signaling and DNA damage response, respectively.

Conclusion

We describe a new approach and cell model to study large scale genomic aberrations and 8pLOH in cancer. State-of-the-art genomic technologies were integrated to explain increased patient mortality by the loss of cooperating metastasis suppressors. Extending the view to patient treatment, we identified novel synthetic lethalties as potential targets and therapeutic options in patients with 8pLOH.

EACR22-0793

The E3 ubiquitin-ligase HAKAI is associated to CMS2 colorectal cancer and stemness

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Introduction

HAKAI is an E3 ubiquitin-ligase (CBLL1) that induces the ubiquitination and subsequent degradation of E-cadherin at cell-cell contacts in phosphorylation dependent manner, inducing epithelial-to-mesenchymal transition (EMT), tumour progression and carcinoma metastasis. Moreover, HAKAI expression is significantly increased in human colon adenocarcinoma, compared with healthy colon tissues. HAKAI has been proposed as a potential new therapeutic target to block tumour development and metastasis.

Material and Methods

Using robust bioinformatic and machine learning methods, we have analysed HAKAI expression on a large integrated cohort of 1273 primary tumour samples from colorectal cancer (CRC) patients. We have collected genome-wide expression data and clinical survival data for this large CRC cohort to achieve accurate survival analysis and patient risk prediction. HCT116 colon cancer cell lines were grown in 2D monolayer and 3D tumorosphere, in an enriched stemness conditions. Viral transduction of shRNA-Hakai silencing in an inducible system of HT29 were used to analyse RNA and protein expression of pluripotency and differentiation RNA and protein genes.

Results and Discussions

Transcriptomic mRNA analysis in a cohort of human CRC patients show that HAKAI is gradually increased in clinical stages TNM stages (stage I to stage IV) of colon adenocarcinoma. Moreover, HAKAI expression was analysed in different types of colorectal tumours attending to the classification of Consensus Molecular Subtypes (CMS) of CRC. According to HAKAI gene expression, we observed its association with CMS2 tumours that are reported to show activated WNT- β -catenin pathway. Given the key role of Wnt/ β -catenin signalling in regulating the differentiation of stem cell populations, we analysed HAKAI expression in monolayer colon tumour cells and 3D tumorospheres grown in an enriched stemness conditions. HAKAI is upregulated in 3D conditions compared to monolayer cultures, accompanied by an increase of stemness markers, while the differentiation genes were downregulated. In addition, silencing HAKAI induces the downregulation pluripotency genes and an upregulation of differentiation genes.

Conclusion

Our data suggest that HAKAI could be a potential good marker for CMS2 for CRC signature and that is associated to high stemness in CRC.

EACR22-0847

Germline genetics correlates with aberrant signaling pathways in cancer

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Introduction

Cancer is a complex disease influenced by a heterogeneous landscape of both germline genetic variants and somatic aberrations. Although there is an increasing evidence suggesting an interplay between germline and somatic variants and a large number of somatic aberrations in specific pathways are now used as hallmarks in many well-known forms of cancer, the interaction landscape between germline variants and the aberration of those pathways in cancer is still largely unexplored.

Material and Methods

We conducted a set of genome-wide association studies (GWAS) using >10,000 human samples across 33 cancer types characterized by TCGA, considering 40 traits defined using a large collection of somatic aberration types across 10 well-known oncogenic signaling pathways. A bootstrap approach was used to reduce false positive calls.

Genomic and transcriptomic data were used to establish functional links between associated variants and the corresponding oncogenic signaling pathways. GWAS summary statistics were used to build a set of polygenic somatic scores (PSS) using a cross-validation approach. Predictive accuracy of PSS was validated using PCAWG and CCLE data and an independent cancer specific cohorts and was then tested against cancer subtypes, cancer-specific clinical variables and patient's survival data.

Results and Discussions

We identified 75 genome-wide significant ($p < 5e-8$) associations between 62 SNPs and 17 traits and an additional 217 suggestive ($p < 1e-6$) associations between 213 SNPs and 24 traits. 132 associated SNPs revealed *cis*-associations with genes that were observed functionally correlated with the corresponding traits oncogenic pathways.

TCGA-based PSS of 26 traits individually demonstrated an average AUC > 0.55 and 10 of them, among 15 that could be tested, demonstrated consistent statistically significant shifts across about 2,000 PCAWG patients and/or 1,000 CCLE cell lines based on the

corresponding oncogenic pathway aberration status. In addition, PSS demonstrated prognostic value for clinical OS and PFI endpoints and their integration demonstrated AUC>0.8 in classifying patients' specific cancer subtypes. Finally, using an independent prostate cancer dataset, we show that integration of our PSS can identify patients with cancer-specific aggressive phenotypes.

Conclusion

We showed that germline genetics shapes susceptibility to somatic aberrations in oncogenic signaling pathways and that polygenic scores can describe patients' genetic liability to develop specific cancer molecular profiles.

EACR22-1025

GENOTYPE ANALYSIS OF ESR1, ESR2 AND CYP19A1 GENES OF PAKISTANI BREAST CANCER PATIENTS

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Introduction

Reproductive factors pose a risk for development of breast cancer owing to the lifetime exposure to estrogen, a hormone responsible for cell proliferation in the breast. So, estrogen is a key to breast cancer pathogenesis that produced its effect by binding its receptor (ER), which exists as ER α and ER β , encoded by *ESR1* and *ESR2* genes, respectively while its metabolism occurred through *CYP19A1* gene. Numerous studies have conducted to evaluate the association of *ESR1* and *ESR2* variants, but they have reported mixed outcome. Study was conducted to evaluate the association between of six variants of all three genes and breast cancer and associated features in Pakistani women.

Material and Methods

Retrospective case control study was conducted and ninety breast cancer and 100 case control healthy volunteer patients were included. DNA was isolated from peripheral blood by using the Qiagen DNA kit. After that DNA samples were enriched by performing the PCR and PCR products were subjected to genotyping through Pyrosequencing. Epidemiological, clinical, and reproductive factors were analyzed. Statistical tests conducted included the χ^2 test, Fisher's exact test, and Mann-Whitney and Kruskal-Wallis tests or their parametric equivalents.

Results and Discussions

Minor allele frequencies (MAF) of tagging SNPs rs4775936 (*CYP19A1*) was decreased while MAF of rs6493497 (*CYP19A1*), rs2077647 and rs2228480 (*ESR1*) were significantly higher in breast cancer patients vs. controls. It was observed that there was non-significant increase in rs1256049 and rs1256030 (*ESR2*) was compared to breast cancer patients vs. controls. Patients carrying rs4775936 genotypes had higher risk, while rs1256049 genotype carriers had reduced risk of breast cancer. The association of *ESR1* and *ESR2* gene variants with breast cancer depended on ER and Her-2 status. *ESR1* rs2228480 and *ESR2* rs1256049 were associated with breast cancer in ER-positive cases, and *ESR1* rs2044647, and rs1256030 were associated with breast cancer in Her-2-negative cases, while the association of *ESR2* rs1256049 with breast cancer was seen in Her-2 positive cases.

Conclusion

ESR1 and *CYP19A1* alleles and genotypes, and specific 3-locus *ESR1* haplotypes are related with increased breast cancer susceptibility in Pakistani women. However, *ESR2* variant and specific 1-locus *ESR1* haplotype have a protective effect.

EACR22-1103

Neural-related Hyper-Methylation fingerprint in various cancer types. Probable role in neural stemness

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Introduction

Promoter DNA hypermethylation is a frequent epigenetic abnormality in cancer. DNA hypermethylation plays a key role in modulating gene expression, and consequently promoting tumorigenesis. Previously, we identified a neural-related DNA methylation fingerprint in colon cancer, with most of the top significantly hypermethylated and downregulated genes having functions in the nervous system. Here, we aim to further characterize DNA methylation neural signature in cancer, and its relationship to cancer biology.

Material and Methods

In silico analysis on 15 solid cancer types available in The Cancer Genome Atlas (TCGA) was performed. A linear model was fitted for each probe to determine differential methylation in each cancer type. Gene ontology analysis was then performed. Chi-squared test was used to verify enrichment of neural-related genes, and to correlate this with gene bivalency.

Results and Discussions

DNA methylation levels did not differentiate between neural-related and non-neural-related genes. However, all tested cancers showed enrichment in neural-related terms in the respective hypermethylated genes. This neural-related DNA methylation fingerprint was partially explained by the bivalency status of chromatin and was also found in premalignant tissue samples. Genes normally overexpressed in neurons, rather than those overexpressed in neural progenitor cells, were mainly affected by DNA methylation in cancer.

Conclusion

Our study confirms the presence of a neural-related DNA hypermethylation fingerprint in various cancer types and brings new insights into its contribution to carcinogenesis.

EACR22-1235

(TA)n-dinucleotide repeat expansions in MSI cancers and implications for Werner synthetic lethality

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Introduction

Microsatellite instability (MSI) is caused by deficient DNA mismatch repair (MMR) and is a ubiquitous feature of cancer. Werner syndrome (WRN) helicase is involved in genome stability and DNA repair. We identified WRN as a synthetic-lethal target in dMMR/MSI cancers and highlighted WRN inhibition as a therapeutic option for dMMR/MSI cancers refractory to available therapies. A previously unappreciated genetic feature of dMMR/MSI cancer cells, DNA (TA)_n-dinucleotide repeat expansions, were recently reported to cause vulnerability to WRN depletion. Our mechanistic understanding of TA-dinucleotide repeat expansion biology is limited and their potential therapeutic implications are unclear.

Material and Methods

To investigate the landscape of (TA)_n-dinucleotide repeat expansions in cancer, we inferred (TA)-dinucleotide repeat expansions by performing coverage analysis in a collection of hundreds of preclinical cancer models and human tumors profiled by whole genome sequencing (WGS). We validated these findings in a set of cancer cell lines and organoid cultures by performing long-read WGS. Furthermore, we investigated TA-expansions in single-cell-derived clones from human MSI tumors and cancer organoids. Finally, we inferred TA repeats in laser capture microdissection (LCMB)-derived samples obtained from patients affected by familial cancer predisposition syndromes.

Results and Discussions

Our analysis unveils the landscape of TA-repeats alterations in a large collection of tumors and preclinical models, informing on the level of intertumor heterogeneity and their association with variable levels of WRN dependency. In addition, we investigated intra-patient tumor heterogeneity of TA-repeats length both within clonal organoids expanded from normal and neoplastic colorectal stem cells, and within different subclones derived from MSI cancer organoids. Furthermore, analysis of non-neoplastic and neoplastic tissues from patients affected by familial cancer predisposition syndromes revealed the pattern of TA-repeats expansions associated with various DNA-repair pathway alterations. Finally, we will discuss the clinical implications of our findings, as TA-repeats heterogeneity may affect sensitivity and resistance to the future generation of WRN inhibitors.

Conclusion

Our data provide fresh insights into the inter and intra-tumoral heterogeneity of TA-dinucleotide repeat expansions in human cancers. These data contribute to understanding the role of WRN in cancer and exploiting Werner as a therapeutic target in cancer.

EACR22-0752

ORAL PRESENTATION

New epigenetic players in diffuse intrinsic pontine glioma

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Introduction

Diffuse intrinsic pontine glioma (DIPG) is the most aggressive brain tumour found in children with a peak incidence of 5-7 years of age, and a median survival of less than one year. In

more than 60% of DIPG cases, there is a driving somatic mutation of the H3F3A gene, that leads to a lysine 27 to methionine mutation at the histone variant H3.3 (H3.3K27M). This mutation causes a global loss of H3K27me3 by the inhibition of the PRC2 complex. Physiologically, histone variants H3.3 and H2A.Z are frequently found in the same nucleosome and cooperate to regulate transcription. We and others have identified that acetylation of H2A.Z (H2A.Zac) can be an oncogenic driver in adult cancer through the mislocalization of H2A.Z at promoters and enhancers of cancer-associated genes. However, the role of H2A.Z in DIPG has never been studied, and we hypothesized that H2A.Zac cooperates with H3.3K27M to drive DIPG oncogenesis. Here we aim to unravel the molecular relationship between H2A.Zac and H3.3K27M in DIPG and their link to oncogenesis.

Material and Methods

We have profiled H2A.Zac by mass spectrometry and ChIP-seq in patient-derived DIPG cell lines. In addition, ChIP-seq of the different histone marks and H3K27M to identify chromatin regulatory regions and its association with H3K27M and H2A.Zac.

Results and Discussions

Histone mass spectrometry revealed the level of H2A.Zac is significantly higher in samples with H3K27M compared to H3.3WT. In addition, the comparison between H2A.Zac with H3.3K27M ChIP-seq data in several DIPG cell lines, showed that around 30% of H3.3K27M peaks overlapped with H2A.Zac marked regions, a similar proportion found between H3.3 and H2A.Z under physiological conditions. Interestingly, active enhancers are the most enriched regulatory regions for H3.3K27M/H2A.Zac overlapping regions and those enhancers are associated with genes involved in pathways commonly altered in H3.3K27M gliomas.

Conclusion

These data suggest H2A.Zac levels are altered in DIPG and H2A.Zac may be involved in aberrant enhancer activation in DIPG and thus constitute a novel therapeutic target for H3.3K27M+ DIPG.

EACR22-0676

Functional investigation of somatic non-coding variants in a DNA regulatory element affecting MCF2L gene expression in neuroblastoma

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Introduction

Somatic variants in non-coding DNA are mostly unexplored referring to tumorigenesis, although disease-associated genomic variation in cancer may be found in regulatory regions. In our recent work, whole genome-sequencing (WGS) data from 151 neuroblastoma (NB) primary tumors identified an enrichment of non-coding somatic mutations in DNA elements regulated by tumorigenic transcription factors (TF), suggesting that they could contribute to deregulation of specific pathways in NB pathogenesis. Specifically, two variants (chr13:114058179:T:C, chr13:114058184:G:A) in a putative cis-regulatory element (CRE) affect *MCF2L* expression, whose lower levels correlated with disease unfavorable markers. *MCF2L* is a RhoGEF involved in the Rho/Rac pathways acting in cytoskeleton dynamics, differentiation and malignant transformation, mainly through its interaction with RhoA and

Cdc42. In a study evaluating metastatic NB, stage 4 patients with unfavorable prognosis showed a significantly reduced expression of *MCF2L* and other genes participating in Rho signaling, suggesting that this pathway could determine a poor outcome in advanced disease.

Material and Methods

The identified variants have been functionally characterized through luciferase assay in NB cell lines. Motif analysis with the R Bioconductor package “motifbreaker” verified if the variants altered TFs binding motifs. Three-siRNA silencing experiments have been performed to evaluate if the selected TF (STAT3) affects *MCF2L* expression.

Results and Discussions

The two non-coding variants induced a decreased activity of the putative CRE, with chr13:114058179:T:C showing a higher effect. In accordance with *MCF2L* levels in mutated samples, the variants could either increase a repressor or disrupt an activator binding. Motif break analysis on chr13:114058179:T:C and expression data of 498 NBs from R2 database identified STAT3 as putative TF involved in *MCF2L* regulation.

Indeed, *STAT3* and *MCF2L* showed a positive correlation, consistent with STAT3 role as transcriptional activator. *STAT3* silencing confirmed this correlation *in vitro*; indeed, upon STAT3 depletion, *MCF2L* showed a strong decrease in expression.

Conclusion

Our preliminary findings show that non-coding somatic variants may affect a putative CRE regulating *MCF2L* expression due to STAT3 binding alteration. Allele-specific ChIP-qPCR to confirm TF binding *in vitro* are ongoing. We are further investigating the chr13:114058184:G:A variant and *MCF2L* role in differentiation and in driving NB aggressiveness.

EACR22-0806

Functional characterisation of Bromodomain and Extra-Terminal motif (BET) inhibition in anti-endocrine resistant invasive lobular carcinoma

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Introduction

Approximately 10% of all breast tumours are invasive lobular carcinoma (ILC). ILC tumours tend to be estrogen receptor-positive (ER+). Despite the histological differences with other ER+ tumours, ILC patients are treated in the same manner as ER+ patients. However, approximately half of all tumour recurrences develop resistance. As such, there is a pressing need to develop tailored therapeutic options for anti-endocrine resistant ILC patients.

Previously our lab has shown that high BRD3, a bromodomain and extra-terminal (BET) motif family member, is an ILC specific marker for poor prognosis.

Material and Methods

2D and 3D cultures of SUM44PE(SUM44) and MDA-MB-134-VI(MM134) anti-endocrine resistant ILC cell lines were used to assess BRD inhibition using BET inhibitors (BETi). RNA-Sequencing was carried out to study gene expression changes caused by BETis and BRD3 knock out (KO). Genome enhancer upstream analysis was applied to identify master transcription regulators (MTRs). These drugs were further tested in ILC cell-derived xenograft (CDX) models established by mammary intraductal implantation (MIND).

Results and Discussions

We found that ILC cell lines that do not respond to anti-endocrine therapy are sensitive to a panel of BETis in both 2D and 3D assays. Out of 9 BETis, JQ1 and Mivebresib showed the highest potency and were selected for further research. Both drugs downregulated genes which were enriched in pathways such as sequence-specific DNA binding and downregulated MTRs included oncogenes such as AURKA and TNF. In contrast, upregulated genes were enriched in pathways such as negative regulation of growth. Among the MTRs of upregulated genes, we identified potentially pro-tumorigenic MTRs including FGFR3, UBE2S, TRIM24, UBA1, SRXN1 and MMP15 as candidates for dual targeting. Further analysis of BETi combined with erdafitinib, an FGFR inhibitor, drives increased cell inhibition compared to either agent alone. Among all MTRs, 37 were affected by both, BETi and BRD3 KO. Finally, *in vivo* studies showed that JQ1 alone and in synergy with tamoxifen significantly decreases tumour burden in this tamoxifen resistant ILC CDX model.

Conclusion

Taken together this data characterises the function of BETis in ILC and highlights the use of BET inhibition in the anti-endocrine resistant ILC setting.

EACR22-0245

Breast cancer disparities in South African populations

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Introduction

There is scarcity of clinical, genetic and demographic data on South African (SA) breast cancer (BC) patients due to limited research. Subsequently, SA diagnostic and management strategies are extrapolated from comparable populations such as African Americans and Europeans. While this approach works for the SA White population, the opposite can be said for Blacks and Indians, suggesting a different aetiology for the African population. This study investigated differences and similarities among SA population groups, and other Western populations.

Material and Methods

We retrospectively examined clinical data of 737 BC and high-risk patients presenting at Inkosi Albert Luthuli Central Hospital between 2011 and 2021. Data collected included diagnosis, age of onset, tumor characteristics, racial classification, family history and genetic screening results. Race and family history were self-identified while clinical and genetic data were as described by healthcare workers.

Results and Discussions

The cohort constituted 40.8% Indians, 40.5% Blacks, 16.4% Whites and 2.4% Coloureds, which were consistent with the province's composition. The average age of onset was 46.1

years, specifically 51.1, 46.8, 43.3 and 36.2 for Indians, Whites, Coloureds and Blacks, respectively. This is comparable with global trends, but SAs present ~10yrs earlier. At least 87.5% were BC patients while 10% were high-risk cases. Genetically, 11.8% of the patients carried 31 pathogenic *BRCA1/2* variants including founder mutations, and large genomic rearrangement. Blacks and Indians carried variants that are considered uncommon in SA and are excluded from the first-line screening panels by SA laboratories.

Most triple-negative BC patients were Black which explains their high mortality. Surprisingly, Black patients did not present with a family history despite disease characteristics of hereditary nature. The males constituted 3.5% and were mostly Indian. Overall, there was a gradual increase in the number of annual new cases, but an exponential increase was noted among Blacks.

Conclusion

The SA populations vary among themselves and the world. These differences greatly affect the diagnostic and treatment outcomes, hence, must be taken into consideration. Currently, strategies in place favor 10% of the population, but with better understanding of non-Whites, more can be done to improve the clinical outcomes for all affected patients and their families.

EACR22-0583

Functional characterization of the 11p11.2 risk locus identifies *HSD17B12* as a susceptibility gene in neuroblastoma

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Introduction

Genome-wide association studies (GWAS) identified dozens of common genetic risk variants and genes associated with susceptibility to develop neuroblastoma (NB). Among the risk loci identified in our GWAS study on 2101 cases and 4202 controls of European-American origin, 11p11.2 risk locus remained unexplored. The study aims to functionally characterize the 11p11.2 NB predisposition locus by evaluating how the risk variants can regulate their target genes and how these genes can act in NB initiation.

Material and Methods

To identify functional variants, we annotated 72 putative causative SNPs with ATAC-Seq and ChIP-seq data from 7 NB cell lines. The most functionally significant SNP was characterized by luciferase reporter assays in NB cells and, since it was predicted to map inside a GATA3 binding motif, GATA3 allele-preferential binding was assessed by ChIP-qPCR experiments. The SNP regulatory role was verified performing expression quantitative trait loci (eQTL) analysis using RNAseq and SNP array data from adrenal gland tissues. Gene expression was correlated with clinical parameters using 498 tumors RNAseq data. MTT and invasion assays in combination with gene silencing were performed to evaluate *HSD17B12* role in NB tumorigenesis.

Results and Discussions

The functional annotation analysis identified rs2863002 T>C as the candidate regulatory variant of 11p11.2 NB risk locus, with the minor allele C associated with NB risk in GWAS. rs2863002 maps within *HSD17B12* first intron, in an open chromatin region with enhancer

features. The C risk allele, which probably disrupts GATA3 binding, exhibited significantly higher enhancer activity in *in vitro* assays and was associated with higher *HSD17B12* expression in adrenal gland tissues. The inverse correlation between *HSD17B12* and *GATA3* expression seen in 498 tumors, was confirmed *in vitro* by *HSD17B12* expression increase after *GATA3* silencing in NB cells. Higher *HSD17B12* expression was significantly associated with the high-risk NB group and with poor survival, suggesting its potential oncogenic role in NB. Indeed, *HSD17B12* silencing significantly reduced cell growth and invasion in NB cells and induced an increase of neuronal differentiation markers.

Conclusion

Our results suggested that rs2863002 C risk allele predisposes to NB by altering a GATA3 binding site and leading to an increase of *HSD17B12* expression. This study allowed the identification of a functional variant in an NB risk locus and helped to elucidate the biological role of genes involved in NB development.

EACR22-0675

Functional characterization of somatic noncoding variants identifies CTTNBP2 as putative tumor suppressor in neuroblastoma

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Introduction

The impact of somatic noncoding variants on neuroblastoma (NB) genesis remains poorly investigated. Our recent whole-genome sequencing study on 152 primary NB highlighted an enrichment of somatic variants in NB tissue-specific regulatory regions that may alter the binding of transcription factors (TFs) involved in tumorigenic processes. An interesting cluster of noncoding mutations nearby the transcription start site (TSS) of *CTTNBP2* (cortactin binding protein 2) is predicted to affect the expression of this gene by altering the binding sites of a set of TFs.

Material and Methods

In NB cell lines, luciferase assays on wild-type and mutated cis Regulatory Elements (cREs) were performed to validate the alteration of cRE activity caused by somatic noncoding mutations. Allele specific ChIP-qPCR was used to confirm the predicted TF binding alteration. Silencing experiments by three-siRNA are used to verify the correlation between TF and CTTNBP2 expression. CTTNBP2 role in NB tumorigenesis and differentiation was evaluated by cell invasion assay, cell viability assay and through treatment with all-trans retinoic acid (ATRA).

Results and Discussions

Two noncoding variants (chr7:117513318:G>C and chr7:117513582:G>T) in CTTNBP2 cRE were predicted to affect STAT3 and SIN3A motifs using the R Bioconductor package “motifbreaker”, respectively. In NB cells, chr7:117513318:G>C decreased the cRE activity, while chr7:117513582:G>T increased it, altering SIN3A binding. SIN3A silencing correlated with an increase of CTTNBP2 levels, suggesting the regulatory role of this TF on CTTNBP2. CTTNBP2 silencing increased NB cell invasion capability and viability, suggesting that CTTNBP2 acts as tumor suppressor in NB. This latter observation was further supported by the analysis of 498 NBs profiled by RNAseq (freely available data) that found low CTTNBP2

mRNA levels associated with poor survival and unfavorable clinical parameters. Interestingly, we also observed that CTTNBP2 mRNA and protein levels increased at 72h while decreased at 6 to 21 days after ATRA treatment. Further experiments to elucidate the role of CTTNBP2 in NB differentiation are ongoing.

Conclusion

These results highlight that the somatic noncoding variants can have a role in NB initiation and they might represent novel biomarkers for prognostic, diagnostic and therapeutic applications.

EACR22-1145

Key mediators of TNF-alpha transcriptional response identified by arrayed and pooled sgRNA library Perturb-seq screens

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Introduction

We performed a single-cell 10X Perturb-Seq screen on HEK293-Cas9 cells with an 84-sgRNA pooled library, aimed at identifying genes involved in TNF α -induced transcriptional response. A parallel screen was performed with cells transduced in arrayed format with the same set of sgRNAs as the pooled library. The 10X Perturb-Seq screen was carried out in triplicate with increasing amounts of cells/10X reaction, to (1) determine the minimum number of cells/sgrNA needed to cover the full complexity of a pooled library, (2) determine the maximum allowed number of cells/10X reaction before degradation of single-cell resolution occurs.

Material and Methods

Perturb-Seq screen: cells were transduced with the pooled sgRNA library, puromycin selected & expanded for 10 days, then split into 2 samples for 72h TNF α treatment/mock-treatment. After treatment, cell samples were loaded on the 10X Genomics Chromium Controller per manufacturer's specifications at 2,000, 7,500 & 15,000 cells/reaction. Arrayed screen: cells were transduced with individual sgRNAs, puromycin selected and expanded for 10 days, then split into 2 samples for 72h TNF α treatment/mock-treatment. Genome-wide transcriptome analysis was performed for the arrayed screen. For the Perturb-Seq screen, 10X Cell Ranger software was used.

Results and Discussions

Both screens identified TNFRSF1A, IKBKG, CHUK and RELA as mediators of TNF α transcriptional response. Increasing the number of cells per 10X reaction from 2,000 to 7,500 & 15,000 (corresponding to 23, 90 & 180 cells/sgrNA ratios, respectively) showed a positive effect on sgRNA library coverage: while the 23 cells/sgrNA ratio proved too low, the 90 cells/sgrNA was adequate to cover the full complexity of the library. At the same time, raising the number of cells/10X reaction from 2000 to 7,500 and 15,000 did not adversely affect the magnitude of the measured effect of TNFRSF1A, IKBKG, RELA, CHUK KO on TNF α -mediated transcriptional activation.

Conclusion

Based on the results obtained with the small sgRNA library used, we conclude 1) To cover the full complexity of any pooled sgRNA library, a minimum coverage of ~100 cells/sgrNA is advised, 2) With a loading limit of 15,000 cells/10X reaction (Chromium Controller), a minimum of one 10X reaction will be needed for every 150 sgRNAs in the library, 3) the NGS sequencing depth needed to analyze the results of the Perturb-Seq experiment will be

directly tied to the complexity of the library (each sgRNA in the library will need full genome-wide transcriptome coverage).

EACR22-1199

Genome editing for stem cells

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Introduction

With years of experience in high-end technologies, Cellecta Inc. has developed genome editing and transcriptome profiling services for stem cells. Due to their self-replicating ability and capacity to differentiate into multiple types of cells, induced pluripotent stem cells (iPSCs) have significant potential for biological and therapeutic applications. CRISPR/Cas9 technology can be easily applied to efficiently edit and screen immortalized cell lines. However, iPSCs--which can provide more relevant, patient-derived models for human pathology--are much more difficult to engineer as CRISPR/Cas9 delivery can be inefficient and toxic.

Material and Methods

We used WTC11 human iPS cells from Coriell Institute for Medical Research. Lentiviral transduction was used to generate a Cas9 line as well as to deliver CRISPR sgRNA libraries. Cas9 activity was assayed and confirmed using the relevant Cellecta's CRISPRtest kits. The pluripotency of WTC11-Cas9 cells was confirmed by flow cytometry using conjugated Ab for Oct3/4, TRA1-60, SSEA-4. StemDiff Trilineage Differentiation Kit from StemCell Technologies was used to validate the ability of the WTC11-Cas9 cell line to differentiate to the three germ layers: ectoderm, mesoderm and endoderm. Genome-wide transcriptome analysis was performed using the DriverMap™ Human Genome-Wide Gene Expression Profiling Assay.

Results and Discussions

After screening multiple viral and mammalian promoters, we successfully identified EF1a as the most efficient promoter for sustained Cas9 expression, while viral promoters like CMV and SFFV proved to be quickly inactivated a few days after transduction. We were able to generate both polyclonal and clonal validated iPSC-Cas9 lines with confirmed Cas9 activity and differentiation potential. We were also able to confirm retained Cas9 activity after differentiation

Conclusion

We showed that iPS cells can be successful engineered to express active Cas9, for the use in CRISPR/Cas9 functional genetic screens, as well as transcriptome analysis using Cellecta DriverMap™ Gene Expression Profiling platform. Clonal iPSC-Cas9 lines are preferred over polyclonal lines, due to higher heterogeneity of Cas9 expression and activity in the latter.

EACR22-0274

POSTER IN THE SPOTLIGHT

THE SHEDDASE DOMAIN OF ADAM10 AUGMENTS THE INTERACTION OF LEUKEMIA CELLS WITH THE BONE MARROW NICHE IN VIVO AS SHOWN BY RECONSTITUTING PDX LEUKEMIA CELLS WITH CRISPR-CAS9-INDUCED KNOCKOUT

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Introduction

Tumor-microenvironment interactions are critical for leukemia formation and maintenance. Their interruption therefore represents an attractive therapeutic strategy. We developed a CRISPR-Cas9 screening approach for functional analysis of surface molecules in patient-derived xenograft (PDX) models of acute leukemias (AL) *in vivo* in order to decipher common and individual tumor specific vulnerabilities and gene dependencies.

Material and Methods

CRISPR library size was determined by genetic barcoding. Stable expression of Cas9 and sgRNA constructs in two PDX samples. Enrichment by MACS and injection into NSG mice. Gene depletion analysis using MAGeCK algorithm to screen and functional competitive *in vivo* assays to validate candidates. Characterization of the ADAM10 Knockout (KO) or inhibitor treated cells for engraftment capacity by homing assay, frequency of leukemic stem cells by competitive limiting dilution transplantation assay (LDTA), sensitivity towards routine chemotherapy by *in vivo* competitive chemotherapy trials. Rescue assay by reconstitution of ADAM10 variants in competitive *in vivo* assays.

Results and Discussions

Running a customized CRISPR-Cas9 screen targeting about 100 cell surface molecules in two AL PDX samples, sample-specific as well as commonly depleted genes were identified. Candidates were confirmed using a competitive *in vivo* approach testing the PDX cells with and without KO in the same mouse. Among other candidates, ADAM10 was depleted in both PDX models. *In vivo* competitive experiments confirmed its essential role in PDX models from 6 additional patients with either acute lymphoblastic leukemia (ALL) or acute myeloblastic leukemia (AML). Treating PDX cells with ADAM10 inhibitor reduced the engraftment capacity into the bone marrow significantly. KO of ADAM10 reduced the frequency of leukemia stem cells and ADAM10 KO PDX samples in both lineages showed increased sensitivity towards routine chemotherapy treatments. Reconstitution of ADAM10 KO PDX cells with recombinant wildtype variant *in vivo* rescued the phenotype, while an enzymatic domain lacking variant did not, highlighting the importance of ADAM10's sheddase function in leukemia maintenance.

Conclusion

We established CRISPR-Cas9 drop-out screens in PDX models *in vivo* as technology to explore patient-specific tumor dependencies. Our data revealed a yet unknown role of ADAM10 to maintain patient leukemic cells in the bone marrow niche. ADAM10 thus represents an attractive future therapeutic target for the treatment of acute leukemia.

Spatial genomic analysis provides evidence for submucosal glands as smoke-protected niche in the proximal airway

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Introduction

The influence of tobacco on lung tumorigenesis has been extensively described as driven by genomic aberrations related to carcinogens in cigarette smoke. We have begun investigating the genomic consequences of smoking in phenotypically normal bronchial epithelium in a variety of individuals with various smoking histories. This work found that the majority of epithelial cells accumulate DNA damage proportionally to the individuals smoking habits. However, the analysis of whole genomes of single bronchial epithelial cells from previously smoking patients indicated the abundance of a population of cells with a near-normal mutation burden, mirroring populations found in never-smoking individuals. This observation raises the question, how a proportion of basal cells evades the mutagenic effect of tobacco exposure.

Material and Methods

To explore the origin of this phenomenon and to assess the spatial distribution of epithelial clones in the lung, we performed laser-capture microdissection to isolate submucosal glands and epithelial microbiopsies from 14 individuals with pre-malignant lung disease or cancer. Whole genome sequencing of these biopsies was utilised to assess the landscape of single nucleotide variants (SNVs), insertions and deletions (indels) as well as copy number and structural variations (SVs). In addition, we leveraged SNVs to create phylogenetic trees for each patient, analysing the relationship between clones at distinct locations in the proximal airway.

Results and Discussions

Distinct histological locations demonstrated strong differences in burden of SNVs and indels. Submucosal glands and ducts showed a 4.1- and 2.4-fold decrease in SNV burden compared to bronchial epithelium. Commonly observed driver mutations of squamous cell carcinoma, including NOTCH1, TP53 and SETD2, were found under positive selection within phenotypically normal cells. Moreover, genes not previously identified in lung cancer, including MAGEC1 and HCN1, were found to be significantly positively selected as identified by dNdScv. By closely examining the phylogenetic relationship between submucosal glands, ductal and surface epithelium, our results highlighted that clones in the airway are direct descendants from the cells residing in glands.

Conclusion

Ultimately, our results provide evidence for a protected niche within the submucosal glands in the proximal airway, where tobacco carcinogens cannot penetrate and which give rise to clonal populations that repopulate the airway upon smoking cessation.

RNA sequencing reveals unique and overlapping transcriptional targets of GLI1, GLI2 and GLI3 in melanoma cell lines

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Introduction

Melanoma is known as the most aggressive and deadliest of all skin cancers. Understanding the interplay of different molecular pathways leading to development and progression of melanoma is important for developing therapeutic strategies. One of the recent approaches in cancer therapy is targeting the Hedgehog-GLI (HH-GLI) signaling pathway. This pathway is important during cellular proliferation, differentiation and stem cell maintenance. HH-GLI signaling pathway has been associated with skin tumors, but its mechanism differs between melanoma and non-melanoma tumors. In order to elucidate the role of GLI proteins in melanoma, we performed RNA sequencing on human melanoma cell lines with over-expressed GLI1, GLI2 or GLI3.

Material and Methods

Melanoma cell lines A375, MEL224 and CHL1 were transfected with GLI1, GLI2 or GLI3 plasmids. Non-transfected cell lines were used as controls. Total RNA was isolated 48h post transfection and sample quality was checked on Agilent 2100 Bioanalyser. RNA sequencing was performed on Illumina Novaseq 6000. The quality of sequencing reads was checked with FastQC tool and reads were mapped to the human reference genome (hg38) with STAR. Mapped reads were quantified with RSEM. Differential gene expression was computed using edgeR in R software. In order to select GLI target genes for further validation, a list of differentially expressed genes (DEGs) was filtered according to FDR values and log₂F_C. DEGs were then selected for further investigation according to their role in diseases, role in different signaling pathways and their importance in melanoma. 12 genes were chosen for validation by qPCR. The validation was performed on three cell lines used for RNA sequencing and on 11 additional melanoma cell lines.

Results and Discussions

After filtering DEGs according to FDR<0,01 and log₂F_C>1, we have found 1806 genes with significantly changed expression. Our results confirmed GLI1 and PTCH1 as known targets of HH-GLI pathway. None of the 12 chosen targets are yet reported as GLI targets, and only half of them are recently reported to have a role in melanoma pathogenesis. Majority of the genes are overlapping targets of GLI1 and GLI2, which was expected, while GLI3 regulates a small number of separate gene targets.

Conclusion

In this study we have identified unique and overlapping targets of GLI1, GLI2 and GLI3 in melanoma cell lines and confirmed 12 of them by qPCR on an expanded set of melanoma cell lines, suggesting these genes are novel targets of HH-GLI signaling in melanoma.

Investigating how Human Papilloma Viruses (HPVs) affect prostate cancer progression

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Introduction

Prostate cancer progression is highly concerning as five-year survival rates rapidly drops from 98% to 30% in metastatic disease. Recognised risk factors associated with prostate cancer development include family history, advanced age, testosterone, African American descent, diet and environmental exposure. Sexually transmitted infections (STIs) may also play a key role in the onset and progression of prostate cancer. Indeed, recent evidence suggests that the STI Human Papillomavirus (HPV) is linked to prostate cancer pathogenesis, with 18.93% of prostate cancer cases positive for HPV infection worldwide.

Material and Methods

To investigate the link of HPV in prostate cancer progression both experimental and bioinformatic analysis methods are being complemented. The presence of high-risk HPVs will be investigated during prostate cancer progression histologically, using immunohistochemistry of formalin-fixed paraffin-embedded (FFPE) human prostate sections at various clinical stages of prostate cancer progression. Customised pipelines are also being built to identify the presence of HPV viral strains in next-generation sequencing patient sample datasets.

Results and Discussions

This is work in progress and updates on the two-pronged project regarding both histology and bioinformatics customised pipelines for RNA-seq and WGS datasets, should be provided during EACR 2022.

Conclusion

The data obtained would be highly useful in understanding the link of HPV and prostate cancer, which is currently highly debated in literature.

EACR22-0384

Multimodal Analysis of DNA and Proteins in Single Cells

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Introduction

Cancer is a disease driven by genomic mutation and selection. While the advent of next generation sequencing has revolutionized genomic analysis, the data obtained from bulk sequencing do not represent the heterogeneity in cancer. Single-cell RNA sequencing has advanced the knowledge of cellular heterogeneity, but this method is limited to the analysis of transcriptomes. As proteins are the key functional machinery of cells, capturing the differential protein expression from cell to cell would benefit the study of cancer biology. Multimodal approaches such as CITE-seq only partially address this problem by allowing simultaneous analysis of transcriptome and surface proteins, but not intracellular proteins that play vital roles in cellular functions. Additionally, these methods do not directly analyze DNA, hence do not provide a readout of genotypic information such as single-nucleotide variants (SNVs) and copy number variations.

Material and Methods

To perform single-cell multiomic DNA and protein analysis, cells are first treated with oligonucleotide-barcoded antibodies targeting surface proteins, then fixed and permeabilized, followed by incubation with barcoded antibodies for intracellular proteins. The resulting cells are processed on the Mission Bio Tapestry® platform that enables encapsulation of single cells in droplets. Targeted DNA sequencing libraries are generated from the single cells using a multiplex panel of primers targeting regions of interest. Protein sequencing libraries are separately generated from the oligonucleotides off the antibodies.

Results and Discussions

Cell types are successfully identified based on SNVs from the single-cell DNA-seq results. Analysis of cell surface proteins (e.g., CD3, CD19, CD34, and CD38) allows for classification of cells based on surface markers. Intracellular protein detection enables measurement of pivotal proteins in cancer mechanisms, including development (GATA3), apoptosis (BCL2 family proteins), proliferation regulators (TP53), and phosphorylated proteins involved in cell cycle signaling pathways (phosphorylated ERK). Protein expression UMAP resolves the cells into populations that correlate with the results from DNA analysis.

Conclusion

This is the first method to effectively link surface and intracellular protein measurement with targeted DNA analysis. This approach enables the correlation of genotypic and phenotypic readouts from single cells, allowing for concurrent multimodal analysis of cancer evolution and the protein expression that drives it.

EACR22-1158

Ribosome composition and translation regulation in EMT and bone metastasis

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Introduction

The ribosome has long been considered as a stable machinery which synthesizes proteins from mRNAs. However, it appears that its composition varies depending on the physio-pathological context, including in cancer. These data lead to the emerging concept of “specialized ribosomes” referring to ribosomes with distinct translational activities that open up novel opportunity in understanding cancer.

More than 90% of cancer related deaths result from metastasis. Bone is one of the most frequent sites of metastasis from various primary carcinomas, especially breast and prostate. Therefore, understanding the molecular mechanisms underlying bone metastasis is a major issue. These past years, the focus have been directed onto the epithelial-to-mesenchymal transition (EMT) and its influence on the bone marrow microenvironment in supporting the outgrowth of bone metastatic lesions.

EMT is a dynamic and reversible physiological process during which epithelial cells transfer to a mesenchymal morphology. It is driven by a profound transcriptional reprogramming

induced by EMT transcription factors (EMT-TFs), such as Zeb, Snail or Twist family members. They drive the replacement of expression of epithelial markers (e.g. E-cadherin) by mesenchymal ones (e.g. Vimentin). In primary tumors, this change allows cells to acquire mobility and the capacity to migrate from the primary site.

Material and Methods

Here, using an integrated omic approach, we analysed the change in ribosome composition and translation occurring in EMT thanks to human mammary epithelial cells (hMEC) overexpressing or not the EMT-TF Zeb1.

Results and Discussions

Using RiboMethSeq, we identified change in ribosome composition at the level of ribosomal RNA. Moreover, using single-end RNA-seq and Ribo-seq analyses, we show by Gene Ontology analyses, that genes implicated in the ossification process are highly translated in mesenchymal hMEC-Zeb1 cells in comparison to epithelial hMEC cells. Up to date, no study has ever investigated the relative importance of translation compared to transcription in EMT nor the involvement of ribosome-mediated translational reprogramming in EMT.

Conclusion

Our working model is that alteration of ribosome composition occurs during EMT to induce a change in ribosome structure/function thus promoting a translational reprogramming contributing to the establishment and/or maintenance of EMT, thus directly impacting metastasis formation.

EACR22-0167

Transcriptional and post-transcriptional network analyses in colorectal cancer subtypes and their reflection in surrogate biospecimens.

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Introduction

The high inter-tumoral heterogeneity of colorectal cancer (CRC) is one of the main obstacles in its diagnosis and prognosis. The classification of CRC has been improved by the development of the Consensus Molecular Subtypes (CMS) classification system based on gene-expression data, but little is known about the gene regulatory networks driving each subtype. This is relevant also to identify novel and accurate non-invasive molecular biomarkers. Here, we sought to integrate data from CMS classification with small RNA sequencing and pan-cancer target assay to better describe CRC heterogeneity. The outcomes will be explored also in the stool miRNome of patients, to identify novel biomarkers in this surrogate tissue mirroring tissue alterations.

Material and Methods

Paired tissue specimens were collected from 115 CRC patients (tumor and adjacent normal mucosa). RNA-seq and small RNA-seq were performed on each sample. After the CMS

classification, each subtype was genomically characterized with the TruSight Oncology 500 (TSO) cancer-panel. Specific genes and miRNA signatures of each CMS were identified using differential expression analysis.

Results and Discussions

Based on RNA-seq data, individuals were assigned to CMS1 (n=9), CMS2 (n=24), CMS3 (n=26) and CMS4 (n=24). Differential expression analyses revealed 1096 differentially expressed (DE) genes in CMS1, 4218 in CMS2, 27 in CMS3 and 190 in CMS4. Notably, only *CEMIP* was DE in all subtypes. CMS3, the most represented group, showed the highest number of coding variants (n=683) of which 384 were unique missense variants. CMS1 was the least represented subtype but it included almost all subjects with microsatellite instability. *APC* harboured the most coding variants in all CMS groups but CMS1.

In total, 380 miRNAs were differentially expressed in tumor tissue compared to adjacent mucosa. Based on these results CMS-specific miRNA-target interactions will be defined based on validated annotations and co-expression analysis. Putative upstream regulators will also be identified by functional enrichment analysis.

Finally, the stool miRNome of CRC patients will be analysed to identify in this surrogate tissue the similarity with the obtained outcomes.

Conclusion

Integration of TSO, RNA-seq and small-RNA-seq data is a feasible approach to shed new light on CRC heterogeneity and reveals a potential role for stool as less invasive biomarker of CRC.

EACR22-0705

A single cell mRNA atlas of rhabdomyosarcoma: identifying foetal developmental targets

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Introduction

Rhabdomyosarcoma (RMS) is a highly aggressive skeletal muscle cancer that primarily affects children. Traditionally, RMS has been classified into embryonal rhabdomyosarcoma (ERMS) and alveolar rhabdomyosarcoma (ARMS) for prognostic purposes. The oncogenic fusion of *PAX3* or *PAX7* with *FOXO1* is expressed by most ARMS, the more lethal subtype. While the overall outlook for children suffering from RMS has improved in recent decades, metastatic and relapse RMS remains incurable. RMS is thought to form from foetal mesenchymal cells that differentiate aberrantly along a trajectory of striated muscle cells. We hypothesise that within these trajectories of aberrant development lie novel therapeutic opportunities.

Material and Methods

Fundamentally, there may be mRNA transcripts that are present exclusively during foetal development, yet persist postnatally in RMS. These represent formidable targets for

chemical compounds or immune therapy approaches. In order to identify these targets and unravel the foetal roots of RMS, we have performed single-cell gene expression profiling of 10 RMS tumours.

Results and Discussions

In total, 29,765 single cells passed quality control and were annotated using canonical lineage markers. Uniform manifold approximation and projection (UMAP) visualisation showed a global separation of ARMS and ERMS subtypes. As expected based on previous literature, foetal myogenic markers *MYOG* and *MYOD1* were more highly expressed in ARMS, compared to ERMS which expressed *MYF5* and *PAX7*. We investigated which stage of skeletal muscle development cancer cells recapitulate by performing cancer to normal cell comparisons, using methodology based on logistic regression. We found that ARMS most resembled mature foetal myoblasts and myofibers, while ERMS resembled immature *PAX3+* and *PAX7+* skeletal muscle progenitors.

As RMS cells use transcripts of foetal skeletal muscle cells, like *MYOD1* and *MYOG*, we interrogated the expression of genes relatively restricted to foetal development that may represent novel oncofetal targets. We uncovered three groups of genes in this analysis: (1) genes expressed in RMS and CNS tissues, (2) foetal myogenic genes, (3) and genes with restricted expression patterns, including IGF binding proteins. Together, these analyses reveal a number of foetal cancer genes that may lend themselves as targets in RMS.

Conclusion

We have built a single cell developmental atlas of RMS, thus providing a quantitative unbiased account of foetal transcription underpinning RMS.

EACR22-0795

Exploring the effect of Body-Mass-Index (BMI) and Gender on Gene Expression in human colorectal epithelium

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Introduction

Colorectal cancer (CRC) is now the second most common cause of cancer death globally. GWAS studies have established that cancer risk mediated through common genetic predisposition can be linked to variance in gene expression. Since obesity and male gender impart substantially elevated CRC risk, we studied transcriptional profiles of normal colorectal mucosa using RNAseq to better understand the effect of these risk factors on gene expression levels.

Material and Methods

Normal colorectal mucosa was sampled from 365 subjects (208 males, 157 females) in Edinburgh (SOCCS study) and Oxford, UK (InterMPHEN study). 103 samples were taken from a single site during surgery (stripped mucosa) and 515 samples were collected through rectal biopsy from cancer patients and patients with other unrelated conditions. In total, we obtained 29 transverse colon samples, 131 caecum samples, 270 rectum samples, 139 sigmoid colon samples and 49 descending colon samples. RNA was extracted from normal mucosa using a proprietary RNA extraction kit. Illumina HiSeq 4000 platform was used for RNA sequencing on 150bp (SOCCS) and 75bp (InterMPHEN) paired-end reads. Data

processing and gene filtering followed the GTEx Project pipeline v8. The DESEQ2 package with raw counts in R was used to build models for the Differential Gene Expression Analysis (DGEA). The models for Linear Mixed Effect Models (LMEM) were built with TPM counts using the R package “lme4” in R. Models were adjusted for sex, BMI, cancer status, age, sample site and batch. An absolute log2 fold-change > 1.2 was required and a BM adjusted p-value < 0.05 considered statistically significant.

Results and Discussions

RNAseq generated a median of 35 million reads/sample in SOCCS and 26 million reads/sample in IntermPHEN. Following GTEx filtering, there were 15,465 (SOCCS) and 13771 (IntermPHEN) genes available for analysis. Following exclusion of sex chromosome genes, DGEA identified five genes that were significantly associated with gender. Five additional genes were also associated with BMI by DGEA analysis. However, whilst these nominal signals are of interest, none of these genes remained significant when further interrogated by LMEM.

Conclusion

We found no systematic differences in gene expression in normal colorectal epithelium between males and females, nor did we find an effect of BMI. Although sample size and statistical power may limit our analysis, we are reassured that genetically predicted gene expression may not be heavily confounded by gender and BMI effects.

EACR22-1124

ORAL PRESENTATION

Genomic characterization of colorectal cancer across the world by de novo extraction of mutational signatures with SigProfilerExtractor

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Introduction

All cancers are caused by somatic mutations imprinted by the activities of different mutational processes, with each process leaving a characteristic pattern of mutations termed mutational signature. Characterizing mutational signatures can help understand the processes behind the onset and progression of tumors, with potential application as biomarkers in clinical practice. Colorectal cancer (CRC) is one of the most common and lethal cancers worldwide, although with substantial epidemiological differences among countries. CRC has been previously associated with mutational processes related to aging and different DNA repair deficiencies, although these findings have been limited to specific populations. Accordingly, the main goal of the present study was to characterize the genomic landscape of CRC across different countries with varying incidence rates.

Material and Methods

Whole-genome sequencing was performed for 150 colorectal adenocarcinomas from seven countries of different incidence and associations with previously known risk factors. Mutational signature analysis was performed using SigProfilerExtractor, a state-of-the-art tool for *de novo* extraction of mutational signatures. The performance of SigProfilerExtractor was compared to thirteen other tools by using 33 scenarios encompassing more than 60,000 synthetic tumor genomes.

Results and Discussions

SigProfilerExtractor outperformed all other computational approaches to perform *de novo* extraction of mutational signatures by identifying between 20% and 50% more true positive signatures while keeping the highest precision among all tools assessed using synthetic data reflecting high-quality genomic datasets. Seventeen different mutational signatures were found to be contributing to CRC mutations, showing significantly different activities among countries. Although most signatures were previously identified and indexed in the COSMIC database, two potential novel signatures were detected. Interestingly, two COSMIC signatures were identified for the first time in CRC: SBS10c, associated with *POLD1* exonuclease domain mutations and previously detected in normal colorectum, and unknown etiology SBS93, recently found in gastric cancers from the PCAWG cohort.

Conclusion

SigProfilerExtractor has proved to be the most accurate tool for *de novo* extraction of mutational signatures. By applying SigProfilerExtractor to the largest and most geographically diverse CRC cohort sequenced so far, we have uncovered new mutational processes active in this cancer type.

EACR22-0062

A Robust and Comprehensive Workflow for Extraction of DNA, RNA, Protein and Chromatin from FFPE Samples

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Introduction

Confident characterization and monitoring of alterations occurring to DNA, RNA, proteins and chromatin during onset and progression of any disease type is critical for optimizing therapeutic regime. However, inefficient sample preparation methodologies often impede the process of developing a robust protocol that can be applied by researchers regardless of the matrix or analyte complexity. In this study, we report development and optimization of a robust and comprehensive solution that allows reliable and reproducible isolation of DNA, RNA, proteins, and chromatin from Formalin Fixed Paraffin Embedded (FFPE) samples, scalable to 96-well format.

Material and Methods

This report will showcase the use of Adaptive Focused Acoustic® (AFA®) technology for deparaffinization and rehydration of FFPE tissue without the need of any hazardous, organic solvents enabling multiOMIC analysis from archived samples. We have developed workflows to isolate RNA and DNA from the same sample scalable to 96-well format and integration into lab-automation as well as workflows to characterize the proteome and chromatin landscape.

Results and Discussions

For nucleic acids, a workflow demonstrating isolation of DNA and RNA from the same sample is proposed, which can enable direct comparison of gene expression to DNA mutations and methylation profiles and can be automated in a 96-well format. For protein analysis, an efficient and fast protein extraction from FFPE samples suitable for downstream analyses (such as LC-MS) was developed, which in comparison to fresh frozen input samples demonstrates improved protein solubilization and high recovery for low abundance or difficult to isolate proteins without any technical bias. Furthermore, a collaborative study will demonstrate the development of a rapid and scalable extraction method to isolate chromatin from archived FFPE tissues suitable for ChIP. This part of the workflow allows confident analysis of histone modifications and transcription factor binding events from FFPE

archived material, which in turn enables thorough understanding of how the epigenetic landscape changes during course of a disease enabling stratification of patient subgroups.

Conclusion

In summary our workflows for FFPE samples allow for comprehensive, scalable and reliable analysis of biomolecules from archived material enabling integral understanding of underlying molecular characteristics of diseases.

EACR22-0170

An integrative in silico data analysis discloses a previously unidentified molecular subtype of colorectal cancer potentially eligible to immune checkpoint immunotherapy

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Introduction

Historically, the molecular classification of colorectal cancer (CRC) was based on the global genomic status, which identified microsatellite instability (MSI-H or MIN) in mismatch repair deficient (dMMR-MSI-H) CRC, and chromosomal instability (CIN) in MMR proficient (pMMR-MSS/MSI-L) CRC. With the introduction of immune checkpoint inhibitors (ICIs), MIN/CIN classification regained momentum as the dMMR-MSI-H condition predicted sensitivity to ICIs in clinical trials, possibly due to both high tumor mutation burden (TMB) and high levels of infiltrating lymphocytes. Conversely, pMMR-MSS/MSI-L CRC are mostly resistant to ICIs.

To better understand the relationship between MIN/CIN, and eventually discover additional CRC subgroups relevant for therapeutic decisions, we performed an integrative *in silico* dataset analysis.

Material and Methods

Copy number variation (CNV), mutational, transcriptomic, weighted gene co-expression network analysis (WGCNA), and base substitution fingerprints analysis were performed using R programming language, on 520 CRC samples' data downloaded from the TCGA database.

Results and Discussions

By performing unsupervised hierarchical clustering analysis of CNVs versus hypermutation status (HM), we identified two main tumor clusters: (A) CRC with few CNVs enriched in HM samples and (B) CRC with a high number of CNVs mostly including non-HM samples. Within (A), we noted a group of samples (7.8% of the entire dataset) with low CNVs and low TMB, which shared clinical-pathological features with HM CRC (HM-like CRC). Analyses of the mutational profiles and base substitution fingerprints indicated that HM-like tumors are distinct from both HM and non-HM CRC and are likely to develop and progress through different genetic events. WGCNA further highlighted differences amongst the groups at the gene expression level, and revealed HM-like tumors bear high expression of immune

response genes. Analysis of immune/inflammatory infiltration signatures confirmed that HM-like tumors showed T and NK cells signatures similar to HM samples.

Conclusion

Our work highlights HM-like tumors as a previously unidentified CRC subgroup potentially responsive to ICIs. If validate with additional *in silico* and experimental work, these finding can lead to expanding the fraction of patients eligible to ICI treatment.

EACR22-0328

A Study of the Anterior Gradient Homolog 2 (AGR2) Protein in Central Nervous System Tumors' Cell Lines

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Introduction

Glioblastoma (GBM) is the most highly malignant brain tumour in the human central nervous system (WHO grade 4) and comprises 80% of all gliomas. The pro-oncogenic anterior gradient 2 (AGR2) is an endoplasmic reticulum-related protein with disulfide isomerase features, shown to be involved in tumour growth and drug resistance in GBM.

Material and Methods

Meta-analysis was performed to determine AGR2 expression level in the CNSt vs normal brain and pathways linked with AGR2 high expression. Primary GBM tissue and cell lines were stained with immunofluorescence. To evaluate the drug response treatments, cells were treated with various drugs and concentrations. Impact of siRNA suppression on AGR2 expression was examined, were cells transfected with prepared AGR2 RNAi against Exome1,2,5/6, incubated, fixed and stained with anti-rabbit for AGR2. A combined value of drugs with siRNA was used to measure the inhibitory impact on AGR2 expression. After 24h transfection with AGR2 RNAi against Exome2, cells were treated with drugs for 3days.

Results and Discussions

AGR2 is highly expressed in meningioma and GBM compared to normal brain tissue and several clinically relevant genes are deregulated in AGR2-high tumours. AGR2 expression was detected in several glioma primary cell lines, both AGR2 and GRp78 were co-localized in tissues and cell lines. It has been linked to drug resistance, a series of cytotoxic studies were conducted to evaluate the link between AGR2 expression and drug resistance founding that AGR2 is significantly expressed in surviving cells. siRNA against AGR2 Exome 2 exhibited the greatest inhibitory impact, demonstrating the significance of its existence. Using double hits results in significant survival inhibition. When siRNA for AGR2 was used in conjunction with Temozolomide or Irinotecan, there was a substantial change in cell density compared to drugs alone, surviving cells are those that express AGR2.

Conclusion

AGR2 was highly expressed in high-grade GBM and meningioma. It's one of the CSC markers in the CNS system tumours and part of the UPR machinery. AGR2 expression was shown in the remaining survival cells following drug treatments or using siRNA, indicating AGR2 importance for cell proliferation and survival. Targeting, blocking AGR2 using antibodies and peptides is one way to eliminate cancer in the brain, among other cancer types.

A comprehensive study characterizing intra-tumor heterogeneity and genomic alterations under evolutionary pressure induced by chemotherapy in small cell lung cancer.

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Introduction

Small cell lung cancer (SCLC) is an aggressive, early metastasizing tumor with a 5-year overall survival of 5%. An initial good response to chemotherapy is followed by a rapid relapse of a drug-resistant tumor. Despite recent insights into the molecular biology of SCLC, the dynamic temporal and treatment-associated alterations require further investigation to elucidate how different genomic alterations affect lineage differentiation and oncogenic signaling. The goal of our research is to gain an in-depth understanding of the evolutionary biological processes underlying the adaptation of cancer to therapeutic interventions.

Material and Methods

We currently have generated a repertoire of 76 patient-derived xenograft models from biopsies or circulating tumor cells and characterized all cases at genomic and transcriptomic levels. Out of these, further functional characterization was performed for 22 models established from treatment-naïve patients, and for 8 models obtained at chemotherapy relapse, including multi-regional and longitudinal models. Patient-derived xenograft models were treated with two cycles of cisplatin/etoposide per week until the maximum volume of 1500mm³ was reached. Tumor samples were subjected to genome sequencing and single-cell transcriptome analyses.

Results and Discussions

Our patient-derived cancer models capture a multitude of genetic backgrounds, molecular subtypes and phenotypes regarding clinical courses and therapy response. The treatment response mostly resembled the clinical response of the donor patient, whereas models established from different tumor sites differed in their response. Some models of relapsed tumors maintained a good response; likewise, tumors treated *in vivo* that were rechallenged. Genome sequencing revealed mutation clusters with cancer cell fractions that point to the selection of clones following therapeutic pressure. In agreement with the genomic profiles, single-cell transcriptome sequencing of chemotherapy-treated tumors confirmed a dramatic shift in expression profiles and the distribution of subpopulations.

Conclusion

Here, we demonstrate a functional characterization of multi-regional tumor sites and longitudinal specimens, combined with a coupled analysis of therapy-induced genomic and transcriptional heterogeneity. Our work will yield deep mechanistic insights into the pathogenesis of small cell lung cancer under treatment and will provide an important foundation in the search for alternative treatment strategies for chemotherapy-resistant patients.

Optical Genome Mapping: Unravelling the genomic landscape of Solitary Fibrous Tumor

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Introduction

Solitary fibrous tumour (SFT) is a rare mesenchymal neoplasm most often occurring in adults. Although most cases are benign, the behaviour of SFT can be unpredictable. In 2013, the presence of NAB2:STAT6 gene fusion was identified in nearly 100% of cases. This rearrangement results from an inversion at the 12q13 locus, overlapping genes NAB2 and STAT6. Apart from this, few point mutations potentially promoting oncogenic properties have been described, and no clinical correlations with other structural variants (SVs) have yet been identified. Thus, the aim of this study is to elucidate the presence of additional SVs in SFTs and evaluated their association with the clinical outcome of SFT patients.

Material and Methods

Here we report the characterization of SFT samples with a new cutting-edge cytogenetic technique (Optical Genome Mapping, OGM) enabling comprehensive characterization of SVs in a discovery set of SFT patients. Selected tumor specimens were confirmed by H&E staining and STAT6 immunohistochemistry by two expert pathologists. For each sample, ultra-high molecular weight DNA was purified from 10mg of tissue and genomic DNA was labelled using a Direct Label and Stain DNA Labelling Kit. The Saphyr chip was ran to reach a minimum yield of 320 Gbp. *De novo* assembly and variant annotation pipeline were executed on Bionano Solve V3.7 and reporting and direct visualization of SVs was done on Bionano Access v1.7.

Results and Discussions

Preliminary results show that gross SV are not common in SFTs. A few copy number variations, Absence/Loss of Heterozygosity or aneuploidies were observed. Nevertheless, focal SVs such as short insertions, deletions, inversions and duplications were identified. Interestingly, many of these SVs overlap coding regions that could be disrupting the expression of certain genes or generating chimeric proteins, promoting oncogenic behaviour. Other SVs overlap non-coding regions, potentially affecting the transcriptional regulation of specific genes. Further analyses by total RNA sequencing are pending, in order to integrate both analyses and identify if these SVs are key in generating aberrant transcripts and/or inducing transcriptomic changes.

Conclusion

SFTs present a noteworthy stable genome with a few identifiable SVs. The integration of OGM and total RNA-sequencing with further *in vitro* assays, will allow us to confirm if these SVs play a role in the oncogenesis of this rare neoplasia.

EACR22-1234

DGCR8 and the new model of schwannomatosis

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Introduction

Familial multinodular goiter (fMNG) is a rare disorder. To date, the only bona-fide susceptibility gene for fMNG is *DICER1*, a gene encoding for a key protein in microRNA biogenesis.

Schwannomatosis (SWN) is a genetic disorder of Schwann cells and the least common form of neurofibromatosis. The accepted model for SWN tumorigenesis involves multiple hits over three steps to inactivate *LZTR1* or *SMARCB1* together with *NF2*. Following this pattern, most of *LZTR1*/*SMARCB1*-schwannomas acquire a somatic loss of chr22, deleting the three genes, and a somatic mutation affecting the remaining wildtype *NF2* copy on the germline pathogenic variant (GPV) allele.

In 2020, we characterized a GPV in the *DGCR8* microprocessor gene (c.1552G>A;p.E518K), as responsible for a familial form of MNG that manifests together with peripheral SWN by studying a three-generation family.

Material and Methods

To characterize the role of *DGCR8* as a tumor susceptibility gene, we selected patients following the next criteria: A) Patients from families with a constellation of peripheral schwannomas and thyroid nodules or thyroid tumors B) Cases with schwannomas and at least one of the following: Wilms tumors, choroid plexus tumors or pineoblastomas within the same patients or a first degree relative. Whole exome sequencing was performed using germline DNA from family members and DNA from eleven tumors. Two additional tumors were subjected to a Next generation sequencing targeted panel.

Results and Discussions

We identified a new case with the p.E518K GPV presenting multiple schwannomas, thyroid nodules, and papillary thyroid cancer with no family history reported, pointing to a *de novo* character of the variant. From our collection of 13 schwannomas, all tumors analyzed showed loss of heterozygosity expanding throughout chr22 which aligns with the most frequent scenario seen in other SWN models. Four schwannomas retained a wildtype *NF2* copy, while the remaining nine schwannomas (69.23%) acquired a secondary somatic mutation in *NF2*, thus representing a 3rd alteration on top of the loss of a copy of chr22.

In addition to the 13 schwannomas, the choroid plexus papilloma, and thyroid nodules analyzed showed a complete loss of chr22, supporting the pro-tumorigenic feature of the p.E518K variant and reinforcing the role of *DGCR8* as a novel susceptibility gene.

Conclusion

Here, we propose a model of *DGCR8*-schwannoma tumorigenesis consisting of six-hits three-steps and expand on the phenotypic spectrum associated to the *DGCR8* GPV c.1552G>A;p.E518K.

Using Single Cell Multiomics to Characterize Cancer

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Introduction

The single cell sequencing and spatial transcriptomic technologies are new and exciting technologies in cancer research. In opposition to “bulk” methods, these technologies allow studying biological processes from an unbiased perspective. With these methods it is possible for example to identify rare cell types, characterize the tumour heterogeneity or describe its spatial context. The Josep Carreras Leukaemia Research Institute (IJC) has recently opened a Single Cell Unit fitted with state-of-the-art equipment to carry out single cell sequencing and spatial transcriptomics experiments.

Material and Methods

The Single Cell Unit at the IJC provides researchers the possibility of performing multiomic single cell and spatial analysis with cutting-edge instrumentation. The Tapestry Platform from MissionBio is a droplet-based microfluidic device which allows single cell multiomic analysis at genomic and proteomic level. The Chromium Controller from 10x Genomics is also a droplet-based microfluidic equipment used to analyse gene expression, chromatin accessibility and cell surface proteins studies alone or in a multiomic perspective. In addition, it is possible to combine whole transcriptome spatial analysis with protein detection by using spatial transcriptomics.

Results and Discussions

A study performed by the Cancer Epigenetics group from the IJC has used the Tapestry Platform and the Chromium Controller to study myelodysplastic syndromes (MDS). Samples from patients with MDS treated with hypomethylating agents (HMAs) at different stages of the treatment have been assessed. The goal of the study is to improve MDS treatments and go into detail about this heterogeneous disease through a multiomic approach involving transcriptomics, genomics and immunoproteomics.

The Cancer Immunogenomics group has used Visium Spatial Transcriptomics to study different biological processes involving cancer. Specifically, they have studied haematopoiesis in mouse embryos or ovarian cancer before and after chemotherapy.

Conclusion

The applications available in the single cell unit of the IJC are allowing single cell studies to further characterize cancer from a single cell multiomic perspective.

Pan-cancer whole genome comparison of primary and metastatic solid tumors

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Introduction

Most cancer deaths are associated with the metastatic stage and poor treatment response. Hence, there is an urgent need to characterize the genomic differences between primary and metastatic tumors in order to understand the evolutionary process as well as to improve clinical interventions

Material and Methods

To address this, we performed a harmonized analysis of 5,751 whole genome sequenced tumors from 1,916 primary and 3,835 metastatic patients from 22 cancer types.

Results and Discussions

Cancer type specific comparison revealed that metastatic tumors displayed higher clonal fraction and a generally conserved karyotype compared to the primary counterparts. We also observed that the tumor mutational profile is primarily determined by the tissue of origin and that only a few cancer types showed an increased tumor mutation burden at the metastatic stage, which was mainly linked with increased APOBEC activity and treatment exposure. Remarkably, metastatic tumors displayed greater genome instability rates resulting in a significant increase in structural variants (SV) load across multiple cancer types. We found that this increase is strongly associated with higher genome ploidy, treatment exposure and inactivation of certain tumor suppressor genes such as TP53, CDKN2A or TSC2. The metastatic cohort also displayed an average increase of +1.5 driver alterations per patient, explained both as a selective pressure towards cancer treatment survival and to adapt to the higher aneuploidy rates. In fact, we found that 50% of treated patients have at least one driver alteration linked to treatment resistance.

Conclusion

Taken together our results show that except for a few cancer types, the genomic landscape of metastatic tumors is strongly shaped by the primary and the main differences lie in increased genome instability rates and the genomics scars during or after exposure to evolutionary bottlenecks.

EACR22-0367

Early Gastric Cancer: Molecular Characterization of Submucosa-Penetrating Tumors with Different Prognoses

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Early gastric cancer (EGC) incidence is characterized by a great geographical variability, e.g. in North-Eastern Italy, it represents nearly 25% of all surgical resected gastric cancers. According to Kodama classification, submucosa-penetrating tumors (Pen) can be divided into Pen A and Pen B. Pen A tumors present more aggressive characteristics and are associated with lower survival compared to Pen B and the other EGC subtypes. In the present study, we performed a molecular characterization of Pen tumors to verify if gene alterations are associated with relapse.

Material and Methods

The tumor tissues of 27 patients with a diagnosis of EGC, equally distributed between Pen A and Pen B, were analyzed. Clinical-pathological characteristics and follow-up data were collected for all the cases. Among the cases, 9 patients (33%) had a recurrence and/or died for EGC and 18 (67%) were disease-free/alive within 10 years of follow-up. The patients' DNA was extracted from Formalin-Fixed Paraffin-Embedded slices and characterized at the molecular level using Trusight Oncology 500 (Illumina®), including genetic alterations of 523 genes, as well as microsatellite instability (MSI) and tumor mutational burden (TMB) status. The pathway enrichment analysis was performed on Reactome database.

Results and Discussions

The most frequent alterations were **TP53**, **ARID1A** and **RNF43** mutations, as well as **ERBB2** and **c-MYC** amplifications. Mutations in **ARID1A** ($p=0.023$), **FBXW7** ($p=0.030$), **RNF43** ($p<0.001$), **PTPRT** ($p=0.030$) and **APC** ($p=0.007$) were associated with TMB status. No significant associations with any clinical-pathological characteristics, including the relapse status, were found except for **TP53** mutations that were more frequent in **intestinal-type EGC** ($p=0.041$). Of interest, mutations in **ARID1A** and **POLE** genes were significantly more frequent in **Pen A tumors** ($p=0.033$ and $p=0.041$, respectively). Finally, the enrichment analysis revealed that genes involved in apoptosis, necrosis, and signaling are significantly more altered in relapsed patients, as well as genes belonging to the DNA repair pathway.

Conclusion

The ongoing analyzes suggest that **ARID1A** and **POLE** mutations are more frequent in Pen A with respect to Pen B tumors, assuming a potential role in tumor aggressiveness. Finally, pathways such as cell death, signal transduction and DNA repair seem to influence the risk of relapse. Further analyzes on molecular markers and disease-free survival will allow us to better understand the molecular mechanisms underlying different prognoses in EGC.

EACR22-0403

Selecting non-cycling cells for single-cell DNA sequencing

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Introduction

New advances in single-cell DNA sequencing technology allows to decode the genomic complexity of cancers by analyzing their structural and numerical variants. These alterations can only be accurately evaluated in non-duplicating cells (at G1 phase) since DNA imbalances caused during cell cycle progression introduce noise to sequencing data. There are available markers for selecting cells in the G1 phase, but their suitability for posterior single-cell DNA sequencing is unknown. Hence, we aimed to identify an optimal marker

compatible with single-cell DNA sequencing technology for selecting non-cycling cells of experimental human cancer models.

Material and Methods

For isolating non-duplicating single-cell populations, we tested different combinations of cell cycle immunofluorescent markers: EdU + Cyclin A + DAPI, GEMININ + DAPI, and CDT1 + DAPI. Isolation of cells in the G1 phase was done using a high-accuracy image-based platform. Then, we performed shallow WGS to generate copy number profiles. Duplicating cells (in S and G2/M phases) were also collected, sequenced and profiled to confirm the accuracy of our G1-phase isolation procedure.

Results and Discussions

G1-phase profiles were successfully obtained from cells isolated using EdU + Cyclin A + DAPI staining. However, the accuracy of the GEMININ + DAPI and CDT1 + DAPI staining protocols showed to require optimization since their selection also included cells in S and G2/M phases. In addition, we observed that FACS-sorting protocols cannot be directly used with high-accuracy imaged-based platforms, needing previous adaptation steps, specially in the context of fluorescence discrimination.

Conclusion

We have demonstrated that high-quality copy number aberrations data can be generated from non-duplicating cancer cells selected with EdU + Cyclin A + DAPI staining, and that can be extended to other cancer models. Nevertheless, this combination of markers considerably restricted the availability of fluorescence channels of the isolating platform. Therefore, we suggest that the number of markers used for G1 phase selection should be further optimized, broadening the selection capacity to additional cell processes (i.e. apoptosis).

EACR22-0517

Genome-wide copy-number profiling using NGS targeted panels

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Introduction

NGS targeted capture panels are routinely used in the clinic allowing for small variant detection in a considerable number of genes which vary depending on the panel design. The capture efficiency of these panels is not optimal, resulting in off-target DNAs being sequenced along with the target DNA. The off-target percentage can range from 8-50% of the total number of reads. These off-target reads are uniformly distributed across the genome, therefore generating a low-coverage whole genome that can be used to derive copy number profiles, an extra layer of information that is often disregarded when analyzing this type of data. Here I assess the ability to extract copy number profiles from clinical samples, with varying ratios of overall success depending on the technology used, read depth, sample purity and preservation method.

Material and Methods

Three different NGS protocols were tested for copy-number profiling: Illumina TruSight Tumor (TST170), Illumina TruSight Oncology (TSO500) and Agilent SureSelect Exome (WES). We also compared differences in FFPE vs Fresh Frozen tissues by getting off-target read metrics. Finally, we assessed the number of samples that could be considered for further analyses such as copy-number signatures extraction.

Results and Discussions

About one third of FFPE samples subjected to TST170 were considered valid for copy number profiling, with a median off-target read number of 4.9M and a conserved ratio of off-target reads vs on-target reads (median 12.3%, standard deviation 2.8%). On the other hand, the same set of samples undergoing TSO500 panel sequencing yielded a lower amount of off-target reads (median 3.1M), with a lower ratio (median 7.5%, standard deviation 1.1%) and in this case only 15% of the samples were considered useable for further analyses. Also, we noticed an overall better performance when analyzing Fresh Frozen tissues vs FFPE, and a much higher number of off-target reads in all WES samples (>10M).

Conclusion

NGS targeted capture panels offer the possibility of deriving genome-wide copy-number profiles from off-target DNA. They can be theoretically considered as a shallow whole genome sequencing, that is often disregarded in the clinical context as these panels were conceived initially to only provide information on genetic variations in certain genes. Not all designs have the same efficiency and this impacts in the ability of extracting reliable copy-number profiles.

EACR22-0577

Tumor Mutational Burden in Male Breast Cancers characterized for germline mutations in DNA repair genes.

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Introduction

Tumor mutational burden (TMB) is a quantitative measure of the total number of somatic mutations per coding area of a tumor genome. Tumors with genetic alteration in DNA repair genes are hypothesized to have an increased TMB and tumors with high-TMB are more likely to express neoantigens which could enhance T-cell immune response. In breast cancer (BC), TMB has been shown to correlate with response to immunotherapy, specifically in the triple negative subtype. Compared with female BC, male BC (MBC) is rare and shows different biological and clinical characteristics. Here, we investigated TMB in MBCs characterized for germline mutations in DNA repair genes to obtain a molecular classification with possible clinical implications.

Material and Methods

A series of 73 Formalin-Fixed Paraffin-Embedded MBC samples, including 26 cases with *BRCA1/2* and *PALB2* germline mutations, was analyzed by gene panel sequencing with TruSight Oncology 500 that targets 1.94 Mb of the genome in 523 cancer-relevant genes. MBCs were classified as having high-TMB if they had ≥ 10 mutations/Mb. PD-L1 and PD-1 expression was obtained by RNA-expression data. Associations between TMB status and clinical-pathologic characteristics were also evaluated.

Results and Discussions

TMB values in MBCs range from 0 to 23.5 (median=3.8 mutations/Mb). High-TMB levels were observed in about 10% of MBCs. Overall, MBCs with germline mutations in *BRCA1/2* and *PALB2* showed higher TMB values ($p=0.03$), compared with wild-type MBCs. This finding is in line with evidences of high-TMB in other cancer types with alterations in DNA repair genes. MBCs with high-TMB were also more frequently estrogen-receptor negative ($p=0.02$), thus in line with evidences in triple negative female BCs. Furthermore, MBCs with high-TMB showed a higher expression of PD-1 ($p=0.006$) and a worse overall survival ($p=0.0001$), compared with low-TMB MBCs.

Conclusion

Our data indicate that MBCs with germline mutations in DNA repair genes and estrogen-receptor negative status may be characterized by high-TMB levels. These findings suggest that alterations in DNA repair genes and estrogen-receptor status in combination with TMB may be potential biomarkers to predict response to immunotherapy in MBCs. Further studies with larger sample size and patient follow ups are ongoing to provide more data for more individualized therapeutic approaches in MBC patients.

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EACR22-0709

The Growth Regulation by Oestrogen in Breast Cancer 1 (GREB-1) rs10929757 polymorphism and plasma omega-3 PUFA five years post-diagnosis of breast cancer.

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Introduction

The GREB1 gene is a gene often studied to assess estrogen-stimulated tumor progression, but its function outside the cancer setting is not well known. Polyunsaturated fatty acids (PUFA) influence many physiological functions. In vitro, in vivo studies as well as clinical trials have reported that a diet rich in omega-3 fatty acids can modulate GREB1 expression. In the present report we aim to evaluate the association of GREB rs10929757 with blood levels of omega-3 PUFA in healthy postmenopausal women, and in those who have survived breast cancer.

Material and Methods

We conducted a cross-sectional study on 49 Spanish women survivors of breast cancer and 63 healthy controls, to evaluate associations between blood omega-3 PUFA (α -linolenic acid (ALA; 18:3 ω -3), eicosapentaenoic acid (EPA; 20:5 ω -3) and docosahexaenoic acid (DHA; 22:6 ω -3). Lipids were extracted from plasma samples, and fatty acids were isolated and separated by gas chromatography with flame ionization detection. The Hardy-Weinberg equilibrium (HWE) test was performed. HWE P values and comparisons of genotype frequencies were estimated using the chi-square test. The associations of single-nucleotide

polymorphism with blood omega-3 PUFA in the subgroups were investigated by the analysis of covariance.

Results and Discussions

Genotype distributions in both groups conformed to HWE ($P > 0.05$). Genotype distribution was AA 11 (17.5%) AC, 33 (52.4%) and CC 19 (30.2%) in the control group while AA 12 (24.5%) AC, 30 (61.2%) and CC 7 (14.3%) in the breast cancer group. The genotype frequencies of rs10929757 were not significantly different ($P=0.133$). No differences in linolenic acid levels were observed in the two groups of women ($P=0.524$) or based on GREB1 rs10929757 genotype ($P=0.865$). Similar result was observed for eicosapentaenoic acid ($P=0.676$ for breast cancer group vs control and $P=0.459$ for GREB1 rs10929757) and for docohexaenoic acid ($P=0.732$ for the groups studied and $P=0.544$ for rs10929757).

Conclusion

Our study is the first to report that GREB1 rs10929757, is not associated with serum omega-3 PUFA in breast cancer survivors. The small sample size prevents us from definitively discarding a possible association due to the risk of type II error. We recommend that future studies should be developed to increase the sample size.

EACR22-0777

Whole-genome optical mapping reveals a complex genetic architecture involving translocations in pheochromocytomas

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Introduction

Genetic testing affects both treatment selection and clinical follow-up in patients with pheochromocytomas (PCCs), rare neuroendocrine tumours of the adrenal medulla. The genetic testing is mainly based on mutational analysis, limited data on complex genomic landscape in affected adrenal medulla in PCCs exists.

Material and Methods

Whole-genome optical mapping (Saphyr, Bionano Genomics) and whole exome sequencing were used to analyse the genome architecture of frozen adrenal medulla tissues from seven patients with PCCs (5 male/2 female, mean age 55 years).

Results and Discussions

Of enrolled patients, the germline mutations in PCC-associated genes were found only in one patient (P6; *NF1* gene, c.3871-2A>G). Using a whole-genome optical mapping on high-weight DNA (coverage ~250X), we revealed complex genetic rearrangements, including numerous translocations (median 7 translocations/patient, range 0-9), large deletions (14-54 deletions/patient; deletion size 0.5–589 kbp), and large inversions (0-4 inversions/patient; 9.1–230 kbp) in analysed tissues. In four patients (P2/P3/P5/P6), the translocations were detected on chromosome 1 in region 1q21.3-1q44, encompassing PCC-associated genes *SDHC*, *FH* and *CSDE1*, and these translocations occurred with various partners (chromosomes 3, 5, 8, 17). One of these patients (P3) had additional t(9;17)

affecting *NF1* gene. Other patient (P1) carried two translocations: t(3;10) affecting *RET* gene and t(2;7) affecting *MDH2* and *MET* genes, all found associated with PCCs. Two patients (P4, P7) carried no translocations, whereas both patients had numerous large deletions/inversions (P4: 14/3; P7: 54/1); none of these structural variants affected PCC-associated genes. The confirmation of genetic aberrations by long-read whole-genome (TELL-Seq®) and Nextera XT® (Illumina) sequencing is ongoing.

Conclusion

This study utilising optical mapping reveals a complex genome architecture in the affected adrenal medulla in PCCs, involving numerous translocations, large deletions and inversions often affecting the genes associated with PCCs. Refining the genome architecture of PCCs could contribute not only to the understanding of pathogenesis but also to the management of patients.

EACR22-1251

Analyses of multiple gene expression data implicate specific pathways in pancreatic cancer

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Introduction

Pancreatic cancer is one of the deadliest malignancies. Several gene expression profiling studies have been performed to determine the molecular drivers of pancreatic cancer. To facilitated drug target identification and biomarker discovery, we aimed to determine genes and pathways consistently underlying pancreatic cancer.

Material and Methods

We analyzed differential gene expression in five published PDAC microarrays and defined a gene as 'consistent' if 'upregulated' or 'downregulated' in ³4 cohorts (adjusted P<0.05). We further tracked the consistent genes in >20 additional datasets, including tumor and blood cell microarrays, bulk tumor and single cell RNA sequencing data, gene expression data of normal tissues and in over 40 cancer cell lines. We interrogated the association of the genes with tumor subtypes, proliferation, metastasis, mutation and tumor grade, and patient survival outcome.

Results and Discussions

Cell cycle, extracellular matrix receptor interaction, focal adhesion, p53 are among the topmost upregulated pathways in pancreatic cancer, which also included glycolysis and cholesterol metabolism. Cell cycle, p53 and proteosome pathways emerged in proliferation-high tumors. Further, focal adhesion, ECM-receptor interaction, and PI3K-Akt pathways emerged top in metastasis samples and were among the topmost pathways represented by genes that predicted poor overall survival in pancreatic cancer.

Conclusion

Our study has pinpointed specific cluster of pathways that underlie pancreatic cancer and thus highlight these pathways are high priority axes for further research.

EACR22-0774

POSTER IN THE SPOTLIGHT

Integrative genomic analysis of Basal Cell Carcinoma subtypes reveals the routes of skin carcinogenesis.

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Introduction

Basal Cell Carcinoma (BCC) of the skin is the commonest human malignancy with a lifetime risk of 30% and increasing incidence. BCC is characterised by constitutive activation of the Sonic hedgehog pathway and by hyper-mutator phenotype due to UV. The main histological subtypes of this cancer type (nodular, morpheiform and superficial) have not been characterized by genomic methods yet.

Material and Methods

We have assembled the largest to date BCC cohort representing major histological subtypes for genetic analysis by WGS (n=89), WES (n=310) and RNA-seq (n=360).

Results and Discussions

Over 20 million point mutations allowed unprecedentedly detailed analysis of UV-induced mutagenesis in skin cancer and revealed differences between BCC and melanoma. The mutational profile of BCC is determined by UV-light exposure however, it differs from melanoma by a high fraction of COSMIC signature SBS7b mutations. We explained it by the high contribution of GC-rich early replicating regions to mutagenesis in BCC, which are characterized by more condensed and difficult to repair chromatin in BCC than in melanoma. We report that loss of *TP53* is typically the first event in keratinocytes and precedes the loss of *PTCH1*. Moreover, we estimated from 17p copy neutral LOH regions that loss of *TP53* results in a dramatic increase of mutation rates in BCC. In line with that, BCCs with *TP53* mutations have a 2.5-fold higher mutation burden than BCC with wt *TP53*. We discovered novel BCC driver genes outside the Sonic Hedgehog pathway. 65% of BCC harbor mutations in HIPPO-YAP and Contact Inhibition of Proliferation pathways: (*FAT1* (30%), *NF2* (9%), *ARHGAP35* (18%), *CREBBP* (21%), *PTPN14* (21%), *LATS1* (9%)). Chromatin remodelling genes including *KMT2D*, *ASXL3*, *ARID1A* and others were frequently mutated in 70% of BCCs. The role of these genes was validated in the BCC cell line by siRNA screening followed by migration and proliferation assays. Promoter mutations or amplifications of *TERT* were identified in 48% of BCC and resulted in overexpression of the gene. High-risk morpheiform BCCs were different from low-risk nodular BCCs by transcriptional activation of HIPPO-YAP pathway confirmed on the protein level and fibrotic types of tumor microenvironment.

Conclusion

This study provides new insights into the role of epigenetics in UV-induced mutational signatures, suggests that BCC might originate from preneoplastic skin lesions, and reports the particular role of HIPPO-YAP pathway in BCC tumorigenesis and aggressiveness.

EACR22-0730

POSTER IN THE SPOTLIGHT

Detecting ongoing chromosomal instability using single cell DNA sequencing of clinical samples

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Introduction

Chromosomal instability (CIN) causes genetic chaos and increased intratumoral heterogeneity, which translates into rapid progression and treatment resistance. Accurately detecting CIN is emerging as an important biomarker for prognosis, diagnosis and therapy selection. Shallow whole genome sequencing of clinical samples provides a readout of the copy number landscape of tumours facilitating detection of CIN. However, standard bulk sequencing does not allow distinction between historical CIN (sometimes occurring up to 20-years prior to diagnosis) from ongoing CIN, currently operating in a tumour; which is crucial for developing better biomarkers.

Single cell DNA sequencing facilitates identification of recently acquired copy number events, allowing detection of ongoing CIN. Nonetheless, applying this to formalin fixed paraffin embedded (FFPE) tissues is technically challenging. Here we present an optimised protocol for single cell DNA sequencing of clinical samples and detection of ongoing CIN.

Material and Methods

Cells were dissociated using a customized protocol for paraffin removal and tissue digestion. Single cells (nuclei) were then isolated on a high-accuracy sorter according to multi-fluorescence image-based technology, and a unique barcoding library preparation was performed and sequenced. Sequenced data passed through an analysis pipeline where copy number segmentation was performed. By applying our own bespoke computational approach, copy number profiles were generated by counting reads at 30kb genomic window resolution.

Results and Discussions

Our preliminary results show successful isolation and generation of DNA libraries from single nuclei. However, cells that did not fulfil minimum quality standards were discarded. Sequencing data from single cells were sufficient to generate copy number profiles and thus discriminate ongoing from historical CIN, especially in tumours with low overall ploidy. Nevertheless, we observed that higher number of reads per genomic window is needed to evaluate tumours with extreme chromosomal instability and higher overall ploidy.

Conclusion

We demonstrate the feasibility of decoding genome complexity at single cell level from FFPE material, although protocols are still being optimized for standardisation and maximal efficiency. Moreover, deeper sequencing is needed for exploring more complex tumours. To select optimal depth while limiting costs, we are aiming to perform an initial super-shallow bulk sequencing which will allow us to estimate tumour ploidy and complexity.

EACR22-1146

ORAL PRESENTATION

Stream-lined single sample multi-omics workflow including phosphoproteome for therapy response prediction in cancer

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Introduction

Personalized cancer therapy bases medical care on patient-specific characteristics and individual data to decide which available treatment options fit the patient best. Current efforts focus on DNA and RNA sequencing data only. However, the drug targets are mainly proteins which are thus not directly assessed. Therefore, the most suited information on protein abundance and protein activity, which is modulated via posttranslational modifications (e.g. phosphorylation), is currently missing. This information can be provided by mass spectrometry based (phospho)proteomics, an approach that is not yet routinely integrated in the efforts on personalized oncology but clinically very promising. In this project, we aim to provide a solution for the need of biopsy-scale multi-omic workflows in individualized oncology.

Material and Methods

We compared the phosphoproteome of protein extracts obtained with a urea protocol or an AGPC reagent (RNA-Bee). In a functional context, we also compared coverage of the well-studied phosphorylation response after DNA damage, performing differential analysis of phosphorylation site data from untreated versus irradiated U2 osteosarcoma (U2OS) cells. We subsequently applied to workflow to tumor tissues. We used high-resolution tandem mass spectrometry (label-free single-shot LC-MS/MS, TiO₂ phosphoproteomic workflow, Q Exactive HF mass spectrometer) and recent bioinformatic tools such as inferred kinase activity (INKA) analysis and phosphosite (PS) signature enrichment analysis (PTM-SEA).

Results and Discussions

On average we identified circa 9000 phosphosites per sample with both extraction methods. Moreover, we observed high similarity of phosphosite characteristics with 94% shared class 1 identifications and deduced kinase activities including ATM, ATR, CHEK1/2, PRKDC. We furthermore extended our comparison to murine and human tissue samples yielding similar and highly correlated results for both extraction protocols. We are currently applying our multi-omics workflow to 116 human melanoma biopsies collected in the REPOSIT trial, a phase II, open-label, multicenter trial including patients with BRAFV600E/K mutated melanoma.

Conclusion

Establishing a robust workflow for simultaneous isolation of DNA, RNA and (phospho)protein from single tumor biopsies will strongly facilitate the generation of multi-omics data. Multi-omic data acquisition, integrative analysis and exploitation has the potential to change current clinical practice in the future.

EACR22-1166

Systematic assessment of variants of unknown significance in cancer cell lines nominates new therapeutic targets and drug repositioning opportunities

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Introduction

It is widely accepted that cancer is caused by genetic mutations that lead to uncontrolled proliferation and growth. These mutations occur in a particular set of genes, called “cancer drivers”, conferring on the cells a selective advantage. Cancer drivers are usually identified based on their mutation frequency, thus potentially missing rare functionally relevant variants of unknown significance, which could serve as novel targets for drug repositioning.

Material and Methods

Starting from 865 in vitro models from the Cell Model Passports with available somatic mutation data and high quality CRISPR-derived cancer dependency data, we independently analyse 33 different cancer types, unbiasedly screening for variants conferring dependency on the mutated gene (Dependency Associated Variants or DAMs). To assess the clinical relevance of DAMs, we integrate our results with patients’ data, as obtained from resources such as COSMIC and IntOGen, and screen for their association with altered drug sensitivity.

Results and Discussions

We report 1,784 DAMs – cancer type combinations spanning 1,206 genes, mostly cancer type specific. As expected, the genes with DAMs are enriched in known drivers and cell cycle genes. Importantly, we identify many novel candidate drivers (1111), some of which are also found in patients with the corresponding cancer type. For example, we identify GNB1-I80T, already shown to induce B cell malignancies when expressed in the bone marrow, and several genes previously associated with cancer cell proliferation or invasiveness (e.g. APBB1, EIF2AK3, SSH2, KRT81). Unexpectedly, we find not only oncogenes but also tumour suppressors to be part of our DAMs, which could be explained by inactivating mutations in a gene conferring selective advantage to cancer cells, while at least one functional copy being necessary for cell survival, as previously shown for PP2R2A. Finally, we identify DAMs associated with altered drug sensitivity, such as MAP2K2-Q60P associated with increased sensitivity to the ERK inhibitor PD0325901.

Conclusion

Here we present an unbiased analytical framework to comprehensively characterize genomic point mutations in cancer cell lines. Our approach suggests drug repositioning opportunities, identifies new therapeutic targets with associated markers for precision oncology and it also enables the identification of spurious models harbouring functional driver mutations in a wrong tissue context.

EACR22-0253

Patient-tailored CRISPR/Cas9 screens to selectively detect vulnerabilities in soft-tissue sarcoma

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Introduction

Soft-tissue sarcomas (STS) are rare and highly diverse malignancies originating from mesenchymal precursor cells. Due to the heterogeneity of STS, the high number of tumor recurrences, and the variable response to standard therapies, there is a high demand for novel therapeutic approaches. This project aims to identify new entry points for molecularly targeted STS therapies using CRISPR/Cas9 knockout screens.

Material and Methods

Based on transcriptome data from more than 700 STS patients enrolled in the MASTER (Molecularly Aided Stratification for Tumor Eradication Research) precision oncology program of the National Center for Tumor Diseases and the German Cancer Consortium, we built a customized sgRNA library that can be used to discover essential genes in cell lines whose transcriptional profiles represent that of primary patient tumors. Due to the heterogeneity of the patient cohort, we performed dimensionality reduction and clustering of RNA sequencing data to nominate STS subtypes to focus on. Candidate genes were selected based on their therapeutic accessibility in addition to their expression and predicted activity in patient tumors. The screening library consists of sgRNAs against 819 candidate genes and several controls. Screen hits are validated using competition assays, shRNA knockdowns, and drug sensitivity tests.

Results and Discussions

To date, we have performed seven screens that met stringent quality control criteria. We classified seven genes as hits in all screens with synovial sarcoma cell lines. Five of the genes were not identified in previous, less sensitive genome-wide functional screens of synovial sarcoma cell lines and none of them was annotated as a core essential gene. Preliminary hits were also identified in leiomyosarcoma cell lines. Validation experiments are ongoing, and confirmed hits will be presented.

Conclusion

In summary, we generated a hypothesis-driven CRISPR/Cas9 knockout library to detect novel vulnerabilities in STS and performed a series of functional genomic screens. The results potentially provide new insight into essential genes in synovial sarcoma and leiomyosarcoma, whose validation and pathway-related analysis will help understand their utility as novel therapeutic targets. In the future, our pipeline will be used to investigate potentially druggable functional dependencies of additional sarcoma subtypes, in particular leiomyosarcoma, clear cell sarcoma, and desmoplastic small round cell tumor.

EACR22-0637

Deregulated lncRNAs in T-cell Lymphoblastic Lymphoma key Notch pathway

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Introduction

T-cell lymphoblastic lymphoma (T-LBL) is a rare aggressive neoplasm of precursor T cells mainly affecting children and adolescent. T-LBL is closely related to acute lymphoblastic

leukemia (ALL), the most common pediatric cancer, although much less characterized at a molecular level. Over the recent years, some biomarkers have contributed to explain the oncogenic landscape of this disease. However, studies analyzing the non-coding part of the genome are scarce. In this context, this work aimed to elucidate the implication of long non-coding RNAs (lncRNAs), a class of long transcripts without protein-coding capacity but with a crucial regulatory role in a wide variety of biological processes, in modulating T-LBL key pathways.

Material and Methods

Whole transcriptome analysis of nine pediatric T-LBL samples and three non-cancer controls was performed. RNA libraries were generated using TruSeq Stranded Total RNA Library Prep Kit and sequenced on the NovaSeq 6000 system (Illumina). The paired-end reads were aligned to hg19 human genome using HISAT2, and featureCounts was used to get the raw counts for each gene annotated in LNCipedia v5.2 (for lncRNAs) or GENECODE release 19 (for protein coding genes). RStudio was used for differential expression analysis (DEA) using DESeq2, and for Pearson's correlation analysis. A cut-off of $p < 0.01$ was considered statistically significant.

Results and Discussions

T-LBL patients vs. T-controls DEA resulted in the identification of 3,542 differentially expressed protein coding genes and 7,744 lncRNAs ($p_{adj} < 0.01$; $\log_2FC > 2 / < -2$). Correlation analysis showed, among the most interesting results, a strong correlation ($r > 0.8$) between five lncRNAs (*lnc-SBDS-2*, *lnc-RFESD-6*, *lnc-SLC25A15-1*, *lnc-ADAM10-1*, and *lnc-DNAJC19-4*) and Notch pathway related genes (*NOTCH1*, *NOTCH3* and *FBXW7*). These five lncRNAs were significantly upregulated in T-LBL patients, as well as *NOTCH1* and *NOTCH3*. Meanwhile, *FBXW7*, downregulated in T-LBL patients, was negatively correlated to the rest of genes. This lncRNA-Notch pathway deregulation would be in line with recent results showing that activated Notch/PI3K-AKT signaling axis together with alterations in cell cycle regulators constitute the core oncogenic program for T-LBL. However, how these five lncRNAs modulate the expression of these key genes at functional level remains unknown.

Conclusion

These results suggest that long non-coding RNAs modulate the expression of key genes present in pathways involved in the pathogenesis of T-LBL, such as Notch signaling.

EACR22-0703

Inhibition of Pttg1 as a novel mechanism of aspirin antitumoral effect

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Introduction

The antitumoral role of aspirin has been investigated in colon cancer, but there is scarce information about its role in endocrine tumors. Our aim was to investigate the genomic and epigenomic effects of Aspirin in pituitary neuroendocrine tumors (PitNETs).

Material and Methods

DNA methylome by HPLC-MS/MS and whole transcriptome profile were evaluated by NGS in pituitary cell lines upon aspirin treatment. Effects of aspirin and demethylation agent, decitabine were further tested *in vitro* by RT-qPCR, western blot and functional (proliferation, viability, migration, luciferase promoter reporter) assays. DNA methylome was also correlated with *PTTG1* expression in 41 human PitNET samples. Gene and protein expression data (obtained from Gene Expression Omnibus and literature mining) of 77 PitNET with 35 control samples were also analyzed.

Results and Discussions

Increased level of *Tet* enzymes and their cofactor *Uhrf2* was identified behind the global DNA demethylation (increase of 5-hydroxymethylcytosine, 5hmC) in pituitary cells upon aspirin treatment. Transcription factor regulatory network and *in vitro* functional assays showed that aspirin increased p53 and decreased E2f1 activities. The increased p53 activity was due to its acetylation. *Pttg1* was identified as downregulated upon aspirin treatment and was verified by reporter assay. 5hmC positively correlated with *Tet1-3* and *Tp53* expression, and negatively correlated with *Pttg1* expression that was also reinforced by the effect of decitabine.

In addition, high overlap (20.15%) was found between aspirin-regulated genes and dysregulated genes in PitNET tissue samples. The functions of the common genes were mostly related to cell proliferation and p53 function but cell migration and genome stability were also detected.

Conclusion
Along with complex genomic and epigenomic effects, we described a regulatory network where Aspirin regulated global methylation, p53 activity and *Pttg1* expression in pituitary cells along with decreased cell proliferation and migration. Based on our previous findings that showed the interdependence of DNA demethylation in human blood samples of PitNET patients with proliferation and differentiation of PitNET tumors and the significant involvement of the classical *Pttg1* oncogene, our data may suggest the potential beneficial effect of aspirin in PitNET.

Drug Resistance

EACR22-0297

ORAL PRESENTATION

Stromal Regulation of Patient-Derived Organoid Drug Responses

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Introduction

Colorectal cancer (CRC) is characterised by genetic and cellular heterogeneity, where stromal constituents, such as cancer-associated fibroblasts (CAFs) in the tumour microenvironment (TME), play an important role in tumour progression and therapy responses. Patient derived organoids (PDOs) are powerful biomimetic cancer models that

preserve the pathological and genomic profiles of the parent tumour. PDOs can mimic the patient response to anti-cancer treatments, demonstrating a high predictive value. With the development of highly multiplexed single-cell technologies, it is possible to mechanistically study cell-cell communication within the TME of PDOs to understand response and resistance to cancer therapies.

Material and Methods

Using thiol organoid barcoding in situ mass cytometry (TOBis MC) (Qin et al., Nature Methods, 2020 and Sufi et al., Nature Protocols, 2021), we developed a single-cell screening platform to study the response of CRC PDOs to several treatments alongside colonic CAFs. This analysis allows us to measure >40 post-translational modification (PTM) signalling and cell-state parameters in a cell-type-specific manner across 126 PDO co-cultures in a single MC run. Using PDOs from 10 CRC patients, alone and in co-culture with CAFs, alongside 11 titrated therapies, we have generated a dataset of >2,500 organoid cultures containing single-cell information of 30 million cells (PDOs and CAFs). This dataset contains >4,000 cell-type specific signalling networks that provide information on how CAFs can influence the response of different patients to various anti-cancer therapies.

Results and Discussions

To compare single-cell PTM signalling data from thousands of organoid co-cultures, we developed 'Trellis' – a highly-scalable method for hierarchically-paired treatment effect analysis. Trellis revealed PDO drug response is highly-patient specific. We observed three primary patient specific responses to 5-Fluorouracil, Irinotecan, and Oxaliplatin chemotherapies: chemosensitive apoptotic, chemorefractory with no apoptosis but a strong DNA-damage response, and chemorefractory with minimal DNA-damage response. In addition, we demonstrate CAFs ability to protect cancer cells from these therapies by modifying signalling pathways involving P90RSK and NDRG and by modifying their cell cycle.

Conclusion

These results suggest CAFs are major regulators of cancer cell drug response and should be included when using organoids as avatars of personalised medicine.

EACR22-0002

ORAL PRESENTATION

AKT pathway as a therapeutic target to constrain lineage plasticity leading to histological transdifferentiation

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Introduction

In lung adenocarcinomas (LUADs), lineage plasticity drives neuroendocrine (NE) and squamous cell (LUSC) transdifferentiation in the context of acquired resistance to targeted therapies, with up to 14% and 9% incidences in tumors relapsed on EGFR inhibitors, respectively, and leading to poorer survival than those of either LUAD or *de novo* LUSC patients. To date, little is known about the molecular effectors driving histological transdifferentiation due to the paucity of transdifferentiating clinical samples amenable for molecular analyses. Currently no specific therapies for LUSC or NE transdifferentiation prevention are available for patients at high risk of transformation.

Material and Methods

We performed multi-omic profiling of NE- and LUSC-transdifferentiating clinical samples, including genomic (whole exome sequencing), epigenomic (bisulfite sequencing), transcriptomic (RNAseq) and protein (antibody arrays) characterization. Clinical findings were validated in preclinical models including cell lines as well as LUSC- and NE-transdifferentiation patient-derived xenograft models.

Results and Discussions

Our data suggest that histological transdifferentiation is driven by epigenetic -rather than mutational- events, and indicate that transdifferentiated tumors retain molecular features of their previous LUAD state. Integrative epigenomic, transcriptomic and protein analysis revealed biological pathways dysregulated specifically for distinct histological outcomes, including downregulation of RTK signaling and Notch-related genes in NE-transformed tumors, and upregulation of genes involved in Hedgehog and Notch signaling and MYC targets in LUSC-transdifferentiated tumors. Most interestingly, these analyses revealed commonly dysregulated pathways for transdifferentiated tumors, including downregulation of immune-related pathways and upregulation of AKT signaling and PRC2 epigenetic remodeling complex genes. Concurrent activation of AKT and MYC overexpression induced a LUSC phenotype in LUAD preclinical models. Pharmacological targeting of AKT in combination with osimertinib delayed both squamous and NE transformation in *EGFR*-mutant patient-derived xenograft transdifferentiation models.

Conclusion

These results identify common and histology-specific drivers and dysregulated pathways in NE and LUSC transdifferentiation, and nominate AKT as a therapeutic target to constrain lineage plasticity and prevent the acquisition of resistance to EGFR-targeted therapies through histological transdifferentiation.

EACR22-0012

Evolution of topoisomerase inhibitor resistance in cancer cells involves recurrent mutations in non-coding genome that reduce DNA breaks

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Introduction

Resistance to chemotherapy is a leading cause of treatment failure. Mostly attributed parameters that defines the cell fate transition to a drug treatment involves persister cells, cancer stem cells and tumor microenvironment. Although its not clear as of how persister like cells could be selected in multiple rounds of drug treatment. It gives an impression that possibly there are resistant driving mutations that are created and selected over time in a dose dependent scheme. Also in our primary experiments we observed that if genetically homogenous population is taken it does get selected in dose escalation steps without pre defined capability in terms of differential expression of MDR or any other drug response genes.

Material and Methods

Using Irinotecan as test drug on colon cancer line (HCT116), we performed major study where we utilized single cell cloning, dose escalation steps for resistant mutant selection, Performed in-depth analysis on transcriptome and human whole genome sequences. Also utilized DNA damage response assays to support our findings.

Results and Discussions

We picked up very interesting characteristics of adaptation where mutations are created in certain non-coding region of genome, gets positively selected. Homologous recombination repair creates this mutation and lowers the affinity of drug target protein binding to the DNA sequence. This lowers cutting of DNA thereby protecting the resistant variants in further dose selection steps.

Conclusion

Repair of DNA breaks at sites following initial irinotecan exposures reverts cleavage-sensitive cancer-related sequences to cleavage-resistant reference genome sequences, thus gradually reducing generation of DNA breaks upon subsequent exposures and increasing irinotecan-resistance. Mechanistically similar mechanism may be involved in radiation therapy or any other DNA damaging drug treatments.

EACR22-0064

ST6Gal1 Targets the Ectodomain of ErbB2 in a Site-Specific Manner and Regulates Gastric Cancer Cell Sensitivity to Trastuzumab

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Introduction

The therapeutic efficacy of the monoclonal antibody trastuzumab (TTZ) in the treatment of ErbB2-positive unresectable gastric cancer (GC) is undermined by the emergence molecular resistance. TTZ targets the extracellular domain of the oncogenic cell surface receptor ErbB2, which undergoes extensive post-translational glycosylation. We have previously shown that ErbB2 is modified with the α 2,6NeuAc tumor-associated glycan antigen by the Golgi-residing ST6Gal1 sialyltransferase. However, the ErbB2 site-specific glycome, and the molecular mechanisms through which specific glycan signatures actively support the acquisition of TTZ resistance, remain elusive.

Material and Methods

CRISPR/Cas9 genome-editing technology was used to establish *ERBB2*-amplified *ST6GAL1* K.O. (α 2,6NeuAc(-)) GC cell *in vitro* models. The structural site-specific glycome of ErbB2 purified from wild-type (WT) and *ST6GAL1* K.O. GC cells and cryopreserved tissues was comprehensively characterized by mass spectrometry (MS)-guided glycomic and glycoproteomic analytical workflows. The molecular proximity between ErbB2 and α 2,6NeuAc was analyzed by *in situ* Proximity Ligation Assay (PLA) in a clinical cohort of GC tissues. The ErbB2 functionality and TTZ sensitivity of WT and *ST6GAL1* K.O. GC cells was evaluated.

Results and Discussions

MS-based glycomic and glycoproteomic analysis of ErbB2 ectodomain disclosed a microheterogeneous and site-specific glycosylation profile in GC cells. Strikingly, the ST6Gal1-mediated terminal addition of the α 2,6NeuAc motif is highly restricted to two *N*-glycosylation sites (N530 and N629) located at ErbB2 TTZ-binding domain. Silencing of *ST6GAL1* reshaped the cellular and ErbB2-specific glycomes and expanded the

receptor's membrane half-life. *ST6GAL1* K.O. GC cells depicted increased sensitivity to TTZ-induced cytotoxicity through the cell surface stabilization of ErbB dimers, and a downregulation of ErbB2/EGFR tyrosine phosphorylation. PLA analysis identified ErbB2 as an *in situ* molecular carrier of α 2,6-sialylated glycans in GC tissues.

Conclusion

ST6Gal1-mediated aberrant α 2,6-sialylation actively tunes the resistance of ErbB2-driven GC cells to TTZ. Specific ErbB2 glycoforms may serve as novel predictive biomarkers for the rational therapeutic stratification of ErbB2-positive GC patients.

EACR22-0116

Non genetic mechanisms of ER+ HER2- breast cancer resistance to the combination of Palbociclib and Fulvestrant.

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Introduction

Combination of CDK4/6 inhibitors with hormone therapy (HT) is the actual first-line treatment for ER+HER- metastatic breast cancer (MBC). Thus, combination of Palbociclib and Fulvestrant (PF) doubles the PFS in comparison with HT alone, but all patients progress in less than one year and the reason of this resistance remains unknown. This work aims at deciphering mechanisms of cell survival during treatment and of resistance to the cytostatic action of PF.

Material and Methods

We developed 2 cell lines resistant to PF by applying a continuous treatment for 2 years in vitro. RNA sequencing was performed by Diagenode®. The transcriptome of the resistant cells was compared to cells treated with PF for 20 days and to untreated cells. Biomarkers of interest were validated by western blot (WB) or immunofluorescence. Inhibition tests were performed using small interfering RNA or pharmacological inhibitors. All cell proliferation assays that assess resistance to PF and effectiveness of new drugs were carried out with Incucyte®. Biopsies (n=31) from patients were collected before treatment and after resistance. Validation of biomarkers on human samples was performed by immunohistochemistry.

Results and Discussions

Transcriptomic analysis revealed that EGFR and ER α signaling were upregulated in resistant cells. Alteration in the estrogen pathway was related to upregulation of STS, an enzyme involved in the increased availability of estrogens.

Treatment with PF induces an early increase in ROS level and an accumulation of lipid droplets within the cells. Moreover, resistant cells were characterized by an up-regulation of lipid transporters, which suggest a possible role of lipid transporters in the relief of stress induced by treatment. Interestingly, we found that refractoriness to PF was associated with a mechanism of resistance to ferroptosis in cancer cells.

These results suggest that targeting estrogen metabolism and ferroptosis resistance could be an effective strategy to treat PF resistant cells. We validated this strategy *in vitro* by showing that the use of an estrogen metabolism inhibitor and a ferroptosis inducer effectively reduced the proliferation of PF resistant cells. Confirmation of these results *in vivo* is in progress. **Conclusion**

Alterations in lipid and estrogen metabolism as well as ferroptosis resistance mechanisms are implicated in the resistance of ER+HER- breast cancer cells to PF. Targeting estrogen metabolism and ferroptosis could be an effective strategy to overcome resistance of ER+HER- MBC to PF.

EACR22-0300

Anti-angiogenic therapy resistance promotes angiogenesis and metastasis of clear cell renal cell carcinoma

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Introduction

The introduction of anti-angiogenic therapy, especially multi-targeted tyrosine kinase inhibitors (TKIs), has been a major breakthrough in clear cell renal cell carcinoma (ccRCC) treatment. However, despite positive initial response, most of the patients develop drug resistance and disease progression occurs after one year. Despite significant progress in ccRCC treatment the biological mechanism underlying TKIs resistance remains unclear. The main aim of this work is to explain the mechanism of the acquisition of resistance to antiangiogenic drugs used in ccRCC therapy, sunitinib and sorafenib.

Material and Methods

ccRCC cell lines, Caki-1 and Caki-2, were constantly stimulated with sunitinib, sorafenib or DMSO (control) for 7-21 days to obtain resistance. Resistant cells were analyzed with Western blot, IF staining, qPCR, and conditioned media (CM) were collected. CM and mice plasma were used for ELISA assays, and for stimulation of endothelial cells HUVEC (Human umbilical vein endothelial cells). For animal studies GFP positive Caki-1 cells were treated 5 weeks with the drug and injected sc. into NODSCID mice. After approximately 6 weeks tumors were harvested and analyzed with Western blot and IHC staining. To examine the effect of MCPIP1, ccRCC cells were transduced to overexpress MCPIP1 (pLIXMCPIP1) and used in *in vitro* and *in vivo* studies.

Results and Discussions

We provide evidence that the commonly used antiangiogenic drugs, sunitinib and sorafenib, increased the level and activation of c-Met and decreased MCPIP1 leading to promotion of the metastatic potential of resistant cells. Drug resistance also enhanced tumor vascularization due to the secretion of IL-6, IL-8 and MMP9 and the activation of endothelial cell migration through phosphorylation of VE-cadherin. Furthermore, we showed that MCPIP1 overexpression decreased the phosphorylation of the receptor c-Met, Src kinase and the transcription factor STAT3 even after sunitinib and sorafenib stimulation. Additionally, a high MCPIP1 level reduced tumor growth, decreased micrometastasis in lungs and expression of factors involved in tumorigenesis.

Conclusion

Our data indicate that TKIs resistance is regulated by multiple factors, including the MCPIP1 protein. Therefore, modulation of MCPIP1 activity may alter cell behavior and partially

overcome sunitinib and sorafenib resistance and could be used as a novel therapeutic strategy for patients with ccRCC.

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EACR22-0362

IKK γ /NEMO as a guide-dog for the recognition of damaged DNA upon chemotherapy

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Introduction

Colorectal cancer (CRC) remains the second leading cause of cancer-related death in Europe, which highlights the need for novel therapies to treat advanced disease. Others and we have previously identified several elements of the NF- κ B-inducing IKK (by I kappa B kinase) complex for their involvement in tumor initiation and promotion, therapy resistance and metastasis in various human cancers including CRC. However, general inhibitors of IKK are extremely toxic what preclude their use for patient treatment.

Material and Methods

We have generated BiID-IKK α fusion proteins and IKK γ /NEMO knockout colorectal cancer cells, generated by CRISPR-Cas9, to identify specific IKK γ /NEMO-dependent or -independent IKK α interactors that are modified following DNA damaging treatment. We confirmed candidate interactors by coelution in Superdex S200 columns, double IF and Co-IP assay.

Results and Discussions

Our results indicated that IKK γ /NEMO is in a cytoplasmic complex with IKK α and ATM under basal conditions. Importantly, whereas activation of Ataxia Telangiectasia Mutated (ATM) activation upon damage is IKK α -dependent but IKK γ /NEMO-independent, IKK γ /NEMO is required to drive IKK α and activated ATM to the sites of DNA damage leading to efficient damage resolution. Cells defective in IKK γ /NEMO function show reduced number of γ H2A.X foci upon chemotherapy treatment and defective ATM and p-ATM recruitment to the chromatin associate with a massive increase in the amount of damaged DNA.

Conclusion

IKK α - and IKK γ /NEMO contribute at different stages of the DNA Damage Repair pathway but are both essential for DNA damage resolution upon exposure of cells to damaging agents. We propose that defective DDR may contribute to the skin phenotype observed in ectodermal dysplasia patients, which are characterized by defective IKK γ /NEMO function.

EACR22-0466

Low levels of anti-cancer drugs may contribute to drug resistance and cancer aggressiveness by increasing HER2 expression and release of extracellular vesicles

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Introduction

Human epidermal growth factor (HER2) is frequently amplified and/or overexpressed in breast cancer and so tucatinib (Tuc), is an approved tyrosine kinase inhibitor for HER2-overexpressing breast cancer. Unfortunately, resistance often limits the benefit of such drugs. Previously our group discovered that extracellular vesicles (EVs) released from cancer cells are associated with transmitting drug resistance. Here, we aimed to investigate if exposure to low dose of Tuc (Tuc_{low}) -which may be all that is achieved depending on the tumour size, location, heterogeneity, etc.- might actually stimulate HER2 expression and/or EV release and so unintentionally contribute to tumour aggressiveness.

Material and Methods

Tuc's IC₁₀ for HER2-positive breast cancer cell lines, HCC1954, SKBR3 and EFM192A, was established using acid phosphatase assay. Effects of cell exposure to this Tuc_{low} for 48h, was evaluated by analysing cellular HER2 by both immunoblotting and flow cytometry (FC). To get an indication of any changes in EVs release, the corresponding conditioned media (CM) was centrifuged (300g for 5 min, thrice) and the presence of both HER2 and the EV's tetraspanin marker CD9 was analysed by imaging flow cytometry (IFCM).

Results and Discussions

HCC1954 (IC₁₀=747.4nM) was found to be innately resistance to Tuc, in comparison to SKBR3 (IC₁₀=1.2nM) and EFM192A (IC₁₀=2.6nM). Immunoblotting of lysed cells following Tuc_{low} treatment showed significantly (p=0.043) increased HER2 levels only in HCC1954 cells, although FC analysis of the HER2 indicated that Tuc significant increased expression by both HCC1954 (p=0.033) and SKBR3 (p=0.042) cells. Regarding analysis of CD9+ and HER2+ events in corresponding CM, IFCM showed an upward trend of CD9+ and HER2+ events in all cases, following treatment, although only CM from treated HCC1954 cells showed a significant (p=0.0058) increase in detectable HER2+ events.

Conclusion

Interestingly, the most consistent and solid effects of Tuc_{low} treatment were manifest on the innate resistant cells. While further studies, including comprehensive analysis of EVs and the functional relevance of the increased HER2, are warranted -including more cell lines and tumour samples- this suggest that sub-optimal treatment of tumours that are innately drug-resistant may actually increase their aggressiveness. Given that Tuc is indicated for breast cancer where at least two prior anti-HER2 treatment regimens have been used and failed, this may have important implications when choosing optimal therapies.

EACR22-0502

p53 context-dependent role of miR-9/KLF5/SP1 axis in modulating response to therapy in Head and Neck Squamous Cell Carcinoma.

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Introduction

Head and Neck Squamous Cell Carcinoma (HNSCC) is the sixth most common cancer worldwide classified as HPV- or HPV+. TP53 is the most frequently mutated gene in HPV- HNSCC, playing an uncontested role in its pathogenesis. HPV+ HNSCC are mostly TP53wt and usually less heterogenic with a more favorable prognosis compared to HPV- ones. Radiotherapy (RT) *plus* Cetuximab (CTX) may represent an effective curative therapy for selected HPV-/TP53mut patients. To date, no validated biomarker exist to predict the response to RT+CTX in HNSCC patients. We recently demonstrated that miR-9 was strongly associated with recurrence risk in HNSCC and in this study we aim to identify if miR-9 could represent a novel potential prognostic/predictive biomarker for RT+CTX activity in the context of HPV- and or HPV HNSCC patients.

Material and Methods

Lentiviral approaches were used in *in vitro* and *in vivo* HNSCC models to modify miR-9 expression and study tumor onset, progression and response to therapies. Stably silenced/overexpressed miR9 cells were tested in proliferation-, survival- 2D/3D-growth and in response to drugs used in clinics. RT was administered to our models using a linear accelerator for external beam radiation therapy. Luciferase assay and other molecular biology experiments allowed us to identify new molecular miR-9 targets acting in a p53-dependent manner. TP53wt and TP53mut cells were used to investigate the role of KLF5/SP1 and a shRNA approach was used to silence KLF5 *in vitro*.

Results and Discussions

We discovered that a miR-9/KLF5/SP1 signaling axis behaved differently depending on the TP53 status. In p53mut HNSCC cells, MiR-9, targeting KLF5, regulated the expression of SP1 that, in turn, stimulates tumor growth and confers resistance to RT+CTX, *in vitro* and *in vivo*. However, SP1 regulation by miR9/KLF5 axis worked in a p53 context-dependent manner: when p53 was mutated KLF5 downregulated SP1. Conversely, when p53 was WT, KLF5 had opposite effects on SP1 expression. Consequently, KLF5 differently affected the responsiveness to therapies: in HNSCC TP53wt cells KLF5 increased the resistance to RT+CTX. Yet it re-sensitized TP53mut ones to this combination therapy.

Conclusion

We propose that the miR-9/KLF5/SP1/p53 axis may represent a novel potential prognostic/predictive marker for RT+CTX activity that could explain why only a subset of HNSCC patients benefits from RT+CTX therapy eventually stimulating the development of novel targeted approaches for resistant patients.

EACR22-0602

Discovering new key drivers of cancer persistence.

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Introduction

Tumour recurrence is associated with a relative rapid acquisition of resistance to drugs and remains a key obstacle to successful cancer therapy. Treatment with chemotherapy is increasingly recognized to promote the emergence of resistant cells. Suppression of proliferation that can manifest as quiescence, dormancy, or *slow-cycliness*, has been revealed as one of the non-mutational mechanisms of chemoresistance ruled by this cell reprogramming. The slow-cycling or dormant state is characteristic of cancer cells that delay their cell cycle or remain quiescent independently of the use of any therapeutic drug. This differs from the drug-tolerant persister (DTP) cancer cells that emerge following the use of

anti-cancer agents. In any case, whatever the event that promotes reduced proliferation or quiescence, there are two key characteristics common to all of them: drug resistance and reversibility of their state. Therefore, these resistant cancer cells, in either the primary tumour site or distant metastatic sites, emerged by intrinsic or extrinsic stimuli, can recover and re-enter into an actively dividing state being responsible for disease recurrence. Thus, slow-cycling/dormant/DTP cancer cells are considered the cause of cancer persistence.

Material and Methods

Our laboratory used a doxycycline-inducible H2BeGFP system in colorectal cancer (CRC) cell line and patient-derived cells to identify and characterise slow-cycling cancer cells (SCCCs). We used xenograft models to evaluate chemoresistance. We analysed cohorts of chemotreated CRC patients to confirm our results, applying a gene-expression signature and performing immunohistochemical staining.

Results and Discussions

We observed that DPPA3 is highly expressed in SCCC. We demonstrated that DPPA3 promotes the acquisition of a slow-cycling phenotype by a G2/M cell cycle delay in CRC cells. We also discovered that DPPA3 is involved in therapy resistance and uncover its value as an early predictive biomarker of patients' disease relapse and progression.

Conclusion

We identified the role of DPPA3 in the regulation of *slow-cycliness* and chemoresistant phenotype of CRC cells. Our study highlights a new potential drug target for slow-cycling DTP cancer cells eradication, giving novel therapeutic opportunities to overcome cancer persistence and improve patients' survival.

EACR22-0736

DNA repair in colorectal cancer therapy response: The role of MRE11

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Introduction

Cancer therapy failure, mainly represented by the development of chemotherapy resistance or outgrowth of metastasis, is the major complication for cancer patients. Cancer chemotherapy resistance is the ability of cancer cells to blunt and counteract the effects of chemotherapeutics. The important mechanism in cancer therapy and chemoresistance is an alteration of DNA damage response (DDR) pathways. The MRE11-RAD50-NBS1 (MRN) complex plays an important role in DNA damage response and the repair pathway of double-strand breaks (DSBs) and telomere homeostasis. Colorectal cancer (CRC) is the third most common type of cancer worldwide. CRC therapy response still faces many obstacles for having a successful outcome. Besides the side effect of chemotherapy, acquired resistance is one of the most serious issues of CRC therapy. The overall survival for patients with metastatic CRC (mCRC) remains poor (approximately 14.0%). Therefore, there is a crucial clinical need for a better understanding of cellular processes associated with acquired chemotherapy resistance.

The main aim of our research is to define the role of DNA repair pathways, mainly homologous recombination represented by MRE11, in CRC therapy response to oxaliplatin.

Material and Methods

On a cohort of 50 paired samples of tumor tissue and adjacent mucosa, we defined the effect of *MRE11* expression on disease free-survival of CRC patients. For studying the effect of MRE11 in CRC therapy response, *in vitro*, we developed oxaliplatin resistant CRC cell lines as well as CRISPR/CAS9 mediated *MRE11* knock-out cell line.

Results and Discussions

Our findings on the cohort of CRC patients showed an association of MRE11 with progression-free survival in CRC. qPCR analysis on CRC cells with acquired chemoresistance to oxaliplatin (3.5 times higher IC₅₀) showed an increased expression level of MRE11 ($p < 0.05$). Subsequent analysis of CRC cells with downregulation of MRE11 indicates accumulation of DNA damage (γ H2AX, comet assay) and increasing of the sensitivity of the CRC cells to oxaliplatin ($p < 0.01$).

Conclusion

Our results suggest that downregulation of MRE11, could be associated with a better therapy response in oxaliplatin-treated CRC patients.

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EACR22-0776

Epidermal Growth Factor Receptor (EGFR) hyper-activation promotes resistance to new generation selective RET inhibitors in NSCLC

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Introduction

Non-small cell lung cancer (NSCLC), the most common cause of cancer-related deaths, harbors *RET* rearrangements in up to 2% of cases. Novel selective RET inhibitors, BLU-667 and LOXO-292, showed promising anti-tumor activity in clinical trials so that FDA and EMA granted accelerated approval to both. Despite the positive clinical outcomes, not all patients benefit from RET inhibitors and a significant fraction eventually progress, underscoring the need to discover novel therapeutic approaches that circumvent such resistance.

Material and Methods

We chronically exposed Lc-2/AD NSCLC cells (harbouring CCDC6/*RET* fusion) to increasing doses of BLU-667 to generate cells resistant to the drug (BluR). RNA-Sequencing analysis of BluR and sensitive Lc2/AD cells was used to define transcriptional profiles sustaining resistant phenotypes. To validate the relevance of gene expression alterations, we employed specific inhibitors and siRNA-mediated knock-down to re-sensitize resistant cells. We stable over-express candidate genes by lentiviral particles infection in sensitive cells to induce resistant phenotype. We further tested combination therapies *in vivo*.

Results and Discussions

RNA-Sequencing studies revealed a significant enrichment of Epidermal Growth Factor Receptor (EGFR) signaling pathway in BluR cells compared to sensitive parental cells. We hypothesized that EGFR rebound could prompt compensatory mechanisms to RET inhibition, in turn pushing signalling pathways (i.e. Mitogen-activated protein kinase, MAPK) responsible for cell proliferation. Furthermore, Integrated System for Motif Activity Response

Analysis (ISMARA)-based investigation of RNA-Seq data revealed Fos1 as transcriptional mediator of EGFR over-expression in BluR cells. Interestingly, si-EGFR, as well as siFos1, led to a drastic reduction of cell viability and a significant decrease of MAPK activation of BluR cells, overcoming the resistant phenotype. Further, EGFR over-expression reduced sensitivity of NSCLC cells to BLU-667. Since combination study revealed a synergistic effect of BLU-667 and afatinib in vitro, we injected Lc-2/AD-BluR xenografts in Balb/c mice and we verified that tumor growth resulted impaired by combination of afatinib and BLU-667. These data strongly support our hypothesis of a pivotal role for EGFR in anti-RET resistance.

Conclusion

Our findings suggest that targeting EGFR signaling pathway in combination with RET inhibitors therapy may provide a novel treatment strategy for patients unresponsive to RET inhibitors in NSCLC.

EACR22-0888

FETAL CONVERSION AND METABOLIC ADAPTATIONS IN DRUG-TOLERANT PERSISTENT CELLS

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Introduction

Colorectal cancer (CRC) remains the second leading cause of cancer-related deaths worldwide. First-line treatment for CRC currently involves surgery in combination with radiotherapy and/or chemotherapy (CT), which are designed to eradicate tumors by inducing DNA damage to highly proliferative cancer cells. However, a recently demonstrated mechanism of drug resistance involves the acquisition of a slow-proliferating phenotype that allows cell survival after treatment. Cell acquiring this phenotype are termed Drug Tolerant Persister cells (DTP cells) and are capable of re-enter the cell cycle under certain stimuli, which could contribute to tumor relapse after a variable period of latency. Thus, we aimed to study the transcriptional and metabolic changes of DTP cells after sublethal CT treatment in CRC cells.

Material and Methods

We took advantage of our in-house biobank of CRC patient-derived organoids (PDOs) to analyze the effects of sublethal CT treatment on cell growth, tumor initiation, metastatic activity and transcriptional and metabolic profiles.

Results and Discussions

Sublethal CT induces the emergence of DTP cells displaying a quiescent-like phenotype in the absence of sustained DNA damage with increased in vitro and in vivo tumor initiation and metastatic capacity, specifically in TP53 WT PDOs. Moreover, we identified a fetal intestinal stem cell (fISC) signature, dependent on YAP1, that is acquired by DTP cells. Importantly, this fISC signature can be also identified in a subset of CRC patients from three different public cohorts. and is predictive of reduced disease-free survival. We have also discovered that DTP cells acquire metabolic changes such as increased glutamine consumption and glutamate production linked to higher expression of specific amino acid transporters (i.e. SLC6A14 and SLC2A2), which could facilitate their adaptation to the metastatic sites. We will also present our ongoing studies on the dependence on glutamine

or glucose uptake by DTP cells and its impact in their capacity to survive under stress conditions.

Conclusion

Our study indicates that sublethal CT induces specific changes in cancer cells including acquisition of a DTP and fetal-like phenotype, higher metastatic activity and specific metabolic adaptations. We propose that generating the tools to identify these adaptations at the time of diagnosis or in the course of patient treatment would represent a valuable tool for improving patient follow-up and for therapy prescription.

EACR22-0904

Global proteomics profiling to understand potential mechanisms of neratinib-resistance in HER2-positive breast cancer

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Introduction

Human epidermal growth factor (HER2)-overexpression occurs in ~20% of breast cancers and confers aggressive behaviour and poorer prognosis. Although several drugs such as neratinib have been developed to target HER2, it is estimated that up to 70% of patients with HER2-overexpressing breast cancer do not gain benefit, due to innate or acquired drug-resistance. This study investigated if differences in proteome occurred between HER2-positive breast cancer cell lines representing three patients' tumours and their neratinib counterparts, in order to shed light on the neratinib resistance mechanism.

Material and Methods

Mass spectrometry-based quantitative proteomics was used to analyse the protein expression profile of HER2-positive breast cancer cell lines (HCC1954, SKBR3 and EFM192A) and their neratinib-resistant (NR) counterparts developed in our laboratory (HCC1954-NR, SKBR3-NR and EFM192A-NR). These experiments were repeated n=3 times. The differential expression analysis of the three parental cell lines individually, versus their resistant counterparts was investigated.

The workflow run in R was based on (DEP) package developed by Zhang et al. (2018) [doi:10.1038/nprot.2017.147] and a total of 18 samples from HER2+ breast cancer cells were analysed. Gene Ontology and pathway analysis were performed by *enrichr* web tool. Principal component analysis (PCA) was also performed. Differentially expressed proteins (DEP) were defined as FDR < 0.05 and |log₂(fold change)| ≥ 1.5.

Results and Discussions

The conditions and replicates were clustered together accurately and independently of being resistant or sensitive to neratinib. A total of 105 differentially expressed proteins were found. Of those, 57 were DEP between HCC1954 variants, 43 between SKBR3 variants and 7 between EFM192A variants. Notably, only 2 DEP were shared between two different cell line pairs and no DEP was shared between the three different cell line pairs.

Conclusion

Interestingly, no DEP were found between the three cell line pairs. These findings suggest that the three different cell lines developed neratinib resistance through divergent

mechanism(s) that may be cell-specific. Determination of possible targets to elucidate the mechanism(s) of onset of resistance and establishing reliable biomarkers are crucially needed to improve diagnosis and patients' outcomes.

EACR22-1084

Targeting glutaminolysis to breakdown resistance to targeted therapy in metastatic melanoma

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Introduction

Metastatic melanoma is associated with a poor prognosis. Melanoma cells have a high mutational potential and can activate alternative signalling pathways for survival leading to the development of acquired resistance to targeted therapies based on MAPK inhibitors.

Cancer cells have a high metabolic rate to sustain their growth and can adapt their metabolism to treatment. In melanoma, MAPK inhibitors block glycolysis and cancer cells need to switch their metabolism to become resistant.

Material and Methods

We studied glucose, glutamine and lipid metabolisms in 3 melanoma lines with different mutational profiles (BRAF, NRAS or cKIT) and either sensitive or with acquired resistance to MAPKi. We performed ¹H-NMR and RPPA, IHC, clonogenic assays, qPCR and flow cytometry. We also worked on tissue samples from patients responsive or unresponsive to MAPKi and we developed an animal model.

Results and Discussions

Integrating metabonomic and proteomic findings revealed some metabolic pathways (i.e., glutaminolysis, choline metabolism, GSH production) and key proteins (i.e., EPHA2, DUSP4, HIF-1A) as potential targets to discard resistance. Flow cytometry demonstrated a higher mitochondrial pool, consistent with glutaminolysis process, in the resistant cells, and an increase in ROS levels in the sensitive cells treated with MAPKi. Finally, qPCR analyses on patient biopsies prior treatment with BRAFi indicated, in unresponsive patients to BRAFi, a significant increase of mRNA levels of GAC and GDH, both playing key roles in glutaminolysis, as well as of TFAM, NRF2 and GSH, implicated in mitochondrial biogenesis and anti-oxidative mechanisms. GAC levels were assessed by IHC in 62 biopsies of melanoma and revealed a significant association between high GAC expression and short patient survival ($p=0.037$, $HR=1.97$, Cox regression). Finally, we used the glutaminase inhibitor CB-839 to target such metabolic changes in resistant cells to BRAFi and found inhibition of cell proliferation in vitro and tumor growth in vivo.

Conclusion

Our data indicate a switch in melanoma cells with acquired resistance to targeted therapies that relies on increased glutaminolysis, OXPHOS, mitochondrial content and anti-oxidative properties. Patient samples confirmed the metabolic switch when non-responding to BRAFi, while animal model shows that the glutaminase inhibitor CB-839 blocks tumor growth. Clinical study could be proposed targeting GAC by CB-839 in patients while GAC expression could be used as a prognosis marker in melanoma patients.

EACR22-1184

The role of ISG15 in cancer and IFN-treatment resistance

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Introduction

ISG15 has been widely studied for its antiviral function. However, recent research has implicated ISG15 in many more biological processes, both when conjugated to target proteins and when in free form. Regarding a role in cancer, researchers are divided between those that suggest ISG15 is a tumour suppressor and immune system enhancer, and those that claim it relates to tumour aggressiveness and treatment resistance. The variety of roles and modification targets of ISG15 makes it challenging to determine its precise molecular mechanisms. The aim of this study is to shed light on the mechanisms of action and interactions of ISG15 as they relate to interferon (IFN) sensitivity and resistance in terms of cancer cell growth.

Material and Methods

Gene-editing was used to ablate ISG15 expression in a number of cell backgrounds including tumour cell-lines and patient derived glioblastoma stem cells (GSC). Both cell pools and individual clones were generated and validated. Cell cycle analysis and cell growth assays were used to characterise the effects of type 1 and 2 IFNs on wild-type (WT) and ISG15 knock-out (KO) cell-lines. The phenotypic analysis of isogenic ISG15 cells was explored at the molecular levels using mass spectrometry approaches.

Results and Discussions

Although real time cell growth analysis showed no intrinsic differences between ISG15 KO and WT growth rate in preliminary studies, loss of ISG15 specifically affected the response of both tumour cell lines and cancer stem cells to various IFN α subtypes (but not IFN γ), most strikingly in colony formation assays. Pulse and dynamic SILAC mass spectrometry were used to provide insight into how increases in free ISG15 (24 hours post-IFN α treatment) and conjugated ISG15 (48 post-IFN α treatment) relate the ISG15 KO phenotype to protein expression and turnover at the whole cell level. Interferon stimulated genes that were most affected by ISG15 loss, such as IFIT1 and MX1, were defined and validated.

Conclusion

The data presented here shows ISG15 to play a key role in determining the outcome of IFN α -treatment on the growth of both tumour-lines and cancer stem cells, providing the foundation for further studies to 'dive-deeper' into the ISG15 pathway network using proteomic approaches to explore the role of ISG15 in cancer cell growth and IFN α resistance. Current research is focused on studying the relationship of ISG15 to IFIT1 and MX1, which could be key to understand the biochemical processes that lead to IFN sensitivity and resistance in cancer cell growth.

EACR22-1238

Proteomic profiling of gallbladder cancer patient-derived organoids (PDOs) identifies potential predictive protein markers related with gemcitabine resistance

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Introduction

Gallbladder cancer (GBC) is an aggressive disease characterized by high chemoresistance which limits the therapeutic effectiveness and contributes to poor prognosis. The molecular mechanisms underlying chemoresistance in GBC are poorly understood. Moreover, the lack of patient-specific cell models has been an obstacle to study drug resistance in GBC and establish more effective and personalized therapeutic strategies. In recent years, Patient-Derived Organoids (PDOs) have emerged as a promising preclinical model because they recapitulate the in vitro pathophysiological features of the original tumor and have the potential to predict individual drug response. The aim of this study was to characterize the proteomic profiles of gallbladder cancer PDOs (GBC-PDOs) that exhibit different sensitivity to gemcitabine, the first-line treatment in GBC, and thus identify potential protein markers for predicting response or resistance to gemcitabine in GBC.

Material and Methods

Total proteins obtained from GBC-PDOs sensitive (n=4) and resistant (n=2) to gemcitabine were analyzed by Tandem Mass Tag (TMT)-based quantitative proteomics. Then, the proteomic profiles of each phenotype and DEPs between both groups ($FC \geq 1$, p-value ≤ 0.05) were functionally characterized using the Gene Set Enrichment Analysis (GSEA) method and the hallmarks gene set of Molecular Signature Database (MSigDB) in order to identify the enriched pathways based on the normalized enrichment score (NES).

Results and Discussions

We found 43 up-regulated and 31 down-regulated proteins in gemcitabine-resistant PDOs compared to sensitive organoids. Relevant proteins included VIM, FN1, CAV1, TGM2, IGTAG2, TGFB1, among others. GSEA analysis of differentially expressed proteins revealed significantly enriched pathways in resistant organoids such as Epithelial Mesenchymal Transition (EMT), hypoxia, TGF- β signaling, xenobiotic metabolism, inflammatory response and glycolysis. Deregulation of these processes has been associated with drug metabolism, cell dedifferentiation and tumor metabolic reprogramming, which induce the acquisition of a mesenchymal phenotype and resistance to anti-neoplastic drugs.

Conclusion

The gemcitabine-resistant GBC-PDOs showed a plethora of differentially expressed proteins involved in relevant cellular process that may promote chemoresistance and tumor aggressiveness. These proteins may be potentially useful for therapeutic decision-making in GBC. This work was supported by FONDECYT grants 1201734, 1171463, 1221253, 11180987.

EACR22-0942

Understanding the basis for sensitivity and resistance to KRAS-G12C inhibitors

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Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide. KRAS is the most frequent mutated driver gen in non-small cell lung cancer (NSCLC) and, until recently, it was considered undruggable. However, inhibitors against KRAS-G12C have been recently developed, and have shown efficacy in NSCLC patients.

KRAS mutant NSCLC has high genomic complexity, including co-mutations, and biological heterogeneity. Tumor response to direct KRAS-G12C inhibition is also heterogeneous in several *in vivo* models, and in the early clinical trials. A deep understanding of the mechanism of resistance to KRAS-G12C inhibitors is of prime relevance in order to predict which patients may benefit from these therapies, and to propose alternative treatment strategies.

Material and Methods

The efficacy of the inhibitors Sotorasib and Adagrasib was tested *in vitro* in 3D proliferation assays using 8 KRAS-G12C cell lines and 6 KRAS-G12C PDX-derived organoids (PDXDO) and *in vivo* using 4 PDX, and we classified them in sensitive and partially sensitive. Using the exome and transcriptome of these cell lines, PDXDO and PDX, we looked for predictive response biomarkers.

Additionally, we generated resistant KRAS-G12C cell lines to G12Ci, exposing cells to increasing concentrations of drugs. The resistant cell lines were characterized at the molecular levels looking for resistant mechanisms.

Results and Discussions

In both *in vitro* and *in vivo* experiment, using established cell lines, PDXDO and PDX, we observed that not all models are equally sensitive. Thus, we were able to group them into sensitive and partially sensitive models and compare their molecular profile to propose potential predictive response biomarkers.

Additionally, we generated 11 resistant cell lines and we characterized them at the molecular level. In many resistant cell lines we observed commonly differences in the expression and/or activation of some tyrosine kinase receptors. These alterations could explain the resistance and they could be used to propose alternative combinatorial strategies to overcome the resistance.

Conclusion

1. Not all cell lines or PDX-derived organoids are equally sensitive despite having the same KRAS mutation. This indicates that there are other factors that influence sensitivity to these inhibitors.
2. *in vitro* are EMT and changes in the expression and/or activation of some tyrosine kinase receptors.

EACR22-0049

Host-dependent reversible resistance mechanisms to EGFR-TKIs in non-small cell lung cancer.

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Introduction

Although tyrosine kinase inhibitors of epidermal growth factor receptor (EGFR-TKIs) show great success to treat lung cancers patients with mutation of EGFR, patients invariably develop resistance to EGFR-TKIs several months later due to secondary mutations such as EGFR T790M mutation. So far, several mutational-dependent and irreversible resistance mechanisms have been reported, while non-mutational and reversible resistance mechanisms are largely unknown. Here, we tried to elucidate the mechanisms of non-mutational resistance by utilizing dacomitinib, a newly approved covalent EGFR kinase inhibitor.

Material and Methods

We established dacomitinib-resistant (DR) cells by means of gradually increasing dacomitinib concentrations for 6 months using PC9 lung adenocarcinoma cells. After long period of drug exposure, PC9DR cells acquired no secondary mutations in kinase domain of EGFR. We also established PC9DTPs by exposing high concentration (100 nM and 1 μ M) of dacomitinib for 9 days.

Results and Discussions

Firstly, we confirmed the characteristics of PC9DR cells. Results showed that PC9DR cells are resistance to other EGFR-TKIs and acquired epithelial mesenchymal transition (EMT) characteristics, including upregulation of AXL receptor. Next, to explore the behavior *in vivo*, we implanted PC9DR cells to immunocompromised mice and evaluated the response to dacomitinib treatment. Unexpectedly, PC9DR-derived tumors reverted to a dacomitinib-sensitive state in mice. Furthermore, expression of EMT-related protein including upregulated AXL receptor in PC9DR derived tumor were reverted to the almost the same as PC9 parental cells. Nevertheless, ex vivo cell lines from PC9DR-derived tumor displayed renewed resistance to dacomitinib. These findings imply that host-dependent factor (s) could revert the resistance of PC9DR cells. We evaluated the relevance of host-dependent factors such as hypoxia, tumor microenvironment including fibroblast and immune cells, and three-dimensional tumor structure. These analyses indicated that three-dimensional culture of PC9DR cells might alter the EMT-related gene expression and AXL expression.

Conclusion

In summary, PC9DR cells (reversible resister) exhibit the unknown step of resistance process toward the irreversible, secondary mutation-driven non-reversible resister state.

EACR22-0207

Docetaxel-induced downregulation of Survivin sensitizes metastatic prostate cancer to platinum-based treatment

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Introduction

Docetaxel is the most effective chemotherapy agent in metastatic castration-resistant prostate cancer (mCRPC) patients; yet, resistance occurs almost inevitably. Taxane-platinum combinations have shown clinical benefit in a subset of patients, but predictive biomarkers remain elusive. In a previous work, we demonstrated that the CXCR2/Bcl-2 antiapoptotic axis was markedly reduced in docetaxel-exposed mCRPC, representing an emerging vulnerability to platinum-based treatment. Survivin, a member of the inhibitor of apoptosis proteins, is a bi-functional protein that acts as both a mitotic key regulator and an apoptosis inhibitor thereby inducing chemoresistance.

The aim of this study was to elucidate the potential role of Survivin as a predictive biomarker of platinum-based treatment response in docetaxel-resistant mCRPC.

Material and Methods

NPK genetically-engineered mouse models as well as PC3, DU145 and their derived docetaxel-resistant cells PC3-DR and DU145-DR were used in this study. Survivin protein expression was assessed by western blot. The effect of platinum and Survivin inhibitor YM155 treatment on cell proliferation was determined by MTT assay. G2M checkpoint pathway single sample GSEA scores were compared with Survivin expression and taxane exposure status using a multiple linear regression model with interaction effect. G2M and Survivin comparisons were performed using nested ANOVA model on a taxane resistance expression profiling dataset (GSE33455).

Results and Discussions

First, we analysed the effect of docetaxel treatment on Survivin expression. We observed an immediate downregulation of Survivin in docetaxel-treated DU145 and PC3 cells. In agreement, tumors from the docetaxel-treated NPK mice showed a marked reduction in Survivin compared with non-treated controls. Consistently, mRNA and protein levels of Survivin in untreated DU145-DR and PC3-DR cells were significantly reduced. Survivin expression levels were highly associated with the "G2M checkpoint pathway", especially in taxane-exposed patients ($p=0.0086$). Moreover, this pathway was negatively enriched in DU145-DR ($p=0.005$) and PC3-DR ($p=1.06e-06$) as compared to its parental cell lines. Interestingly, treatment with YM155 sensitized DU145 and PC3 cells to cisplatin and carboplatin exposure.

Conclusion

Our findings showed that Survivin is markedly reduced in docetaxel-exposed mCRPC preclinical models and suggest that Survivin expression levels might predict therapeutic response to platinum-based treatment in taxane-resistant mCRPC patients.

EACR22-0211

Mechanical stress induces chemoresistance through ER stress/autophagy activation in pancreatic cancer cells

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Introduction

One of the most fundamental problems that oncologists are currently facing in pancreatic cancer patient care is the high resistance to all types of chemotherapies or targeted therapies. A common characteristic among most pancreatic cancer patients that could serve as potential target for treatment, is the biomechanically abnormal tumor microenvironment (TME). This altered TME is characterized by an increased stiffness which among others, is responsible for the development of mechanical stresses in the tumor interior. Even though several studies have investigated the effect of mechanical stress on cancer cells, it has not yet defined whether it can affect drug sensitivity. Based on our recent findings, obtained by a Reverse Phase Protein Assay, we observed that mechanical stress activates a resistance response, employing Endoplasmic Reticulum (ER) stress response and autophagy activation, in pancreatic cancer cells. To this end, we hypothesize that mechanical stress developed during tumor growth, can induce ER stress/autophagy activation to eventually promote drug resistance in pancreatic tumors.

Material and Methods

We use two pancreatic cancer cells lines, MIA PaCa-2 and PANC-1, to apply mechanical stress, either in a 2D experimental setup or by employing tumor spheroids in a 3D agarose matrix. In both cases, cells are exposed to the most common chemotherapeutic agents used for pancreatic cancer treatment, gemcitabine and 5-Fluorouracil (5FU). The cytotoxic effect of each drug, as well as ER stress-, autophagy- and signaling pathway- activation are then analyzed. Finally, *in vitro* assays and treatments, and *in vivo* animal models of pancreatic tumors, are performed to test the efficacy of an autophagy inhibitor combined with chemotherapy and a mechanotherapeutic that modulates intratumoral mechanical stresses.

Results and Discussions

Our results revealed that mechanical stress activates autophagy to impair cell apoptosis and promote cancer cell survival. This stress-adaptation mechanism in turn impairs the cytotoxic effect of both gemcitabine and 5FU. A combined treatment employing chemotherapy with an autophagy inhibitor, and a mechanotherapeutic agent, showed promising results in increasing the overall-survival of pancreatic tumor-bearing mice.

Conclusion

Our results provide evidence that alleviation of intratumoral mechanical stresses with mechanotherapeutics combined with autophagy inhibitors can improve the efficacy of chemotherapeutic agents in pancreatic cancer.

EACR22-0279

Overexpression of ezrin is associated with acquired resistance to vemurafenib in BRAFV600E mutated colon cancer cells

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Introduction

Approximately 12% of patients with metastatic colorectal cancer (mCRC) have BRAFV600E mutation that confers poor prognosis and resistance to standard therapies. Opposite to metastatic melanoma, single-agent vemurafenib, selective BRAFV600E kinase inhibitor, did not prove effectiveness in patients with BRAFV600E mutant mCRC. Better understanding of the mechanisms governing the development of resistance to vemurafenib would improve clinical outcomes in BRAFV600E mutant mCRC. In this study, we aimed to identify novel

molecular features of vemurafenib-resistant colon cancer cells using integrated proteomics and bioinformatics approach.

Material and Methods

Comparative proteomics profiling of vemurafenib-resistant and sensitive RKO colon cancer cell lines harbouring BRAFV600E mutation was carried out by two-dimensional gel electrophoresis coupled with MALDI-TOF/TOF mass spectrometry (MS) and label-free liquid chromatography-MS/MS. KEGG database was used for pathway enrichment analysis. The identification of potential drug targets in resistant cells based on the protein expression profiles and co-expression links was performed by Genevestigator. Western blot analysis and quantitative RT-PCR were used for validation of proteomics results. MTT assay was performed to measure cell proliferation.

Results and Discussions

Bioinformatics analysis of significantly up-regulated proteins in resistant RKO colon cancer cells suggested EZR (Ezrin) as the first order target. KEGG pathway analysis revealed that the up-regulated proteins were significantly associated with the regulation of actin cytoskeleton including ezrin, actin and several F-actin cross-linking proteins. Increased baseline levels of EZR in resistant cells were additionally confirmed by western blot and qRT-PCR. Importantly, treatment with vemurafenib significantly up-regulated EZR expression at both protein and gene level in resistant in comparison with sensitive cells after 72 hours. Expectedly, treatment of resistant cells with the inhibitor of ezrin (NSC305787) for 72 hours significantly reduced cell viability ($IC_{50} = 7,82 \mu M$). In addition, mRNA expression analysis of colorectal adenocarcinoma in the TCGA dataset using cBioPortal revealed higher expression of EZR in BRAFV600E mutated CRC in comparison with unaltered group.

Conclusion

Up-regulation of ezrin is a specific molecular feature of vemurafenib-resistant BRAFV600E mutated colon cancer cells whose pharmacological targeting could provide an opportunity to reverse vemurafenib resistance.

EACR22-0503

ITGA6 is a key molecule in driving metastatization of platinum resistant epithelial ovarian cancer

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Introduction

Recurrent Platinum (PT) resistant Epithelial Ovarian Cancer (EOC) grows as a metastatic disease in the abdomen/pelvis. EOC cells disseminate as clusters/spheroids within the abdominal cavity and then root and thrive on the mesothelium lining the peritoneal organs. Spheroids interact with the mesothelium engaging specific adhesion molecules, such as integrins. If and how the acquisition of a PT-resistant (PT-res) phenotype also contributes to the ability of EOC cells to spread and grow on the peritoneum through specific integrin-mediated interactions is still not clearly understood.

Material and Methods

Citofluorimetric, western blot and qRT-PCR analyses were used to map integrin expression on PT-sensitive and PT-Resistant isogenic cell lines. Pharmacological and genetic (CRISPR/Cas9 technology) inhibition and overexpression studies were used to characterize the role of selected integrins. Functional in vitro assays were used to study the phenotype of genetically and pharmacologically modified EOC cells. In vivo studies in NSG mice were used to confirm in vitro results. Patients' derived models were used to establish the significance of the in vitro/in vivo findings.

Results and Discussions

Using isogenic models, we found that PT-res EOC cells had a greater adhesion ability to the mesothelium and commonly upregulated integrin alpha 6 (ITGA6) expression. PT-treatment activated a transcriptional program leading to ITGA6 overexpression. Both genetic (ITGA6KO) and pharmacological approaches demonstrated that ITGA6 mediated the greater adhesion and dissemination capacity of PT-res EOC cells. Phospho-proteomic studies identified the pathways activated by ITGA6 to ensure the growth and survival of PT-res EOC cells on the mesothelium. Interestingly, PT-treatment, by inducing ITGA6 secretion by PT-res cells, primed mesothelial cells to form a pro-metastatic niche favouring the dissemination also of PT-sensitive EOC cells. Intraperitoneal injection of ITGA6KO cells or the in vivo use of anti-ITGA6 blocking antibody supported that ITGA6 was essential in driving EOC metastatization. Cancer, ascites and blood samples collected from PT-sensitive and PT-res EOC patients confirmed that ITGA6 expression is induced by PT-treatment in vivo and mediate the dissemination of EOC.

Conclusion

Overall, we describe a novel druggable PT-induced positive loop that, by stimulating ITGA6 expression and secretion enable EOC cells to disseminate and grow in the abdomen/pelvis, opening new opportunities of intervention.

EACR22-0515

Sequential redox phenotypes with differential therapeutic vulnerabilities during the acquisition of drug resistance in BRAFV600E LUAD

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Introduction

The current targeted treatment for lung cancer patients harbouring BRAF^{V600E} consists of a dual blockade of RAF and MEK kinases often combining dabrafenib with trametinib (hereafter referred as D/T). This regimen results in extended survival when compared to

single agent treatments but, as with other targeted treatments, disease relapse is a frequent clinical outcome. A significant fraction of these resistant tumours display mutations in other members of the RAS-ERK pathway resulting in reactivated signalling. These alterations co-exist with the original BRAF^{V600E} driver and it is so far unknown whether, as observed in melanoma patients, the oncogenic combination could drive relapse at the expense of acquiring novel targetable vulnerabilities. Furthermore, there is limited information of how BRAF^{V600E}-driven LUAD tumours adapt to D/T treatment before overt relapse can be detected.

Material and Methods

In this work, we have combined naïve BRAF^{V600E} cell lines as well as PDX-derived cell lines with acquired D/T resistance in the clinic, CRISPR/Cas9 screening and pharmacological treatments to identify mechanisms of resistance as well as associated vulnerabilities that could potentially be implemented to fight disease relapse.

Results and Discussions

BRAF^{V600E} mutant LUAD undergoes a non-mutational drug tolerance period that precedes the emergence of secondary alterations giving rise to full blown resistant clones. Our results suggest that oxidative stress is a prominent feature boosted by D/T treatment with concomitant induction of antioxidant responses. Yet, the nature of the oxidative damage and the detoxification systems engaged by cancer cells display substantial temporal differences. Drug tolerant cells suffer from lipid peroxidation and strongly rely on GPX4 to prevent ferroptosis-driven cell death whereas D/T resistant tumours harbouring NRAS secondary mutations enhance cystine transport to boost antioxidant responses. Accordingly, targeting these detox programs by GPX4 or HDAC inhibitors decreased cell viability and extend therapeutic efficacy.

Conclusion

Different redox profiles define stage specific vulnerabilities of BRAF^{V600E} mutant LUAD upon D/T treatment that can extend therapeutic response *in vivo*.

EACR22-0775

THE HYPER-ACTIVATION OF EPIDERMAL GROWTH FACTOR RECEPTOR 1 AND 2 PATHWAYS INDUCES TUMOR RESISTANCE TO ENDOCRINE THERAPY AND CDK4/6 INHIBITORS IN ER+ BREAST CANCER

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Introduction

CDK4/6 inhibitors have been approved in combination with endocrine therapy for the treatment of estrogen receptor positive (ER+) metastatic breast cancers. However, the onset of drug resistance represents the main cause of cancer relapse, disease progression, and breast cancer patient mortality. In this study, we aimed to identify novel mechanisms of resistance to ER antagonists in combination with CDK4/6 inhibitors.

Material and Methods

We generated two ER+ breast cancer cell lines T47D and MCF7 resistant to the combination of fulvestrant (ER down regulator) and abemaciclib (CDK4/6 inhibitor), T47D-FAR and MCF7-FAR, after 6 months of exposure to increasing doses of drug combination. Whole exome sequencing and RNA-sequencing were performed to identify genomic mutations or

differential gene expression patterns between sensitive and resistant tumor cells. Then, loss- and gain-of-function approaches, through siRNA transfection or lentiviral ORF clone transduction, were employed to modulate tumor cell sensitivity to the combination of fulvestrant and abemaciclib.

Results and Discussions

Genomic mutation analysis revealed no acquired mutations in resistant cells compared to parental ER+ breast cancer cells. Of note, Gene Set Enrichment Analysis (GSEA) showed that drug resistance in both MCF7-FAR and T47D-FAR was associated with altered growth factor signalling such as EGFR, HER2 and KRAS, ERK/MAPK. Gene knockdown of EGFR or HER2 through RNAi restored tumor sensitivity to fulvestrant and abemaciclib in FAR spheroid models, strongly affecting ERK activation. Conversely, the stable over-expression of epidermal growth factor receptors in both parental MCF7 and T47D increased tumor spheroid growth in presence of fulvestrant and abemaciclib. Furthermore, through HB-EGF ligand, conditioned media (CM) from FAR cells induced tumor drug resistance in sensitive ER+ breast cancer spheroids in an indirect co-culture system. Finally, the concurrent inhibition of EGFR and HER2 receptors through tyrosin kinase inhibitor (TKI), lapatinib, reverted tumor resistance to fulvestrant and abemaciclib in ER+ breast cancer spheroids.

Conclusion

In conclusion, our findings suggest EGFR and HER2 as novel therapeutic targets in ER+ breast cancer models resistant to endocrine therapy and CDK4/6 inhibitors.

EACR22-0799

Elevated expression of receptor activator of nuclear factor κ B (RANK) induces autocrine IFN γ signaling in luminal breast cancer

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Introduction

The receptor activator of nuclear factor κ B (RANK) signaling pathway is a key mediator of several physiopathological processes, including mammary gland development and carcinogenesis. Recently, we reported the first portrayal of RANK-mediated phenotype in luminal breast cancer (BC), characterized by staminal and mesenchymal traits and resistance to chemo and endocrine therapy. Additionally, the transcriptomic analysis of tumor xenografts disclosed IFN γ signaling as a major hallmark of RANK overexpressing tumors. Since autocrine IFN γ signaling has been implicated in resistance to therapy, this study aimed to investigate autocrine IFN γ signaling pathway in RANK overexpressing luminal BC cells.

Material and Methods

Our experimental model included MCF-7 and T47D luminal BC cell lines and their derivatives with constitutive overexpression of RANK (RANK OE). Standard methods were used for quantification of cell viability and gene and protein expression.

Results and Discussions

RANK OE BC cells presented higher baseline expression of *INFG* and interferon-stimulated genes (ISGs) as well an autocrine production of IFN γ , when compared with parental counterparts. Although the expression of IFN γ is classically associated with pro-inflammatory and pro-apoptotic phenotype the constitutive activation of IFN γ signaling has also been

linked to tumor growth, metastasis and resistance to therapy, namely chemo and endocrine therapy. In accordance with a non-canonical phenotype, RANK OE cells were less sensitive to exogenous IFN γ , unless RANK-pathway was inhibited with osteoprotegerin-Fc (OPG-Fc). Moreover, JAK/STAT pathway downstream of IFN γ was also deregulated in RANK OE cells; and RANK pathway activation with exogenous RANKL directly induced the phosphorylation of STAT1 and STAT3, also described as having both pro and anti-tumor effects.

Conclusion

Overall, our results demonstrate a link between RANK expression and autocrine IFN γ signaling in luminal BC cells. Chronic expression of IFN γ can be associated with pro-tumor effects and resistance to therapy, warranting further studies on RANK-mediated resistance to therapy in BC.

EACR22-0881

Changes in Rictor expression levels affects the metabolic landscape and therapeutic responses of BRAFV600E melanoma cells to targeted therapy

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Introduction

mTORC2 is a key regulator of cell survival and metabolism. Melanoma cells rely heavily on metabolic rewiring to acquire resistance to targeted therapies but the specific role of mTORC2 in this context still needs to be defined. This issue is especially relevant in the field of cancer therapy considering that mTOR inhibition, alone or in combination with other drugs, has shown so far a limited success in this context.

Rictor is an essential component of mTORC2 that is required for its signaling activity and integrity. To study how changes in mTORC2 activity affect melanoma cells responses to targeted therapy (BRAF/MEK inhibitors, BRAFi/MEKi) we analyzed therapeutic responses and metabolic profiles of Rictor-deficient and –proficient BRAF^{V600E} melanoma cell lines.

Material and Methods

Stable downregulation of Rictor was achieved by shRNA lentiviral transduction and by CRISPR/Cas9 technology in different BRAF^{V600E} human melanoma cell lines.

Sensitivity of melanoma cells to BRAF inhibition was evaluated by clonogenic survival assay *in vitro* and by analysis of tumor xenograft growth *in vivo*; the catalytic activity of selected metabolic enzymes was measured with specific enzymatic assays; 2D migration pattern of selected metabolic enzymes was evaluated as well.

Results and Discussions

Changes in Rictor expression are tightly coupled to melanoma cell metabolic reprogramming and sensitivity to BRAFi/MEKi. Most prominent changes observed include alteration of enzymes involved in glucose metabolism, glutaminolysis and NAD metabolism. Pharmacological inhibition of these pathways differently affected the sensitivity of BRAF^{V600E} cells to BRAFi both in cultured cells *in vitro* and in tumor xenografts *in vivo*.

Comparison between total protein profiles and 2D migrations patterns of selected candidate enzymes in Rictor-proficient and –deficient cells evidenced specific changes in protein levels

and/or protein motility in a subset of them. This suggests that Rictor/mTORC2 deficiency, either directly or indirectly, affects the post-translational modifications (PTMs) of these molecules. Functional and molecular analyses are currently ongoing to determine both the nature of these PTMs and their connections with Rictor/mTORC2. **Conclusion**

This work identifies several novel interconnections between Rictor/mTORC2 and the metabolic reprogramming of BRAF^{V600E} melanoma cells, which may represent new therapeutic vulnerabilities of BRAFi-resistant melanomas.

EACR22-0883

Targeting the CCL5-CCR5 axis to tackle chemoresistant malignant pleural mesothelioma

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Introduction

Malignant pleural mesothelioma (MPM) is an aggressive cancer with limited treatment options. Despite advances in immunotherapy, the standard treatment consists of chemotherapy combining pemetrexed and cisplatin. Unfortunately, in a majority of cases, tumor chemoresistance limits average patients' survival to one year. CCL5 is a chemokine overexpressed in various types of cancer and interaction with its main receptor, CCR5, increases tumor development. Pharmacological inhibitors are currently studied in clinical trials. Evidence for a potential role of CCL5 in tumor resistance to chemotherapy is still scarce.

Material and Methods

First, we generated cisplatin-resistant human and mouse mesothelioma cell lines. In order to better understand the potential mechanisms leading to cisplatin resistance and to determine if CCL5 and CCR5 are implicated, we performed several cellular and biochemical analysis both *in vitro* and *in vivo*.

Results and Discussions

In vitro, measurement of CCL5 expression in conditioned media of murine mesothelioma cells (AB12) and human cells (MSTO-211H) showed that cisplatin-resistant cells secreted higher amounts of CCL5 as compared to parental cells.

BALB/C mice and Nude mice were subcutaneously injected in both flanks with cisplatin-resistant and parental AB12 and MSTO-211H cells. Cisplatin-resistant tumors showed increased growth rate and higher CCL5 and CCR5 expression when compared to their parental counterparts. Flow cytometry analysis of dissociated cells from these tumors showed an increased recruitment of F4/80 macrophages with a M2 phenotype (CD206 positive cells) in resistant tumors compared to parental tumors. M2 macrophages present in resistant tumors express CCR5. Treatment with Maraviroc, a CCR5 inhibitor, decreased the growth rate of tumors compared to the control group. **Conclusion**

In conclusion, our results suggest that the CCL5-CCR5 axis might contribute to cisplatin resistance in MPM. These results also underline the potential involvement of changes in tumor microenvironment in resistance to chemotherapy via the recruitment of CCR5+ M2 macrophages. Furthermore, *in vivo* treatment with Maraviroc significantly decreased the growth of resistant tumors which reinforces our initial hypothesis.

Releasing BOK from transmembrane MCL1 interaction: a novel strategy to induce cell death in tumours

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Introduction

Induced myeloid leukemia cell differentiation protein (MCL1) is an anti-apoptotic member of the B cell Lymphoma-2 (BCL2) family of proteins, overexpressed in more than 22 different tumours. Its overexpression is associated with poor prognosis and resistance to therapies. MCL-1 inhibitors, currently in clinical trials, target the cytosolic Bcl-2 homology 3 (BH3) domain and induce cell death mediated by apoptotic BAX / BAK executors. However, there are several tumours in which this therapy is not effective, such as those that decrease Bax expression or overexpress BCL-xL. We have recently discovered that MCL-1 interacts with Bcl-2-related ovarian killer (BOK), another cell death effector member of the Bcl-2 family, through the transmembrane domain (TMD). In a high throughput screen we have identified a first-in-class drug that disrupts the transmembrane interaction between MCL-1 and BOK, releasing BOK to induce cell death.

Material and Methods

A high throughput Bimolecular Fluorescence Complementation Interaction (BIFC) assay was used for the screening of molecules targeting MCL1/BOK transmembrane interactions (MBoINs). Caspase-3 enzymatic and cell viability assays have been done to demonstrate cell death induction in colon carcinoma cells (HCT116). Cells transfected with siRNA BOK have been used to demonstrate BOK dependent - cell death. BAX/BAK dependence has been also studied in BAX/BAK HCT116 double knockout cells.

Results and Discussions

In this study we have identified one molecule that specifically inhibits the interaction between the MCL1/BOK TMDs (MBoIN) without affecting other unrelated TM interactions. Preclinical studies demonstrate that MBoIN treatment induces the translocation of BOK from the endoplasmic reticulum to the mitochondria and activates cell death by apoptosis in a BOK dependent and BAX/BAK independent manner in colon carcinoma cells.

Conclusion

Our data indicate that MBoIN could offer a new therapeutic strategy for tumours that overexpress MCL1. This novel mechanism of action would represent a new opportunity to avoid cardiotoxicity, one of the main drawbacks of current MCL1 inhibitors to reach the clinic.

SCF(β -TrCP) modulates the response to cisplatin-induced DNA damage through the degradation of BRCA1 and CtIP

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Introduction

Cisplatin is one of the most widely used chemotherapy agents for different types of cancer, but over time, patients become resistant to the treatment and survival drops dramatically. Therefore, the study of the resistance mechanisms of tumor cells can help to develop new strategies to increase sensitivity and prevent cancer progression. In this study, we have investigated the role of the E3 ubiquitin ligase SCF(β -TrCP) in the acquisition of cisplatin resistance in different tumor cell lines, analyzing its influence on the regulation of proteins involved in DNA damage repair by homologous recombination (HR) such as BRCA1 or CtIP.

Material and Methods

We have used cell lines from different tumors, such as muscle-invasive bladder cancer HT1376, U2OS or HeLa cells. We have also generated stable lines overexpressing wild-type β -TrCP or a dominant negative mutant, and we have also silenced β -TrCP transiently by siRNA. To study the cisplatin response, cell viability assays, clonogenicity and cell death analysis by western blot have been performed. In addition, we performed western blot, RT-qPCR, immunofluorescence and co-immunoprecipitation assays in order to investigate the involvement of the indicated proteins.

Results and Discussions

We show that SCF(β -TrCP) plays a key role in the cisplatin response, since overexpression of wild-type β -TrCP increased DNA damage and enhanced cisplatin-induced apoptosis, while overexpression of the β -TrCP mutant or siRNA-mediated downregulation of β -TrCP decreased the damage and conferred resistance to the treatment. Furthermore, we demonstrate that BRCA1 interacted with β -TrCP, and its levels increased or decreased when we modulated β -TrCP, so BRCA1 may be a new substrate of β -TrCP. We also describe that β -TrCP-mediated degradation of BRCA1 could involve both lysosome and proteasome pathways. In addition, we show that β -TrCP also interacted with and induced the degradation of another HR protein, CtIP. Mechanistically, the failure of β -TrCP to regulate the degradation of BRCA1 and CtIP enables a more efficient DNA damage repair and thereby the acquisition of resistance to cisplatin treatment.

Conclusion

SCF(β -TrCP) sensitizes to cisplatin treatment by impairing DNA damage repair through the control of BRCA1 and CtIP degradation.

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EACR22-0258

Study of 14-3-3 σ , a protein potentially involved in chemoresistance in head and neck squamous cell carcinoma

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Introduction

Treatment of head and neck squamous cell carcinoma (HNSCC) has not changed much in the last decades. The main chemotherapeutic used for treatment of HNSCC is cisplatin (CDDP). Unfortunately, resistance to CDDP and subsequent relapse of HNSCC patients is common and explains HNSCC high mortality. The aim of this study is to identify target proteins involved in the acquisition of CDDP resistance to improve HNSCC treatment.

Material and Methods

A comparative proteomics study analyzing HNSCC cells CCL-138 (parental), CCL-138-R (CDDP-resistant) and cancer stem cells (CSC) was performed to find proteins deregulated in CDDP-resistant cells. The study revealed that 14-3-3 σ was upregulated in CCL-138-R and CSC in comparison to CCL-138.

Results and Discussions

We found that 14-3-3 σ depletion decreased cell proliferation and induced apoptosis. However, 14-3-3 σ depletion did not sensitize HNSCC to CDDP. In addition, p53 protein levels decreased on the first day of 14-3-3 σ depletion by siRNA technology, which correlates with the theory of a feedback loop between these two proteins. Moreover, in HNSCC patients it was demonstrated that 14-3-3 σ was overexpressed in tumor tissue compared to normal one. TMAs staining analysis showed that 14-3-3 σ expression was negatively associated with tumor stage, and positively associated with p21 and active Src proteins.

Conclusion

Overall, this study suggests that 14-3-3 σ is important for cell response to DNA damage, proliferation and cell survival, whereas its role in HNSCC chemoresistance should be further elucidated.

EACR22-0296

Hedgehog-GLI and MAPK signalling pathway activity in GANT61-resistant melanoma cells

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Introduction

Hedgehog-GLI (HH-GLI) signaling can be activated in a noncanonical manner due the interaction with other signaling pathways, like MAPK signaling. BRAF and NRAS are known for activating GLI proteins directly regardless of the upstream membrane events, but the exact signaling order in which this occurs, and final outcomes are still not fully understood. This interaction may be crucial in establishment and maintenance of drug resistance, which is a known issue for metastatic melanoma.

Material and Methods

We established three melanoma cell lines with different BRAF/NRAS mutation status resistant to GANT61, a GLI specific inhibitor, to explore in more depth the mechanism that underlays HH-GLI and MAPK signaling interaction. Cells were treated with increasing GANT61 concentrations in duration 8-12 months and validated with MTT viability test. HH-GLI and MAPK protein expression was examined by Western blot. Potential GLI transcription targets connected to MAPK signalling were identified with ChIP-seq for endogenous GLI1, GLI2 and GLI3 proteins in these cell lines, and validated by qPCR. Guided by the results, we focused on primary cilia visualization using immunofluorescence in parental and resistant cell lines.

Results and Discussions

GANT61 resistant cell lines were successfully established and validated. By gaining GANT61 resistance, cell lines also changed response to other HH-GLI inhibitors beside GANT61. Resistant cell line showed upregulated MAPK signaling, downregulated HH-GLI signaling and a newly identified GLI transcription target RAB34. RAB34 is essential for primary cilia formation and plays a central role in signal transduction of several signaling

pathways, including HH-GLI. We successfully induced primary cilia with serum deprivation in parental cell line, but only few primary cilia were detected in the resistant cell line, suggesting primary cilia loss in the resistant cell line.

Conclusion

GANT61 resistant cell lines present valuable *in vitro* models which can bring new insights into HH-GLI-MAPK interplay. Based on our current results, we believe that primary cilia present a potential link which can explain switching from HH-GLI on MAPK signaling. Our further experiments will be based on proteins important for regulation of ciliogenesis.

EACR22-0854

Mitochondrial phenotyping of doxorubicin-resistant osteosarcoma cells

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Introduction

Doxorubicin is a chemotherapeutic agent and a first-line treatment for osteosarcoma. Although the benefits of doxorubicin are largely recognized, its therapeutic effectiveness is limited by the onset of resistance. Resistance to chemotherapeutic treatment is one of the major causes that hampers the therapeutic efficacy, inducing cancer relapse, failure of subsequent treatments and eventual patients' death. Several molecular mechanisms, such as changes in drug transport and accumulation, increase of DNA repair and detoxification systems as well evasion from apoptotic cell death, have been described to explain doxorubicin resistance. Accumulating evidence highlighted deregulation of cellular energy as a feature of drug resistance. In particular, mitochondrial alterations play a central role in cancer development and progression.

Material and Methods

Doxorubicin-resistant clones (HOS DXR 10-30-100 ng/mL and MG63 DXR 30-100 ng/mL) were selected by increasing exposure to the chemotherapeutic agent. Crystal violet and Trypan blue assays were exploited for cell viability determination. Confocal microscopy and flow cytometry were used to analyze mitochondrial network, membrane potential, and mass. To better investigate the molecular mechanisms involved in diminished mitochondrial mass, western blot and RT-PCR were performed to assess protein and gene expression.

Results and Discussions

Doxorubicin-resistant osteosarcoma cells showed a different mitochondrial phenotype with respect to their sensitive clones. In particular, resistant cancer cells present reduced mitochondrial membrane potential and mitochondrial mass with decreased protein expression of TOM20 and VDAC1. mRNA levels of genes involved in mitochondrial biogenesis and mitophagy appear to be reduced, besides further studies will be necessary to determine which process is more affected by doxorubicin resistance. Moreover, doxorubicin-resistant clones had diminished ROS levels and appeared to be much less sensitive to hypoxia than parental cells.

Conclusion

The characterization of the mitochondrial phenotype of sensitive and doxorubicin-resistant osteosarcoma cells highlights differences between clones. These alterations could be exploited for new pharmacological approaches that selectively target resistant cells with the final aim of overcoming drug resistance. However, further studies will be necessary to better elucidate molecular mechanisms involved in doxorubicin resistance.

EACR22-1070

N-of-one clinical trials: introducing a high-fidelity primary tissue-engineering platform to assess cellular responses ex vivo

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Introduction

To fully investigate cellular responses to stimuli and perturbations within tissues, it is essential to replicate the complex molecular interactions within the local microenvironment of cellular niches. Here, we introduce ALTEN, a biomimetic tissue platform that allows *ex vivo* analysis of explanted tissue biopsies. This method preserves the original characteristics of the source tissue's cellular milieu, allowing multiple and diverse cell types to be maintained over an extended period of time. As a result, ALTEN enables rapid and faithful characterisation of perturbations across specific cell types within a tissue. Using single-cell genomics, this approach provides integrated cellular response analysis at the resolution of individual cells.

Material and Methods

We use a combination of cellular (flow cytometry, microscopy) and molecular (IHC, IF, scRNA-seq) assays in ALTEN-engineered tumouroids of mouse models of cancer and clinical tumour biopsies to demonstrate tissue architecture, cell viability and diversity preservation. We analysed cellular responses to therapy *ex vivo* using scRNAseq.

Results and Discussions

Our patented ALTEN technology preserves the integrity and tissue architecture of explanted tumours *ex vivo*. ALTEN-engineered tumouroids maintained the original extracellular matrix and faithfully preserved the cell viability of the different lineages within the tissue microenvironment, thus maintaining the cellular niches of the original tumour biopsy. Using scRNA-seq we demonstrated the preservation of cancer cell clonality and classified cancer sub-clones according to their response to chemotherapy and immunotherapy, identifying differential fates of response and resistance within the same tumours. Additionally, we showed ALTEN's scalability using automated microfluidic devices for tissue encapsulation, enabling centralised high-throughput analysis of samples gathered by large-scale multi-centre studies.

Conclusion

ALTEN is a powerful tool for the analysis of cellular responses upon exposure to cytotoxic agents and immunomodulators, with a potential application for personalised medicine as n-of-one clinical trials and drug target validation pre Phase I/II.

Role of cytochrome P450 3A5 in the chemoresistance to antineoplastic drugs in soft tissue sarcoma cell lines

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Introduction

In Mexico, soft tissue sarcomas (STS) are the fourth leading cause of cancer-related death in the pediatric population. Patients with STS suffer relapses after presenting an initial response to chemotherapy, with a 5-year survival of approximately 30%, being the development of resistance to antineoplastic drugs in tumor cells one of the major causes of the low success rates of chemotherapy.

Cytochrome P450 3A subfamily (CYP3A) has the greatest contribution in drug metabolism and takes part in the bioactivation of antineoplastic prodrugs to cytotoxic metabolites, so the characterization at the molecular level of the expression profile of these enzymes can be a predictive tool of cellular response to chemotherapy and contribute to the choice of the best treatment scheme (greater therapeutic effect with less toxicity) for pediatric patients with STS.

Material and Methods

DepMap 20Q1 Public V2 and GDSC databases were used to determine the association between CYP3A4 and CYP3A5 expression with chemosensitivity of 10 antineoplastic agents in STS cell lines by Pearson correlation analysis ($p < 0.05$). The prognostic significance of CYP3A expression was evaluated by survival analysis via OncoLnc webserver. STS cells (ATCC CRL-2061) were exposed to cyclophosphamide (CF, IC₅₀ at 72 h = 2 mM) or vehicle (sterile water) for 7, 14, 21 and 28 days. Cytotoxicity was determined by MTT and trypan blue assays. In viable cells (resistant to CF-induced toxicity), expression levels of CYP3A4 and CYP3A5 were quantified by qRT-PCR. Comparisons between untreated and CF-treated cells were performed by Student's t-test ($p < 0.05$).

Results and Discussions

In bioinformatic analysis, a low expression of CYP3A5 in STS cells correlated with increased resistance to CF and the poor prognosis of patients with STS. For vincristine, higher CYP3A4 expression correlated with higher drug resistance.

Because the cell lines used in the bioinformatic analysis come from both pediatric and adult patients, cell culture was used to determine the relevance of CYP3A in sensitivity to antineoplastic agents in the pediatric population. From day 21 of treatment, cytotoxicity of CF decreased to 35%. CYP3A4 gene expression levels showed no statistically significant differences. CYP3A5 was upregulated from day 14, with the highest level of change (6.8-fold) on day 28.

Conclusion

CYP3A5 is important for acquired resistance to CF in pediatric STS cells. Also, CYP3A5 may be an important prognostic biomarker for assessing response to chemotherapy in pediatric patients with STS.

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Introduction

Despite the advances in early detection and underpinning the molecular bases of breast cancer, almost 30% of patients suffer from recurrence. Common systemic treatments include hormonal, cytotoxic and immunotherapies. Generally, these therapies are effective at the beginning, but resistance to therapy is common.

Several molecular pathways have been identified as main culprits in drug resistance. NF- κ B signaling plays a role in tumorigenesis, drug resistance and inflammation, and has often been addressed as a molecular target in cancer research. Nevertheless, its epigenetic regulation is still unclear though nucleosome remodeling deacetylase (NuRD) complex and metastasis-associated protein 2 (MTA2) have been associated with tumor progression. The present study aimed to determine the levels of MTA2 and NF κ B1 in MCF-7 cells after chemotherapy, and in breast tissues of patients receiving neoadjuvant therapy.

Material and Methods

MCF7 breast cancer cells were transfected with MTA2 overexpression vector and then treated with Doxorubicin or Paclitaxel to determine their cytotoxicity. The expression of main cancer-related genes was screened. Finally, RNA was isolated from FFPE samples of breast cancer patients from the University Hospital in Alexandria, Egypt. Tissue sections were stained for the expression of NF- κ B and MTA2.

Results and Discussions

Cells were treated with increasing doses of the Doxorubicin and Paclitaxel for 48hrs. The IC₅₀ values were 4 μ g/ml for Doxorubicin and 10 μ M for Paclitaxel. The expression level of NF κ B1 and RELA was downregulated with increasing doses of Doxorubicin and Paclitaxel and the expression level of MTA2 was upregulated in all doses of Doxorubicin and Paclitaxel. Transfection of MTA2 into MCF7 cells affected the sensitivity of cells to Doxorubicin and Paclitaxel as seen by MTT assay, wound healing assay and gene expression. MTA2 overexpression increased the proportion of cells entering early apoptosis phase in treated cells. In addition, NF κ B family members were downregulated upon MTA2 overexpression suggesting a repressive function of MTA2. Staining breast tumor sections of patients revealed co-localization of MTA2 with NF κ B1 in the nucleus of neoplastic cells. These results suggest that MTA2 levels play an important role in the response to chemotherapy.

Conclusion

The epigenetic regulation of NF κ B1 using MTA2 mimics has shown to increase the sensitivity of breast cancer cell lines to neoadjuvant chemotherapeutic drugs. This may present a promising solution to overcome drug resistance.

EACR22-0087

C/EBP- β LAP regulates chemo-immuno-resistance in hypoxic non-small cell lung cancer

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Introduction

Solid tumors subjected to intermittent hypoxia are characterized by resistance to chemotherapy and immune-killing by effector T-lymphocytes ^[1].

The aim of this work is to investigate the molecular circuitries determining this double resistance during intermittent hypoxia and to identify novel chemo-immuno-sensitizer approaches.

Material and Methods

From an initial screening on 28 human non-small cell cancer (NSCLC) cell lines, we focused on H2228 NSCLC cell line, that had the highest ratio between ABCB1-ABCB1 and ABCA1, to investigate the molecular mechanisms underlying the divergent expression of these transporters in intermittent hypoxia.

Results and Discussions

Intermittent hypoxia induces a stronger chemo-immuno-resistance than continuous hypoxia in NSCLC cells, by up-regulating the drug efflux transporters ABCB1 and ABCC1, and down-regulating ABCA1, necessary for the anti-tumor activation of V γ 9V δ 2 T-lymphocytes. Cells exposed to intermittent hypoxia have impaired electron transport chain and high mitochondrial reactive oxygen species (ROS) that stabilize the Hypoxia-Inducible Factor 1 α (HIF-1 α) and favor its binding to the mRNA of the CCAAT/Enhancer Binding Protein- β (C/EBP- β) transcription factor. This interaction increases the production of C/EBP- β LAP splicing variant that transcriptionally induces ABCB1 and ABCC1, favoring the efflux of cisplatin and docetaxel, and limiting their efficacy. LAP also decreases ABCA1, limiting the efflux of isopentenyl pyrophosphate (the endogenous activator of V γ 9V δ 2 T-cells), and inducing the escape to the immune-killing. The role of ROS/HIF-1 α /LAP axis in chemo-immuno-resistance was validated by knock-out experiments and using pro-oxidant or antioxidant agents targeting mitochondrial metabolism.

Conclusion

This work demonstrated that the impairment of mitochondrial metabolism induced by intermittent hypoxia increases the ROS-dependent stabilization of HIF-1 α /LAP axis that determines chemo-immuno-resistance. Mitochondrial ROS scavengers may be repurposed as new chemo-immuno-sensitizer agents in hypoxic solid tumors, resistant to conventional treatments.

[1] Muz B., de la Puente P., Azab F., Azab A.K. The role of hypoxia in cancer progression, angiogenesis, metastasis, and resistance to therapy. *Hypoxia*. 2015;3:83. doi: 10.2147/HP.S93413

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EACR22-0333

A model to investigate drug-tolerant persister and resistant cells in melanoma targeted with MAPK inhibitors

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Introduction

The BRAFV600E mutation in melanoma is generally associated with activation of the MAPK/ERK signaling pathway, leading to tumor cell proliferation. Targeted therapy with combined BRAFV600E and MEK inhibitors has improved patient survival, but resistance to

this treatment is very frequent and represents a serious clinical challenge. Resistance can be intrinsic or can be based on newly-acquired genetic alterations. Moreover, drug tolerant/persister (DTP) clones arising without genetic changes from tumor cells that adapt and survive the initial phases of therapy, can be another source of resistance. The aim of our study is to characterize the mechanisms of resistance to MAPK-targeted therapy in melanoma.

Material and Methods

We have used the mouse melanoma YUMM cell model to *in vitro* and *in vivo* (C57BL/6 mice) generate cells resistant to combined BRAF and MEK inhibitors. Both putative DTP and resistant cell populations have been selected from the *in vitro* and *in vivo* platforms, and cell survival and proliferation, and cell signaling and function are being investigated.

Results and Discussions

DTP and resistant cells display significant changes in growth and in several signaling properties and transition markers, and potential gene differences are currently examined by RNAseq and their functional implications investigated.

Conclusion

Our *in vitro* and *in vivo* models represent useful platforms to mechanistically study drug adaptation and resistance mechanisms in DTP and resistant melanoma cell populations, as well as a system to assess new inhibitors targeting these populations.

EACR22-0603

Gene expression signature as a predictive tool to select the best therapeutic choice in BRAF-mutated cutaneous melanoma patients' candidate to receive either target therapy or immunotherapy

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Introduction

The recent development of new therapies like target therapy (BRAFi + MEKi) and immunotherapy have meant a paradigm shift in the treatment of BRAF-mutated melanoma patients. In the clinical practice, both therapies can be administered, but the superior efficacy of one respect the other has not been proven and there is no molecular or translational evidence to choose the best therapeutic option at first line of treatment. Currently, the choice between the two therapies is based only on clinical criteria. Hence, the aim of this project is to identify biomarkers for predicting the best therapeutic option for these patients, through the determination of gene expression profiles.

Material and Methods

We analyzed 19 paraffin-embedded tumor tissues from BRAF-mutated melanoma patients treated with BRAFi + MEKi (Dabrafenib and Trametinib, respectively) and 9 samples treated with immunotherapy (Pembrolizumab, Ipilimumab or Nivolumab). RNA expression screening was performed using Nanostring PanCancer IO 360 panel, a panel against 770 genes involved in the interplay between the microenvironment, the tumor and the immune response in cancer. Data analysis was performed using nSolver software and comparing responder (R: complete and partial response) versus non-responder (NR: stable and progressive disease) groups.

Results and Discussions

Comparing R vs NR groups, and taking selection criteria into account, a set of genes were identified as good biomarkers for the selection of the best therapeutic choice. Preliminary analysis of samples from patients treated with BRAFi + MEKi revealed a gene network related to antigen presentation. We found a cluster of genes associated to B cells function that was upregulated in responders (like MS4A1 and CD19). Preliminary analysis of immunotherapy treated patients shows an interesting enrichment of genes related with the angiogenesis that differentiates responder vs non-responder, despite the low number of collected cases. Importantly, putative target therapy predictive biomarkers do not recapitulate in immunotherapy cohort, suggesting that they could be of vital importance to select best therapeutic choice in BRAF-mutated melanoma.

Conclusion

Focusing on changes in transcriptome, we have been able to determine a set of genes related to response to target therapy and to immunotherapy at independent manner. However, further analysis and validations are warranted to elucidate the potential of these selected biomarkers.

EACR22-0737

Behind the scenes of the metachronous metastatic colorectal cancer

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Introduction

The resistance to systematic therapy and in consequence the ability to form metastases is the most serious complication of colorectal cancer (CRC) treatment and thus the major cause of the high mortality. The identification of clinicopathological and molecular predictive and/or prognostic factors represents currently one of the most challenging tasks in oncology. Significant efforts are currently being dedicated to identifying patients who will or will not benefit from chemotherapy. In fact, drug resistance is a limiting factor of the efficacy of chemotherapy in CRC treatment. However, despite modern surgical techniques and adjuvant systemic therapy, only 20% of patients with distant metastasis achieve long-term remission, while 60–70% of patients develop local or distant recurrence.

In the presented study, we aimed to describe transcriptomic profiles of the metachronous

metastases of CRC and evaluate different sources of gene deregulation, including DNA mutations, methylation, and small RNA regulation.

Material and Methods

We analyzed 10 metastatic metachronous liver samples from CRC and adjacent non-malignant tissue for RNA sequencing, whole-exome sequencing, and small non-coding RNA sequencing. Additionally, analysis of the global methylation profiles was added using the Illumina EPIC methylation microarray. The bioinformatical analysis was performed using open-source tools for quality control (FastQC), data trimming (Trimmomatic), mapping (BWT, STAR, Bowtie), variant calling (GATK, SNPeff, PureCN) gene expression calculation (RSEM, EdgeR). Data manipulation and additional statistical analysis were performed in the R programming language.

Results and Discussions

We discovered more than 6000 differentially expressed genes and over 300 differentially expressed microRNA between metastatic and non-malignant tissues. Among the most affected pathways belonged: Extracellular matrix organization (GO:0030198, $p = 8.9e-08$), Cell adhesion (GO:0007155, $p = 1.3e-05$) or SRP-dependent cotranslational protein targeting to membrane (GO:0006614, $p = 1.3e-05$). There were on average over 600 somatic homo/heterozygous somatic mutations in the metastatic tissue. The most mutated genes were *APC* (75% of the samples), *TP53* (50%), and *SYNE1* (50%).

Conclusion

We believe that our results will contribute to the clarification of pathological processes in resistance development.

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EACR22-0741

Identification of gene signatures and potential therapeutic targets for acquired chemotherapy resistance in neuroblastoma

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Introduction

Patients with high-risk neuroblastoma (HR-NB) are treated with intensive multimodal therapy. Approximately 60% of HR-NB patients that initially respond to chemotherapy will eventually suffer disease relapse and/or develop resistance to therapy. Acquisition of resistance to chemotherapeutic drugs is thus a major problem, leading to treatment failure. To date, the molecular mechanisms underlying drug resistance in NB are still greatly unknown. There is a pressing need to investigate therapy-induced resistance mechanisms in HR-NB, in order to identify aberrantly activated signaling pathways that may be targeted by specific pharmacological compounds.

Material and Methods

NB cell models resistant to cytotoxics (doxorubicin, cisplatin and vincristine) included in current chemotherapy protocols for HR-NB, were generated by long-term exposure to

increasing concentrations of drugs. Differentially expressed genes were identified by gene expression profiling analysis (Affymetrix array). *In vitro* and the *in vivo* functional assays were performed to explore the malignant behavior of therapy-resistant NB cells.

Results and Discussions

Drug-resistant NB cells showed a marked increase of the IC₅₀ values for all the tested drugs, ranging from 10 to 80-fold in *MYCN*-amplified cell lines. Gene expression profiling analysis identified a set of differentially expressed genes between drug-resistant and non-resistant cell lines. This gene signature was enriched in genes involved in cell differentiation, cell migration and DNA damage repair processes. Moreover, drug-resistant NB cells showed increased cell survival, improved invasion capacity and exhibited a significant increase of tumor growth capacity when were injected subcutaneously in immunodeficient mice. Ongoing experimental work is focused on the validation of our resistance-related signature in a cohort of paired diagnosis-relapse NB samples from patients of our institution.

Conclusion

We have identified a set of differentially expressed genes and signalling pathways potentially underlying acquired resistance to doxorubicin, cisplatin and vincristine in our *in vitro* NB model. Our findings contribute to a better understanding of the mechanism mediating treatment failure in NB. Ongoing studies will provide further insights into the mechanisms that protect NB cells from drug-induced death, that will be relevant to improve therapeutic response of drug-resistant NB.

EACR22-0999

In vitro study of temozolomide resistance in glioma cells

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Introduction

Glioblastoma multiforme (GBM) is a highly malignant brain tumor and one of the most dangerous cancer types with poor prognosis. In addition to surgery and radiation, a current treatment strategy includes temozolomide (TMZ). TMZ is an alkylating substance which interferes with cell proliferation by methylating the DNA, especially guanine, at the O⁶ position. This leads to a false inserted base nucleotide during transcription, which subsequently activates miss match repair (MMR), resulting in numerous single strand breaks and finally in cell death. Despite all efforts, TMZ treatment is not effective in the long run, given the fact that most tumors show resistance towards TMZ after some time. This resistance is mainly based on the O⁶-methylguanine-DNA-methyltransferase (MGMT) expression as well as base excision repair (BER). Clinical studies show that low expression of MGMT in glioma patients resulted in a better therapeutical outcome. To increase TMZ efficiency in GBM, a combined treatment with highly specific MGMT inhibitors like Lomeguatrib (LG) is used.

Material and Methods

U373, T98G (resistant) and U373-R (resistant) glioma cells were treated with increasing concentrations of TMZ from 10 µM to 1000 µM for 48 to 96h. Additionally, all cell lines were treated with LG alone for the same time with concentrations up to 50 µM as well as in a combinational treatment with TMZ. U373-R (R=resistant) glioma cells arose from U373 which were treated by us with an increasing concentration of TMZ over a period of 3 months. Cell viability was determined by MTT and SRB assay. MGMT occurrence was examined via Western blot.

Results and Discussions

U373 show a significant decrease in cell viability after TMZ treatment compared to U373-R and T98G. For T98G, it can be explained by a generally high expression of MGMT. Surprisingly, that explanation cannot hold true for U373-R, as these cells shut down MGMT expression completely. In that regard, a further TMZ treatment even with higher concentrations becomes ineffective and other/novel treatment options have to be in focus. In that context, our preliminary data showed that LG exhibited a significant decrease in the cell viability in U373-R even though its target enzyme MGMT is no longer present.

Conclusion

The data indicate that there are different resistance mechanisms in GBM cells to overcome TMZ toxicity. Those mechanisms must be evaluated to get a better understanding of the origin of the resistance. These data may support the development of new treatment strategies.

EACR22-1233

Contribution of methylglyoxal stress to acquired resistance to Gemcitabine in pancreatic ductal adenocarcinoma

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a lethal disease, with a poor 5-year survival rate. Upon receiving gemcitabine chemotherapy, initially sensitive PDAC tumors often develop resistance. Enhanced aerobic glycolysis has been associated with gemcitabine resistance in PDAC. Methylglyoxal (MG), a highly reactive dicarbonyl compound, is spontaneously generated during glycolysis. MG glycates lysine and arginine residues to form MG-adducts called argpyrimidine (Argp). MG can be detoxified through the glyoxalase system (GLO1), or scavenged by exogenous molecules, including metformin and aminoguanidine. Dysregulation between MG production and detoxification results in cellular MG stress. We have previously described tumor promoting effects of MG in breast and colon cancers. Here we investigate for the first time the potential role of MG stress in acquired gemcitabine resistance in PDAC.

Material and Methods

Gemcitabine resistant (GR) PDAC cells (T3M4, MiaPaca2 and Patu-8988T) were generated from parental cells *in vitro*. Western blotting was used to measure abundance of Argp, GLO1, glycolysis enzymes, and heat shock response (HSR) associated proteins. Maximal GLO1 activity was determined by measuring S-D-lactoylglutathione production, and rates of glycolysis were assessed by extracellular flux analysis. Lastly, GR PDAC cells were treated with gemcitabine alone or in combination with MG scavengers (metformin and aminoguanidine) and confluency was assessed using Incucyte-life cell imaging system.

Results and Discussions

When compared to parental counterparts, GR-T3M4, but not GR-MiaPaca2 and GR-Patu-8988T cells, have elevated Argp and GLO1 expression, and higher GLO1 activity. GR-T3M4 cells have elevated rates of glycolysis, whilst glycolysis remains mostly unchanged in GR-MiaPaca2 cells, suggesting that increased MG stress in GR-T3M4 is likely a consequence of elevated glycolytic capacity. Interestingly, HSP27 and HSF-1 expression are elevated in GR-T3M4 potentially implicating the HSR in MG stress associated GR. Co-treatment with gemcitabine and metformin/aminoguanidine, significantly increased GR-T3M4, but not GR-

MiaPaca2, cell sensitivity to gemcitabine, suggesting an important role of MG in promoting resistance to gemcitabine.

Conclusion

This study highlights a cell-line dependent association between MG stress, HSR and GR in PDAC. Moreover, sensitivity to gemcitabine may be rescued using MG-scavengers in GR PDAC cells that show elevated MG stress.

EACR22-0696

ERK Activity as a Proxy for Targeted Therapy Resistance in Colorectal Cancer Organoids

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Introduction

The EGFR-RAS-RAF-MEK-ERK mitogen-activated protein kinase (MAPK) signalling cascade holds fundamental relevance for colorectal cancer (CRC) biology and therapy response. MAPK is a key target of CRC therapy, as blockade of EGFR is the first-line therapy for patients with metastasised MAPK-wildtype CRC. Besides, combined blockade of EGFR and BRAF is first-line therapy for patients with advanced CRC containing oncogenic BRAF mutations. A common aim of targeted cancer therapies in CRC is to directly or indirectly block ERK, and the emergence of resistance is generally linked to ERK reactivation.

Material and Methods

In this project, we evaluate multiple layers of ERK regulation and how they impact CRC targeted therapy resistance. We analyse CRC cell line and organoid single-cell transcriptomes and signalling networks to assess the effects on MAPK inhibition. Currently, we use ERK reporters to evaluate ERK regulation separately in the nucleus and the cytoplasm.

Results and Discussions

We find that therapy-related perturbation of the MAPK-ERK pathway has unique effects on CRC cell phenotypes. Furthermore, our data suggest that MAPK and Wnt pathways are negatively correlated in CRC cells and that MAPK-ERK inhibition can induce or stabilise cancer stem cell traits. Our ongoing experiments aim at disambiguating effects of nuclear versus cytoplasmic ERK activity in the formation of therapy-resistant CRC cell populations.

Conclusion

In this project, we aim to elucidate the relationship between ERK activity and ERK localisation, cell differentiation state, and signalling network behaviour in emerging resistant cell populations under targeted therapy.

EACR22-0238

Combinations of ATR, Chk1 and Wee1 inhibitors with olaparib shows synergistic effect in olaparib resistant Brca1 deficient murine ovarian cell lines.

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Introduction

High-grade serous ovarian carcinoma (HGSOC) is the most frequent histotype of ovarian cancer. More than half of HGSOC is characterized by homologous recombination deficiency (HRD) due to mutations in genes involved in this pathway, including *BRCA1/2*. Olaparib is a poly(ADP-ribose) polymerase inhibitor (PARPi) recently approved in front line and maintenance therapy in platinum-sensitive *BRCA* mutated ovarian cancer patients. Despite its clinical benefits, this treatment is associated with the development of resistance. A better understanding of the mechanisms at its basis could help in finding strategies to delay or overcome/counteract it.

Material and Methods

We generated an olaparib resistant cell line (Brca1-/- OlaR), starting from the *Brca1* and *TP53* deleted murine ID8 cells (Brca1-/-) by step-wise increasing drug concentrations treatment. Sensitive and resistant cell lines were biologically and pharmacologically characterized and the possible molecular mechanisms at the basis of olaparib resistance investigated. Cellular viability was assessed by MTS assay, for molecular studies real-time PCR, western blot and immunofluorescence were performed.

Results and Discussions

We generated an olaparib resistant cell line (Brca1-/- OlaR), starting from the *Brca1* and *TP53* deleted murine ID8 cells (Brca1-/-) by step-wise increasing drug concentrations treatment. Sensitive and resistant cell lines were biologically and pharmacologically characterized and the possible molecular mechanisms at the basis of olaparib resistance investigated. Cellular viability was assessed by MTS assay, for molecular studies real-time PCR, western blot and immunofluorescence were performed.

Conclusion

We generated an olaparib resistant cell lines deficient in HR with multiple mechanisms of resistance. In both resistant and parental sublines, the combination of olaparib with different DDR inhibitors showed a synergistic effect.

EACR22-0254

Phosphorylation of SHP2 at Tyr62 enables acquired resistance to SHP2 allosteric inhibitors in FLT3-ITD-driven AML

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Introduction

The protein tyrosine phosphatase SHP2 is crucial for oncogenic transformation of acute myeloid leukemia (AML) cells expressing mutated receptor tyrosine kinases (RTKs). Downstream of RTKs, SHP2 is required for full RAS-ERK activation to promote cell proliferation. In 2016, Novartis developed a first-in-class potent and selective allosteric, non-covalent SHP2 inhibitor, SHP099, which stabilizes SHP2 in its inactive conformation. The therapeutic potential of SHP2 allosteric inhibitors is currently investigated for solid tumors in several clinical trials, alone and in various drug combinations. Acute myeloid leukemia (AML) is a bone marrow malignancy characterized by a blockade of differentiation and an increased proliferation of myeloid hematopoietic progenitor cells. To investigate the sensitivity of human AML to SHP099, we focused on AML cell lines with internal tandem duplication (ITD) in the juxta-membrane domain of the RTK FLT3. This mutation is present in

twenty-five percent of patients with AML and leads to constitutive activation of its tyrosine kinase activity.

Material and Methods

We established cells with acquired resistance to SHP099 from two FLT3-ITD-positive AML cell lines, MV-4-11 and MOLM-13. To elucidate the molecular basis of SHP099 acquired resistance, we analyzed the proteome and phosphoproteome changes induced by SHP099 treatment in both cell lines at early (1h and 24h) and late (72h and 21 days) time points.

Results and Discussions

We demonstrated that AML cells can restore phosphorylated ERK (pERK) in the presence of SHP099, thus developing adaptive resistance. Mechanistically, SHP2 inhibition induced tyrosine phosphorylation and feedback-driven activation of the FLT3 receptor, which in turn phosphorylated SHP2 on Tyr62. This phosphorylation stabilized SHP2 in its active conformation, preventing SHP099 binding and conferring resistance. Combinatorial inhibition of SHP2 and MEK or FLT3 prevented pERK rebound and resistant cell growth. The same mechanism was observed in a FLT3-mutated B-ALL cell line and in the inv(16)/KitD816Y AML mouse model, but allosteric inhibition of Shp2 did not impair the clonogenic ability of normal bone marrow progenitors.

Conclusion

These findings suggest that combined inhibition of SHP2 and FLT3 effectively treat FLT3-ITD-positive acute myeloid leukemia, highlighting the need for development of more potent SHP2 inhibitors and combination therapies for clinical applications.

EACR22-0334

Sequential combinations with BH3 mimetics enhance rhabdomyosarcoma treatment

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Introduction

Rhabdomyosarcoma (RMS) is the most common childhood soft tissue sarcoma. Currently, all RMS risk-groups are treated with chemotherapy, radiation, or surgery. However,

chemotherapeutic agents do not achieve high cure rates, but they decrease the quality of life of patients due to secondary effects. Thus, targeted therapies are currently studied to foster RMS treatment, as they may improve survival and decrease undesired toxicities.

Pro-survival adaptations upon treatment are a common feature in RMS and other pediatric cancers, frequently mediated by anti-apoptotic proteins of the BCL-2 family. Consequently, targeted therapies often fail to reach clinical efficacy and patients' relapse. In this study, we used a novel functional assay to evaluate targeted therapies effectiveness in RMS, identify anti-apoptotic adaptations and pharmacologically exploit specific inhibitors (BH3 mimetics) to improve its treatment.

Material and Methods

We used dynamic BH3 Profiling (DBP) to predict treatment cytotoxicity, and to identify RMS acquired anti-apoptotic dependence to guide the use of BH3 mimetics. We confirmed DBP predictions *in vitro* by Annexin V/PI cell death analyses. Molecular mechanisms underneath treatment-induced resistance were analyzed by immunoprecipitation and immunoblotting assays. The identified therapeutic strategies were confirmed *in vivo* in an PDX model of RMS.

Results and Discussions

In this study we first established the excellent predictive capacity of DBP by comparing its predictions ($\Delta\%$ priming) with later cytotoxicity (% cell death) using several therapies in three different RMS cell lines. Correlating with previous observations from the laboratory with vincristine (Alcon et al., CDDis 2020), we found a rapid MCL-1 adaptation after trametinib treatment in RMS cell lines and PDX-RMS tumors. The underlying mechanism is a decrease of NOXA expression that leads to increased MCL-1 dependence. We then confirmed that the combination of trametinib with the MCL-1 inhibitor S63845 was synergistic *in vitro* and *in vivo* in a RMS PDX model.

Conclusion

Functional DBP assay can rapidly predict the efficacy of targeted therapies and their anti-apoptotic adaptations in RMS. We found a new sequential combination of trametinib with S63845 that could improve RMS treatment and avoid side effects in the clinic.

EACR22-0344

Pin1 inhibition as an encouraging strategy to maximize platinum-based chemotherapy in Ovarian Cancer

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Introduction

Ovarian Cancer (OC) is one of the most lethal gynecological malignancies due to its insidious nature and the ineffectiveness of screening tests. Primary cytoreductive surgery and platinum (PT)-based chemotherapy represent the standard treatment for OC-bearing patients. Nonetheless, chemotherapy is challenged by the insurgence of drug resistance. Thus, restoring PT-sensitivity is an urgent need in OC. Interestingly, numerous studies documented the implication of Notch receptors in PT-resistance. Among the four Notch receptors, it has been demonstrated that Notch3 (N3) is altered in a wide panel of OC, and it confers PT-resistance to OC cell lines. Given its crucial role, effective targeting of N3 may revert PT-resistance. In this scenario, we previously demonstrated that Pin1 positively regulates N3 in T-cell acute leukemia (T-ALL) and we wondered whether this relationship occurs also in OC context. Collectively, the main aims of the present work are to

investigate *in vitro* and *in vivo*: **1.** the N3-dependent PT-resistance and **2.** the potential role of the down-modulation of the Pin1-N3 axis to restore PT-sensitivity.

Material and Methods

N3-positive and N3-negative established OC cell lines were used. We performed: 1. *in silico* analysis on mRNA data collected by OC-bearing patients; 2. immunohistochemistry (IHC) on OC tissue samples; 3. lentiviral transductions; 4. *in vitro* studies such as pharmacological treatments, cell viability and colony formation assays; and 5. *in vivo* experiments: xenografts in NOD/SCID immuno-deficient mice.

Results and Discussions

Firstly, *in vitro* studies on OC cell lines and stable N3-overexpressing and inducible shN3 clones showed the involvement of N3 in the promotion of PT-resistance. Notably, these findings were consistent with xenografts experiments. Moreover, we demonstrated that the expression of N3 intracellular domain is sustained by Pin1 in OC cell lines. Interestingly, we documented a significant direct correlation between Pin1 and N3 gene expression levels in a cohort of OC-bearing patients by *in silico* analysis, which was in line with IHC performed on primary lesions from OC-bearing patients. These observations corroborate our hypothesis that the pharmacological inhibition of Pin1 may defect N3 signaling also in OC context.

Conclusion

To sum up, the present work will provide evidence in support of N3 targeting via Pin1 inhibition to restore PT-sensitivity, finally improving ovarian cancer treatment.

EACR22-0376

Restricting extracellular Ca²⁺ on gefitinib resistant NSCLC cells reverses altered epidermal growth factor mediated Ca²⁺ response, which consequently enhances gefitinib sensitivity

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Introduction

Non-small cell lung cancer (NSCLC), one of the leading causes of cancer-related death, has a low 5-year survival rate owing to the inevitable acquired resistance toward antitumor drugs, platinum-based chemotherapy, and targeted therapy. Epidermal growth factor (EGF)-EGF receptor (EGFR) signaling activates downstream events leading to phospholipase C/inositol trisphosphate (IP3)/Ca²⁺ release from IP3-sensitive Ca²⁺ stores to modulate cell proliferation, motility, and invasion. However, the role of EGFR-mediated Ca²⁺ signaling in acquired drug resistance is not fully understood

Material and Methods

We analyzed the intracellular calcium ([Ca²⁺]_i) signals in PC-9 cells and PC-9/GR (Gefitinib Resistant) cells under following conditions. We observed hEGF (200ng/ml)-mediated [Ca²⁺]_i oscillations response. Calcium influx, endoplasmic reticulum (ER) calcium contents, hEGF (200 ng/ml)-mediated [Ca²⁺]_i oscillations, and effects of inhibitors were studied in absence of extracellular calcium ([Ca²⁺]_e). We compared gefitinib-induced cytotoxicity and cell viability according to the presence or absence of [Ca²⁺]_e.

Results and Discussions

we found that acute EGF treatment elicited intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) oscillations in PC-9 cells but not in PC-9/GR cells. PC-9/GR cells presented a more sustained basal $[\text{Ca}^{2+}]_i$ level, lower endoplasmic reticulum Ca^{2+} level, and higher spontaneous extracellular Ca^{2+} ($[\text{Ca}^{2+}]_e$) influx than PC-9 cells. Notably, restricting $[\text{Ca}^{2+}]_e$ in both cell types induced identical $[\text{Ca}^{2+}]_i$ oscillations, dependent on phospholipase C and EGFR activation. Consequently, restricting $[\text{Ca}^{2+}]_e$ in PC-9/GR cells upregulated gefitinib-mediated poly (ADP-ribose) polymerase cleavage, an increase in Bax/Bcl-2 ratio, cytotoxicity, and apoptosis. In addition, nuclear factor of activated T cell (NFAT1) induction in response to EGF was inhibited by gefitinib in PC-9 cells, whereas EGF-mediated NFAT1 induction in PC-9/GR cells was sustained regardless of gefitinib treatment. Restricting $[\text{Ca}^{2+}]_e$ in PC-9/GR cells significantly reduced EGF-mediated NFAT1 induction.

Conclusion

These findings indicate that spontaneous $[\text{Ca}^{2+}]_e$ influx in NSCLC cells plays a pivotal role in developing acquired drug resistance and suggest that restricting $[\text{Ca}^{2+}]_e$ may be a potential strategy for modulating drug-sensitivity.

EACR22-0456

Identification of basal lysosomal levels as potential biomarkers for drug resistance in pediatric neuronal tumor models

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Introduction

Despite the discovery of novel cancer drugs and the development of effective cancer treatment strategies, multidrug resistance is still a major problem faced in cancer research. Lysosomes are involved in many cellular processes that cancer cells depend on and there is an emerging role for lysosomes in drug resistance. The aim of the project is to understand the contribution of lysosomes to drug resistance in pediatric tumors.

Material and Methods

High throughput drug screening: To analyze the drug resistance of a panel of 31 high risk pediatric neuronal tumor cell lines, cells are seeded in 384-well plates with 75 anti-cancer drugs in 5 concentrations covering the clinically relevant concentration range of the drugs.

Cells grow as spheroids for 72h. The viability of the cells is measured with an ATP- based luminescence readout and compared to negative and positive controls. Therapy response is described with a drug sensitivity score (DSS) to make drug response comparable among different cell lines.

High content imaging: To characterize the lysosomal compartment of the tumor cell lines at basal levels and in response to drug treatment, lysosomes are stained with functional and immunofluorescence stainings and analyzed with confocal high content imaging. Measurements of the lysosomal stainings in untreated cells are combined into a basal lysosomal score.

Results and Discussions

We generated drug sensitivity profiles for each cell line and correlated them with basal lysosomal scores. Unsupervised clustering analysis based on the DSS, grouped the cell lines into drug resistant and drug sensitive clusters. The cell lines with high basal lysosome score are mostly in the resistant cluster. For many chemotherapeutic drugs we observed a correlation between basal lysosome score and DSS.

Since neuroblastoma was the most represented tumor type in our panel of cell lines, we then compared the basal lysosomal score to molecular features relevant for characterization of neuroblastoma tumor biology. We found that basal lysosome levels correlate with expression of genes that are specific markers for the mesenchymal neuroblastoma tumor subtype.

Conclusion

Understanding the mechanisms of underlying resistance mechanisms in high-risk pediatric tumors is essential to develop novel treatment approaches. Natural variations in lysosome numbers of cancer cell lines emerge as a potential indicator for intrinsic drug resistance and create new vulnerabilities to break resistance by targeting lysosomes.

EACR22-0520

Influence of hypoxia and acidosis on invadopodia activity of low pH-adapted and non-adapted pancreatic cancer cells and its inhibition by both gemcitabine and its lipophilic derivate gemcitabine-C18

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a deadly disease with high mortality due to its late diagnosis, early metastatic development and limited response to chemotherapy. Due to its highly invasive phenotype and dense, hypoxic, and acidic tumor microenvironment, PDAC is one of the most chemoresistant cancers. Dissemination of cancer cells and their spread to distant sites is a multistep process that represents the deadliest aspect of cancer. Cancer cells form F-actin-rich protrusions known as invadopodia, which degrade the extracellular matrix, allowing them to invade surrounding tissues. Gemcitabine is an FDA approved drug used as first-line therapy in PDAC and is a nucleoside analogue which replaces triphosphate cytidine during DNA replication to arrest tumor growth. However, gemcitabine is quickly deaminated to its inactive metabolite, exported to the extracellular compartment and excreted in the urine. Several gemcitabine prodrugs have emerged to

improve its metabolic stability and cytotoxicity. Here we analyzed how the tumor microenvironment can modulate the effect of both gemcitabine and its C-18 prodrug in reducing PDAC cell invadopodia activity.

Material and Methods

PANC-1 and MIA PaCa-2 cells were adapted to pH 6.6 or not for 1 month and cultured on Matrigel containing a quenched bodipy linked to BSA. The cells were exposed overnight to 50 μ M gemcitabine or gemcitabine-C18 in the presence and absence of acidosis and/or 100 or 200 μ M deferoxamine, a chemical hypoxia inducer. Focal proteolysis produced fluorescence on a black background, which was used to quantify the cell's extracellular matrix proteolytic activity.

Results and Discussions

While exposure to low extracellular pH (6.7) increased invadopodia activity only in the acidic pH-adapted cells, hypoxia increased invadopodia activity in both cell lines. Indeed, in pH-adapted cells hypoxia and acidosis together increased invadopodia activity synergistically. Importantly, gemcitabine-C18 was much more effective than gemcitabine in reducing invadopodia activity and, consequently, the digestion of extracellular matrix. Moreover, the C18 prodrug was more efficient in inhibiting PDAC cell invadopodia activity also under the joint, synergistic hypoxia and acidosis condition.

Conclusion

By targeting the early steps of metastasis, C-18 might be a very promising drug candidate for future PDAC therapy.

EACR22-0550

SCO-101 causes Irinotecan (SN-38) re-sensitization in resistant colorectal cancer cells

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Introduction

Each year, 500.000 patients die from Colorectal Cancer (CRC). Chemotherapy resistance represents the main cause of cancer-related deaths and drugs that target chemotherapy resistance cover a high unmet medical need. We have explored the potential of the small molecule SCO-101 to restore sensitivity to irinotecan/SN-38 through inhibition/degradation of the efflux pump ABCG2 and to increase the clinical exposure of SN-38 through inhibition of the enzyme UGT1A1 that inactivates SN-38.

SCO-101 is an oral drug which is currently being tested in a clinical Phase II trial in patients with metastatic CRC and a Phase Ib trial in patients with pancreatic cancer.

Material and Methods

We used pairs of sensitive and SN-38-resistant human colon cancer cell lines (HT29 and LoVo). ABCG2 overexpression and effects of SCO-101 on ABCG2 was investigated by western blot, qPCR, dye-flux assays, bi-directional flux assays, 3H-SN-38 accumulation assays and vesicular uptake assays. The effect of SCO-101 on SN-38 re-sensitization was investigated by cell viability assays and clonogenic assays. The inhibition of UGT1A1 was investigated by enzyme assays.

Results and Discussions

Flux assays demonstrated that SCO-101 inhibited the activity of ABCG2. Protein analysis demonstrated that SCO-101 causes degradation of ABCG2 and in silico docking predicted SCO-101 to bind in the part of ABCG2 where also SN-38 binds. Exposing SN-38-resistant cells to the combination of SCO-101 and SN-38, had a synergistic inhibitory effect on cell viability and colony formation compared to either drug alone. Furthermore, SCO-101 was demonstrated to be a potent inhibitor of UGT1A1.

Conclusion

SCO-101 can re-sensitize SN-38-resistant colon cancer cells to SN-38 through inhibition/degradation of the ABCG2 drug efflux pump. Importantly, SCO-101 is also a potent inhibitor of UGT1A1, leading to increased and prolonged exposure of SN-38 in patients receiving irinotecan containing treatment, as observed in the ongoing Phase II clinical trial CORIST.

In conclusion, SCO-101 represents a unique drug with an innovative dual mechanism of action.

Scandion Oncology has reported the outcome of the first part of the ongoing Phase II clinical CORIST trial with metastatic CRC patients, who are resistant to irinotecan containing treatment. These patients were re-exposed to irinotecan containing treatment in combination with SCO-101 (www.clinicaltrials.gov/ct2/show/NCT04247256?term=scandion&draw=2&rank=2).

EACR22-0720

RAS(ON) inhibitors overcome clinical adaptive resistance to KRAS G12C covalent blockade

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Introduction

The recent development of direct inhibitors of KRAS^{G12C} (G12Ci) that bind the target in its inactive conformation and lock it in off-mode (RAS “OFF” inhibitors) such as adagrasib and sotorasib has initiated a new era in the clinical management of various KRAS-driven solid tumors, including lung adenocarcinoma (LUAD). However, despite high initial efficacy, tumors develop secondary resistance in almost all patients. Although several genomic alterations have been described in patients with acquired resistance to G12Ci, so far, adaptive mechanisms of resistance to G12Ci have been limited to pre-clinical observations only. Therefore, the identification of additional resistance mechanisms and the elucidation of alternative treatments to circumvent them is an urgent medical need.

Material and Methods

In this work, we have used patient derived lung adenocarcinoma cell lines *in vitro* and *in vivo* to demonstrate that disease progression following treatment with the KRAS^{G12C} specific inhibitor sotorasib can be mediated by an adaptive mechanism in patients.

Results and Discussions

Our preclinical results suggest that secondary resistance can be driven by adaptive mechanisms, resulting in higher KRAS-GTP fraction, as an alternative to acquired genomic

alterations. We demonstrate that RM-029, a recently developed compound targeting GTP-bound active KRAS (RAS(ON) inhibitor) is an effective treatment to re-sensitize resistant LUAD to KRAS inhibition and overcome secondary resistance. Our results confirm the therapeutic anti-tumor activity of such treatment by greatly reducing the growth of sotorasib-resistant PDX implants *in vivo* and identify the sequential use of RAS(OFF) and RAS(ON) drugs as a potential therapeutic strategy to extend anti-tumor responses.

Conclusion

Given the limited duration of the clinical response to the current G12Ci(OFF) inhibitors, finding alternative therapies is a pressing medical need. Therefore, our preclinical findings can help explain the onset of clinical resistance in those patients where acquired alterations are not detected. We propose that G12Ci-ON inhibitors targeting GTP-bound KRAS may be an effective alternative to treat disease relapse due to adaptive mechanisms of signal reactivation.

EACR22-0794

Zebrafish patient-derived xenograft models as a model for co-clinical development of a novel ovarian cancer treatment

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Introduction

Today, Pharma companies face a drug discovery challenge in successfully selecting the most reliable and translational in-vivo models for the preclinical testing of new oncology therapies. Unfortunately, oncology has one of the highest preclinical to clinical drug failure rates, with 95% of preclinical drugs failing during clinical development. The concept of co-clinical trials has gained increased attention to overcome this challenge. In a co-clinical trial, the preclinical and clinical development is done simultaneously, allowing fast identification of patient groups or sub-populations that will be more likely to respond to the tested treatment.

Material and Methods

15 tumor samples from the primary and/or metastatic tumor site from ovarian cancer patients were included. The tumors were dissociated into a single cell solution, dyed, and then injected into zebrafish larvae. The larvae were treated for 3 days with a novel small molecule, that has been shown to enhance the efficacy of standard chemotherapy, either alone or in combination with Cisplatin or Carboplatin. Photos were taken at the day of the injection and 3 days later to compare tumor growth and dissemination of metastatic cells to the tail.

Results and Discussions

Results showed that 4 out of 14 samples were responsive to Carboplatin and the drug candidate treatment alone, and only 1 out of 8 samples was sensitive to Cisplatin treatment alone. In addition, treatment with Cisplatin and the drug candidate displayed a greater effect on primary tumor size (4 out of 9 samples) than the combination treatment of Carboplatin and the drug candidate (1 out of 14 samples). Moreover, treatment with Cisplatin and the drug candidate showed to be more effective against tumor samples collected from the metastatic site and tumor samples with advanced cancer stages.

Conclusion

In conclusion, here we show that the zebrafish model can be used in co-clinical trials to stratify patients into resistant or sensitive subtypes for a specific treatment, allowing those that are more likely to respond to be selected for clinical trials and thereby reducing the number of patients needed and improve the outcome of the clinical trial, saving both time and money.

EACR22-0818

Senescence or poliploidy are induced by chemotherapeutics in human glioma cells with knockout of the BLM helicase

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Introduction

DNA damage response is one of the most important biological processes to maintain the genome stability and fidelity. One of proteins participating in this response is the Bloom Syndrome helicase (BLM), the member of RecQ helicases family. Exploration of glioma transcriptome TCGA dataset showed that the level of *BLM* mRNA is increased in diffusive gliomas WHO grades 3 and 4 (high grade Gliomas). Glioblastoma (GBM, WHO grade 4) is a common, most lethal primary brain tumor in adults and remains incurable by conventional therapies. Current GBM therapy includes surgical resection, radiation and chemotherapy with an alkylating agent temozolomide (TMZ), but at least 50% of TMZ treated patients do not respond to TMZ. This is primarily due to over-expression of O⁶-methylguanine methyltransferase and/or dysfunctions of DNA repair pathways in GBM cells. We hypothesized that knockdown of BLM may improve glioma susceptibility to therapy.

Material and Methods

The expression of BLM was explored in TCGA (the Cancer Genome Atlas), tissue microarrays and glioma cell lines. BLM knockout cells were generated with CRISPR/Cas9 technique and evaluated for their basal characteristics and responses to chemotherapeutics using MTT viability test, flow cytometry and β -galactosidase staining.

Results and Discussions

LN229 BLM KO cells were more susceptible to TMZ than the wild-type (WT) cells. We tested cell sensitivity to olaparib (OLA), an inhibitor of PARP-1, another DNA repair enzyme. BLM KO cells were more resistant than WT cells to the combined TMZ+OLA treatment. Evaluation of cell cycle (using propidium iodide staining and flow cytometry) showed that most of BLM KO cells were arrested in the G2/M phase, however, in LN18 BLM KO cells the underwent DNA polyploidy in response to chemotherapeutics. β -galactosidase staining, which is a marker of senescent cells, showed the elevated activity only in treated LN229 cells, suggesting the induction of cellular senescence. Differential responses were due to a different status of the tumor suppressor TP53, as overexpressing p53 affected the response.

Conclusion

Our findings confirm a role of BLM helicase in glioma progression and explain a resistance of certain cells to the combined TMZ/OLA treatment.

EACR22-0820

Characterization of the combination of gemcitabine and its derivate gemcitabine-C18 with nab-paclitaxel as a valid therapeutic option for targeting pancreatic cancer stem

cells

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive tumors, characterized by a five-year survival rate of about 10%. It is usually diagnosed at a late stage and despite of the development of new drugs and the use of multiple therapeutic combinations, no treatment option is viable in patients in the advanced stages. Emerging evidence has suggested that PDAC stroma is heterogeneous and composed of a small population of cells that are responsible for tumor initiation and development, termed cancer stem cells (CSCs). CSCs are the cell type with the most aggressive phenotype, since they have aberrantly activated developmental signaling pathways, are resistant to chemotherapy and radiation, and have upregulated signaling cascades that are crucial for tumor metastasis. Given that CSCs have a role not only in tumor initiation and progression, but also in drug resistance and relapse, thus becoming excellent targets for effective novel therapeutic approaches. Gemcitabine is used as first-line therapy in PDAC, however it has a high cytotoxic effect in the normal cells and a high chemoresistance in the CSCs. Hence, several prodrugs have emerged to improve its efficacy targeting CSCs and its cytotoxicity against normal cells. Here we analyzed how the tumor microenvironment can modulate the effect of gemcitabine, its C-18 prodrug and nab-paclitaxel in CSCs growth, death and normal cells cytotoxicity.

Material and Methods

The normal pancreatic cell line, HPDE, and CSCs derived from PANC-1 and MIA PaCa-2 cells were cultured as an organotypic model growing on Matrigel. The cells were exposed to 50 μ M gemcitabine or gemcitabine-C18 and to 30 nM of nab-paclitaxel alone and as combined therapy for 7 days. Cell growth and death in the presence and absence of acidosis (pH 6.7) were evaluated at different time-points (1, 2, 3, 5 and 7 days of drug treatment).

Results and Discussions

In HPDE cells we found that gemcitabine and C-18 have a similar cytotoxicity profile at both pHs, eg. equivalent induced cell death and growth. However, C18 alone and in combination with nab-paclitaxel was much more effective than gemcitabine in increasing CSCs death and reducing its growth in both cell lines at both pHs. Importantly, gemcitabine was the drug that exhibited a higher chemoresistance at low pH in both CSCs cell lines when compared with C-18 and nab-paclitaxel either alone or combined.

Conclusion

C-18 alone or as a combined treatment might be a very promising drug candidate for targeting CSCs as a novel PDAC therapy.

EACR22-1004

Co-targeting PI3K to overcome anti-EGFR cetuximab resistance in head and neck squamous cell carcinoma

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Introduction

Resistance to therapies targeting the epidermal growth factor receptor (EGFR), such as cetuximab, remains a major roadblock in the search for effective therapeutic strategies in head and neck squamous cell carcinoma (HNSCC). Aberrant signaling of the PI3K/Akt pathway could be involved in resistance to cetuximab treatment. Therefore, this study aims to overcome cetuximab resistance in HNSCC by co-targeting of EGFR (cetuximab) and PI3K (buparlisib) as novel therapeutic.

Material and Methods

Five HPV-negative HNSCC cell lines with different sensitivities to cetuximab were used (the cetuximab sensitive SCC22b-S and SC263-S; the acquired cetuximab resistant SCC22b-R and SC263-R; and the intrinsically resistant SQD9). A phosphorylation analysis was performed after 2h of cetuximab treatment. Cells were grown as 2D monolayers and as 3D multispheroids. Cytotoxicity of cetuximab (2D: 0-50 nM for 72h; 3D: 0-100 nM for 96h) plus the PI3K inhibitor buparlisib (2D: 0-1 μ M for 72h; 3D: 0-2 μ M for 96h) was assessed using the Tecan's Spark Cyto by automated counting of fluorescent cells following staining with Hoechst 3332 and Cytotox Green. Possible synergism between cetuximab and buparlisib was determined using the zero interaction potency reference model in the SynergyFinder 2.0 web application (score: synergism > 10 > additive > -10 > antagonism).

Results and Discussions

Increased Akt phosphorylation was observed after cetuximab treatment in acquired resistant cell lines. This indicates that activation of the PI3K/Akt pathway serves as a compensatory mechanism in acquired resistant cell lines. Co-targeting by simultaneous exposure to 25 nM cetuximab and 0.5 μ M buparlisib revealed a synergistic interaction in all HNSCC cell lines grown as 2D monolayers except for SC263-R (additive). Incubation with 50 nM cetuximab plus 0.5 μ M buparlisib led to similar results in all 2D cell lines. Furthermore, the percentage of cell death was the highest in the combination treatments, with a maximum of $30.33 \pm 4.18\%$ for 50 nM cetuximab plus 1 μ M buparlisib in sensitive cell lines. Combination treatment in 3D spheroids was synergistic in SCC22b-S, additive to synergistic in SCC22b-R, SC263-S and SC263-R and only additive in SQD9 spheroids. Next, these results will be validated in immunodeficient CB-17 SCID mice.

Conclusion

In conclusion, these preclinical results support our rationale for a novel therapeutic strategy combining buparlisib with cetuximab to overcome cetuximab resistance.

EACR22-1016

New strategies based on the use of engineering exosomes as drug delivery system to increase efficacy and overcome resistance to BRAF/MEK inhibitors

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Introduction

Combination therapy with BRAF and MEK inhibitors (BRAF/MEKi) have *substantially improved survival in patients with advanced or metastatic melanoma* with BRAF mutations. However, resistance to these targeted therapies is a frequent cause of therapy failure. Cx43 is a transmembrane protein involved in cellular senescence, tumour microenvironment and tumour progression via channel-dependent and independent functions. This protein is also found in exosomes or small extracellular vesicles (EVs) but its role in EVs released by tumour cells has not been explored yet.

Material and Methods

Different human BRAF-mutated cell lines (melanoma, breast and colon cancer cell lines) were used in this study. Expression vectors and EVs-enriched in Cx43 from different sources were used in this study. EVs were isolated by ultracentrifugation and characterized by NTA, electron microscopy and WB. IP was performed to study protein interactions and samples were analyzed by LC-MS/MS. RNA-seq was used to identify sRNAs in EVs. Standard methods were used to study cellular senescence, cell proliferation and cell death by apoptosis among other assays.

Results and Discussions

The restoration of Cx43 using engineering EVs from different sources, including from tumour cells genetically modified, in different BRAF-mutant tumour cells, significantly decreased cell growth and proliferation and increases cellular senescence and cell death by apoptosis. The combination of sEVs-enriched in Cx43 with BRAF/MEKi significantly increased treatment efficacy and avoid drug resistance in 2D and 3D models. Proliferation assays, LC-MS/MS and RNA-seq showed that Cx43 changes the function of these EVs, the protein content and the recruitment of small RNA. Using these EVs, we have designed a new advanced therapeutic strategy to deliver Cx43 and to increase the efficacy of BRAF/MEKi, preventing drug resistance by reinforcing cellular senescence and enhancing cell death by apoptosis, alone and in combination with the senolytic drug, navitoclax.

Conclusion

Our results indicate that engineering EVs-enriched in Cx43 resulted to be a promising strategy to deliver transmembrane proteins such as Cx43 to increase the anti-tumour activity in target cells and the efficacy of the BRAF/MEKi in order to prevent drug resistance. These results have been protected in an EU patent (PCT).

EACR22-1026

Comprehensive study of Src as a potential therapeutic target in BRAF mutated CRC

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Introduction

Oncogenic mutation BRAFV600E is present in 10% of metastatic colorectal cancer (mCRC), conferring resistance to conventional therapies and a poor prognosis. Activation of Src Family Kinases is associated with an aggressive phenotype and could be involved in chemoresistance mechanisms. We aim to explore the role of Src in BRAFV600E CRC and to investigate the response of this subgroup of tumors to Dasatinib, a potent Src inhibitor.

Material and Methods

BRAFV600E and wild type (WT) CRC cell lines, as well as Src Knock-Out (KO) and Src overexpressed cells, were treated with Dasatinib. Afterwards, Src expression was evaluated by Western Blot and cell viability was analysed by crystal violet and MTS assay. Phospho-kinase proteins screening was performed using the Proteome Profiler Human Phospho-Kinase Array kit.

Results and Discussions

Src and phospho-Src (pSrc) expression displayed an inverse correlation with IC50 Dasatinib values in a panel of 7 CRC cell lines (BRAFV600E and WT). All these doses inhibited Src activation in all cell lines. Focusing on HT29 cells (BRAFV600E), inhibition of p-Src occurs in a dose-dependent manner and in shortened periods of treatment, and Dasatinib showed a significant inhibition of proliferation and migration. Moreover, stable Src-OE HT29 cells showed higher clonogenicity, migration and proliferation capacities, as well as higher resistance to Dasatinib. Conversely, HT29 Src KO cells were more sensitive. In line with those results, silencing Src in other BRAFV600E cells showed decreased clonogenicity and migration abilities. Xenograft mouse model of SRC depletion showed that the growth of SRC KO tumors was slower than WT tumors. Phospho-kinase array quantification in basal and Src-OE HT29 cells showed up- and downregulated proteins related to classical tumorigenic signaling pathways such as MAPK or PI3K/Akt.

Conclusion

To conclude, lower p-Src expression at baseline is related to a higher resistance to Dasatinib, which showed a significant anti-proliferative activity in a subset of CRC cell lines and regulates the activity of Src in a time and dose-dependent manner. Modified expression of Src in BRAFV600E CRC cells significantly regulates clonogenicity, proliferation and migration abilities *in vitro*. Src overexpression is responsible of the acquired resistance to Dasatinib and regulates several pathways that could be involved in the tumorigenesis of BRAFV600E CRC, although further studies to explore Src-mediated resistance mechanisms are required.

EACR22-1128

Patient derived organotypic cultures (PDOCs): an ex vivo model to study the impact of tumor microenvironment on therapeutic resistance

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Introduction

The complexity of tumor tissue may account for the difficulties in using *in vitro* models for preliminary studies. Since the tumor microenvironment is an essential factor modulating the behavior of cancer cells, we developed in our lab patient derived organotypic cultures (PDOCs) as novel and innovative ex vivo system, closely recapitulating tumor patient biology

Material and Methods

Melanoma tumor fragments derived from patients go through a mechanical and a short enzymatical dissociation. PDOCs generated after collecting the filtered fraction <70um are put in 1.5mg/ml rat tail collagen type I in microfluidic device (AIM Biotech) or in 3D collagen beads in 96well-plate with complete culture medium. PDOCs in chips are stained with a live/dead dye and with antibodies specific, for the immune cells compartment (CD45, CD4, CD8) and are analyzed by confocal microscopy; PDOCs in 3D beads are dissociated to single cells, stained with a panel of antibodies recognizing stroma and immune cells and analyzed by FACS

Results and Discussions

PDOCs are generated from fresh and frozen, treatment naïve or therapy-resistant patients, making them suitable for drug testing experiment. Resistant PDOCs are subjected to the same drug regimens as the corresponding patient and results, compared with the clinical response, validate the experimental system. PDOCs undergo also alternative target treatments, identified by a drug screen performed on our cohort of melanoma PDXs, in combination with already approved immune check point inhibitors. Immune cell composition and markers of viability are assessed upon treatments and compared with the standard of care therapy. PDOCs retain a comprehensive representation of the immune cell subset up to 6 days.

Conclusion

PDOCs maintain key features of the native tumor immune microenvironment. This ex vivo system allow to stratify collected melanoma samples according to genotype and drug sensitivity, and to model the tumor microenvironment, thus representing a feasible model to identify specific changes of different cell populations during microenvironment perturbation. Transcriptional and soluble factor analysis could help to understand regulation of cancer survival, proliferation, immune response and to find some markers with prognostic potential.

EACR22-0039

Tumor Microenvironment soluble factors confer platinum chemoresistance to ovarian cancer (OC) cells

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Introduction

Ovarian cancer (OC) ranks as the second most common type of gynecological malignancy, has poor survival rates, and is frequently (>75%) diagnosed at an advanced stage. Platinum-based chemotherapy, such as carboplatin, represents the standard of care for OC. However, toxicity and acquired resistance to therapy have proven challenging in the treatment of patients with OC. Approximately 85% of patients will experience relapse, mainly due to chemoresistance. Chemoresistance, a principal obstacle to durable response in OC patients, is attributed to the alteration within the cancer cells and can also be mediated by the tumor microenvironments (TME).

Material and Methods

Cleavage of PARP protein used as a marker of apoptosis induction in OC cells (A2780 or A2780CisR) by platinum compound (Platinum (IV) prodrug). In-vitro TME (condition media) was collected from MSC cells. OC Patient-derived TME consists of malignant ascites fluid. Nonmalignant TME consists of ascites fluids collected from cirrhosis patients.

Results and Discussions

Previously, we showed that direct co-culture (CC) of OC cells with mesenchymal stem cells (MSC) resulted in reduced drug sensitivity and platinum chemoresistance. To explore the effect of soluble factors originated from the TME, we utilized conditioned media (CM) collected from MSC and monitored the ability to affect drug sensitivity and chemoresistance. Our data showed that CM confers chemoresistance to OC cells. In addition, we explored the content of CM by monitoring levels of different growth factors and cytokines and correlated that to platinum chemoresistance. Interestingly, combination platinum with different modulators resulted in partial or complete restoration of platinum drug sensitivity to OC cells. In addition, malignant ovarian cancer ascites fluids promoted platinum chemoresistance in OC cell line, while ascites fluids collected from cirrhosis patients mostly did not induce chemoresistance.

Conclusion

Our results showed the significance of soluble factors found in TME in promoting platinum chemoresistance and the potential of combination therapy to restore chemosensitivity to OC cells.

EACR22-0071

The effect of different levels of branched-chain amino acids on paclitaxel sensitivity

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Introduction

Breast cancer is the most diagnosed cancer among women worldwide. Drug resistance is the major challenge in breast cancer management. Paclitaxel, a first-line chemotherapeutic drug for certain types of breast cancer, often develops resistance with time. Our group has identified the genes that contribute to paclitaxel resistance. One of these genes was BCKDK. Inhibition of BCKDK with siRNA or drugs increases the sensitivity of breast cancers cell lines to paclitaxel. The development of BCKDK inhibitors for clinical use may help to overcome the drug resistance and lead to improved patient survival rates

Material and Methods

EBSS was supplemented with BCAA to obtain a media equivalent to DMEM with a range of BCAA concentration. The effect of growing the cells in different media and measuring the level of IC50 of paclitaxel were tested.

Results and Discussions

The results showed the effectiveness of our method in the manipulation of intracellular BCAA and emerged the difference of breast cancer cell growth corresponds to the cell culture BCAA level. Also, the lower level of BCAA in the cells growing media is causing the cells to be more sensitive to paclitaxel

Conclusion

That BCKDK enzyme is a promising target to overcome the cancer cell resistance to paclitaxel

EACR22-0193

BET INHIBITORS: A NOVEL EPIGENETIC APPROACH TO TACKLE GLIOBLASTOMA TREATMENT RESISTANCE

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Introduction

Bromodomain and extra-terminal tail (BET) proteins are epigenetic regulators of gene transcription and have been identified as potential targets in glioblastoma (GBM), the most common and malignant primary brain tumor in adults. Differential gene expression profiling of GBM-derived spheres revealed significant downregulation of cancer-relevant genes upon BET inhibitor (BETi) treatment. Interestingly, the O-6-methylguanine-DNA methyltransferase gene (*MGMT*) was among them. *MGMT* is a DNA repair enzyme whose function is to remove alkyl-groups from the O6-position of guanine, thereby blunting the treatment effect of alkylating drugs such as temozolomide. Hence, BETi mediated downregulation of *MGMT* expression may sensitize GBM patients with an unmethylated *MGMT* promoter to temozolomide treatment, which is part of the current standard of care.

Material and Methods

We investigated the effect of the BETi tool-drug JQ1 in combination with temozolomide in GBM cells, and explored respective underlying mechanisms via multiple assays, including: (1) *MGMT* and Mismatch Repair (MMR) components RNA and protein expression analysis, (2) BET proteins occupancy at the *MGMT* promoter region via chromatin immunoprecipitation analysis, (3) DNA damage assessment via immunocytochemistry for γH2AX, (4) cell cycle stages analysis by flow cytometry, (5) cell viability testing via proliferation assays and (6) *MGMT* and MMR genes expression modulation with inducible Tet-On systems and shRNAs.

Results and Discussions

We observed that BET inhibition reduced BET proteins occupancy at the *MGMT* promoter in GBM cell lines, resulting in a direct downregulation of *MGMT* expression and prohibited *MGMT* induction typically observed upon temozolomide treatment. BET inhibition impaired repair of temozolomide-induced DNA damage, increased S and G2/M phase cell cycle arrest and decreased GBM viability upon temozolomide treatment. Additionally, BET protein inhibition did not compromise the MMR system in GBM, a key component for TMZ-mediated cell killing.

Conclusion

Altogether, we have demonstrated that temozolomide resistance typically observed in GBM patients with an unmethylated *MGMT* promoter can be counteracted with the use of BETi, which directly prohibits *MGMT* expression and induction. Therefore, novel small molecule inhibitors of BET proteins that cross the blood brain barrier may counteract *MGMT* mediated resistance to temozolomide treatment in GBM patients with an unmethylated *MGMT* promoter, potentially improving overall survival.

EACR22-0235

Decoding the molecular determinants that mediate autophagy in slow-cycling cancer cells.

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Introduction

Relapse is the leading cause of death in cancer, mainly driven by drug resistance. *Slow-cycliness* is recognised as one of the non-genetic mechanisms of resistance against cancer therapies, therefore targeting slow-cycling cancer cells (SCCCs) should be considered for future treatment strategies to tackle cancer relapse. Autophagy is a lysosome-based degradation pathway that protects cells from diverse stress conditions such as chemotherapy. In this context, autophagy has been presented as a possible mechanism enhancing SCCC survival. Therefore, strategies to block this phenomenon have been proposed to eradicate SCCC. However, despite the promise of autophagy modulators as pharmacotherapeutic agents, clinical results show that toxicity remains as a major limitation. In this regard, it is imperative to discover selective autophagy inhibitors that specifically target autophagic proteins that are defective only in a disease context to maximise therapeutic effects and minimise side effects.

Material and Methods

To identify, isolate and characterize SCCC, we used the ability of these cells to retain a doxycycline (DOX)-inducible H2BeGFP label in their genome (Addgene ID: 165494). A doxycycline-inducible overexpression system was used to generate stable SW1222-DPPA3 colorectal cancer (CRC) cell line. To obtain SCCC, we grown the different cellular models as 3D structures while immunofluorescence and western blot analysis were performed in cells grown in 2D. We examined cell survival and autophagy dynamics by western blot in cells after being exposed to 5-fluorouracil (5FU) during 24 hours and leading to recover at different time-points. We analysed SCCC survival in response to chloroquine and 5FU by flow cytometry.

Results and Discussions

DPPA3 is abnormally overexpressed in SCCC. Our results demonstrated that DPPA3 overexpression promotes cell survival after 5FU treatment. We also observed that DPPA3 regulates autophagic flux, enhancing its function as a protective cell survival mechanism against therapeutic stress. Finally, we detected a synergistic effect of the combination of a lysosomal inhibitor and 5FU in enhancing the death of SCCC.

Conclusion

We propose DPPA3 as a new autophagy-specific component involved in the particular context of drug resistance and SCCC, providing an innovative target autophagy-based therapeutic intervention for cancer relapse.

SDCBP modulates stemness and chemoresistance in head and neck squamous cell carcinoma through Src activation

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Introduction

Drug resistance is the principal limiting factor to achieving good survival rates in patients with cancer. The identification of potential biomarkers for diagnosis and prognostic prediction, as well as the design of new molecular-targeted treatments, will be essential to improving **head and neck squamous cell carcinoma** (HNSCC) patient outcomes. The aim of this study is to reach the sensitization of **resistant cells** and **cancer stem cells** (CSCs) by inhibiting crucial proteins involved in cancer resistance.

Material and Methods

To characterize the mechanisms that govern chemoresistance, we performed a comparative proteomic study analyzing HNSCC cells: CCL-138 (parental), CCL-138-R (cisplatin-resistant), and CSCs. **Syntenin-1** (SDCBP) was upregulated in CCL-138-R cells and CSCs over parental cells.

Results and Discussions

SDCBP depletion sensitized biopsy-derived and established HNSCC cell lines to cisplatin (CDDP) and reduced CSC markers, being Src activation the main SDCBP downstream target. In mice, SDCBP-depleted cells formed tumors with decreased mitosis, Ki-67 positivity, and metastasis over controls. Moreover, the fusocellular pattern of CCL-138-R cell-derived tumors reverted to a more epithelial morphology upon SDCBP silencing. Importantly, SDCBP expression was associated with Src activation, poor differentiated tumor grade, advanced tumor stage, and shorter survival rates in a series of 382 HNSCC patients.

Conclusion

Our results reveal that SDCBP might be a promising therapeutic target for effectively eliminating CSCs and CDDP resistance.

TSPAN1 inhibition: a new therapeutic alternative for the future of HNSCC?

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Introduction

Head and Neck Squamous Cell Carcinoma (HNSCC) is a particularly aggressive cancer type since approximately 60% of patients have the locally advanced disease upon diagnosis and approximately half of affected individuals do not survive more than five years after diagnosis. Sensitization of resistant cells and cancer stem cells (CSCs) represents a major challenge in cancer therapy.

Material and Methods

A comparative proteomic analysis of the following cell lines was performed: CCL-138, CCL-138-R and CCL-138-CSCs by using 3 biological replicates for each of them. In addition, we

studied the inhibition of TSPAN1 in primary culture biopsies from HNSCC patients. On the other hand, the study of TSPAN1 protein expression was performed in 106 biopsies from HNSCC patients by immunohistochemistry method, which was further verified by western-blot.

Results and Discussions

The study revealed TSPAN1 as a protein involved in resistance acquisition to cisplatin (TSPAN1 increased in CDDP resistant cells and biopsies from HNSCC patients TSPAN1 depletion in parental and CDDP resistant HNSCC cells reduced cell proliferation, induced apoptosis, decreased autophagy, sensitized to chemotherapeutic agents and inhibited several signaling cascades, where the phospho-SRC (p-Src) inhibition was the major common target. *In vivo*, TSPAN1 downregulation decreased size and proliferation rates of parental and CDDP resistant tumors and also reduced metastatic spreading. Notably, CDDP resistant tumors showed EMT features that disappeared upon TSPAN1 inhibition, suggesting a link of TSPAN1 with EMT and metastasis. Immunohistochemical analysis of HNSCC specimens revealed that TSPAN1 expression was correlated with p-SRC, and inversely with E cadherin, reinforcing the TSPAN1 association with EMT. We are currently conducting an *in silico* study to determine possible drugs capable of inhibiting TSPAN1 *in vitro* and *in vivo*.

Conclusion

Overall, TSPAN1 is a novel oncogenic protein and a promising target for HNSCC therapy.

EACR22-0353

CDH17 as a biomarker and target of chemoresistance to irinotecan

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Introduction

Cadherin 17 (CDH17) plays a key role in colorectal cancer (CRC) metastasis. CDH17 binds and activates $\alpha 2\beta 1$ integrin, through its RGD motif, promoting cell adhesion and proliferation. Blocking this interaction using RGD-specific monoclonal antibodies (mAbs) inhibited the metastatic capacity in mouse models. In a previous report, an overexpression of CDH17 in CRC cells treated with irinotecan was observed. Here, our goal was to investigate the participation of CDH17 in chemotherapy resistance mechanisms and to test the efficacy of RGD CDH17 mAbs in irinotecan-resistant cells.

Material and Methods

We generated irinotecan-resistant CRC cell lines (COLO205, KM12SM, SW620) by increasing the irinotecan dose from 0.05 to 0.5 mM. Cells were analyzed by Western Blot (WB), flow cytometry (FC) and qPCR assays to study expression of CDH17 and other proteins involved in chemoresistance (i.e. MDR1, ABCG2) or epithelial–mesenchymal transition mediators. In addition, cell cycle and the adhesive and proliferative properties of resistant cells were analyzed in the presence or absence of RGD-specific mAbs to test its efficacy. Then, CDH17 was silenced to determine its role in irinotecan chemoresistance. Finally, we performed a label-free proteomic quantification to survey global changes in proteins of wt cells compared to resistant cells

Results and Discussions

CRC cell lines resistant to 0.5 μ M irinotecan increased their epithelial phenotype with an overexpression of CDH17, ITGA2, CDX2, MDR1, ABCG2 and ALDH1A markers as determined by WB and FC. In agreement with these results, an increase in adhesion was observed in irinotecan-treated cells. Cell cycle in resistant cells showed preferential arrest in G2/M phase, as irinotecan inhibits the action of topoisomerase I. Proteins involved in chemoresistance, tumorigenesis or modulation of tumor response were found dysregulated after proteomic analysis of resistant cells. Interestingly, resistant cells were more sensitive to the inhibitory effects of RGD-specific mAb. However, CDH17-silencing experiments in resistant and parental cells indicated that CDH17 was not involved in the mechanisms of resistance to irinotecan

Conclusion

CDH17 has shown to be a specific marker of irinotecan-resistance and, therefore, a good target for CDH17 RGD-specific mAb. Therefore, patients that develop resistance to irinotecan could benefit from this therapy

EACR22-0399

Adaptive mutability induces an increase of mutation rate of cancer persister cells and pinpoints new therapeutic vulnerabilities.

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Introduction

Persisters represent a major limit to the long-term efficacy of targeted therapies. Persister cells are able to survive lethal doses of therapeutic agents and represent a reservoir from which heterogeneous mechanisms of drug resistance arise. We recently demonstrated that adaptive mutability stress response boosts, through an error-prone DNA replication process, the acquisition of drug resistance to targeted therapy in surviving persisters. Although persister cells have been described across multiple cancer types in response to different therapies, the phenotype and population dynamics of cancer persister cells remain unclear.

Material and Methods

Multiple integrated approaches, including EdU labeling, CFSE staining and time-lapse live microscopy, were employed to characterize population dynamics in cancer persisters during prolonged treatment with targeted therapies. By integrating biological characterization with mathematical modeling, we developed a modified two-steps fluctuation assay to quantify the effect of targeted therapies on the mutation rate of surviving cancer cells. We then performed a Time To Progression (TTP) assay to monitor the efficacy of the inhibition of translesion synthesis error-prone DNA polymerase in preventing adaptive mutability and therefore the acquisition of resistance to targeted therapies.

Results and Discussions

We found that, in colorectal cancer, a fraction of persisters slowly replicate. Data from the imaging assay unveiled that in some instances, the cell division was successful and vital,

while in others the cells died concomitantly. In parallel, we detected cell death, either after a cell division event, either in non-dividing cells.

By developing a novel methodology for fluctuation assay in cancer cells, we quantitatively infer that clinically approved anticancer therapies induce a temporary increase of several folds in the mutation rate of colorectal cancer persister cells.

Importantly, we found that inhibition of mutagenic translesion synthesis significantly delays, or even prevents, the acquisition of secondary resistance to EGFR/BRAF inhibitors in colorectal cancer, thereby suggesting novel strategies to restrict the emergence of drug resistance.

Conclusion

Our strategy might be broadly used to systematically measure mutation rates in cancer cells exposed to a wide range of environmental stressors and to define new drug combination designed to restrict the emergence of therapeutic resistance, a major limitation to the efficacy of targeted therapies in many cancer types.

EACR22-0769

A novel in vitro model mimicking the dynamic of prostate cancer under therapy opens the window to breakthrough anticancer nanozymes

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Introduction

Chemotherapy-treated castration-resistant prostate cancer (CRPC) after initial remission often undergoes cancer repopulation and acquired cell-resistance (CRAC), leading to disease progression and fatal outcomes, highlighting the urgent need to develop strategies to improve therapy efficacy. We propose here that nanomedicine, familiar to oncologists for its drug delivery and bio-imaging potentials, may help stabilizing therapy-induced cancer remission in a new way, by exploiting the intrinsic bioactivity of cerium oxide nanoparticles (CNPs), promising anticancer nanozymes [Corsi *et al.* 2018, Front Oncol].

Material and Methods

To dissect the biological events leading to CRAC, we setup a novel purely epithelial *in vitro* model in CRPC cells, performing long-term (>20 days) observation of PC3 cells upon a 24h-pulse of etoposide. We could thus follow not only the initial phase of cell loss (cancer remission), but also the ensuing repopulation phase (cancer relapse), establishing a reliable system in which to study the anti-CRAC potential of therapeutics.

Results and Discussions

After a robust apoptotic phase induced by etoposide, surviving cells set up a pro-inflammatory milieu and coordinate a “tissue-like” response to cope with the damage and support the repopulation of a tiny fraction of aggressive cells. Molecularly, PC3 repopulation was shown to a) depend on epigenetic reprogramming, b) require strong cyclooxygenase-2(COX2) and 5-lipoxygenase (5LOX) activation, and c) imply epithelial-to-mesenchymal transition (EMT) and resistance to further etoposide insults. The results suggest that therapy-induced CRAC is theoretically druggable, providing possible molecular targets [Corsi *et al.* 2022, IJMS]. In this context, we administered CNPs to etoposide-treated cells.

CNPs counteracted cancer repopulation, prevented EMT and abrogated chemoresistance by switching COX2 and 5LOX activities from inflammatory (prostaglandins and leukotrienes) to resolving (lipoxins), thereby preventing the signalling necessary to CRAC development. Notably, CNPs exert the anti-CRAC activity selectively on cancer cells, whereas protect non-cancer cells from etoposide toxicity, due to differential cell permeability.

Conclusion

We established a reliable system mimicking the dynamic biology of CRPC undergoing chemotherapy, and demonstrated the potential clinical application of CNPs as therapy adjuvants to keep all the best from chemotherapy (cancer shrinkage) avoiding its drawbacks (cancer relapse and resistance).

EACR22-0821

Caffeine and synephrine affect the activity of the multidrug resistance protein, P-glycoprotein, in breast cancer cells.

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Introduction

Complementary and alternative medicines (CAMs) are commonly used by cancer patients to improve treatment outcomes, or alleviate chemotherapy induced adverse effects. There is a concern about the potential of CAMs to contribute to the development of multidrug resistance, a major contributor to treatment failure in cancer. Chronic fatigue and a decline in cognitive ability are common consequences of chemotherapy. They have a detrimental effect on the quality of life of patients and hinder their ability to function. This pilot study evaluated the effects of two substances, caffeine and synephrine, found in high concentrations in energy booster drinks, on the efficacy of doxorubicin, using the MCF-7 breast cancer cell line as a model.

Material and Methods

The effects of caffeine and synephrine on cell viability, and the combined effect of doxorubicin with caffeine or synephrine were evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The activity of the multidrug resistance protein, P-glycoprotein (PgP), was measured using the calcein-AM fluorometric assay. The effects of doxorubicin, caffeine, synephrine and combinations thereof, on the expression of PgP protein, was determined by immunofluorescence microscopy.

Results and Discussions

Doxorubicin had a low IC₅₀ value of $0.98 \pm 0.09 \mu\text{M}$, whilst caffeine and synephrine were poorly active against MCF-7 cells with IC₅₀ values of $167.4 \pm 1.9 \mu\text{M}$ and $149.3 \pm 3.2 \mu\text{M}$, respectively. Combination studies indicate that both caffeine and synephrine caused small but significant decreases in the IC₅₀ value of doxorubicin ($0.82 \pm 0.05 \mu\text{M}$ and $0.47 \pm 0.02 \mu\text{M}$ respectively) indicating improved efficacy of doxorubicin. Doxorubicin caused a dose dependent increase in PgP activity which was efficiently inhibited by caffeine at all concentrations tested. Synephrine showed a similar effect but was less effective than caffeine at decreasing PgP activity. Doxorubicin strongly induced the expression of PgP protein in the nucleus, whilst caffeine and synephrine showed no meaningful increase in the expression of PgP. At the doses tested, combination treatments did not significantly decrease PgP protein expression.

Conclusion

This data indicates that the effects of commonly consumed substances, like caffeine and synephrine, on patient treatment outcomes cannot be overlooked and should be further investigated. Patients should be educated on the potential risk of commercially available substances that may affect their chemotherapeutic outcome and wellbeing.

EACR22-0861

Mechanisms of acquired resistance to trastuzumab, pertuzumab and palbociclib in HER2+ breast cancer

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Introduction

The dual HER2 blockade (trastuzumab and pertuzumab) and CDK4/6 inhibition (palbociclib) combination (palboTP) has promising molecular (Ki67 drop) and clinical activity in the NA-PHER2 trial in HER2+ breast cancer (BC) patients. Little is known about acquired resistance mechanisms to this triplet. We aim to identify mechanisms of resistance to palboTP in HER2+ BC and to explore treatments that could prevent or delay resistance.

Material and Methods

We have generated palboTP acquired resistant HER2+ SK-BR-3 BC cells. mTOR inhibitors (everolimus, TAK-228) and BCL-2 inhibitor (ABT-199) activities were tested on resistant cells by assaying cell growth (MTS assay, crystal violet staining), cell cycle and apoptosis (flow cytometry), and signaling (western blot). Senescence was analyzed by senescence-associated β -galactosidase (SA- β -gal) staining and senescence-associated secretory phenotype (SASP) by qPCR. Differential gene expression was explored from resistant and parental cells (Affymetrix microarrays).

Results and Discussions

PalboTP increased G1 arrest and reduced retinoblastoma (RB) and AKT phosphorylation compared to each drug alone. At 7 days of exposure, palboTP induced in a subset of parental cells a senescence-like phenotype with increased SA- β -gal activity and the secretion of the cytokines IL-6 and IL-8, both linked to senescence. After 5 months of palboTP continuous exposure, cells became resistant. *GSEA revealed* that palboTP downregulated the mTOR pathway in parental cells but not in resistant cells, and demonstrated SASP-related gene signatures in resistant cells. Two markers suggested mechanisms of resistance concomitant with this phenotype: S6RP phosphorylation (a surrogate of mTOR pathway activation) and BCL-2 protein overexpression (anti-apoptotic). TAK-228 and everolimus were active in SK-BR-3 cells resistant to palboTP. ABT-199, known as senolytic drugs that selectively kill senescent cells, reduced RB phosphorylation, and also induced G1 arrest and apoptosis in resistant cells.

Conclusion

Our data suggest potential preclinical molecular mechanisms underlying the acquisition of resistance to trastuzumab, pertuzumab and palbociclib in HER2+ BC. This warrants further testing of mTOR and BCL-2 activation in breast cancer specimens from patients progressing to this triplet.

Newly established ovarian cancer model of acquired carboplatin resistance reveals new targets to fight drug resistance and metastasis

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Introduction

Despite the notable progress in the development of new technologies and methods for early tumor diagnostic, ovarian cancer is still the deadliest gynecological disease worldwide. Lack of early-stage symptoms and specific biomarkers results in late-stage diagnosis when cancer already spread. Even a small number of the initially responsive ovarian cancer patients usually develop drug resistance upon therapy. Here, we present an *ab ovo* ovarian cancer model to study acquired resistance to carboplatin (CBP) and CBP-induced epithelial-mesenchymal transition (EMT).

Material and Methods

Parental MES-OV cells were repeatedly treated with increasing doses of CBP. Resistant variants, once stabile, were characterized in terms of carboplatin and paclitaxel (TAX) response by resazurin survival assay, migratory potential by scratch assay, and invasive potential by the transwell assay. Expressions of EMT markers were checked by RT-qPCR. Transcriptome analysis was performed on all established variants. The data were analyzed by two different approaches, the first one based on the differentially expressed genes (DEG), and the other one focusing on the genes that correlate with sensitivity to CBP (CBP-correlating genes, CCORG). Functional and clinical roles of selected candidates were examined by siRNA transfections *in vitro* and using bioinformatics tools *in silico*, respectively. The roles of enriched pathways were checked by survival assays on cells pre-treated with a set of specific inhibitors.

Results and Discussions

Gradual increase in CBP and TAX resistance, as well as an increase in migration and invasion rates, were reported in CBP resistant cell lines. Two approaches in data analysis resulted in groups of completely different genes highlighting different signaling pathways. Functional analysis revealed that *HES7* probably plays a role in the development of CBP resistance, while *SERPINE2* probably plays a role in metastases. *TMEM200A*, *PRKAR1B*, and *FBLN5* seem to be involved in both phenomena, while *TMEM200A* could also serve as a biomarker of resistance. The significance of enriched signaling pathways is yet to be determined.

Conclusion

The characterized cellular model, along with two distinct approaches to transcriptome analysis, presents a novel platform in research of drug resistance and drug-induced EMT. They enabled the exploration of novel candidate genes, possible therapeutic targets, and biomarkers, and support the narrative of two phenomena being interconnected.

Anti-EGF antibodies potentiate the effects of BRAF inhibitors and delay the emergence of resistance in colorectal cell line models

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Introduction

Progression-free survival of *BRAF*-mut CRC patients to BRAF inhibitors is short, due to feedback reactivation of EGFR pathway, and the combination of encorafenib with the anti-EGFR antibody cetuximab has been recently approved. Vaccination against EGF represents an alternative to the administration of monoclonal antibodies against *EGFR*.

Material and Methods

The effects of anti-EGF antibodies generated by vaccination (anti-EGF VacAbs) in combination with encorafenib and binimetinib were investigated by MTT and Western blotting and compared with cetuximab. Emergence of resistance was determined by microscopic examination in low density cultures, gene expression by the nCounter® and genetic alterations by Next Generation Sequencing.

Results and Discussions

Anti-EGF VacAbs suppressed EGF-induced proliferation more efficiently than cetuximab and significantly enhanced the antitumor activity of encorafenib and binimetinib in the *BRAF*-mut CRC cell line HT29. Also, although anti-EGF VacAbs and cetuximab showed similar inhibitory effects on EGFR, Akt, Erk and PRAS40 phosphorylation; anti-EGF VacAbs were more effective than cetuximab in delaying the emergence of resistance to encorafenib. Ten resistant colonies per treatment (encorafenib, encorafenib+cetuximab, encorafenib+anti-EGF VacAbs) were analyzed by NGS, no acquired mutations or copy number variations were detected in any case. In contrast, Western blotting revealed significant changes in the activation levels of key mediators of signal transduction pathways. In the case of encorafenib single agent, reactivation of the EGFR pathway was prevalent. In contrast, by-pass mechanisms were frequent in colonies resistant to the combination of encorafenib and cetuximab. Finally, mixed mechanisms of resistance were observed in colonies resistant to encorafenib combined with anti-EGF VacAbs. nCounter® analysis of the resistant clones is ongoing, final results will be presented at the meeting.

Conclusion

Similarly to cetuximab, anti-EGF VacAbs enhance the antitumor effects of BRAF inhibitors in *BRAF*-mut CRC cells growing *in vitro* and effectively block activation of the EGFR pathway. In addition, anti-EGF VacAbs are more efficient than cetuximab in preventing emergence of resistance to encorafenib. Clinical trials combining anti-EGF VacAbs with targeted inhibitors in *BRAF* mutant CRC patients are warranted.

EACR22-0639

Acquired resistance to anti-PD-1 plus anti-LAG-3 via co-existing alterations of MHC class I antigen presentation and interferon-gamma signaling

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Introduction

Anti-PD1 resistance represents a major unmet medical need. Currently very little is known about the durability of immunotherapy responses in the setting of anti-PD-(L)1 resistance, but the dissection of mechanisms behind acquired resistance in post-PD1 immunotherapies may extend clinical responses by uncovering strategies to stop the immune escape.

Material and Methods

We analyzed the genomic, immunologic and functional features of post-PD1 acquired immune resistance by studying longitudinal biopsies of an anti-PD1 resistant melanoma case with response and progression to anti-PD-1 plus anti-lymphocyte-activation gene 3 (LAG-3) antibodies. From each biopsy, tumor cell lines (TCLs, TCL1 – baseline; TCL2 – response; TCL3 – progression) were established and tumor-infiltrating lymphocytes (TILs) were isolated to conduct functional assays. Genomic and immunohistochemistry (IHC) analyses were performed on the biopsies to validate the *in vitro* findings.

Results and Discussions

Functional *in vitro* assays showed that CD8+ (TILs) recognized and killed TCL1 and TCL2, but not TCL3. Further analysis showed that TCL3 had lost surface expression of Major Histocompatibility Complex class I (MHC-I), due to a deleterious mutation in the beta-2-microglobulin gene (B2M) coupled with loss of heterozygosity. Lentiviral transduction of B2M in the TCL3 reinstated CD8+ T cell recognition and CD8+ T-cell mediated killing, showing that B2M-negative cells had been positively selected by CD8+ T cell-mediated immunoediting.

B2M loss was shared by all cells in the TCL3, but a co-existing Janus Kinase 1 (JAK1) signaling defect was identified in a subclone. JAK1 signaling loss determined resistance to Interferon-gamma (IFN γ), associated with decreased CD4+ T cell recognition and abrogated IFN γ -mediated cytotoxicity. Lentiviral transduction of JAK1 in TCL3 restored IFN γ sensitivity and CD4+ T cell-mediated killing, confirming JAK1 signaling loss as the final step of the immune escape, required to completely suppress T cell-mediated antitumor immunity.

Genomic and IHC data directly generated from biopsies confirmed the presence of deleterious mutations on B2M and JAK1 genes as well as loss of MHC-II and PD-L1 expression, hallmarks of the IFN γ resistance.

Conclusion

These findings shed light on the dynamics of acquired resistance following resistance to multiple immunotherapies. Simultaneously targeting diverse co-existing alterations may be necessary to extend the duration of response in the post-PD1 setting and restore CD8 and CD4 T cell recognition.

EACR22-0483

Validation of a DNA methylation microarray for 285,000 CpG sites in the mouse genome.

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Introduction

The mouse has been extensively used as the model organism in many studies to characterize biological pathways, drug effects and to mimic human diseases. The similar DNA sequences between both species facilitates these type of experiments. However, much less is known about the mouse epigenome, particularly for DNA methylation. Progress in delivering mouse DNA methylomes has been slow due to the currently available time-consuming and expensive methodologies.

Material and Methods

Following the great acceptance of the human DNA methylation microarrays, we have herein validated a newly developed DNA methylation microarray (Infinium Mouse Methylation BeadChip®) that interrogates 280,754 unique CpG sites within the mouse genome. The CpGs included in the platform cover CpG islands, shores, shelves and “Open Sea” sequences; and loci surrounding transcription start sites and gene bodies. From the functional standpoint, mouse ENCODE representative DNase hypersensitivity sites (rDHSs) and candidate cis-Regulatory Elements (cCREs) are also included.

Results and Discussions

Herein, we show that the profiled mouse DNA methylation microarray provides reliable values among technical replicates; matched results from fresh frozen versus formalin-fixed samples; detects hemimethylated X-chromosome and imprinted CpG sites; and is able to determine CpG methylation changes in mouse cell lines treated with a DNA demethylating agent or upon genetic disruption of a DNA methyltransferase. Most important, using unsupervised hierarchical clustering and t-SNE approaches, the platform is able to classify all types of normal mouse tissues and organs.

Conclusion

These data underscore the great features of the assessed microarray to obtain comprehensive DNA methylation profiles of the mouse genome and murine tumors.

EACR22-0713

DNA METHYLATION PROFILING OF HIGH RISK NEUROBLASTOMA: POTENTIAL EPIGENETIC BIOMARKERS FOR MOLECULAR RISK STRATIFICATION

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Introduction

Neuroblastoma (NB), the most frequent extracranial pediatric solid tumor, accounts for 15% of cancer-related deaths in children. High-risk NB (HR-NB) is a heterogeneous group, where

patients can response to treatment and have long-term outcome or develop early progressive, chemoresistant disease with poor outcome. To date, HR-NB patients are treated with no stratification. A molecular risk stratification has been proposed based on telomere maintenance mechanisms, and RAS or TP53 pathway mutations. However, risk-stratification of HR-NB tumors is still challenging.

We explored the DNA methylation (DNAm) profile of HR-NB at diagnosis to identify risk-stratification biomarkers and identify epigenetic alterations related with prognosis and response to treatment. Finally, identify potential therapeutic targets. **Material and Methods**

We analyzed DNAm and gene expression (GE) datasets of HR-NB at diagnosis. Discovery of potential biomarkers was performed using Cox-regression models and Machine Learning analysis. Survival curves were estimated by Kaplan-Meier method and compared among groups by log-rank test. Pathway analysis was performed using KEGG Pathway Database. Validation of the most significant results was performed by pyrosequencing, RT-qPCR and immunohistochemistry.

Results and Discussions

The analysis of DNAm of HR-NB revealed two distinct methylation patterns which defined two subgroups with diverse clinical evolution. By using Cox-regression models and Machine Learning analysis, we identified a reduced set of differentially methylated CpG-sites that enabled us to discriminate a rapidly progressing, chemorefractory tumors subgroup (UHR-NB). Our results were consistent with the molecular risk stratification based on telomere maintenance mechanisms.

Correlation of the identified methylation patterns with GE using matching patient data, revealed differences in cellular metabolism, purine biosynthesis and AKT/mTOR cell signaling. Our findings suggested that the heterogeneous behavior of HR-NB is mediated by the metabolism of purines and the activation of oncogenic pathways such as AKT/mTOR, contributing to tumor survival and progression profiles. **Conclusion**

Our findings show that HR-NB behavior is associated with DNAm changes associated with clinical evolution. We have identified a set of CpG-sites that enabled stratification of HR-NB tumors at diagnosis. We also provide further understanding of the biology underlying the behavior of HR-NB and reveal altered pathways for potential therapeutic options.

EACR22-0745

Direct quantification of CpG methylation of natural, non-converted DNA using novel qPCR analysis

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Introduction

DNA methylations and demethylations of cytosines in promoter regions are used to regulate gene expression and often play a role in cancer development. Detection and quantification of DNA methylations have until now relied on methods that require a modification of the DNA prior to analysis. The most applied method is a chemical conversion using bisulfite, which turns non-methylated cytosines into uracil while not converting methylated cytosines. This method is time-consuming, labor demanding, and requires high quantity of DNA to be successful. In this paper, we present a novel method for direct quantification of DNA methylation using Intercalating Nucleic Acid (INA[®]) modified oligoes applied in qPCR without pretreatment of the DNA.

Material and Methods

Implementation of INA[®] molecules in fluorescent oligos were tested for affinity towards methylated and unmethylated targets in melt studies. The INA[®] molecules were then used in a special primer design, which we call a BasePrimer[™]. This primer selectively amplifies methylated DNA at a given temperature in a qPCR. The first BasePrimer[™] was developed to target the MGMT promoter, and afterwards a new BasePrimer[™] was designed and applied to the C-region of the MLH1 promoter. The methylation percentages of the samples were determined using DcT between the reference assays and the assays comprising the BasePrimer[™]. The linear dynamic range of the assays were evaluated using a dilution row of different mixtures ranging from 100% methylated DNA to 100% wild-type genomic DNA. A clinical validation of the two assays is planned in the spring of 2022.

Results and Discussions

With an INA[®] probe covering four CpGs of the MGMT promoter, the difference in melting temperature of methylated and unmethylated DNA was 11 °C. Using the BasePrimer[™] for qPCR, both the MGMT and MLH1 assay showed a linear dynamic range from 100% to 2.5% methylated DNA. The assays will be validated on clinical samples to investigate whether they can be powerful tools to replace the currently applied methods.

Conclusion

INA[®] modified special primers can directly and selectively amplify methylated DNA without any pre-treatment of natural DNA. The novel method can be carried out in less than 1.5 hours and has shown to be adaptable to different biomarkers. Our novel method has the potential to give a real quantitative measure on the methylation level of natural, non-converted DNA and ease the implementation of methylation analysis in clinical routine.

Epigenetics

EACR22-1113

Target enrichment using a complete human methylome specific panel allows for a cost effective and highly sensitive CpG methylation detection system

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Introduction

Whole genome bisulfite sequencing (WGBS) and microarray studies have linked aberrant methylation patterns to a variety of cancers. However, WGBS is costly and includes coverage of uninformative regions, and microarrays only detect a small fraction of all CpG sites. To minimize cost and enable thorough methylome-level measurements at scale, Twist Bioscience has designed a solution to enrich the most current, annotated, and relevant CpG sites within the human genome. The Twist Human Methylome Panel, in combination with the Twist Methylation Detection System, is an optimized end-to-end workflow designed to target more than 3.98 million biologically relevant CpG sites, with an additional 2.61 million observed when shadow coverage is included. Here, we present these products as an end-to-end solution.

Material and Methods

The Twist Human Methylome Panel spans 85Mb (123Mb total probe space) of human enhancers, promoters, transcription factor binding sites, and CpG islands, curated from the most recent database releases. Twist's panel design approach uses predictive modeling to reduce issues that arise due to a lack of sequence complexity related to the conversion process in methyl-seq applications. Using pre-capture enzymatic conversion technology demonstrated by Twist's system, DNA damage is minimized and library mass is increased. Further, the Twist Methylation Detection System is designed to enable tuning of protocol conditions to ensure flexibility in different contexts.

Results and Discussions

At 150x raw coverage, the Twist end-to-end solution achieved a Fold-80 Base Penalty of 1.54; Percent Target Bases at 30X of 90%; and Percent Off-Bait of 5%. 6.59 million CpG sites were detected using a minimum depth of 10X, representing 23% of the available CpG sites found in the human genome. This compares favorably to leading methylation microarray-based methods, which only measures ~850k CpG sites. Finally, the BWA-meth/MethylDackel workflow was found to be the most robust method to align reads, handle the presence of SNPs, and produce high quality CpG calls.

Conclusion

Our study demonstrates an efficient end-to-end method to interrogate genome-wide methylation patterns for various applications. Not only does using this target enrichment system decrease the cost of methylation detection compared to WGBS, but it also provides higher sensitivity for differential methylation detection compared to microarray technology.

EACR22-1252

POSTER IN THE SPOTLIGHT

Expanded information and enhanced accuracy of cell free DNA sequencing - afforded by expanding the information content of next-generation-sequencing from 4 states to 16

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Introduction

Cell free DNA present in blood samples contains information about presence and subtype of tumours. It can be accessed in a relatively non-invasive manner and aid early disease detection, disease monitoring and guide treatment. Hitherto researchers have assessed either genetic or epigenetic information from the limited quantity of cell free DNA available from a standard blood draw. However, it has become evident that combining both genetic and epigenetic information is necessary to fully elucidate tumour subtype and tissue of origin. Moreover, accuracy of measurement is of prime importance, as tumour derived DNA is present in a vanishingly small proportion of already limited available volumes of total cell free DNA. Current DNA sequencing technologies are not able to measure genetic and epigenetic letters simultaneously and at the required accuracy.

Material and Methods

We describe a technology that simultaneously and digitally sequences genetic and epigenetic letters. We demonstrate the technology's capabilities by applying to DNA standards and human genomic DNAs with known genetic and epigenetic states, as well as cell free DNA samples donated by individuals diagnosed with cancer. We perform these analyses using an Illumina Novaseq with no customisation.

Results and Discussions

The described technology is more sensitive and specific to detect epigenetically modified cytosine than state-of-the-art epigenetic sequencing methods. Simultaneously, It is more accurate in sequencing complete genetic information than state-of-the-art genetic sequencing methods. The technology is compatible with the cell free DNA volumes typically available from a standard blood draw.

Conclusion

The expanded information and enhanced accuracy afforded by the described technology enables a decreased limit of detection for the tumour DNA component of blood, which translates to higher sensitivity for detection of new or recurrent cancer. The information is phased and interaction of genetics and epigenetic marks can be observed within single reads.

The technology comprises a pre-sequencing molecular biology kit and post-sequencing software. It is compatible with existing sequencers and compute set-ups. The first product from this platform, 5-Letter seq, is available to early-access customers.

EACR22-0066

The cancer associated 8q24 region: evidence of alterations of specific miRNAs in biofluids from colorectal cancer and bladder patients

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Introduction

Despite the locus 8q24 is a genomic region enriched in cancer-associated polymorphisms, it has been described as a “gene desert” due to sparse presence of protein-coding genes. Nevertheless, this locus hosts the *MYC* oncogene and genetic elements connected to tumorigenesis, such as pseudogenes (*POUF51B*) and long non-coding RNAs (*PCAT1*, *CCAT2*, and *PVT1*). This region hosts also many genes coding for small noncoding RNAs including 30 microRNAs (miRNAs) genes, scarcely investigated so far. Research on such miRNAs may provide new insights to characterize the multiple-cancer associated variants annotated in this genomic region.

Material and Methods

We explored the miRNA expression profiles in the 8q24 region in 120 patients diagnosed with colorectal cancer (CRC) and 80 healthy controls. miRNA profiling was initially performed on cancer and normal adjacent tissues from CRC patients and then on tissue surrogates (stool and plasma extracellular vesicles (EVs)). Results were compared with CRC data from The Cancer Genome Atlas (TCGA) and the function of target genes of differentially expressed miRNAs was further analyzed. To verify if the 8q24 miRNAs altered in CRC are also dysregulated in another cancer type and other biofluids, we evaluated their expression levels in urine and plasma EVs from a cohort of Bladder Cancer (BC) cases and healthy controls and further validated the results in the BC cases of the TCGA dataset.

Results and Discussions

Overall, we identified 15 mature miRNAs displaying a significantly altered expression (FDR <0.05). Among them, miR-30d-5p and miR-151a-3p were dysregulated in CRC tissues but also in biofluids of CRC and BC patients when compared to healthy controls. The

dysregulation of miR-30d-5p was confirmed in the TCGA dataset for both tumor types. Functional analysis of dysregulated miRNA target genes revealed an enrichment in biological processes related to fatty acid metabolism and DNA damage response through the *TP53* network.

Conclusion

In addition to the oncogenic protein-coding genes, the region 8q24 hosts miRNAs that are dysregulated in at least two cancer types. The observed miRNA dysregulation could contribute in initiation and/or progression of cancer.

EACR22-0702

KDM6 demethylases of H3K27 residue regulate critical genetic programs induced by EWSR1-FLI1 in Ewing sarcoma

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Introduction

Ewing sarcoma (EwS) is an aggressive malignant tumor arising in bones and soft tissues that affects children, adolescents, and young adults. The fusion protein EWSR1-FLI1 is the genetic driver that acts as an aberrant transcription factor during early tumorigenesis. EWSR1-FLI1 binds multimeric GGAA microsatellites causing genome wide reprogramming through the formation of *de novo* enhancers with strong enrichment of the active H3K27ac and lack of the repressive H3K27me3 marks. However, these regions are typically decorated with high levels of H3K27me3 prior to EWSR1-FLI1-activation. Moreover, the Polycomb (PcG) protein EZH2, which catalyzes the methylation of this residue, is a direct target of EWSR1-FLI1 and is highly expressed in EwS. Here, we aim to define the enzymatic activities related to H3K27me3 dynamics in EwS.

Material and Methods

A cohort of EwS primary tumors from our institution Hospital Sant Joan de Deu was used to analyze the expression of KDM6 demethylases by immunohistochemistry. Mesenchymal stem cells from the healthy bone marrow of pediatric donors (hpMSC) infected with EWSR1-FLI1 were used to study global changes of H3K27me3 by chromatin immunoprecipitation sequencing techniques (ChIP-seq). The genomic distribution of KDM6 proteins was analyzed in EwS cell lines by ChIP-seq. We performed CRISPR/Cas9-mediated genome editing and used doxycycline-inducible shRNA systems to analyze the underlying transcriptional changes of these enzymes by RNA sequencing (RNA-seq) techniques.

Results and Discussions

Here, we investigated the dynamics of H3K27me3 in the early steps of EWSR1-FLI1-mediated transformation in hpMSC, a putative cell-of-origin of EwS. The genome-wide analysis of H3K27me3 reveals that this mark is redistributed upon introduction of the fusion oncoprotein, suggesting the enzymatic activities regulating this residue might be essential for EwS tumorigenesis. Moreover, we show that KDM6 demethylases of H3K27me3 residue are

highly expressed in EwS tumors and cell lines, and they contribute to the genetic reprogramming mediated by EWSR1-FLI1. We further demonstrate that these proteins are essential for cell proliferation in EwS. Considering EZH2 is necessary for EwS tumor growth, our data suggest that targeting the enzymes controlling H3K27me3 in both directions is critical in this tumor.

Conclusion

Our findings provide further insight into the epigenetic mechanisms underlying EWSR1-FLI1-mediated genetic reprogramming through KDM6 demethylases and reveal new targets for future clinical trials.

EACR22-0834

Exploring TERT methylation, underlying molecular mechanisms and clinic significance in colorectal cancer

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Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide with nearly 50% recurrence after conventional therapies. Most cancers recur by gaining limitless self-renewal achieved by telomerase activation in 90% of human tumors. In the last years we have shown that hypermethylation of a specific region of the Telomerase Reverse Transcriptase (*TERT*) promoter named **TERT Hypermethylated Oncological Region (**THOR**)** was associated with *TERT* expression and patient outcome in other cancers. Here, we aimed to uncover the potential of THOR as a CRC biomarker and explore the underlying mechanisms.

Material and Methods

Gene expression, DNA methylation and clinical data for CRC patients were retrieved from The Cancer Genome Atlas (TCGA) CRC Cohort (n=736). RNA and DNA were extracted from CRC cell lines or cells depleted or silenced for DNA methyltransferases (*DNMTs*). Gene expression was assessed by qPCR and methylation by pyrosequencing. Mann-Whitney test or one-way ANOVA, followed by Dunn's Multiple Comparison tests, were used for groups comparisons and Spearman correlation coefficient for correlation analysis. Area under the ROC curve (AUC) and Kaplan–Meier curves, with respective log-rank test, were used for clinical significance.

Results and Discussions

TERT expression and THOR methylation were increased in CRC malignant tissue ($p<0.0001$). THOR could distinguish early stages of cancer from normal tissue (AUC=0.8627; 81.27% sensitivity and 75.55% specificity, $p<0.0001$). When the entire *TERT* gene was examined, we identified 13 CpG sites differentially methylated between normal and tumor tissues, which were correlated with THOR. The combination of these CpGs with THOR could predict patient's outcome. In the best combination, lowly-methylated patients for THOR, cg14647640 and cg25353384 had a chance of survival around 90% whereas the highly methylated group rapidly decreased to near 20% survival ($p=0.0008$). To further explore the mechanisms behind *TERT* methylation in CRC, we investigated the DNMT responsible for this epigenetic event. In HCT116 cells, *TERT* expression was downregulated upon DNMT1 depletion, but not when DNMT3B or DNMT3A were silenced. Accordingly, THOR methylation status followed the same pattern.

Conclusion

THOR can be a potential biomarker for CRC early screen and, in combination with other methylation events of the *TERT* gene, THOR can predict CRC patients' outcome. Our preliminary data indicate that DNMT1 depletion impacts on *TERT* methylation and consequent activation in CRC cells.

EACR22-0467

Differentially expression of microRNAs in phyllods tumors of the breast

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Introduction

Fibroepithelial tumors of breast, including fibroadenoma and phyllodes tumor, are characterized by proliferation of both epithelial and stromal components. Fibroadenomas are always benign. However, PTs are classified into three grades, benign, borderline and malignant and are mostly treated with surgery. Accurate differentiation between fibroadenoma and PTs, as well as the grading of PT, may sometimes be challenging on diagnosis. Therefore, better understanding of molecular pathogenesis pathways and tumor biology of PTs constitute an essential way for identification of new biomarkers for early diagnosis and for better disease stratification and management. MicroRNAs have been shown to play important roles in tumour. Their expression can be useful for cancer classification and therapy. However, little is known about miRs in fibroepithelial tumors of the breast. Here, we aimed to evaluate by qPCR, expression of six mature miRNA (hsa-miR-21, hsa-miR-155, hsa-miR-182, hsa-miR-34a, hsa-miR-148a and hsa-miR-205) in fibroepithelial tumors of the breast, and to predict targets of these miRNAs through computational tools.

Material and Methods

A total of 64 patients (9 fibroadenoma and 55 phyllods tumours) aged between 17 and 74 years were included. The study was conducted in the Pathological laboratory of Farhat Hached University Hospital, Tunisia. Expression of these selected miRNA was assessed using qRT-PCR and the prediction of miRNA putative targets was realized using in-silico approaches.

Results and Discussions

Analysis of miRNA expression in fibroadenoma and phyllods tumours tissues revealed that miR-21, miR-155 and miR-182 were upregulated in PTs comparing to fibroadenoma and breast normal tissues. Malignant and borderline tissues of PTs showed highest level of upregulation values. Otherwise, we have reported that miR-34a, miR-148a and miR-205 were downregulated in both borderline and malignant PTs compared to fibroadenoma and breast normal tissue. Furthermore, the relative expression analysis in a recurrent tumour case revealed a specific miRNA signature. In-silico miRNA-targets prediction suggested, in the other hand, the involvement of these molecules in a wide context of cell signalling pathways.

Conclusion

Our study highlights the contribution of miRNAs as potential diagnostic and prognostic biomarkers in PTs of the breast. The association between miRNA expression and phyllods tumours, is continuously gaining more importance specially in the wider context of genetic therapy and innovative treatment drugs.

EACR22-0070

Histone Deacetylase 1 Has a Tumor-Suppressive Role in Early T Cell Lymphomagenesis

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Introduction

Histone deacetylases (HDACs) are epigenetic modifiers deregulated in various tumors, which regulate gene expression through chromatin compaction. HDAC inhibitors (HDACi) are approved for treatments of certain T-cell malignancies. However, a tumor-suppressive role of HDACs was suggested in T cells.

Material and Methods

To investigate the function of HDAC1 in T cell lymphomagenesis, a transgenic mouse model that expresses human fusion oncoprotein NPM-ALK and mimics the anaplastic large cell lymphoma was employed. To assess changes in gene expression and chromatin state after *Hdac1* deletion, RNA- and ATAC-seq of ALK+ and ALK+*Hdac1*KO tumors were performed in biological replicates. To identify functional enhancer regions that were correlated with differentially expressed genes and changes in chromatin state, we correlated RNA- and ATAC-seq data with topologically associated domains.

Results and Discussions

T cell specific deletion of *Hdac1* in our model surprisingly accelerated ALK driven lymphomagenesis from 19.6 weeks to 8.2 weeks of age, indicating that the loss of HDAC1 resulted in an early transformation of T-cells. With RNA-seq we identified more than 700 significantly up- and downregulated genes in KO tumors. Surprisingly, *Hdac1* deletion did not result in opening of the chromatin, as assessed by ATAC-seq. However, the distribution of open chromatin regions showed a pronounced shift from distal intergenic to promoter regions in ALK+*Hdac1*KO tumors. Newly opened promoter regions were involved in oncogenic signaling. All together 8539 correlations between gene expression and chromatin opening were found ($p < 0.05$). Clustering of the replicates within the correlations was employed (Dunn index > 0.3) to assess the differences between the two genotypes. Genes upregulated in the ALK+*Hdac1*KO tumors associated with changes in chromatin were mainly part of T-cell specific pathways.

Conclusion

Our data indicate that HDAC1 has tumor suppressive functions in early T cell development. Deletion of *Hdac1* does not result in stochastic chromatin opening, but in a shift of open chromatin regions from intergenic to promoter regions, which promote oncogenic signalling. Gene specific enhancers showing chromatin opening and association with deregulated expression point to T-cell specific pathways. This suggests that deletion of HDAC1 reinforces the existing cell type specific patterns of DNA accessibility and gene activities. Altogether, our data imply that in certain cases the use of HDACi might be contraindicated.

EACR22-0902

Role of the mSWI/SNF chromatin remodeling complex in preserving liver homeostasis

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Introduction

mSWI/SNF complexes are a class of ATP-dependent chromatin remodelers whose role is to slide, insert, or evict nucleosomes from the DNA, thus regulating gene expression. Although mSWI/SNF subunits are mutated in about 25% of human cancers, little is known about their impact. Hepatocellular carcinoma (HCC) is the third cause of cancer-related deaths worldwide and the sixth most frequent cancer type. There is a dire need for novel molecular targets since pharmacological treatments are lacking. Recent studies have identified specific mutations in HCC patients, including Arid1a, a major subunit of the canonical mSWI/SNF complex (cBAF). How cBAF maintains liver homeostasis and contributes to HCC development is still under debate. In this work, we present new insights about the consequences of cBAF loss in mice.

Material and Methods

We used genetically modified mouse models and Next Generation Sequencing (NGS) techniques to thoroughly characterize the role of the cBAF complex in maintaining liver homeostasis and how its loss contributes to HCC development.

Results and Discussions

In this work, we shed light on the crucial role of cBAF in maintaining liver homeostasis and preventing HCC development. We thoroughly characterized Arid1a^{-/-} mice liver tumors by Whole Exome Sequencing (WES) to discover possible co-occurring mutations. Furthermore, we investigated the epigenetic and transcriptional changes occurring upon Arid1a loss. Of interest, we describe a novel mechanism that confirms the tumor suppressor role of Arid1a in liver cancer formation.

Conclusion

We describe novel insights on the role of the chromatin remodeling complex cBAF and its subunit Arid1a in preserving liver homeostasis and preventing HCC development.

EACR22-1014

Characterisation of KRAS-driven epigenomic changes in pancreatic carcinogenesis

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Introduction

Activating mutations in KRAS are found in almost all cases of pancreatic cancer and they are associated with poor prognosis and therapy resistance. In addition to genetic mutations, epigenetic dysregulation, has been shown to play an important role in malignant transformation. However, the interplay between genetic and epigenetic alterations is poorly understood. Mutations in signaling proteins such as KRAS may have direct effects on the epigenome and hence may play an important role in cellular transformation. In this study, we systematically investigate the impact of KRAS signaling on the epigenomic landscape, i.e. on DNA methylation, histone modifications and chromatin architecture.

Material and Methods

The immortalized pancreatic cell line H6c7 and the KRAS wildtype (WT) pancreatic cancer cell line BxPC-3 were lentivirally transduced with expression vectors encoding KRAS-WT, KRAS-G12V (G12V) or the effector domain mutations G12V/T35S, G12V/E37G, or G12V/Y40C, leading to preferential activation of RAF-MEK-ERK, RALGDS, and PI3K-AKT downstream pathways, respectively. The resulting isogenic cell lines were analyzed by Illumina EPIC bead chip arrays, Assay for Transposase-Accessible Chromatin sequencing (ATAC-seq) and Antibody-guided Chromatin Tagmentation sequencing (ACT-seq). Transcriptomic changes were assessed by RNA-seq.

Results and Discussions

The isogenic H6c7 and BxPC-3 cell lines were successfully generated in biological triplicates and showed stable expression of the different KRAS variants at comparable levels. Unsupervised analysis of these cell lines identified genotype specific aberrant DNA methylation patterns that seem to be modulated by slight changes in the KRAS expression levels. Integration with RNA-seq data suggested that the observed epigenetic changes are reflected at the transcriptional level. Currently, we are awaiting the results of ATAC-seq and ACT-seq and we will further inspect in great detail the molecular changes induced in these cell lines by oncogenic KRAS.

Conclusion

Introducing oncogenic KRAS variants into pancreatic cell lines results in circumscribed epigenetic and transcriptional alterations. Further analysis and integration of additional epigenomic data layers may reveal epigenetic switches, which contribute to KRAS-mediated tumorigenesis. At the conference, we will present a gene level analysis and an update on the integration of additional data layers.

EACR22-0061

Epigenomics at the speed of sound: High throughput epigenomic mapping of low cell input samples using acoustic enhanced immunoprecipitation

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Introduction

Mapping the epigenetic landscape of Myelodysplastic Syndrome (MDS) and Acute Myeloid Leukemia (AML) is essential for understanding gene regulatory mechanisms during cell fate changes, disease onset, progression, as well as for optimizing therapeutic regime. In addition, this information holds immense potential for accurate identification of disease biomarkers, cell of origin, and targets for epigenetic therapy. However, existing chromatin immunoprecipitation sequencing (ChIP-Seq) workflows require significant cellular material

and are very time-consuming, requiring cumbersome optimization. Moreover, cellular heterogeneity can influence accuracy of results from the perspective of sample preparation efficiency and population-biased effects. Both normal and malignant bone marrow are heterogeneous in cell type and stage of differentiation that leads to inaccurate results regarding epigenetic determinates.

Material and Methods

In our study, we implemented Covaris' Adaptive Focused Acoustics® (AFA®) technology to miniaturize sample volumes for both chromatin shearing and immunoprecipitation in a 96-well format. This approach enables a standardized, robust, automatable, half-day workflow for both ChIP and Hi-ChIP applications. Additionally, we evaluated low input *in vitro* and *in vivo* samples, where we utilized a range of epitopes including major histone modifications (H3K27ac) and factors (CTCF). AFA was applied to flow sorted stem and progenitor populations from individual mice.

Results and Discussions

The low cell input capability permitted deconvolution of dynamic epigenetic changes in protein binding and chromatin structure during normal lineage specification of hematopoietic stem cells. Epigenetic remodeling of these sites was consistent with established gene expression programs essential for myeloid differentiation. Data were found to be in alignment with standard ChIPseq and Hi-C datasets generated from similar cell populations. The simplified and significantly shortened (Hi)ChIP-seq processes showcased in this study will be highly beneficial for both research and clinical diagnostic applications owing to their requirements for using 96-well plate format and their ability to become integrated into automated workflows.

Conclusion

Taken together, this technology and workflow represents a high throughput epigenetic system for small cell numbers of cells with a multitude of applications enabling laboratories focused on cell biology and translational research.

EACR22-0130

VRK1 role in chromatin remodeling and DNA damage caused by oxidative stress in tumor cells

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Introduction

Histone posttranslational modifications (PTMs) and nucleosome composition modulate chromatin dynamic remodeling, which is required for gene expression regulation and for the DNA damage response after oxidative stress in cells. Alteration of PTMs and chromatin structure leads to defective oxidative stress signaling and to an unbalance between oxidants and antioxidants, causing pathologies like cancer and neurodegenerative diseases. VRK1 (Vaccinia-related kinase 1) is a nucleosomal serine-threonine kinase involved in epigenetic chromatin changes and in the DNA damage response (DDR). VRK1 phosphorylates the acetyltransferase Tip60 in response to DNA damage, which carries out the acetylation of H4K16, a PTM necessary for chromatin remodeling prior to DNA repair. In addition, VRK1 phosphorylates γH2AX and 53BP1, both critical for a proper DDR in different pathways. Furthermore, VRK1 is overexpressed in many cancer types such as breast cancer, colon, lung, liver, and gliomas.

Material and Methods

We used H₂O₂ 200 µM at different times to induce oxidative stress in A549 cells. We studied VRK1 autophosphorylation and activation by performing radioactive kinase assays. For the measurement of reactive oxygen species (ROS) levels, we quantified the intensity of the marker 8-oxo-guanine using immunofluorescence. We used a commercial kit (TUNEL) to detect DNA ends in cells, corresponding with DNA damage levels. Different PTMs (H3K9ac, H4K16ac, H3K27ac, H3K9me3, and H3K4me3), were detected by immunofluorescence.

Results and Discussions

In this work, we have demonstrated that VRK1 is autophosphorylated and activated in response to oxidative stress. Furthermore, we observed that the depletion of VRK1 led to the accumulation of ROS assessed by the increase in 8-oxo-guanine levels, causing an increase in DNA damage in cells. VRK1 knock-down completely altered the epigenetic landscape of histone PTMs. We observed that the absence of the kinase led to a decrease in H3K9ac, H4K16ac, and H3K27ac and an increase in H3K9me3 and H3K4me3 after hydrogen peroxide exposure. These results suggest that the depletion of the kinase impairs chromatin remodeling after H₂O₂ treatment. Therefore, we propose VRK1 as an early modulator of the oxidative stress response.

Conclusion

VRK1 plays a key role in the oxidative stress response by regulating chromatin remodeling after hydrogen peroxide exposure.

EACR22-0460

Identification of an epigenetic mechanism that supervises HR-mediated DSB repair

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Introduction

Cellular senescence is a permanent state of cell cycle arrest associated with an epigenetic reprogramming and the accumulation of DNA damage, due to an impairment of double-strand breaks (DSBs) repair. Indeed, many epigenetic modifications observed in senescent cells correlate with DSBs onset and ongoing or failed repair. However, a lack of the temporal epigenetic resetting observed in correspondence to unrepaired lesions makes it difficult to decipher a direct role of epigenetic modifiers in genome surveillance and maintenance.

We recently demonstrated that a class IIa HDAC, HDAC4, selectively binds and controls the p300-mediated H3K27 acetylation at specific enhancers and super-enhancers involved in supervising the senescent transcriptome. This activity requires the binding of the C-terminus of HDAC4 to HDAC3.

Material and Methods

For this work, we studied the HR impairment in different cellular models, including the induction of the endonuclease I-PpoI, and we investigated the role of HDAC4 in this process. With different techniques, including *in-vitro* assays, we examined the epigenetics modifications and HDAC4 partners involved.

Results and Discussions

HDAC4 is degraded at the protein level during replicative and oncogene-induced senescence onset, in response to a GSK3β mediated phosphorylation. Here, we similarly observed that HDAC4 is proteasomal degraded in HR impaired cells, after the inhibition of MRN complex or the silencing of RAD51. HDAC4 degradation leads to an unbalance of the H2BK120 acetylation/ubiquitylation rates. This results in a deep impairment in HR-mediated

repair efficiency. Curiously, the N-terminus domain of HDAC4 is involved in this activity and controls the binding of an active HDAC1/2 complex on HR-sites.

Conclusion

The senescent-mediated degradation of HDAC4 might be responsible for the accumulation of DNA lesion and the integration of the epigenetic responses triggering the senescent genetic program. Cancer cells need the presence of HDAC4 and more in general of class IIa HDACs to escape senescence and to efficiently repair DSBs.

Our work identifies a new epigenetic mechanism that controls DSB repair and maintain cellular fitness and transcriptome plasticity.

EACR22-0876

Targeting epigenetic machinery in combination with immunotherapy for bladder cancer treatment

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Introduction

The search for new strategies to augment response to immune checkpoint inhibitors (ICI) in bladder cancer (BC) is of major importance, since this therapy has been proven to be unsuccessful in a significant number of patients. Alterations in epigenetic machinery in both cancer and immune cells contribute for cancer development, constituting a promising target to overcome ICI resistance. Thus, the main goal of this study is to explore the effects of CM-1758, a novel dual HDACs/DNMTs inhibitor, in BC cell lines and in an immunocompetent mouse model in combination with ICI.

Material and Methods

Five BC cell lines (253J, 5637, J82, RT112 and TCCSUP) and an immortalized urothelial cell line (SV-HUC1) were treated for 48h with CM-1758. Half maximum inhibitory concentration (IC₅₀), cell cycle using propidium iodine and apoptosis by annexin V were analyzed. PD-L1 expression levels were measured by flow cytometry. H3 acetylation (H3Ac) and acetylation of lysine 9 of histone 3 (H3K9ac) levels were analyzed by western blot. Three million cells (Adeno-Krt5-Cre) previously established from a mouse bladder tumor (*Pten* -/-; *Trp53* -/-) were injected subcutaneously in an immunocompetent mouse model (*Pten* F/F; *Trp53* F/F). Treatment with CM-1758 and an anti-PD-L1 antibody was applied for 2 weeks, and tumors were collected for histology and flow cytometry analysis.

Results and Discussions

CM-1758 showed cytotoxicity in all BC cell lines in the low micromolar range. The mechanism by which CM-1758 exerted its effects depends on cell line: 253J, RT112 and TCCSUP showed high levels of apoptosis after treatment, whereas J82 cells displayed a stoppage in G2/M phase in the cell cycle. Higher H3Ac, H3K9ac and PD-L1 levels were

found after treatment with CM-1758. Mice treated with CM-1758, alone or in combination with ICI blockade, showed a significant delay in tumor growth. A higher tumor infiltration of CD8+ T cells concomitantly with a decrease in CD4+ T cells and macrophages was observed. PD-L1 expression was lower in groups treated with anti-PD-L1 antibody, alone or in combination with CM-1758, in macrophages and dendritic cells. In contrast, treatment with CM-1758 alone increased PD-L1 levels in these cells.

Conclusion

CM-1758 displays cytotoxic effects both *in vitro* and in *in vivo* models of BC, having a direct effect on tumor immune infiltration. Several studies are required to uncover the mechanism behind epigenetic modulation of the immune system in bladder cancer.

EACR22-0127

Regulation of chromatin epigenetic modification by the nucleosomal kinase VRK1, implications for new cancer therapies

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Introduction

Epigenetic alterations are one of the main mechanisms driving different human diseases, including many cancer types and neurological diseases. Histone posttranslational modifications (PTMs) are changes in the amino acid residues of the histone tail which regulate gene expression. The coordination of PTMs requires the strictly regulation of multiple epigenetic enzymes, termed writer and eraser proteins. Chromatin active states are characterized by PTMs such as H3K4me3 and H3K27ac, whereas repressive states are enrichment in H3K9me3 and H3K27me3 among others. Thus, it could be crucial to understand the mechanisms that mediate the coordination of such enzymes. VRK1 (Vaccinia-related kinase 1) is a nuclear kinase implicated in different cellular process like transcription or DNA damage response. In this context, we studied the potential role of VRK1 in the regulation of those epigenetic modifiers.

Material and Methods

We compared the transient VRK1 silencing with the effect of modulators of various epigenetic enzymes. In our study, we evaluated the expression levels of different PTMs (H3K4me3, H3K9ac/me3, H3K27ac/me3, and H4K16ac), by immunodetection in two different human cancer cell lines, lung adenocarcinoma A549 and osteosarcoma U2OS. We studied the interaction of VRK1 with the histone-modifying enzymes of this PTMs by co-immunoprecipitation.

Results and Discussions

Our results showed that VRK1 depletion caused an alteration of post-translational histone marks in cells. We observed that VRK1 depletion promotes a decrease of chromatin active marks (H3K4me3, H3K9ac, H3K27ac, and H4K16ac), and an increase of H3K9me3 and H3K27me3. Treatments with drugs targeting histone-modifying enzymes also remodel the acetylation and methylation pattern. HAT (C646 and MG149) and KDM (JMJD2i and ORY-1001) inhibitors mimic the effect of VRK1 depletion. HDAC (Vorinostat, Entinostat, Panobinostat, Selisistat, Thiomyristoil, AGK2, and AK7) and KMT (Chaetocin and Tazemetostat) inhibitors mimic the effect of VRK1 on histone marks. VRK1 forms a stable protein complex with KDM1a, KDM3a, KDM4a, HDAC1, Sirt1/2, and SETDB1.

Conclusion

Our findings revealed that permissive chromatin marks decreased (H3K4me3, H3K9ac, H3K27ac, H4K16ac) while repressive marks (H3K9me3, H3K27me3) increased in VRK1-depleted cells. Therefore, the loss of VRK1 expression in the cells had the same effect than the inhibition of these enzymes, suggesting that VRK1 might be a regulator of those epigenetic enzymes and used as new target in synthetic lethality strategies.

EACR22-0213

CDK6 regulates the epigenome in AML

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Introduction

Acute myeloid leukemia (AML) is a highly aggressive disease of the hematopoietic system. Cyclin-dependent kinase 6 (CDK6) levels are recurrently increased in various human cancers and CDK6 has been shown to be a crucial regulator of AML. CDK4/6 kinase inhibitors have been widely studied in many cancer types and first evidence indicates beneficial effects for AML therapy. AML is frequently accompanied with aberrant DNA methylation and recent research identified CDK6 as a regulator of DNA methyltransferases (DNMTs) in acute lymphocytic leukemia and breast cancer. The involvement of CDK6 in regulating the epigenetic landscape including DNA methylation and histone modification in AML remain to be elucidated. Understanding the mechanisms initiated by CDK6 inhibition will help to reveal novel combinatorial treatment strategies for AML patients.

Material and Methods

The recently established hematopoietic progenitor cells (HPC^{LSK}) allowed us to generate genetically defined AML lines harboring either MLL-AF9 or AML1-ETO fusion proteins. To study CDK6-targeted therapy in AML we used pharmacological CDK4/6 kinase inhibitors and CDK6-specific degraders. We performed RNA-Sequencing to identify global transcriptional changes. A CDK6 Co-IP followed by Mass Spectrometry revealed CDK6 interactors linked to epigenetic regulation. We analyzed cell cycle, proliferation and viability using flow cytometry after combinatorial inhibition of CDK6 and BRD4.

Results and Discussions

Pathway analyses of RNA Sequencing revealed that kinase inhibition or protein degradation of CDK6 downregulates genes assigned to chromosomal organization. The histone deacetylase 5 (Hdac5) is significantly downregulated after CDK4/6 inhibition and Hdac7 is downregulated after CDK6 degradation. The lysine-specific histone demethylase 4A (Kdm4a) and Kdm5b are significantly upregulated after CDK6 inhibition or degradation. To verify epigenetic interaction partners, a CDK6 Co-IP followed by Mass Spectrometry revealed that CDK6 binds to components of the polycomb complexes, BRD4, Kdm3a/b, Hdac1/3, Paf1, and subunits of the RNA polymerases 1 and 2. Combinatorial inhibition of CDK6 and BRD4 synergistically inhibits cellular growth in MLL-AF9+, but not AML1-ETO+ AML.

Conclusion

These data reveal a CDK6 mediated regulation of a complex epigenetic interplay among histone modifiers in AML. Our data suggest novel oncogene-specific treatment opportunities targeting CDK6 and epigenetic regulators in AML.

Understanding The Role of mTOR in H3K27me3 Landscape in Cancer

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Introduction

Changes in gene expression, including those caused by epigenetic dysregulation, represent a hallmark of cancer. mTOR coordinates a variety of signals, including nutrient and oxygen availability, growth factors and hormones to stimulate cell growth and proliferation, whereby mTOR is frequently hyperactivated in neoplasia. The role of mTOR in epigenetic reprogramming in cancer remains largely unknown.

Material and Methods

Using mTOR inhibitors (mTORi; Ink128 and rapamycin) and physiological mTOR inhibition by serum-starvation, histone methylation was examined in two cancer cells harbouring activating mutations in PI3K (MCF7 and HCT116). Chromatin immunoprecipitation (ChIP) with H3K27me3, followed by sequencing (ChIP-seq), and transcriptomic and metabolomic profiling was performed.

Results and Discussions

mTOR inhibition significantly increased two repressive histone marks, H3K9me3 and H3K27me3. Importantly, these effects were not secondary to the inhibition of the cell cycle by mTORi. As the change in H3K27me3 was more pronounced than H3K9me3, we focused on H3K27me3. Using the ChIP-seq technique, we confirmed that mTOR inhibition results in a global increase in H3K27me3. We next targeted the PRC2 complex which methylates H3K27, by depleting its key enzyme EZH2. This revealed that mTOR inhibition increases the H3K27me3 levels independently of EZH2. Furthermore, we monitored the SAM/methionine ratio, which is indicative of methylation capacity, and found that mTOR inhibition decreases it. This suggests that mTOR inhibition does not increase de novo H3K27 methylation. To this end, we are in the process of determining the potential role of H3K27me3 demethylases (KDM6A/6B) in the observed increase of H3K27me3 upon mTOR inhibition.

Conclusion

Our result shows that mTOR inhibition induces H3K27me3, and this regulation is independent of EZH2. Investigating how mTOR alters the epigenome may help to improve understanding of the complex role of this signalling pathway in neoplasia.

DNA methyltransferases drive gastric cancer growth and present a therapy target for gastric cancer

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Introduction

Gastric cancer (GC) remains the third leading cause of cancer-related death worldwide. While inflammation is a well-established driver of gastric tumorigenesis, only a small subset of GC patients responds to immunotherapy. One proposed mechanism of immune escape is silencing tumour-antigen expression, and thereby avoiding immune recognition. DNA

methyltransferases (DNMTs) are the enzymes responsible for epigenetic silencing of gene expression and are often overexpressed in solid tumours. Epigenetic drugs inhibiting these DNMTs have shown anti-tumour effects in combination with immune checkpoint inhibitors. Here we are studying the role of DNMTs in GC mouse models and the possibility of combining DNMT inhibitors with immunotherapy for the treatment of GC.

Material and Methods

This project utilises various mouse models of GC. We established a *Dnmt3a*-overexpressing inflammation-driven GC mouse model (*gp130^{FF}*, *A33^{Dnmt3a}*). In a second mouse model, mutant *Kras*, *Pi3kca* and *Tp53* expression results in highly advanced invasive gastric carcinoma formation (KPT model). We successfully established GC organoids of one of these triple mutant tumours, which can be injected subcutaneously into wild type mice and result in allograft tumour formation.

Results and Discussions

Gastric adenomas of our *gp130^{FF}*, *A33^{Dnmt3a}* mouse model have a 10-fold elevated *Dnmt3a* expression. Importantly, tumour-specific *Dnmt3a* overexpression significantly increased gastric tumour burden. Treatment with the DNMT inhibitor decitabine reduced tumour growth in the inflammation-driven *gp130^{FF}* gastric adenoma mouse model. In the KPT model, we have identified DNMT3A as being highly expressed in the invasive front of tumours, their liver metastases as well as in the allograft tumours established by the KPT organoids. In this model of advanced GC, treatment with the DNMT inhibitor decitabine significantly decreased tumour growth and we are currently investigating the efficacy of decitabine in combination with anti-PD-1 and/or anti-CTLA-4 immunotherapy.

Conclusion

Taken together, we provide evidence for a driver function of *Dnmt3a* in gastric tumorigenesis and gastric tumour growth. In addition, our findings encourage further studies to investigate the potential of DNMT inhibitors for the treatment of GC.

EACR22-0047

Interplay between SWI-SNF alterations and canonical cancer pathways in lung cancer development

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Introduction

Despite a great research effort, lung cancer remains the major cause of cancer-related deaths worldwide and shows one of the lowest survival rates. Therefore, there is an urgent need for the identification of new therapeutic targets.

Genes encoding subunits of the SWI/SNF chromatin remodeling complex are collectively mutated in almost 25% of all human tumor types, including lung cancer, what places SWI/SNF as the second most frequent alteration in cancer, just after *TP53*. Nevertheless, the molecular mechanisms through which these alterations foster cancer development remain to be fully understood.

Material and Methods

To decipher a possible relationship between SWI/SNF alterations and canonical cancer promoting pathways, we have generated a collection of stably-transduced human lung

adenocarcinoma (LUAD) cell lines deficient for the mainly mutated subunits of SWI/SNF, which are as well representative of the main mutational backgrounds found on lung cancer patients (*TP53*, *KRAS* or *EGFR* mutated). We have studied the transcriptional and chromatin landscape, as well as the sensitivity of these cell lines to the main treatments used in lung cancer. Additionally, we have analysed the genomic and transcriptomic data from LUAD samples deposited on the public database TCGA.

Results and Discussions

SWI/SNF genes are collectively mutated in nearly 20% of LUAD patients, with *SMARCA4* (9%), *ARID1A* (5%), *ARID2* (5%) and *SMARCA2* (4%) being the most commonly altered subunits. Additionally, SWI/SNF mutations tend to be mutually exclusive among the different subunits of the complex and with the ones in *KRAS* and *EGFR*.

SWI/SNF deficiency results in the development of a pro-tumoral transcriptional program, which provides an explanation to the increase in the proliferation, migration and invasion capacities of cells, both *in vivo* and *in vitro*.

We have also identified that SWI/SNF deficiency is accompanied with a general loss of accessibility at chromatin structure, which is significantly more profound in enhancers than in the rest of the genome and it is essential to maintain the expression of specific gene networks.

Finally, we have demonstrated that *ARID2* deficiency results in impairments in DNA repair mechanisms, which predispose cells to the action of chemotherapeutic agents and PARP inhibitors.

Conclusion

SWI/SNF-altered lung tumor accounts for 20% of the cases and its deficiency alters cell sensitivity to different antitumoral treatments, suggesting that these alterations could be therapeutically exploited in lung cancer patients.

EACR22-0148

Nanoformulation to enhance safety and bioavailability of the anticancer candidate ZZW-115

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Introduction

Pancreatic cancer is a deadly cancer without any efficacious treatment options. We previously identified a drug candidate, ZZW-115, which exhibited an excellent anticancer effect on pancreatic cancer by targeting the nuclear protein 1, a stress-associated protein overexpressed in cancers tissues. However, ZZW-115 is not soluble in water, and binds to the hERG channel, which may cause Q-T interval prolongation.

Nanotechnology-based drug delivery is widely expected to improve drug solubility hence bioavailability, while at the same time, reducing drug toxicity and increasing drug efficacy via

Enhanced Permeability and Retention (EPR)-based passive tumor targeting. Recently, self-assembling supramolecular dendrimer nanosystems have been explored for drug delivery with the unique advantages of high drug loading and small nanosize for deep tumor penetration and accumulation via EPR effect.

Material and Methods

We firstly prepared ZZW-115-carrying nanomicelles using the thin film dispersion method, and then studied these nanomicelles for their size, surface potential, morphology, drug loading and encapsulation efficiency. We also evaluated their cellular uptake, intracellular trafficking and the consequent anticancer activity. Finally, we assessed their in-vivo biodistribution using tumor-xenograft mice.

Results and Discussions

ZZW-115-carrying nanomicelles exhibited a high drug loading of 38% and an encapsulation efficiency of 95%, with the size of 30 nm and zeta potential of +18 mV. The ZZW-115-loaded nanomicelles showed effective and rapid cell internalization, with macropinocytosis, clathrin-mediated, and caveolae-mediated endocytosis being all involved. Also ZZW-115 nanomicelles were trafficked through the endosomal/lysosomal system for effective release it in the cytosol, resulting in potent antiproliferative activity. Further in-vivo real-time imaging demonstrated that the nanomicelles preferentially accumulated in tumor via EPR effect.

Conclusion

Using self-assembly supramolecular dendrimer nanosystems, we successfully encapsulated ZZW-115 within small and stable nanomicelles, which accumulated preferentially in tumor via EPR effect. In addition, these nanomicelles showed rapid cell uptake and potent anticancer activity, highlighting the promising potential as safe and effective anticancer drug candidate. We are currently working for the related preclinical evaluation in treating pancreatic cancer.

EACR22-0394

New Anti-tumour Natural Product Screening Platform: transition from 2D cultures to 3D spheroids miniaturized drug discovery

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Introduction

Cancer is one of the leading causes of death worldwide, being breast cancer the second cause of cancer-related mortality among women worldwide. Natural Products (NPs) are one of the main sources for drug discovery. In the past 30 years, the percentage of NP or NP-inspired new chemical entities (NCEs) has risen to approximately 50 % of all drugs, and beyond, up to 74 %, in the anti-tumour field. In the 2000s traditional natural product screenings were transiently abandoned mostly due to redundant discoveries, technical difficulties associated to the isolation of active constituents and the incompatibility of NP extracts with HTS platforms. Nonetheless, recent technological advances running on highly sensitive equipment have successfully been implemented in NP-based drug discovery workflows, facilitating the rapid identification of novel bioactive molecules and structure elucidation, rewardingly returning a substantial pharmaceutical momentum to NPs, over synthetic molecules. At Fundación MEDINA, we have implemented a new anti-tumour HTS platform integrating high-content imaging systems, miniaturization of phenotypic cell-based assays and bio-guided chemical fractionation of microbial extracts. A pilot screening was developed using the implemented platform.

Material and Methods

A primary screening of 5,000 microbial extracts was carried out against the human breast cancer cell line MCF-7 and after the candidate hit selection, a bioactivity-guided fractionation procedure and confirmation of the isolated compounds was developed. New systems such as the Echo® 550 Acoustic Liquid Handler (Labcyte) and Operetta CLS High Content Analysis System (PerkinElmer) were used in this screening.

Results and Discussions

Six compounds with chemically related structures were identified and isolated and their anti-tumoral activity was confirmed in a 3D breast cancer cell model. High-content cellular imaging has increasingly met the challenges of high-throughput needs and facilitates the integration of disease-relevant models and screens at early stages of the drug discovery process.

Conclusion

Miniaturization in 384-well plates, nanolitre-acoustic dispensing with Echo 550 (Labcyte), which is powered by Acoustic Droplet Ejection (ADE), and bioimaging of 3D spheroid cultures were successfully implemented in this pilot screening.

EACR22-0500

Proteogenomics and ADMETox profiling for drug repurposing

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Introduction

Drug repurposing reveals new molecular targets, expands pharmaceutical research and development portfolios, and eases efficacy/toxicity burdens. In precision oncology, drug repurposing is ready for prime-time, translating information growth into knowledge growth.

Material and Methods

We exploited the 3D cell microenvironment as the means to a. define the druggable key-players, b. identify and validate candidate biomarkers and c. characterize cell phenotypes. For this, LC-MS/MS and high-resolution particle-by-particle 3D fluorescent microscopy measurements were coupled in our 3-tier strategy.

A-tier; The cell microenvironment in 3D is a multifaceted ecosystem that is dynamic and exhibits profound cellular heterogeneity as well as complex cross-talk and interactions. For this, we used soft agar, spheroids, and hanging drops. Cell phenotypes have been characterized and next, their spatial organization and inter-cellular cross-talk were reconstructed.

B-tier; An *in silico* and *in vitro* platform was set for medium-to-high throughput efficacy and ADMETox profiling addressing the pharmacokinetics, pharmacodynamics, and toxicokinetics ("hit-to-lead" screening) via a proteogenomic perspective

C-tier; For companion biomarkers, we focused on the number, nature, and cargo of exosomes.

Results and Discussions

We have established that cell phenotypes secrete "messages" mediating short- or long-range communication within the microenvironment and beyond. Exosomes also serve as a toolbox for drug resistance and drug-drug interactions. Our pipeline indicates that cellular heterogeneity and plasticity empowers drug repurposing as the 3D cell microenvironment is

a key determinant of cell behaviour and function in (patho)physiology. Overall, exosomal profiles show distinct features.

Conclusion

Our pharmaco-centric (and hence, based on the drug) strategy facilitates a better-informed druggable proteome for drug repurposing in precision oncology.

This research has been co-financed by the European Regional Development Fund of the European Union and Greek national funds through the Operational Program Competitiveness, Entrepreneurship and Innovation, under the call RESEARCH – CREATE – INNOVATE (project code:T2EDK-03153)

EACR22-0528

New insights on melanoma treatment: how can seriniquinones benefit?

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Introduction

Melanoma is the most aggressive type of skin cancer and, once it metastasizes, most patients develop drug-resistant tumors. Considering the limited treatment options for these tumors, the natural product seriniquinone (SQ1) showed promising results as prototype for a new anticancer class. SQ1 is a secondary metabolite from a marine bacterium (*Serinicoccus* sp.) and presents unique cytotoxic properties: selectiveness for melanoma cells, a novel molecular target (dermcidin) and induction of cell death through autophagy followed by apoptosis. Previous results demonstrated that SQ1 is cytotoxic to melanoma cell lines carrying BRAF and NRAS mutations – the first and the second most incident mutations, respectively. This work aims to elucidate the role of autophagy in the cytotoxicity of serinoquinones and the susceptibility of vemurafenib-resistant cells to these compounds.

Material and Methods

Studies with SQ1 were performed in parallel with SQ2, a more water-soluble synthetic derivative. After treatments with bafilomycin A1 (autophagy inhibitor), rapamycin (autophagy inducer), Z-VAD-FMK (apoptosis inhibitor) or doxorubicin (apoptosis inducer), cells were submitted to MTT assay to determinate the respective IC₅₀ (half maximal inhibitory concentration) and to Western blotting analyses to evaluate key-protein expressions from autophagy and apoptosis pathways. Resistant cells SK-MEL-28R (BRAF^{V600E}; vemurafenib-resistant) were also treated with SQ1 and SQ2 treatment then followed by MTT and clonogenic assays.

Results and Discussions

SK-MEL-28 and SK-MEL-147 displayed different basal activation of autophagy and apoptosis: the first with higher autophagy activation, suggesting distinct modulation of cell death induction. Both cell lines triggered caspase-mediated apoptosis following SQ1 and SQ2 treatments. As expected, BRAF mutants were more sensitive to the cytotoxic treatments after autophagy inhibition, however, NRAS mutants revealed a higher resistance thereof. Furthermore, studies using the vemurafenib-resistant cells showed that both

seriniquinones were able to inhibit cell proliferation at micromolar concentrations and reduce their clonogenicity.

Conclusion

Due to its novel mechanism of action, seriniquinones have a great potential not only to inaugurate a new anticancer drug class but also to become a pharmacological tool to understand the role of dermcidin in the context of carcinogenesis and chemoresistance. This way, more studies are necessary and dermcidin silencing may bring new insights on this relationship.

EACR22-0808

A Targeted Catalytic Nanobody (T-CAN) with Asparaginolytic Activity

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Introduction

Asparaginase (ASNase) is the gold standard drug for the treatment of Acute Leukemia. It is a protein drug with enzymatic activity capable of depleting circulating asparagine and to induce metabolic stress in asparagine-dependent leukemia cells. ASNase has been successfully used in the clinics for over 50 years, but, despite its efficacy, it presents some side effects that hamper its use. One of the most frequent and unfavourable side effect is its immunogenicity due to its bacterial origin. Systemic reduction of asparagine and glutamine cause mainly liver and pancreas disfunctions due to off-target activity. Innovative solutions to improve on these drawbacks can exploit protein engineering to obtain functional molecules with reduced or absent immunogenicity and targeted activity. In this work, we present a miniaturized, functional version of ASNase built on a VHH scaffold and targeted onto the cell surface receptor CD19 by a scFv antibody fragment. We named this nanobody-based antibody-drug conjugate "Targeted CAlytic Nanobody" (T-CAN).

Material and Methods

The catalytic VHH moiety was engineered by *in silico* modelling using a camelid VHH protein as a backbone and transferring key catalytic residues onto the three hypervariable loops. The recombinant protein, named sdASNase, was successfully produced in bacteria and purified from classical inclusion bodies. A previously described anti-CD19 antibody scFv fragment (FMC63 clone) was cloned at the 3'- of the gene encoding the sdASNase. The obtained construct was expressed in bacteria and purified from non-classical inclusion bodies.

Results and Discussions

The transfer of ASNase catalytic residues onto the VHH was successful. The obtained sdASNase has an ASNase activity of 1.09 ± 0.29 U/mg and can inhibit the growth of the T-ALL cell line MOLT-4 (IC_{50} 0.13 ± 0.02 U/ml). The T-CAN fusion protein also retains ASNase activity (4.30 ± 0.26 U/mg), can bind CD19 and show increased cytotoxic activity on CD19 positive B-ALL cell lines compared to the full-length Kidrolase (percentage of live cells at 0.3 U/mL: $21.42 \pm 3.60\%$ vs. $35.47 \pm 3.40\%$ for REH, respectively, and $76.66 \pm 5.10\%$ vs. $89.02 \pm 4.51\%$ for RAJI cells).

Conclusion

sdASNase and T-CAN represent a strong proof-of-concept for further engineering of new, unexplored platforms for the transfer and targeting of ASNase activity and provide the basis for the formulation of ASNase-based drugs with reduced side effects and improved efficacy.

TES-4207, a First-in-Class NR2F6 Antagonist: in vitro Pharmacology and Preclinical Data of a Potential Next Generation Immuno-Oncology Therapy in Ovarian Cancer

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Introduction

The development of therapies able to block inhibitory proteins called “immune checkpoints” has emerged as key strategy in cancer therapies. Currently approved immuno-therapies are still limited by several factors, as low patient response rate and high costs. Orphan Nuclear Receptor Subfamily-2 Group-F Member-6 (NR2F6) has been recently suggested as a novel immune checkpoint. NR2F6 acts as a transcriptional repressor of specific cytokines pivotal for tumor rejection. Moreover, overexpression of NR2F6 has been recognized as a negative prognostic factor in several human diseases, such as ovarian cancer. So far, the evaluation of NR2F6 as a potential drug target has been limited by the lack of small molecule ligands.

Material and Methods

Binding of TES-4207 to NR2F6 has been assessed using purified recombinant human NR2F6 through label-free technology.

CD4⁺ and CD8⁺ T cells activation was assessed stimulating them with plate bound anti-CD3 and anti-CD28 under T helper differentiation conditions, with or without TES-4207. Cytokine secretions were monitored using BioPlex Luminex Technology or FACS. To assess in vivo efficacy, TES-4207 was administered daily via i.p. in a orthotopic syngeneic ID8-luc ovarian cancer model, starting administration from 14 days after tumour inoculation. Tumor volume was assessed through bioluminescence (BLI) measurement.

Results and Discussions

Our drug discovery project led to the identification of several hit compounds able to interact with NR2F6. The hit-to-lead optimization led to the discovery of TES-4207, a first-in-class NR2F6 antagonist. The latter show capability to specifically interact with NR2F6 in the nanomolar range in both cell-free and cell-based assays. Moreover, small molecule TES-4207 can increase the production of specific cytokines including IL-2 and IFN γ , when added in vitro during CD4⁺ T cells and CD8⁺ cytotoxic T cells stimulation, as well as cytotoxic factors as perforin and granzyme in CD8⁺ T cells. *In vivo*, TES-4207 is able to significantly slowdown cancer growth in a well-established orthotopic syngeneic ovarian cancer mouse model along very good tolerability.

Conclusion

Overall, our data shows a promising profile of the first-in-class small molecule NR2F6 antagonist TES-4207, suggesting that NR2F6 is a valuable novel drug target in immuno-oncology. Further studies are ongoing to complete the preclinical development of TES-4207 for the treatment of ovarian cancer as well as other tumours.

Experimental / Molecular Therapeutics, **Pharmacogenomics**

EACR22-0093

Acridocarpus orientalis Inhibits Cell Proliferation, Induces Autophagy and Cellular Senescence in Human Breast Cancer Cell Line MDA-MB-231

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Introduction

Breast cancer is the most frequently diagnosed cancer in women worldwide. Among the subtypes of breast cancer, triple negative breast cancer (TNBC) is the most aggressive with higher rate of and distant reoccurrence, poorest prognosis and lowest survival rate. Chemotherapy and radiotherapy are the current available options for TNBC patients. However, these options are accompanied with severe side effects. Therefore, there is an urgent need to develop novel and effective anticancer drugs for TNBC. *Acridocarpus orientalis* is a well-known plant native to UAE and Oman and has been historically used in their folk medicine to treat many health conditions. The aim of this study is to investigate the anti-cancer activity of the leaves extract of *Acridocarpus orientalis* (AOEE) and its underlying mechanism, in TNBC.

Material and Methods

Anti-proliferative effect of AOEE was measured with Cell-titer Glo (Promega). Cellular senescence was detected using Senescence Associated- β -galactosidase (SA- β -gal) staining protocol. The expression of proteins of interest were measured using Western blot analysis.

Results and Discussions

In MDA-MB-231, the results revealed that AOEE could significantly reduce cell viability and cell proliferation in a concentration- and time-dependent manner. The anti-proliferative effect of AOEE was found to be accompanied with the induction of cell cycle arrest at the G1/S phase with upregulation of p21^{WAF1} and p27^{Kip1}, downregulation of PCNA, Cyclin D1, phospho-Rb. Moreover, AOEE induces autophagy through upregulation of autophagy related proteins LC3-II, Beclin-1, p62. Cellular senescence was induced in AOEE treated MDA-MB-231 cells confirmed by p16 upregulation and senescence-associated β -galactosidase (SA- β -gal) expression in the treated cells. We showed that AOEE may potentially act via activation of ERK and p38 pathways, which might be involved in autophagy induction and senescence.

Conclusion

In conclusion, AOEE inhibits the proliferation of MDA-MB-231 breast cancer cells through induction of autophagy, cellular senescence and DNA double stranded breaks, suggesting that *Acridocarpus orientalis* could be a promising candidate for development as a therapeutic agent against TNBC.

EACR22-0099

Targeting protein degradation as a novel cancer therapeutic strategy: from research to innovation

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Introduction

EMT process is characterized by a decreased expression of E-cadherin protein, a tumour suppressor responsible for cell-cell adhesion in the epithelium. E-cadherin is regulated at the posttranslational level by the E3 ubiquitin-ligase Hakai, which is important not only at cell-cell contacts, but also in cell proliferation, cell-substrate adhesion and invasiveness. The role of Hakai during cancer progression in vitro and in vivo was previously reported and it has been proposed as a potential new therapeutic target to block tumour progression and metastasis.

Material and Methods

We firstly performed a virtual screening by analysing 5M small-molecules inhibitors to identify novel specific inhibitors of Hakai, by targeting its phosphotyrosine-binding pocket (named HYB domain), where phosphorylated-E-cadherin specifically binds. In vitro analyses were performed to determine the antitumor activity of specific small inhibitor, Hakin-1, by immunoprecipitation and ubiquitination assays. Moreover, MTT assays, proliferation, oncogenic potential, migration and invasive capabilities were also tested in vitro. By using in vivo xenograft mice models, it was determining the effect on tumour growth, and by immunohistochemistry different markers of proliferation, invasion, and EMT were also tested.

Results and Discussions

As a result of virtual screening, some inhibitor compounds were selected. We identified a new compound named Hakin-1 that show an important effect on Hakai-induced ubiquitination. Moreover, E-cadherin results showed that Hakin-1 new compound significantly increased E-cadherin expression at the cell junctions to the DMSO vehicle control. Antiproliferative, anti-invasive and antioncogenic effect, in vitro of Hakin-1 in tumour epithelial cells was also confirmed. Moreover, Hakin-1 inhibits tumour growth in xenografted mice. This represents an important step forward in further development of an effective therapeutic drug that would prevent or inhibit carcinoma tumour progression.

Conclusion

As Hakin-1 exerts an antitumor activity in colon cancer cells, by inhibiting proliferation, colony formation, migration and invasion, accompanied by an elevation of E-cadherin expression at cell-cell contacts, we will discuss the potential of the project to advance into a novel therapeutic strategies to halt cancer.

EACR22-0327

Binding characteristics and preclinical activity of UCB6114, the first fully human anti-gremlin-1 monoclonal antibody in development for the treatment of cancer

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Introduction

Gremlin-1 is a high affinity antagonist of bone morphogenetic proteins (BMPs) 2, 4 and 7, which are a group of signalling molecules that belong to the Transforming Growth Factor β (TGF- β) superfamily of proteins that regulate stem cell proliferation and differentiation. Gremlin-1 has recently been implicated in the initiation and progression of different types of cancer, with increased expression correlated with poor prognosis in colorectal (CRC), gastric, lung, breast and cervical cancers, and cholangiocarcinoma. Here we characterise UCB6114, a first-in-class, human, anti-gremlin-1 IgG4P monoclonal antibody (mAb), along with its murine analogue, Ab7326 mIgG1, which inhibits gremlin-1–BMP binding.

Material and Methods

UCB6114 was generated via a phage bio-panning campaign using recombinant gremlin-1 protein. The chimeric Ab7326 mIgG1, with the same variable regions as UCB6114, was created for mouse model studies. Restoration of BMP signalling was assessed by HEK-Id1 reporter assay using HEK293 cells stably transfected with an Id1-luciferase reporter vector. The affinity of UCB6114 and Ab7326 mIgG1 for gremlin-1 was determined by surface plasmon resonance, and gremlin-1–Ab7326 Fab complex binding was investigated via crystallography. Modulation of BMP signalling via gremlin-1 blockade by UCB6114 was investigated in human CRC cell lines and fibroblast co-cultures using SMAD1/8 phosphorylation assays and qPCR for downstream Id1–3 gene expression analysis.

Results and Discussions

UCB6114 and Ab7326 had similar affinities for human and mouse gremlin-1, with mean equilibrium dissociation constants of 88.8 pM and 53.3 pM, respectively. The gremlin-1–Ab7326 Fab structure revealed a gremlin-1 dimer with a Fab molecule bound to each monomer of gremlin-1, at an epitope discovered to result in Ab7326 mIgG1 sterically blocking BMP binding. UCB6114 at >0.833 μ g/ml fully blocked the activity of 300 ng/ml of recombinant human gremlin-1 and restored BMP signalling pathways in human CRC cell lines. Furthermore, UCB6114 restored BMP signalling in CRC cell lines and fibroblast co-cultures via inhibition of fibroblast-derived gremlin-1, with upregulation of Id1, Id2 and Id3 expression.

Conclusion

UCB6114, the first human, highly selective, IgG4P mAb optimised for activity against human gremlin-1 protein, demonstrated restoration of gremlin-1-ablated BMP signalling. UCB6114 is in a Phase I/II clinical trial in patients with advanced solid tumours (NCT04393298).

EACR22-0373

A new reporter system for high throughput identification and validation of eIF4F inhibitors

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Introduction

The eIF4F translation initiation complex has a critical role in cancer. The complex enhanced activity has been identified as a nexus of drug resistance in cancer cells, making it a promising therapeutic target. However, the spectrum of available eIF4F inhibitors is limited, and none of them is in clinical use. One of the reasons could lie in the relative complexity of techniques used to identify new inhibitors of eIF4F-mediated translation. Here we report a unique cell-based reporter system suitable for high-throughput identification of novel eIF4F inhibitors in small molecule compound libraries.

Material and Methods

We performed a proteomic screen that identified several eIF4F-regulated pathways controlling cancer cell proliferation. We then used a promoter of one of the genes involved in the pathways to build a reporter system, responding to the eIF4F inhibition by changes of luciferase expression in a dose-dependent manner. Subsequently, we validated the system in a panel of cancer and non-cancer cell lines, determining the impact of eIF4F inhibition on luciferase activity. Changes in protein levels were confirmed using western blotting.

Results and Discussions

We assessed the performance of the luciferase-based reporter system and confirmed its high sensitivity to the eIF4F inhibition. The current state-of-the-art eIF4F inhibitor screening assays, such as the proximity ligation assay (PLA), have several limitations in the high-throughput mode. Our new technique is not only specific and suitable for high-throughput screening; it is also less cost-intensive, significantly faster, and does not require expensive fluorescent microscopy/image analysis equipment.

Conclusion

Our new eIF4F activity reporter system is sensitive and suitable for high-throughput drug library screening for novel eIF4F inhibitors. Furthermore, as the translation initiation complex eIF4F appears to be a promising therapeutic target for overcoming drug resistance in cancer, our approach may identify new drugs to increase cancer patient survival rates.

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EACR22-0471

DISCOVERY OF NEW PYRAZOLE DERIVATIVES DISRUPTING MICROTUBULE ASSEMBLY

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Introduction

Pyrazole scaffold possesses a wide range of biological and pharmacological activities, which play important roles in medicinal chemistry. The present study reports *in vitro* biological characterization of nine pyrazoles derived from chalcones as potential anticancer agents for non-small cell lung cancer. Novel compounds were evaluated for their antitumor activity against various lung cancer cell lines, including studies on cytotoxic, antiproliferative, and proapoptotic effects.

Material and Methods

Non-small cell lung cancer cells (A549, H226, H460) were studied for sensitivity to new derivatives of pyrazoles. The cell cycle distribution and apoptotic effect were examined with

flow cytometry. The ability of compounds to regulate anticancer signaling pathways was validated by western blot. The effect of new agents on microtubule organization was analyzed by *in vitro* assay and immunofluorescence.

Results and Discussions

New series of pyrazoles showed potent antiproliferative activity against various lung cancer cell lines at micromolar concentrations. Among these compounds, PCH-1 exhibited the strongest cytotoxic activity and thus was chosen to evaluate its molecular mechanism of action. The antiproliferative effects of anticancer drugs are often linked with the suppression of the cell cycle. Studies on cell cycle distribution showed that PCH-1 induces G2/M cell cycle arrest by disruption of microtubule assembly followed by cellular apoptosis through extrinsic and intrinsic pathways.

Microtubule dynamics are essential in cell migration by providing protrusive and contractile forces, a front-rear polarity allowing movement of organelle and protein through the cells and controlling signaling during motility. We established that PCH-1 efficiently decreased cell migration in a concentration-dependent manner and suppress the epithelial-mesenchymal transition process, by deregulating various proteins, such as N-cadherin or vimentin.

Conclusion

Overall, this study provides novel opportunities to identify anticancer agents in the pyrazole series. Our data indicate that presented derivatives of pyrazoles have a potent antitumor potential for the treatment of lung cancer. The lead compound PCH-1 is a promising antitumor compound that warrants further preclinical development.

EACR22-0481

Antiangiogenic and Antiproliferative Potential of Phenolics from *Annona muricata* Fruit

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Introduction

Annona muricata (L.) (AM), commonly known as Graviola, Soursop and Lakshmanaphala/Hanumaphala in India, has been extensively used in ethnomedicinal practices for the treatment of a variety of diseased conditions, including tuberculosis, urinary tract infections (UTIs) and cancers. All organs of the plant have been reported to have significant biomedical importance. The AM fruit has been found to be a rich source of antioxidants and antitumor agents, such as phenols.

Material and Methods

In the current study, the phytoconstituents of the AM fruit pulp were extracted using methanol solvent. Qualitative analyses revealed the presence of phenols alongside other molecules in the obtained extract. The extract was initially subjected to *in vitro* antioxidant and anti-inflammatory evaluation, followed by antiproliferative assay using MCF-7 and MDA-MB-231 cell lines, and apoptosis as well as cell cycle arrest detection using flow cytometry. Bioactive phenols reported in AM, gallic acid, coumaric acid, cinnamic acid, chlorogenic acid, 4-hydroxybenzoic acid, and neochlorogenic acid, were evaluated for their *in silico* anticancer activity by molecular docking. The *in vivo* antiangiogenic potential of the phenols was estimated according to CAM assay protocols.

Results and Discussions

The observations made were significant and the AM fruit pulp extract showed a dose-dependent antioxidant and anti-inflammatory activity. The tumoricidal behavior of the phytochemicals were in par with the Cisplatin positive control, demonstrating a dose-dependent inhibition of proliferation of both MCF-7 and MDA-MB-231 cell lines, via G₀/G₁ phase cell cycle arrest as determined cytometrically. Chlorogenic acid occurring in the AM fruit pulp was found to be the most potent anti-breast cancer agent acting via pathways involving epidermal growth factor receptor tyrosine kinase (EGFR-TK). The AM phenolics showed time- and dose-dependent antiangiogenic potential which was on par with the Cisplatin standard.

Conclusion

The above observations suggest that the AM phenolics are potent anti-breast cancer agents and further elucidation of their mechanistic behavior coupled with *in vivo* toxicity, tumor reduction and survival studies conducted using suitable animal models.

EACR22-0523

Novel Melphalan Analogs in Hematological Malignancy Treatment- Synthesis, In Silico Analysis and In Vitro Activity

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Introduction

Blood cancers remain a major health issue despite the high therapeutic efficacy of new treatment options and continuous pharmacological developments. A major limitation of chemotherapy, including melphalan treatment, is the lack of drug selectivity, which leads to adverse effects on healthy tissues. Introducing chemical modifications to the melphalan's structure and determining the structure/activity relationship we may develop derivatives with optimal properties. Compounds designed in this way can be used as future anticancer drugs candidates.

Material and Methods

The aim of our study was to synthesize, analyze in silico and determine the biological properties of novel melphalan derivatives. We obtained three methyl esters of melphalan having in their structures amidine moiety substituted with thiomorpholine (EM-T-MEL), indoline (EM-I-MEL), or 4-(4-morpholinyl) piperidine (EM-MORPIP-MEL). To determine whether the test compounds belong to the druglike group, the rules of Lipinski and the definition of Veber were used. The *in vitro* anticancer properties of the new analogs were determined against acute monocytic (THP1), promyelocytic (HL60), and multiple myeloma (RPMI8226) cancer cells. Melphalan derivatives were evaluated for cytotoxicity (resazurin viability assay), and their ability to induce apoptosis (caspase 3/7, 8, and 9 activity measurements; phosphatidylserine translocation).

Results and Discussions

All the test compounds complied with Lipinski's rules and had only one violation of Veber's rules. The number of rotational bonds was slightly greater than the number specified in Veber's rule. We also considered that none of the new compounds was a substrate for P-

glycoprotein. The EM-T-MEL derivative had the highest biological activity, showing higher cytotoxic properties than the parent drug. The EM-T-MEL derivative most potently induced caspase 3/7 activation in all cell lines tested. Moreover, examination of the morphological changes after annexin V-FITC/PI staining suggested that this derivative triggered apoptosis-specific markers more clearly than the unmodified melphalan in all cell lines tested. This compound also had a beneficial effect in peripheral blood mononuclear cells (PBMC).

Conclusion

In conclusion, we verified and confirmed the hypothesis that chemical modifications of the melphalan structure improved its anticancer properties. The conducted study made it possible to select the compound with the highest biological activity and provided a basis for structure/ activity analyses.

EACR22-0567

Copper complexes induce apoptosis and suppress the expression of inhibitor of apoptosis proteins (IAPs) in triple negative breast cancer cells.

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Introduction

Despite the successful treatment of breast cancer, the prognosis for patients with triple negative breast cancer remains poor. Triple negative breast cancer lacks the expression of the receptors for estrogen, progesterone and epidermal growth factor-2. The lack of effective targeted therapies warrant the search for alternative treatments. The ability of copper complexes to induce apoptosis at low micro molar concentrations have been reported in a variety of cancer cell lines. This study investigated the efficacy and the mechanisms of cell death of copper complexes on MDA-MB-231 cells.

Material and Methods

MDA-MB-231 cells were maintained in culture and treated with a copper(II)-theophylline-phenanthroline complex (CuTP), and a copper(II)-diimine complex (CuDI). Doxorubicin was used as a positive control in all experiments. Cytotoxicity was determined with the MTT assay and morphological changes were investigated using Hoechst 33342 and propidium iodide. The presence of phosphatidylserine on the cell membrane was detected with annexin-V. Caspase-3/7 and caspase-9 enzyme activity was detected with fluorescent labelled substrate enzyme assays. The JC-1 cationic dye was used to determine a loss of mitochondrial membrane potential. Effects of the copper complexes on the expression of p21 and phospho-p53(S15) was assessed by immunofluorescence. Changes to the expression levels of apoptotic regulatory proteins were identified using a proteome array.

Results and Discussions

Copper complexes CuTP and CuDI were active with IC₅₀ values of 1.2 and 3.3 µM, respectively, whilst doxorubicin had an IC₅₀ value of 9.45 µM. Preliminary investigation indicated the absence of necrotic cell death with some cells showing features of apoptosis, like fragmented nuclei. Increased annexin-V binding and the activation of caspase-3/7, further supported apoptotic cell death. A loss of mitochondrial membrane potential and an increase in caspase-9 activity confirmed cell death via intrinsic apoptosis. Both copper complexes increased the number of cells expressing nuclear p21. Phospho-p53(S15)

expression was increased in the treated cells and observed mainly in the nucleus indicating DNA damage and apoptosis. Proteome array analysis showed that the copper complexes decreased the inhibitor of apoptosis proteins like survivin, XIAP and livin.

Conclusion

In summary, these two copper complexes show that they are valuable lead compounds and worthy of further preclinical investigations.

EACR22-0579

Evaluation of the anti-cancer activity and the mechanism of cell death of novel platinum(II) complexes

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Introduction

Cancer remains a major cause of death despite the development of targeted treatments. Platinum-based drugs, such as cisplatin are essential in many chemotherapeutic regimens. However, their use is associated with severe adverse effects, resistance and instability in aqueous media. This prompted the search for platinum complexes with different ligands selected to favour a non-covalent interaction with DNA as a target molecule. We previously reported the synthesis and evaluation of platinum (II)-based complexes with two bidentate chelating ligands; 5,6-dimethyl-1,10-phenanthroline (dmp) and various acylthioureas (Lⁿ-O,S). These active complexes showed efficacy on cancer cell lines with IC₅₀ values of active complexes in the low micro molar range (0.68 - 2.28 µM), lower than that of cisplatin (12.5 - 19.4 µM). Herein, we report the cell death mechanisms of these platinum complexes against cancer cells.

Material and Methods

The effects of platinum complexes on cell death pathways were investigated in two lung cancer cell lines (H1975 and A549) and a colorectal cancer cell line, HT-29. The mode of cell death was evaluated using Annexin-V, Caspase-3 and Caspase-8 assays. An apoptotic proteome array was used to determine effects on the protein expression levels of apoptotic regulatory proteins. Immunofluorescence was used to detect changes in the expression of p21 as an indicator for DNA damage

Results and Discussions

In each cell line, platinum complexes [Pt(dmp)(L¹-O,S)]⁺, [Pt(dmp)(L²-O,S)]⁺ and [Pt(dmp)(L³-O,S)]⁺ caused morphological changes consistent with regulated cell death; apoptosis or paraptosis. All cell lines showed an increase in annexin-V binding to phosphatidyl serine. The three active complexes moderately increased caspase-3 activity whilst caspase-8 activation was not observed. The nuclear expression of p21 was increased in the three cell lines indicating DNA damage. An apoptotic proteome array of HT-29 treated cells showed a significant decrease in the expression of anti-apoptotic proteins claspain, HSP70 and survivin. Expression of heme-oxygenase-1 and Trial-R2 was increased indicating activation of cell stress pathways. Immunofluorescence confirmed the increased expression of heme-oxygenase-1 protein in all three cell lines.

Conclusion

The active [Pt(dmp)(acylthiourea)]⁺Cl⁻ complexes induce regulated forms of cell death at low micromolar concentrations and warrant further pre-clinical investigation.

Experimental validation of computational identified drugs targeting breast cancer stem cells

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Introduction

The majority of human tumors are initiated and maintained by a population of cells that display stem-like properties, including the ability to undergo self-renewal and asymmetric division. Such cells, termed cancer stem cells (CSCs), may mediate tumor metastasis and relapse, thus representing a potentially effective therapeutic target against invasive cancers and towards long-term remission. CSCs have been identified in a broad spectrum of tumors, including breast cancer, the most prevalent cancer with high mortality rates among women worldwide. Established breast cancer cell lines possess a CD44⁺CD24^{-/low} subpopulation of self-renewing tumorigenic cells identified as CSCs. The prevalence of this subpopulation is variable in different breast cancer cell lines and is associated with the metastatic phenotype. Consistently, it is relatively higher in metastatic MDA-MB-231 cells than in non-metastatic MCF7 cells.

Material and Methods

Using an innovative deep learning model trained with large collections of gene expression profiles from breast cancer cells at different stages of differentiation and multiple drug-treated cell lines, we selected a list of 50 molecules that have the potential to target CSCs and cause them to differentiate. Here, we tested the individual impact of seven of these molecules on MDA-MB-231 and MCF7 cells. After establishing the optimal working concentration for each drug, deriving dose-response curves and IC50 from cell viability assays, cells were treated for 16-24 hours with two doses for each molecule and then the number of CSCs and their properties, including growth ability and self-renewal, analyzed by mammosphere assay and fluorescence-activated cell sorting.

Results and Discussions

Five of the seven molecules analyzed showed a significant ability to target at least one of the two cell lines analyzed, reducing the CSC subpopulation and inducing their differentiation towards an epithelial-like phenotype. Notably, three molecules were effective on both cell lines, while two others were able to inhibit the CSC potential of MDA-MB-231 or MCF7 cells alone, respectively. Future in vivo validation in experimental models is needed as a further step for the repurposing of these drugs in breast cancer management.

Conclusion

These findings implicate the validity of the computational method used, which could have a significant impact on the management of invasive and chemoresistant breast cancer.

MLK4 activates DNA damage response and promotes TNBC survival upon treatment with chemotherapy

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Introduction

The development of better therapies for triple-negative breast cancer (TNBC) patients remains a major unmet need in breast oncology. In the past few years, multi-omics profiling studies identified kinase-driven molecular alterations across molecular TNBC subtypes, which opened new possibilities for targeted therapeutic strategies. Mixed-Lineage Kinase 4 (MLK4) is a member of MLK family of serine/threonine kinases. Cancer genomics data indicated that MLK4 is amplified and overexpressed in approximately 50% of triple-negative tumors, facilitating the aggressive growth and migratory potential of breast cancer cells.

Material and Methods

We used various *in vitro* phenotypic assays, RNA sequencing, phosphoproteomics and *in vivo* models to study the impact of MLK4 upregulation on TNBC response to chemotherapy.

Results and Discussions

We demonstrated that MLK4 promotes TNBC chemoresistance by regulating the pro-survival response to DNA-damaging therapies. We observed that MLK4 knock-down or inhibition sensitized TNBC cell lines to doxorubicin and etoposide *in vitro*. Moreover, loss of MLK4 enhanced HCC1806 TNBC cell line sensitivity towards doxorubicin in a xenograft mouse model. Our data indicated that MLK4 silencing induced persistent DNA damage and enhanced apoptosis in TNBC cells treated with chemotherapeutics. Furthermore, doxorubicin-induced phosphorylation of several core DNA damage response components, including ATM, was impaired in cells with MLK4 knock-down. These findings were further confirmed using HCC1806 cells with CRISPR/Cas9-mediated deletion of MLK4, which showed markedly reduced phosphorylation of ATM kinase at Ser1981. ATM was shown to activate NF-κB transcription in response to DNA damage through the well-established nuclear to cytoplasmic signaling pathway involving NEMO. We also observed diminished phosphorylation of NEMO and reduced expression of several NF-κB-associated pro-survival cytokines in TNBC cells treated with doxorubicin, showing the molecular details of MLK4-ATM-NF-κB pathway. Finally, our latest results suggest that MLK4 might control autocrine/paracrine signaling loops in cancer cells and tumor microenvironment upon treatment with chemotherapy.

Conclusion

We identified a novel function of MLK4 in the regulation of DNA damage response signaling, highlighting that the inhibition of this kinase could be an effective strategy to overcome TNBC chemoresistance.

EACR22-0164

Mesenchymal stem cells as a system for delivering nab-Paclitaxel in a metastatic model of PDAC

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Introduction

Current therapy for metastatic pancreatic ductal adenocarcinoma (PDAC) is limited by drug toxicity and resistance. A delivery system might improve drug bioavailability. The aim of this study was to investigate mesenchymal stem cells (MSCs) for delivering nab-Paclitaxel (nPTX) in metastatic PDAC. The feasible manipulation of MSCs, their ability to deliver drugs and to do homing in the damaged site make them a suitable candidate for an autologous cell-therapy approach.

Material and Methods

An *in vivo* model of liver metastases was generated by injection of K8484 murine PDAC cells derived from KPC mice into the portal vein of C57BL/6N mice. Firstly, to assess MSCs ability to reach the tumor site, tumor bearing mice were intravenously (*iv*) or intraportally injected with luciferase-transduced bone-marrow derived MSCs for studying their biodistribution by *in vivo* imaging. Secondly, tumor bearing mice were treated with nPTX to assess their sensitivity to this drug, monitoring the metastatic volume by magnetic resonance after 20 days from tumor induction. Finally, the MSCs viability after treatment with nPTX, as well as drug uptake and release, were investigated *in vitro*. *In vivo*, the effect of nPTX-loaded MSCs injected via the portal vein was further evaluated.

Results and Discussions

Biodistribution studies revealed a high and prolonged accumulation of MSCs in the liver after intraportal injection, while *iv* injected MSCs accumulated in the lung. The high MSCs liver accumulation could be explained by the homing ability of MSCs to the tumor site. Concerning the treatment, nPTX significantly reduced the metastatic volume of tumor bearing mice, demonstrating a responsiveness of our model to this drug. *In vitro*, after 24h we could appreciate a high uptake of nPTX by MSCs without a reduction of cell viability. NPTX release by MSCs was higher after 24h but sustained until 72h, as reported by mass-spectrometry analysis. Results obtained *in vivo* reported a significantly higher effect on metastatic reduction in mice treated with nPTX-loaded MSCs respect to control mice treated with not loaded MSCs or with only nPTX.

Conclusion

The ability of MSCs to incorporate nPTX to a high extent and without reporting toxicity is promising. This system allowed to lower the drug dose thus reducing nPTX toxicity and specifically targeting the tumor site reporting an effective reduction of the metastatic burden. Obtained results open new insights in the use of MSCs for delivering nPTX as a suitable and promising therapeutic option for PDAC.

EACR22-0482

Construction of antibiotic-free plasmid, simultaneously silencing Eng and Mcam, and evaluation of its therapeutic potential

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Introduction

Targeting tumor vasculature through specific endothelial cell markers represents a promising approach for cancer treatment. Two prominent targets of tumor vascular endothelium are

endoglin (Eng), a transforming growth factor β co-receptor, and the Mcam, an adhesion molecule present also on many tumors. Based on our previous results, the aim of the study was to construct an antibiotic resistance gene-free plasmid encoding shRNAs simultaneously targeting two endothelial cell markers, Eng and Mcam, and to test its functionality and therapeutic potential *in vitro* when delivered by gene electrotransfer and combined with irradiation.

Material and Methods

Functionality of the novel plasmid, after successful construction, was tested on several cell lines by determining the silencing effectiveness of the targeted genes using qRT-PCR. Anti-proliferative and anti-angiogenic effects were evaluated by cytotoxicity assay, tube formation assay, and wound healing assay in murine endothelial cells 2H-11. The functionality of the plasmid construct was also evaluated in malignant melanoma tumor cell line B16F10. Additionally, potential activation of immune response was evaluated by determining the induction of DNA sensor STING and pro-inflammatory cytokines using RT-qPCR in endothelial cells 2H-11.

Results and Discussions

We demonstrated that the plasmid construction was successful as the plasmid could efficiently silence the expression of the two targeted genes, when delivered by gene electroporation. Due to the efficient silencing of the targeted genes, reduced migration rate and angiogenic potential were confirmed in 2H-11 endothelial cells. Furthermore, induction of DNA sensor STING and pro-inflammatory cytokines were determined, that could add to the therapeutic effectiveness when used *in vivo*.

Conclusion

To conclude, we have successfully constructed a novel plasmid DNA encoding two different shRNAs acting on different signaling pathways in endothelial cells. Simultaneously silencing two main pathways makes this plasmid potentially more efficient than others previously used. Furthermore, we have confirmed its anti-angiogenic and anti-proliferative potential, after being delivered into the targeted cells by gene electrotransfer. The obtained results hold a great promise for further *in vivo* testing that are currently ongoing.

EACR22-0498

Novel Combinatory Therapy in Non-Small Cell Lung Cancer Cells

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Introduction

The PI3K/mTOR pathway is known to be overstimulated in lung cancer and is associated with increased eIF4E activity. AZD2014 (Vistusertib®), a dual mTOR inhibitor, targets both mTORC1 and mTORC2 complexes, both of which are key players in the mTOR pathway. Additionally, translationally controlled tumour protein (TCTP), which is overexpressed in various oncologies, induces additional hyperactivity of the PI3K/mTOR pathway leading to further eIF4E overexpression. Our aim is to achieve sensitisation (30% loss in cell viability) of non-small cell lung cancer (NSCLC) cells with low concentrations of AZD2014, followed by the addition of a TCTP inhibitor. We aim to demonstrate this by combining such reagents into one pharmaceutical conjugate.

Material and Methods

Cell viability assays were carried out with the addition of AZD2014 alone. These results were then used to formulate a single AZD2014-TCTP inhibitor conjugate (AZD2014-TCTP) by combining with a TCTP antisense oligonucleotide. Both AZD2014 and AZD2014-TCTP were administered at a range of concentrations (0 to 25 μ M) to cell lines encompassing the three main NSCLC subtypes: H460 (large-cell), A549 (adenocarcinoma) and H520 (squamous). Cell viability was analysed after 24, 48 and 72 hours of treatment via PrestoBlue® assays.

Results and Discussions

The most significant results were observed for H460 cells. Using AZD2014 alone, IC₅₀ was not obtained at the lower concentration range. However, 30% viability losses at the three different time points were obtained at 18 μ M, 18 μ M and 25 μ M respectively. In contrast, when treated with AZD2014-TCTP, IC₅₀ was observed at 5 μ M, 5 μ M and 9 μ M, following 24, 48 and 72 hours of treatment respectively. Currently, further results using A549 and H520 cells are being collected for a complete analysis of this enhanced combinational therapy on NSCLC.

Conclusion

These results strongly indicate an enhanced efficacy of AZD2014-TCTP, compared to AZD2014 alone. The conjugate exerted enhanced anti-proliferative effects on NSCLC cell lines with a better dose-dependent growth inhibition profile and improved drug efficacy at lower drug concentrations. Therefore, following these promising results, our aim for diversifying pharmacological targets which diverge on a common end-point (eIF4E), via the PI3K/mTOR pathway, may offer enhanced therapeutic outcomes and potentially improved safety profiles in NSCLC.

EACR22-0536

Development of synthetic cell penetrating peptide based RAS inhibitor for RAS-RAF interaction in cancer treatment

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Introduction

Activation of mutations in RAS has been found in high ratio in human cancers, inducing downstream critical effectors corresponding tumorigenesis. The attempts in direct targeting to activated RAS, which is GTP bound, has been somewhat successful in the treatment of certain type of cancer, however, still beset by the resistance. It has been well established that the tumor engagement is quite related with the interaction between mutated RAS protein and RAF kinase in ERK cascade. Here, we developed a synthetic peptide that targets the KRAS(G13D) interaction between RAF, for the treatment of breast cancer.

Material and Methods

Using solid phase peptide synthesis, prepared RAS/RAF binding peptide (RBP) was analyzed by surface plasmon resonance and immunoprecipitation assays for interaction of peptide with RAS/RAF. Cellular effect of peptide was tested for cell viability, cell migration, and protein assays from MDA-MB-231. *In vivo* experiment of RBP was performed to analyze tumor growth, metastasis, and histopathology change. Furthermore, conjugated peptide with anti-mut-KRAS antibody was observed in xenograft model.

Results and Discussions

The cell permeability of the RBP was confirmed by confocal microscopy. The RBP had a higher binding affinity to GppNHp-KRAS(G13D) than RAF protein, which competitively interfere binding of GppNHp-KRAS(G13D) to RAF1. *In vitro* binding to KRAS was also confirmed by immunoprecipitation when treated to MDA-MB-231 cells. The RBP treated MDA-MB-231 decreased cell viability in a concentration-dependent manner, and decreased cell migration in scratch assay and migration assay. Reduced p-ERK1/2 in western blot result shows the mode of action of RBP on MDA-MB-231 reduced KRAS pathway. *In vivo* experiment confirmed that the RBP reduced tumor growth rate and metastasis inhibition. In addition, reduction of Ki-67 was confirmed through histopathology staining test. In addition, RBP has further advantage as a drug delivery carrier. The RBP was further modified with the RAS binding anti-mutant KRAS antibody. The RBP-anti-mut-KRAS antibody conjugates showed inhibition of KRAS-RAF interaction. The RBP fused antibody showed significant tumor uptake. With the tumor bearing xenograft study, the RBP conjugated anti mutant KRAS antibody significantly inhibited tumor volume.

Conclusion

Taken together, these findings demonstrate the potential of the RBP drug discovery for targeting dysregulated RAS/RAF signaling, and it can be further advantageous as a drug delivery carrier in cancer treatment.

EACR22-0614

Repurposing drugs that target the interaction between HPV and TBX3 to treat cervical cancer.

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Introduction

Cervical cancer (CC) is a leading cause of death in women globally with over 80% occurring in low- and middle-income countries. Thus cheap and efficacious therapeutics to treat CC are needed. The Human Papilloma Virus (HPV) is the prime etiological agent of CC and its E6 and E7 oncoproteins cooperate with host factors to induce and maintain the malignant phenotype. An approach that may facilitate rapid and cost-effective drug development is to identify these host factors and to target them with commercially available non-cancer drugs. In this regard, we are interested in the transcription factor TBX3 which is overexpressed in several cancers where it promotes oncogenesis, and it has been validated as a druggable target. Importantly, we have previously shown that the commercially available drugs niclosamide (NS), piroctone olamine (PO) and/or pyrvinium pamoate (PP) target TBX3 and exhibit anti-cancer activity in TBX3-driven melanoma and rhabdomyosarcoma cells. This project aims to determine (1) the status and oncogenic roles of TBX3 in CC; (2) the interaction of TBX3 with E6 and E7; and (3) if NS, PO and PP target the HPV:TBX3 axis and exhibit anti-cancer activity in CC.

Material and Methods

Immunohistochemistry was used to determine the status of TBX3 in HPV positive CC patient-derived tissues. TBX3 was depleted by siRNA in HPV positive (HeLa and CaSki) and HPV negative (C33A) CC cells and proliferation (growth curves) and migration (scratch assay) assessed. CC cells were treated with NS, PO and PP and the impact on endogenous TBX3 was assessed by western blotting and immunofluorescence. NS, PO and PP on their own or in combination with CC chemotherapeutic drugs were investigated for their effect on CC cell survival (MTT and clonogenic assays), cell cycle profile (FACS), migration (scratch

assay), senescence (SA- β -gal/p16) and spheroid growth (calcein-AM stain) and invasion (collagen matrix).

Results and Discussions

We show (1) TBX3 is upregulated in advanced stages of HPV positive CC; (2) TBX3 promotes HPV positive but not HPV negative CC cell proliferation and migration; (3) NS, PO and PP reduced TBX3 levels, inhibited CC short and long-term survival, migration and spheroid formation, induced cell cycle arrests and senescence and were synergistic with first-line CC drugs.

Conclusion

Results from this study suggest that TBX3 co-operates with E6/E7 oncoproteins to promote HPV positive CC proliferation and migration and reveals 3 effective and cheap drugs which can potentially be used to treat CC.

EACR22-1204

Immune checkpoint ligand CD155 synergizes with anti-EGFR cetuximab to provoke a natural killer cell response to head and neck cancer.

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Introduction

Targeting of the epidermal growth factor receptor (EGFR) and PD-1 are now at the forefront of head and neck squamous cell carcinoma (HNSCC) treatment. However, resistance to EGFR inhibition and immune escape mechanisms present major hurdles, limiting durable clinical responses. Interestingly, the EGFR-inhibiting antibody cetuximab could link both therapeutic approaches through antibody-dependent cellular cytotoxicity (ADCC) by natural killer (NK) cells. This study aims to uncover the effects of cetuximab treatment on the immunological profile of HNSCC cells, in order to develop a potent cetuximab-based combination immunotherapy chartering NK cells.

Material and Methods

This study made use of a public genomic dataset of HNSCC patients obtained from The Cancer Genome Atlas (TCGA) and a well established HNSCC cell line panel, comprising cetuximab sensitive as well as intrinsically and acquired resistant cell lines.

Results and Discussions

In silico analysis of TCGA data identified candidate key immune checkpoint ligands in HNSCC. 27 genes were differentially expressed in HNSCC tumors compared to healthy tissues, 11 of which showing prognostic value. Next, we assessed the expression of these candidate genes, and influence of cetuximab thereon in our HNSCC cell line panel. Live-cell protein kinetics revealed 24 hours following cetuximab treatment to be an optimal time point for investigation. Flow cytometry then pointed to a significant decrease in the immune checkpoint profile of HNSCC cells, though irrespective of cetuximab resistance status, and their receptors on NK cells. High expression of the CD155-DNAM1:TIGIT axis made us look further into its potential. Combined targeting of EGFR with cetuximab along with CD155 in

co-cultures of HNSCC:NK cells resulted in synergistic enhancement of the anti-tumor activity by NK cells compared to single-agent treatments. CD16 blocking experiments demonstrated this effect was completely dependent on the ADCC effect of cetuximab.

Conclusion

In conclusion, we show that blocking inhibitory signaling through CD155 synergizes with the approved EGFR-targeting cetuximab to generate a very potent response *in vitro* of NK cells to HNSCC cells, irrespective of their cetuximab resistance status. These data provide a preclinical rationale towards combining oncogenic and immune targeting agents in HNSCC to enhance the clinical efficiency of cetuximab treatment.

EACR22-1064

Targeting Serine/Threonine/Tyrosine Kinase 1 to increase efficacy of EGFR inhibitors in EGFR-mutant non-small cell lung cancer

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Introduction

Non-small cell lung cancer (NSCLC) patients harboring activating mutations in the epidermal growth factor receptor (EGFR) are sensitive to therapy with EGFR tyrosine kinase inhibitors (TKI). Despite remarkable clinical responses, a reservoir of drug tolerant cells survives from which drug-resistant tumours may ultimately emerge. This study addresses the need for improved efficacy for EGFR TKIs by identifying targets involved in functional drug tolerance against EGFR inhibitors.

Material and Methods

A high-throughput siRNA kinome screen was performed in two EGFR TKI-sensitive EGFR-mutant NSCLC cell lines in the presence/absence of afatinib. Cell viability assays, soft agar colony formation assays, and RNA sequencing were performed for further validation and characterization. Co-immunoprecipitation combined with western blot was performed to determine protein-protein interactions.

Results and Discussions

From the screen, the Serine/Threonine/Tyrosine Kinase 1 (STYK1) was identified as a target that, when downregulated, potentiates the effects of EGFR inhibition *in vitro*. We found siRNA-mediated downregulation of STYK1 combined with EGFR inhibitors led to a significant decrease in cancer cell viability and the anchorage-independent cell growth *in vitro*. Further, we show that STYK1 selectively interacts with mutant EGFR and that the interaction is disrupted upon EGFR inhibition. Finally, we identified fibroblast growth factor 1 (FGF1) as a downstream effector of STYK1 in NSCLC cells. Accordingly, downregulation of STYK1 counteracted the afatinib-induced upregulation of FGF1.

Conclusion

We unveil STYK1 as a valuable target to improve the efficacy of initial EGFR-related treatments by diminishing or eradicating the pool of drug-tolerant cells in which constitutive resistance can arise. Co-targeting of EGFR and STYK1 could lead to a better overall outcome for NSCLC patients.

EACR22-0007

DEAD-BOX RNA HELICASE DDX20 PREDICTS STATIN SENSITIVITY IN TRIPLE NEGATIVE BREAST CANCER

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Introduction

Simvastatin, a lipophilic statin used for lowering cholesterol, inhibits 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), the key enzyme of the mevalonate pathway. Studies have shown that cancer cells express deregulated level of HMGCR and statins exert anti-tumoral activities.

Material and Methods

We first assessed correlation between mevalonate pathway genes and DDX20 (DP103, Gemin-3) in 1325 breast cancer patients and observed a positive correlation between DDX20 and the mevalonate pathway genes. Having this data, we then proceeded to explore the effect of statins on DDX20 expression. We used various in vitro cell lines and in vivo statin clinical trial patients specimens, mouse xenograft, mouse intravenous tail injection and *Drosophila* (wild-type vs Gemin-3 knockdown vs Gemin-3 overexpression flies) models.

Results and Discussions

We show exposure to statin decreases the expression of DDX20. Through a series of add-back experiments, we show that the decrease in DDX20 expression by statins is via the mevalonate pathway and downstream of RhoA. In clinical specimens, we observed breast cancer patients with high baseline DDX20 positively correlates with high baseline YAP/TEAD expression. Having known that SUMOylation of YAP maintains its activity and that DDX20 is a critical enhancer of the SUMOylation machinery, we showed through a series of experiments that a physical interaction between DDX20 and YAP is crucial for maintaining SUMOylation of YAP; thereby decreasing its ubiquitination and degradation.

Conclusion

Interestingly, we also identified for the first time that DDX20 is a direct target of YAP-TEAD complex and that maintenance of DDX20 expression is needed as a positive feedback forming an Achilles heel for sustained YAP-TEAD activity.

EACR22-0187

Disrupting Dok-Card interactions for anti-fungal and anti-tumor immune therapies

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Introduction

The Card/Bcl10/Malt1 (CBM) signalosome acts downstream of antigen receptors on B and T cells and innate receptors such as the C-type lectin receptors on macrophages, neutrophils and dendritic cells. It plays a critical role in the activation of these immune cell types via the induction of NFkB signalling. On the other hand, the Dok family of adaptor proteins have been shown to inhibit CBM signalling via the recruitment of phosphatases. For example, we recently demonstrate that Dok3 recruits PP1 phosphatase to dephosphorylate Card9 and attenuate CBM activation. As such, we hypothesize that disrupting Dok-Card interactions via small molecules could heighten immune cell activation and would be useful in the context

of enhancing anti-fungal and anti-tumor therapies. We have therefore established a humanised yeast platform to screen for small molecule inhibitors of Dok-Card interactions as candidates for adjunct therapy in anti-fungal and anti-tumor treatments.

Material and Methods

We have constructed a humanised yeast strain to identify small molecules which can disrupt the interaction between Dok3 and Card9. We have also collated a library of 250,000 small molecules sourced from various companies representing different scaffolds and structures for future refinement. The constructed yeast strain will be screened against this curated library for possible inhibitors of Dok-Card binding.

Results and Discussions

We have successfully expressed human versions of Dok3 and Card9 in yeast. Yeast strain bearing these compounds are viable. We have also validated the utility and robustness of the screen.

Conclusion

The humanised yeast system allows one to screen for possible inhibitor of Dok-Card interactions. Once small molecule candidates have been identified, we will perform in silico modelling and modification of the original hit compound. These lead compounds will be tested in-vitro and in-vivo for efficacy.

EACR22-0295

Nano-encapsulation increases the safety and therapeutic potential of mithramycin A for the treatment of sarcomas

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Introduction

Sarcomas comprise a group of aggressive malignancies with very little treatment options beyond standard chemotherapy. Reposition of approved drugs represents an attractive approach to identify effective therapeutic compounds. One example is mithramycin (MTM), a natural antibiotic which has demonstrated a strong antitumour activity in several tumour types, including sarcomas. However, its widespread use in the clinic was limited by its poor toxicity profile.

Material and Methods

In order to improve the therapeutic index of MTM, we have loaded MTM into newly developed nanocarrier formulations. First, polylactide (PLA) polymeric nanoparticles (NPs) were generated by nanoprecipitation. Also, liposomes (LIP) were prepared by ethanol injection and evaporation solvent method. Finally, MTM-loaded hydrogels (HG) were obtained by passive loading using a urea derivative non-peptidic hydrogelator. The effect of the different MTM delivery systems in cell survival (WST-1 assays and CFU assays), invasion (spheroid invasion assays) and stemness (tumorsphere formation and SORE6 activity) was assayed in myxoid liposarcoma, undifferentiated pleomorphic sarcoma and

chondrosarcoma models. The *in vivo* anti-tumor potential of free and nano-encapsulated MTM was evaluated in a xenograft model of myxoid liposarcoma. Finally, gene expression profiling of MTM-treated sarcoma cells was performed using RNAseq and Western Blotting.

Results and Discussions

MTM-loaded NPs and LIP display optimal hydrodynamic radii between 80 and 105 nm with a very low polydispersity index (Pdl) and encapsulation efficiencies (EE) of 92 and 30%, respectively. All formulations show a high stability and different release rates ranging from a fast release in HG (100% after 30 min) to more sustained release from NPs (100% after 24h) and LIP (40% after 48 hours). *In vitro* assays confirmed that all assayed MTM formulations retain the cytotoxic, anti-invasive and anti-stemness potential of free MTM in models of myxoid liposarcoma, undifferentiated pleomorphic sarcoma and chondrosarcoma. In addition, whole genome transcriptomic analysis evidenced the ability of MTM, both free and encapsulated, to act as a multi-repressor of several tumour-promoting pathways at once. Importantly, the treatment of mice bearing sarcoma xenografts showed that encapsulated MTM exhibited enhanced therapeutic effects and was better tolerated than free MTM.

Conclusion

Overall, these novel formulations may represent an efficient and safer MTM-delivering alternative for sarcoma treatment.

EACR22-0475

A quest for selective drug delivery of highly cytotoxic anticancer diimine complexes: Challenges and progress.

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Introduction

A surge in metallodrug discovery came after the serendipitously discovered antiproliferative properties of cisplatin by Rosenberg in 1965. Many approved and highly effective metallodrugs have been discovered to date as highlighted by Anthony *et al.* recently. However, most reviews on potential metallodrugs acknowledge the gift and the curse associated with metal-based compounds which include multiple targets or mechanisms of action and a molecule that can change properties such as oxidation states, coordination geometry and ligand substitution based on the medium (solution). The challenge is to find an effective compound that induce a favourable mechanism of cell death (such as apoptosis) without having numerous serious adverse effects. For cytotoxic chemotherapeutic molecules, this is a major challenge, even for the currently used platinum drugs which form part of effective treatment regimens in many cancers. We therefore focussed our research efforts towards finding a drug delivery system for our cytotoxic diimine complexes that would ultimately induce some selectivity by having a cancer specific biological targeting molecule incorporated in a delivery system. This will facilitate cytotoxic concentrations in cancer cells and spare non-cancerous cells. Finding suitable molecules to introduce selectivity is a major challenge and will be highly cancer specific.

Material and Methods

The synthesis of complexes have been according to our previously published methods. Host-guest interactions were studied using high-resolution NMR Spectroscopy.

Results and Discussions

Platinum diimine acylthiourea complexes made in our group showed to induce apoptotic cell death with associated IC₅₀ values between 0.68 - 2.28 µM (against lung cancer cell lines, H1975 and A549; a colorectal cancer cell line, HT-29). We have attempted to encapsulate these with a PAMAM G4 dendrimer to ultimately functionalise the dendrimer with selective vectors for specific cancer cell lines. Our study has shown that the complex is in fast exchange between bound and unbound state which could facilitate drug release but could also risk failure if the partitioning into the drug delivery system is too short lived. The next step involves identifying molecules to introduce selectivity and screening them for selectivity.

Conclusion

We have managed to reversibly encapsulate our lead complexes in a functionalised dendrimer as indicated by NOESY and DOSY NMR spectroscopy data as a proof of concept.

EACR22-0641

The anticancer effect of vitamin D analogs is cell line specific.

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Introduction

Vitamin D is well known for its role in maintaining calcium and phosphorus homeostasis. However, vitamin D has many other important functions in human organism. The active form of vitamin D (calcitriol) has been proven to be an antitumor agent with antiproliferative and pro-differentiating properties. As high doses of calcitriol - which are needed to induce these anticancer effects - can cause hypercalcemia, less calcemic and more potent vitamin D analogs have been developed. Our aim was to test the anticancer effect of the several of these novel vitamin D analogs in ovarian and colon cancer cell lines.

Material and Methods

Gene expression and cell viability of three patient-derived primary ovarian cancer cell lines (13781, 14433 and 8714) and one colorectal cancer cell line (Caco-2) were studied after treatment with several analogs in various concentrations for 4 hours or 5 days respectively.

Results and Discussions

Treatment with the analogs increased CYP24A1 gene expression in all tested cell lines, confirming that all tested analogs were active, as CYP24A1 is the main gene target of calcitriol and is responsible for its degradation. In the 13781 cell line, all tested analogs significantly reduced cell viability ($p < 0.01$). This effect was not observed in the other cell lines, indicating that different cancer cell lines have different susceptibilities to vitamin D analogs.

Conclusion

Based on the obtained results it is feasible to reason that the anticancer effect of the tested analogs may be specific for the individual cell line, and, by extension, individual patients –

highlighting the need for personalized diagnostics and therapies. The exact mechanism of the analogs' action in these cancer cells is not known and requires further studies. Our next step will be to investigate differentiating properties of these analogs in the different cancer models.

EACR22-0657

Ascorbyl palmitate-incorporated solid lipid nanoparticles for targeting cancer stem cells

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Introduction

Targeting cancer stem cells (CSC) is significant because they are considered responsible for metastasis formation and relapse after conventional chemotherapy. Ascorbic acid (AA) has been thoroughly investigated as an anticancer agent due to its specific cytotoxic effect on CSC. Because of the instability of ascorbic acid, its derivatives are often used to improve delivery bioavailability. As an alternative, we have used the ascorbyl palmitate (AP), a lipophilic derivative of AA. But for utilization in aqueous environments, AP has to be incorporated in carriers, due to its lipophilic nature.

Material and Methods

For that purpose, we have synthesized solid lipid nanoparticles with AP (SLN-AP) using the microemulsion technique (water in oil). They were analyzed by measuring their size and polydispersity by DLS and zeta-potential by ELS. Also, for evaluation of morphology, they were visualized on TEM. Cellular uptake of SLNs containing fluorescent dye coumarin-6 was analyzed on HEK 293, U2OS and patient-derived sarcoma CSC by fluorescent microscope during 48h. The effect of SLN-AP on the viability of patient-derived sarcoma CSC (n=5) was determined by the MTT test.

Results and Discussions

DLS analysis of SLN-AP showed that nanoparticles have a diameter of 414 nm, the zeta potential of 0 mV and polydispersity of 0,228. TEM revealed round morphology and confirmed the size of nanoparticles. SLN containing coumarin-6 are successfully and gradually uptaken by cell lines HEK293, U2OS and patient-derived sarcoma CSC. However, the high exosome/vesicle activity was noticed on the CSC membranes suggesting possible efflux of SLN. Viability assays show that sarcoma CSC have diverse sensitivity to SLN-AP treatment and they are sensitive to up to 33 µM concentration of ascorbate delivered by SLN-AP.

Conclusion

In conclusion, we have successfully synthesized and characterized ascorbyl palmitate-incorporated solid lipid nanoparticles. The SLN-AP gradually enter the cells and affect the viability of sarcoma CSC. Therefore, our research confirms the potential of SLN-AP for targeting sarcoma CSC.

EACR22-0825

Investigating the role of EGLN1 in non-small cell lung cancer 2D cultures and in

patient-derived 3D spheroids

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Introduction

Lung cancer still causes 20% of overall tumour death. Even though many therapeutic advancements, the prognosis of non-small cell lung cancer (NSCLC) patients remains extremely poor. EGLN1, a proline hydroxylase involved in HIF silencing, has been identified as a dependency in KRAS-mutated NSCLC and its overexpression correlates with a worse prognosis. EGLN1 activity can be inhibited by specific inhibitors, like roxadustat and molidustat, currently in clinical trials for the treatment of anaemia due to chronic kidney failure. These drugs could be exploited also in lung cancer therapy. Tumor organoids represent tumor patient avatar, allowing multiple type of analysis at an increased level of complexity thus offering new opportunities in drug discovery field.

Material and Methods

We performed the knockout (KO) of EGLN1 in NCI-H23, NCI-H2030, and A549 NSCLC cell lines by CRISPR/Cas9, and analysed their proliferation, colony forming and migration potential. Patient primary cultures followed two different protocols to generate CTOS (Cancer Tissue Originated Spheroids) or Organoids. CTOS were obtained through a partial digestion of the sample, filtered to select only multiple cell structures, and cultured in BME matrix for two weeks. Organoids were generated after a complete digestion to single cells, that were stimulated to proliferate in BME matrix domes with DMEM-F12 culture medium enriched with several supplements.

Results and Discussions

The KO of EGLN1 reduced the proliferative and invasive potential in all three KRAS-mutated NSCLC cell lines. Accordingly, treatment with EGLN1 inhibitor molidustat induced proliferation impairment in all cell lines. Molidustat administration in CTOS cultures lowered greatly the CTOS dimensions suggesting an impairment of the cell survival and the promotion of cell death. A deep characterization of organoids cultures demonstrates that they are able to recapitulate the genetics and the histology of the patient tissue, thus offering a precious tool to investigate EGLN1 inhibitors potential as monotherapy and in combination with gold standard treatments for NSCLC.

Conclusion

EGLN1 plays a fundamental role in KRAS-mutated NSCLC and EGLN1 inhibitors represent a valuable possibility that can become part of future therapeutic strategies for this tumor. Preliminary evidence suggest that patient-derived 3D cultures may be useful to assess ex-vivo the response to the molidustat treatment, thus acquiring both a screening and a prognostic meaning.

EACR22-0827

Optimizing photo-assisted eradication of triple-negative breast cancer through novel doped conjugated polymer nanoparticles

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Introduction

Triple-negative breast cancer (TNBC) has a poor prognosis because of the aggressive clinical behavior and limited targeted treatment options. In recent years Photodynamic therapy (PDT) has been examined experimentally in TNBC and it has been proposed as a therapeutic option to bypass and inhibit escape pathways in multidrug resistant breast cancer cells. Here, we investigated the tumor targeting and the antitumorigenic effectiveness of novel photosensitizers-based on conjugated-polymers-nanoparticles (CPNs) whose cancer selectivity is ensured by the conjugation to novel RNA aptamers recognizing human TNBC cells at high efficiency.

Material and Methods

We prepared CPNs by using the CP F8BT due to its exceptional features as a donor antenna to collect excitation energy and funnel it towards molecular dopant PS acceptors, such as the porphyrin PtOEP. Two functional polystyrene-based polymers (PS-PEG-COOH and PSMA) were chosen and incorporated in ~ 20% mass ratio to F8BT mass in order to improve colloidal stability and introduce COOH groups on the surface of CPNs for EDC reaction with NH₂-modified aptamers (the previously validated anti-EGFR CL4 and the recently optimized sTN58 and sTN29 aptamers). Aptamer-decorated CPNs were characterized by size and zeta potential by DLS. Cell targeting/uptake, cytotoxicity and photodynamic evaluation of the nanovectors were tested in TNBC cells and cisplatin-resistant derivatives.

Results and Discussions

Our results show the selectivity of recognition for TNBC membrane receptors and cell uptake of doped conjugated PNPs decorated with CL4, sTN58 or sTN29 aptamer in different TNBC cells. A significant improvement in PDT efficacy was obtained in the presence of CPNs functionalized with the aptamer compared to CPNs unconjugated or scrambled aptamer-conjugated. In cisplatin-resistant cells, sTN58 was the best candidate for improving labelling and PDT efficacy with CPNs. We propose sTN58, sTN29 and CL4 aptamers as valuable tools for selective TNBC cell internalization and therapeutic improvements for CPNs in PDT protocols.

Conclusion

Our study proposes novel and safe photosensitizers-loaded and aptamer-decorated nanosystems with excellent potential for the application in TNBC therapy. The availability of a panel of TNBC cell-type targeting aptamers, will supply multiple tumor-targeting agents with the possibility of using them in different combinations depending on distinct molecular and/or clinical TNBC phenotypes.

EACR22-1133

Protein aggregation in sarcoma as a target for therapy

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Introduction

Sarcoma is a group of rare cancers, characterized by over 100 subtypes. Finding effective therapies remains a challenge, since many types are understudied and poorly characterized. Mutations in p53 are prominent in sarcoma and found in ~80% osteosarcoma cases, ~50% leiomyosarcoma and angiosarcomas and with lower percentages in other subtypes. We investigated whether p53 mutations causing its inactivation through misfolding and aggregation can be targeted by ReACp53, a p53 aggregation-targeting peptide with promising activity in ovarian cancer (Soragni et al, 2016). Protein misfolding and aggregation could involve other proteins besides p53, since many physiological stressors that happen during tumor progression can lead to protein aggregation. We explore the hypothesis that sarcoma cells can increase their fitness through aggregation when exposed to stressors.

Material and Methods

We tested four sarcoma cell lines, established from osteosarcoma, fibrosarcoma, leiomyosarcoma and rhabdomyosarcoma. The cells selected carry p53 mutations as well as control WT p53. We established tumor organoids using our optimized mini-ring method: cells are seeded in 96-well plates in a mixture of medium and Matrigel, allowed to grow for 2 days and followed by a two-days drug treatment (Phan et al, 2019; Nguyen et al, 2020; Al Shihabi et al, 2021). We assess response by measuring cell viability by ATP release assay or fix for histological analysis.

Results and Discussions

We evaluated the effect of ReACp53 on sarcoma organoids. We obtained a drug-response profile for cell lines bearing mutant and WT p53, with WT p53 lines showing a stronger decrease in cell viability. To characterize the p53 aggregation status and elucidate the response to ReACp53, we perform staining and quantification of folded/unfolded p53 pools in these samples. We also tested the effect of different cellular stressors (oxidative, DNA damage and ER) on sarcoma organoids. We determined the response to these stressors by measuring cell viability and assessed if protein aggregation occur. For those conditions that display chaperone overexpression, an indicator of protein misfolding and aggregation, we will proceed with isolation and characterization of protein aggregates.

Conclusion

The discovery of structural alterations of p53, as well as other proteins in sarcoma, could represents a new opportunity for the development of novel therapeutic strategies.

EACR22-0112

Novel benzoprims inhibit growth of mutant RAS-possessing colorectal cancer cell lines

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Introduction

Mutant KRAS is a genetic driver of non-small cell lung cancer (NSCLC), colorectal cancer (CRC) and pancreatic ductal adenocarcinoma (PDAC). Experimental anti-tumour Benzoprims, analogues of the anti-malarial drug pyrimethamine, possess anti-tumour activity against metastatic melanoma cells, mainly by inhibiting dihydrofolate reductase (DHFR), a validated target in cancer therapy. In the NCI 60 cell line panel, methylbenzoprime (MBP) and dichlorobenzoprime (DCB) were found to elicit selective, potent activity in CRC and NSCLC

cell lines possessing mutant KRAS indicating an additional mechanism of action, independent of anti-DHFR activity. The aims of this study are to corroborate previous findings and to test activity of novel benzoprims analogues, interrogating benzoprims structure activity relationships (SAR) and examining anti-tumour activity in colorectal cancer cell lines possessing different RAS mutations, to ultimately gain insight into the benzoprims' molecular targets independent of anti-DHFR activity.

Material and Methods

Growth inhibition of CRC cell lines possessing KRAS^{G13D} (HCT116) and wild-type RAS (HT-29) was studied by MTT assays after 72 h exposure to benzoprims. Isogenic RAS cell lines possessing KRAS^{G12V}, NRAS^{G12V}, HRAS^{G12V}, as well as the parental cell line with wild-type RAS were studied after 6-day treatment with benzoprims. Regulation of protein phosphorylation after 24 h treatment with benzoprims SM1235 was assessed in HCT116 cells by using a Human/Mouse MAPK phosphorylation array.

Results and Discussions

DCB, MBP, SM1245 and SM1235 were found to be more potent in cell lines possessing mutant KRAS^{G13D} compared to a wild-type RAS cell line. SM1235 was the most potent compound in HCT116 cells, with a mean GI₅₀ value of 0.002 μ M $\pm$ 0.0007, 160 times more potent than in HT-29 cells (mean GI₅₀ value 0.317 μ M $\pm$ 0.057). Benzoprims were also found to be less potent in all isogenic RAS cell lines possessing KRAS^{G12V}, HRAS^{G12V} and NRAS^{G12V} mutations, compared to mutant KRAS^{G13D} cells. Interrogating MAP kinase signal transduction using a MAPK array revealed downregulation (>30%) of several proteins by SM1235 in HCT116 cells, including mTOR, MKK3, MKK6 and ERK1/2.

Conclusion

Benzoprims are most potent in CRC cell lines possessing mutant KRAS^{G13D}. Although their mechanism of action independent of DHFR inhibition is still unclear, downregulation of proteins by SM1235 in HCT116 cells indicate that benzoprims inhibit proteins downstream of RAS.

EACR22-0250

PRADIMICIN-IRD, A POTENTIAL ANTITUMOR COMPOUND, PROMOTES TP53-INDEPENDENT MODULATION OF DNA REPAIR GENES

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Introduction

Our previous data has shown pradimicin-IRD as a DNA-damaging agent against colon cancer cells. Cellular and molecular analyzes indicate that pradimicin-IRD induces DNA damage (phosphorylation of H2AX protein and induction of p21 expression) independent of p53 expression, cell cycle arrest, and apoptosis (increased cleaved caspase-3 and PARP-1 expression) in colorectal carcinoma cells. Despite a similar molecular structure to

anthracyclines, including doxorubicin, anticancer activity of pradimicin-IRD differs from that of doxorubicin in terms of potency, p21 induction, cell cycle arrest, and *TP53* independent response. This study aims to investigate the molecular interaction of pradimicin-IRD with DNA and the DNA repair pathways related to the DNA damage response promoted by this molecule.

Material and Methods

Cellular and molecular assays, such as molecular docking, transcriptomic of DNA repair genes (PCR array), PCR, Western Blotting, MTT, and clonogenic assay were carried out.

Results and Discussions

Our results show pradimicin-IRD as a DNA intercalating agent and a potential inhibitor of DNA-binding proteins. Transcriptomic analysis revealed DNA repair functions related to pradimicin-IRD modulated genes, such as nucleotide excision repair, telomere maintenance and double-strand break repair. Validation of genes related to these functions highlighted the PCNA genes for presenting DNA-pradimicin-PCNA (molecular docking) interaction and reduction of their protein levels in pradimicin-IRD treatments. In addition, *hTERT* and *POLH* showed reduced mRNA levels after 6 hours of pradimicin-IRD treatment. Moreover, *POLH*-deficient cells displayed higher resistance to pradimicin-IRD than *POLH*-proficient cells and the compound prevented formation of the *POLH*/DNA complex (molecular docking).

Conclusion

Since the modulation of DNA repair genes by pradimicin-IRD is *TP53*- independent, unlike doxorubicin, dissimilarities between mechanism of action and DNA damage response of pradimicin-IRD and doxorubicin indicate that the latter is a putative pharmaceutical candidate to overcome resistance mechanisms developed through doxorubicin treatment. Therefore, further studies should focus on evaluating the antitumor potential of pradimicin-IRD in doxorubicin resistant cells.

EACR22-0468

Heterogenous response to MET inhibitors in SHH medulloblastoma cells

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Introduction

Medulloblastoma (MB), a cerebellar tumour primarily diagnosed in children, is the most common malignant brain tumour consisting of four molecular subgroups: Wingless (WNT), Sonic Hedgehog (SHH), Group 3 and Group 4. Despite the 70-75% cure rates for MB patients, the risk of morbidities due to the current therapy (surgery, chemo and radiotherapy) remains unacceptably high. Thus, identifying new clinical strategies to overcome medulloblastoma progression and dissemination is crucial in the eradication of the disease. Over the last few years, various tyrosine kinase signalling pathways have been identified to play important roles in medulloblastoma tumorigenesis. Active MET kinase has been

detected as a marker of SHH-driven MB and several inhibitors provided pre-clinical evidence for an effective therapy for SHH-MB patients.

Material and Methods

The efficacy of multiple MET inhibitors against several SHH MB cell lines was investigated *in vitro* by cell viability assays in both 2D and 3D models, alongside with colony and sphere formation assay. Furthermore, cell cycle analysis by flowcytometry was performed to better highlight and compare the effects of the tested compounds. Time-lapse microscopy with single-cell tracking confirmed a difference between the mechanisms of different MET inhibitors. Finally, western blot analysis was performed to define the molecular rational behind the drug effects.

Results and Discussions

We observed that the MET inhibitor tivantinib impairs MET activation and outperforms other MET inhibitors, such as foretinib and crizotinib, in haltering cell proliferation by inducing apoptotic response. By combining time-lapse microscopy with MET inhibitors treatments, we observed a complex and diverse pharmacological response to MET inhibitors. On one hand, tivantinib caused cell death in mitosis whilst foretinib and crizotinib induced mitotic slippage. Interestingly, when challenged with tivantinib, only p53 mutant SHH cells displayed mitotic cell death and high sensitivity to combination with spindle assembly inhibitors. By contrast, p53 wild-type SHH cells, although sensitive to tivantinib, showed a striking level of interline variation in sensitivity to the drug, ranging from prolonged mitosis to slippage, death in mitosis or interphase.

Conclusion

Taken together our results substantiate the rational use of MET inhibitors for SHH-driven MB but the pharmacological heterogeneity in response to MET inhibitors should be highly considered when designing novel effective combination therapies.

EACR22-0732

YPEL2 regulates the efficacy of BRD4-EZH2 dual targeting in EZH2-mutated germinal center-derived lymphoma.

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Introduction

A significant proportion of diffuse large B cell lymphoma (DLBCL) and follicular lymphoma (FL) cases harbor a gain-of-function, heterozygous somatic mutations of the methyltransferase gene, EZH2. Despite an acceptable safety profile and early signs of activity in clinical trials, single-agent treatment with EZH2 inhibitors are unlikely to be curative in these diseases.

Given the known interplay between EZH2 and MYC downstream signaling in malignant B cells, we undertook the simultaneous evaluation of two epigenetic drugs that interfere with EZH2 methyltransferase activity and BRD4-mediated control of MYC transcription, namely

CPI169 and CPI203, using preclinical models of DLBCL and FL with distinct EZH2 mutational status.

Material and Methods

CPI169 antitumor activity was assessed *in vitro* in 10 DLBCL and FL cell lines, including cells expressing basal or ectopic EZH2^{Y641mut}. The molecular bases of its activity were determined by gene expression profiling (GEP), qPCR and western blot, followed by automated exploratory data analysis to identify the most relevant genes associated with response to the epidrug. Biomarkers validation was carried out *in vitro* and *in vivo*, using a mouse xenotransplant model of EZH2^{Y641mut} DLBCL, followed by the identification of factors involved in the simultaneous blockade of MYC and EZH2.

Results and Discussions

We observed a specific and synergistic antiproliferative effect of CPI169 and CPI203 in EZH2^{Y641mut} cells and EZH2^{Y641mut} mouse xenografts, but not in EZH2^{wt} cultures. Loss of H3K27me3 mark upon CPI169 treatment was associated with upregulation of gene sets related to G1 cell cycle blockade, mTOR, P53 and MYC signalling. Accordingly, a synergistic antiproliferative activity was achieved by CPI169 when combined with CPI203, in EZH2^{Y641mut} cultures and xenograft models. Gene expression profile analysis, followed by automated exploratory data analysis and validation by a siRNA screening, further identified the PI3K/AKT-regulated gene and mitosis regulator, YPEL2, as a crucial factor involved in the efficacy of MYC/EZH2 dual targeting *in vitro* and *in vivo*.

Conclusion

These results provide a first preclinical evidence that simultaneous targeting of MYC and EZH2 is a safe and efficient approach that can be monitored by specific biomarkers in aggressive lymphoid tumors of germinal center origin.

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Monastrol shows anti-fascin and anti-invasive properties in colorectal cancer cells

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Introduction

Cytoskeleton rearrangement is a prerequisite for carcinoma cells to develop cellular protrusions allowing these cells to migrate, invade, and metastasize. Fascin (FSCN1) is a key protein in actin and tubulin rearrangement and is expressed in aggressive and invasive carcinomas. Recent *in vitro* evidence suggested that monastrol, an inhibitor of the kinesin-5 protein, can bind fascin.

Material and Methods

Biophysical (differential scanning fluorescence), biochemical (actin bundling), cellular (MTT, migration, invasion on human tissue) assays and animal models (zebrafish invasion) were performed to characterize the effect of monastrol using migrastatin as a typical fascin inhibitor. Confocal microscopy and immunoprecipitation were used to test the monastrol effect on fascin-tubulin binding.

Results and Discussions

The binding of this compound to fascin was validated *in vitro* using fluorescence titration experiments, rendering a dissociation constant in the high microMolar range ($K_D=183\mu M$). In order to choose colorectal cell lines with highest and lowest endogenous fascin expression, a RT-qPCR was performed upon RNA extracted from eight cell lines and we selected DLD-1 and HCT-116 for subsequent experiments. Monastrol reduced cell viability with an IC_{50} in the micromolar range. Monastrol demonstrated effects against filopodia lamellipodium formation and also against cell migration in wound healing assays. Using matrigel invasion assays and cell invasion in a human myoma tissue model we demonstrated that monastrol inhibits depth and area of invasion of HCT-116 compared to control in a comparable magnitude to migrastatin. Purified fascin increased the amount of F-actin in the pellets. However, when we added monastrol, we observed how the compound partially inhibits the fascin-induced bundling of F-actin polymers. Finally, in order to find out whether the anti-invasive properties of monastrol could be extrapolated to an animal system, the well-established zebrafish larvae invasion model was used. A xenograft assay was carried out and we demonstrated that monastrol reduced the percentage of HCT-116 invasion in zebrafish larvae. Moreover, monastrol seems to disrupt the binding between fascin and tubulin.

Conclusion

This study reports, for the first time, the anti-migratory and anti-invasive properties of monastrol on colorectal tumor cells possibly due, but not restricted, to a direct inhibition of fascin.

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EACR22-0940

The small molecule TK216 targets microtubules in Ewing sarcoma cells

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Introduction

Ewing sarcoma (EWS) is a pediatric malignancy driven by the EWSR1-FLI1 fusion protein formed by the chromosomal translocation t(11;22). The small molecule TK216 was developed as a first-in-class direct EWSR1-FLI1 inhibitor and is in phase II clinical trials in combination with vincristine. However, TK216 exhibits anti-cancer activity against cancer cell lines and xenografts that do not express EWSR1-FLI1. Therefore, we sought to uncover the mechanism of action of TK216-induced cytotoxicity in EWS cells.

Material and Methods

We applied a forward genetics screening platform previously reported that can reveal the direct protein targets of cytotoxic small molecules such as TK216. We also used reconstituted microtubule (MT) polymerization *in vitro* and cell-based chemical probe competition assays to investigate the activity of TK216 and to understand synergistic effect with vincristine observed in EWS cells and xenografts.

Results and Discussions

Using an unbiased forward genetics platform that utilizes engineered hypermutation in EWS cell lines, we identified recurrent mutations in *TUBA1B*, encoding α -tubulin. These *TUBA1B* mutations proved sufficient to drive resistance to TK216. To investigate whether TK216 impacted MT dynamics, we reconstituted MT polymerization *in vitro* and observed that TK216 inhibited MT polymerization in a dose-dependent manner indicating that TK216 acts as an MT destabilizing agent. This led to the hypothesis that TK216 and vincristine acted on distinct MT binding pockets potentiating their synergistic effect. We used a cell-based competition assay using a tubulin chemical probe and observed that TK216 and vincristine bind to different binding sites of β -tubulin.

Conclusion

This work defines the mechanism of cytotoxicity of TK216 and explains the synergy observed with vincristine in EWS cells. These results call for a reexamination of ongoing clinical trials using TK216, and additional investigation of potential synergy between colchicine binding pocket agents and vinca alkaloids for cancers sensitive to MT destabilizing agents.

EACR22-1028

Trabectedin is able to restore adipocyte differentiation in myxoid liposarcoma by directly displacing FUS-DDIT3 from its targets

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Introduction

Myxoid liposarcoma (MLPS) is a rare soft tissue sarcoma characterized by the expression of the chimeric fusion protein FUS-DDIT3, which inhibits adipocytic differentiation. Clinically, MLPS shows high sensitivity to trabectedin thanks to its ability to restore adipocytic differentiation. We hypothesize that trabectedin selectively counteracts FUS-DDIT3 ability to bind its DNA targets. On this basis, we have investigated possible modifications of FUS-DDIT3 DNA-binding upon trabectedin treatment, also evaluating whether trabectedin affects

the FUS-DDIT3-dependent transcription by integrating RNA-Seq and ChIP-Seq data in samples obtained from MLPS patient-derived xenograft (PDX) models.

Material and Methods

Tumor biopsies were collected from MLPS PDX models, sensitive to (ML017) and resistant against (ML017/ET) trabectedin. To investigate early and late effects of the treatment, animals were sacrificed at different time-points from trabectedin administration. At least three mice per condition were sequenced and analyzed following the ENCODE guidelines.

Results and Discussions

In both ML017 and ML017/ET at basal conditions, FUS-DDIT3 binds genome regions that span from promoters to distal sites involving 2243 genes in common that were interestingly ascribed to extracellular matrix remodeling and adipogenesis pathways. According to ChIP-Seq data, transcriptional results do not show any significant difference over these genes.

In ML017, at early time point from trabectedin administration, FUS-DDIT3 DNA binding capacity is unaffected and transcriptional modulation is mainly related to the cytotoxic effect of the drug. At later time point, trabectedin induces the displacement of FUS-DDIT3 from its binding sites, leading to the transcriptional activation of genes involved in adipogenesis and extracellular matrix remodeling.

In ML017/ET, no significant changes are identified in FUS-DDIT3 binding sites at both early and late time point. This finding is consistent with RNA-Seq data and provides further evidence of the drug ability to detach FUS-DDIT3 from the DNA, thus regulating the transcription of adipocytic differentiation.

Conclusion

Overall, by highlighting for the first time the role of trabectedin to inhibit FUS-DDIT3 activity at genome-wide scale, we have contributed to shed light on its mechanisms of action in MLPS, providing new basis for novel combination therapies for MLPS.

EACR22-0117

Sensitisation of breast and ovarian cancer cells to PARP inhibitors by PPM1D (WIP1) inhibition

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Introduction

PARP-1 (Poly-ADP Ribose Polymerase-1) is activated in response to DNA damage and inhibition of it is cytotoxic, particularly for BRCA-deficient cancer cells. PPM1D/WIP1 (Wild-type p53 induced phosphatase 1) limits the DNA damage response (DDR) by dephosphorylating several components of the DDR pathway and negatively regulates p53 by de-phosphorylation. We evaluated the combined effect of WIP1 and PARP-1 inhibitors on cell viability and key proteins in the p53 pathway in a panel of breast and ovarian cancer cell lines.

Material and Methods

A breast cancer cell line (MCF-7) and 2 ovarian (A2780, PA1) cancer cell lines were exposed to combinations of the WIP1 inhibitor (GSK2830371) and 3 PARP inhibitors, Rucaparib, Olaparib and Talazoparib for 72 hours. Cell proliferation was assessed by sulforhodamine B (SRB) assay. The effect of combination treatment with GSK2830371 and PARP inhibitors at 4 and 24 hours on key proteins in the p53 pathway, including p21, MDM2, WIP1 and PARP, was evaluated by western immunoblotting.

Results and Discussions

Rucaparib as a single agent inhibited the proliferation of A2780, PA1 and MCF-7 (GI50 0.7 μ M, 6.34 μ M, >10 μ M respectively) cells, while combination treatment with GSK2830371 (2.5 μ M), significantly potentiated the dose dependent effect of Rucaparib on these cell lines (GI50 0.1 μ M, 0.54 μ M, 2.2 μ M, respectively). Olaparib as a single agent also inhibited the proliferation of A2780, PA1 and MCF-7 (GI50 0.712 μ M, 3.308 μ M, 7.785 μ M respectively) cells, and combination treatment with GSK2830371 potentiated the dose dependent effect of Olaparib (GI50 0.048 μ M, 0.112 μ M, 0.05 μ M, respectively). Talazoparib was the most potent PARP inhibitor and as a single agent inhibited the proliferation of A2780, PA1 and MCF-7 (GI50 5.40nM, 17.42nM, 786nM). Combination treatment with GSK2830371, nevertheless also potentiated the dose dependent effect of Talazoparib (GI50 0.604nM, 0.53nM, 77nM). WIP1 inhibition increased p53 serine-15 phosphorylation in the three PARP inhibitor responding cell lines, with corresponding increases in MDM2 and p21 induction for combination treatment of these three PARP inhibitor with GSK2830371. Moreover, GSK2830371 treatment not only inhibited WIP1 phosphatase activity, but also promoted its degradation.

Conclusion

Chemical inactivation of WIP1 potentiates the response of a subset of breast and ovarian cancer cells to PARP inhibitors. This was reflected by corresponding evidence of p53 pathway activation. Similar potentiation effects were obtained with the three PARP inhibitors

EACR22-0119

Enhancing DNA hypomethylating drug efficacy by inhibition of SUMOylation reduces lymphoma outgrowth in an orthotopic xenograft mouse model

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Introduction

Combining drugs is key to improve cancer therapy efficacy and to reduce treatment associated toxicity. Hypomethylating drug 5-Aza-2'-deoxycytidine (5-Aza-2') activates tumor suppressor genes and induces replication errors via entrapment of DNMT1, resulting in DNA-protein crosslinks (DPCs). DPC clearance is regulated by the post translational modification (PTM) Small Ubiquitin-like Modifier (SUMO). 5-Aza-2' is widely used in the treatment of hematologic malignancies. Therefore we combined the novel SUMOylation inhibitor TAK981 with 5-Aza-2' to treat Burkitt Lymphoma in an orthotopic xenograft mouse model.

Material and Methods

We used the orthotopic xenograft model Namalwa-Luc10 (Burkitt Lymphoma) and injected 1×10^5 cells intravenously. One week after tumor cell injection, treatments were started. TAK981 was used in a concentration of 0.025 to 1 μ M *in vitro* and 25 mg/kg for the *in vivo* model. 5-Aza-2' was used in a concentration of 0.01 to 1 μ M and 2.5 mg/kg for the *in vivo* model. Treatments were carried out twice weekly and tumor outgrowth was monitored using bioluminescence.

Results and Discussions

SUMO inhibition prolonged the entrapment of DNMT1 to the DNA, introducing excessive replication errors, resulting in extensive DNA damage. *In vitro* cell proliferation assays, using a Burkitt lymphoma cell panel, demonstrated significant synergism of these drugs. Combining TAK-981 and 5-Aza-2' significantly ($P = 0.0014$) reduced outgrowth of

Burkitt lymphoma in our orthotopic xenograft model, compared to single agent treatment. Our results highlight the potential of tailored combination therapies, employing epigenetic drugs and PTM inhibitor therapy, based on insight in molecular mechanisms.

Conclusion

Since SUMOylation is a key-player in DPC repair, TAK-981 prolongs the entrapment of DNMT1 to incorporated 5-Aza-2', increasing replication errors. Our results demonstrate the potential of tailored combination of drugs, based on insight in molecular mechanisms, to improve the efficacy of cancer therapies.

EACR22-0147

The role of mTOR inhibitors in the tumor immune microenvironment and PD-L1 expression in bladder cancer

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Introduction

Overexpression of the programmed death-ligand 1 (PD-L1) allows cancer cells to evade the immune response. Immunotherapeutic agents targeting the PD-1/PD-L1 pathway are approved for treatment of locally advanced or metastatic urothelial cancer. However, the role of PD-L1 expression by immunohistochemistry as a predictive biomarker still remains controversial. Combination with agents targeting other pathways is an area of active investigation and several clinical trials are now testing the efficacy of anti-PD-1/PD-L1 antibodies combined with PI3K or mTOR inhibitors. The relevance of crosstalk between mTOR and PD-L1 expression pathways remains largely unexplored in bladder cancer (BC) and we aimed to gain insights into this dual targeting strategy.

Material and Methods

We analyzed the effect of the ATP-competitive inhibitor of the catalytic activity of mTOR TAK-228 on PD-L1 expression in BC cell lines *in vitro*; in *ex vivo* tumor tissue explants cultures of xenografted cancer cells, and in patients' samples. Real-time qPCR, western blot, flow cytometry and immunohistochemistry of FFPE samples were used to study PD-L1 expression. Effects of mTOR inhibition on tumor-immune cell interaction were studied with TAK-228 and IFN- γ - co-treatment of cancer cells or by co-culturing them with immune cells (activated peripheral blood mononuclear cells or CD8+ cytotoxic T-cells) isolated from healthy blood donor.

Results and Discussions

We found that mTOR inhibition with TAK-228, significantly upregulated PD-L1 expression on the surface of BC cell lines. This was also seen in patient's tumor samples treated *ex vivo*. Mechanistically, we showed that this effect is mediated at least partially by the induction of EGF and IFN- β expression since it was reverted with the co-treatment of TAK-228 with anti-EGFR and anti- α IFNAR. TAK-228 was able to increase IFN- γ -induced surface PD-L1 and MHC class I molecules (MHC-I) expression and the IFN- γ -cytotoxic effects. Of note, the cytotoxic anti-tumoral effects of CD8+ T-cells was reduced on TAK-228 treated cells with higher PD-L1 that could potentially be overcome by adding PD-L1 inhibitor treatment.

Conclusion

Our preclinical results in BC models show that TAK-228 induced several immunologic changes in BC, including tumor surface MHC-I and PD-L1 up-regulation. The latest could induce local immune suppression in the tumor and could be a potential target for anti-PD-L1 combination therapy. Combining mTOR and PD-L1 inhibitors may be of therapeutic interest for patients with advanced bladder cancer.

EACR22-0171

NUPR1, a new actor preventing PARP1-dependent cell death

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Introduction

Nuclear Protein 1 (NUPR1) is an intrinsically disordered protein of 82 amino acids induced by stress. NUPR1 plays an important role in cancer development and progression, becoming a promising therapeutic target. By using a multidisciplinary approach combining biophysics, chemistry, bioinformatics and biology, we have developed a NUPR1 inhibitor, named ZZW-115 which induces a strong decrease of ATP and OXPHOS metabolism, triggering ROS production and inducing tumor cell death in vitro and in vivo. By proteomic, cellular and biochemical analysis, we have found that NUPR1 binds to PARP1 into the nucleus. PARP1 is a nuclear protein which catalyzes the covalent attachment of PAR (Poly-ADP-ribose) using NAD⁺ as a donor, under stress conditions, to promote DNA repair. However, when PARP-1 activity becomes uncontrolled, it may also lead to PARP1-dependent cell death, named parthanatos. Our objective was to demonstrate that NUPR1 inhibition, by ZZW-115, induces PARP1-dependent cell death.

Material and Methods

We performed proteomic analysis, enzyme activity assays, metabolic assays, NAD⁺/NADH quantification, proximity ligation assays, ROS quantification and cell viability assays.

Results and Discussions

Our results show that NUPR1 binds to PARP1 preventing deleterious and critical hyperPARylation. Moreover, we observed that treatment of the cells with ZZW-115, the NUPR1 inhibitor, abolished the protective effect of NUPR1 as a PARP1 regulator, inducing strong hyperPARylation, loss of cellular NAD⁺ pool, ROS overproduction and strong mitochondrial dysfunction. These effects were reversed by olaparib (a PARP1 inhibitor) or the NAD⁺ precursor nicotinamide mononucleotide (NMN). In agreement, we also demonstrate that the cell survival is improved by olaparib and worsened by inhibiting dePARylation, indicating that the intracellular level of PARylation control the cell survival during stresses. Interestingly, we also found that ZZW-115 induced the PAR polymer release from the nucleus that interact with the mitochondrial membrane, a key event in parthanatos. However, AIF remains completely limited into the mitochondria with no release to the nucleus, thus, our results suggest that cell death induced by hyperPARylation is mediated by a non-canonical pathway.

Conclusion

In conclusion, inactivation of NUPR1 induces hyperPARylation, which in turn, induces a mitochondrial and metabolic catastrophe and consequently a cell death through a non-canonical Parthanatos.

EACR22-0275

Modulating glioma cells survival by proteasome and HDAC inhibitors treatment

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Introduction

Gliomas are the abnormal growth of glial cells, being the most common form of Central Nervous System neoplasm. The high mortality rate is related to the resistance to the currently available treatments. Molecular-profiling studies revealed very complex signatures associated with different types of gliomas. In order to test new therapies for gliomas treatment, we are investigating the individual and combined effects of the proteasome inhibitors (PI) – Salinosporamide A (Sal A) Bortezomib (BTZ) and Carfilzomib (CFZ), and the histone deacetylase inhibitors (HDACI) – Suberoylanilide Hydroxamic Acid (SAHA) and four novel molecules (3a, 4a, 4b, 6a) – in glioma cell lines.

Material and Methods

Initially, the compounds activity was accessed through the SRB assay in four glioma cells - HOG, T98G, U87MG, and U251MG. Among the PIs, CFZ was generally the most potent one followed by BTZ and Sal A, while for HDACIs, SAHA was more potent than the new synthetic molecules. For a better understanding of the molecular mechanisms involved in the cytotoxicity, SAHA and Sal A, alone and as a combined treatment, were tested using HOG and T98G cells. Expression of indirect target proteins of PI and HDACI were evaluated, by immunoblotting and immunofluorescence, as well as apoptosis induction and cell cycle alterations by flow cytometry.

Results and Discussions

Sal A (80 nM) and SAHA (0.4 µM) tested in combination were higher active in T98G than in HOG cells. Cell cycle analyses showed a six-fold increase in T98G cells subG1 population after the combined treatment when compared to the control and SAHA treatment, and two-fold when compared to the Sal A treatment. The cotreatment led cells to apoptotic process, which was reinforced by the decrease in PARP and increase in cleaved caspase-3, besides p62 decrease expression, indicating also autophagy. The protein profile highlighted the increase of reticulum stress markers, HSP70, and ubiquitinated proteins, and of epigenetic modulation, acetyl-α tubulin, and histone H3 accumulation. Moreover, the co-treatment reduced the number of cells and increased the number of vacuoles, which further confirms the incidence of autophagy.

Conclusion

Based on preliminary results, our data suggest that the combination of SAHA and Sal A can be a viable alternative to treat glioma since low concentrations of the drugs can produce relevant cytotoxicity in cancer cells. Additional studies are underway addressing the

mechanisms involved in anticancer effects of the combined use of proteasome and HDAC inhibitors.

EACR22-0325

A novel late-stage autophagy inhibitor that efficiently targets lysosomes and shows potent cytotoxic effect in lung cancer cells

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Introduction

Overcoming resistance to anticancer treatments is one of the most challenging features in current cancer therapy. Autophagy is a regulated process that degrades damaged proteins and cytoplasmic organelles into the lysosome, contributing to cell homeostasis. In an advanced-tumour context, it confers resistance to treatments as it enables cells to adapt to the stress situations provoked by anticancer therapies. Recent studies have shown that the inhibition of this cytoprotective autophagy leads to tumour cells sensitization. In this regard, a novel potent anionophore compound targeting lysosomes, called LAI-1 was chemically designed and tested to evaluate its role in blocking autophagy and its potential use as a future anticancer treatment.

Material and Methods

Confocal microscopy using a lysosomal tracker was done to check the cellular drug localization. Cell viability after 24 h of known autophagy inhibitors (chloroquine and 3-Methyladenine) and LAI-1 treatments in three lung cancer cell lines with different genetic backgrounds, was evaluated by MTT assay. Western blot and immunofluorescence analysis were performed to examine autophagy flux (p62 and LC3 I/II expression), whereas cell death was studied by western blot (pro-caspase 3 and PARP) and flow cytometry. Acridine orange staining was assayed to study lysosomal pH modifications.

Results and Discussions

Our results show that LAI-1 is able to efficiently transport anions in liposomes and specifically target lysosomes in lung cancer cells while basifying their pH. Significantly lower cell viability was observed after LAI-1 treatment compared to other autophagy inhibitors. In a dose-dependent manner, LAI-1 treatment truncated the autophagy flux by increasing p62 and LC3 II/I ratio expression. Similar to chloroquine, no colocalization was observed between LC3 I/II or p62 and LAMP-1 after LAI-1 treatment, indicating the blockade of autophagosomes and lysosomes fusion. Finally, LAI-1 induced cell death by activating apoptosis machinery (enhancing cleavage of caspase-3 and PARP) without affecting the cell cycle in lung cancer cells.

Conclusion

Altogether, our new compound LAI-1 specifically reaches lysosomes in cancer cells, where it transports anions basifying their pH. This effect results in a blockage of autophagy flux in the late-phase stage by inhibiting the fusion of autophagosomes and lysosomes and consequently, producing cell death at lower concentrations than other known autophagy inhibitors.

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EACR22-0527

Bafilomycin A1 induced ER stress associated colorectal cancer cell specific ferroptosis

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Introduction

Ferroptosis is a type of programmed cell death, that is distinct from necroptosis, autophagy, and apoptosis, and characterized by iron-dependent oxidative damage. Because iron plays role of mediation in the production of reactive oxygen species and enzyme activity in lipid peroxidation, ferroptosis is associated, and controlled by iron metabolism, such as iron uptake, storage, utilization, and efflux.

Material and Methods

In this study, we investigated the anticancer effect of bafilomycin A1 (BaMA1), vacuolar type H⁺-ATPase (V-ATPase) inhibitor, on colon cancer cells *in vitro*, and verified it *in vivo* zebrafish model. Cell viability, and proliferation were measured by MTT, cell counting, and colony assays. Cell death was measured using FITC Annexin V Apoptosis Detection Kit I by FACS. Labile ferrous ion pool (LIP) was analyzed using FerroOrange. Lipid peroxidation was analyzed by BODY staining. qRT-PCR, and western blotting were used to measure expression of various molecules.

Results and Discussions

BaMA1 block autophagy and induced colon cancer cell specific cell death, which was associated with PERK pathway caused by ER stress, with very limited cytotoxicity to normal colon fibroblast cell, CCD-18Co. BaMA1 induced cell death was caspase independent, and showed increased cellular lipid peroxidation. And, BaMA1 induced cell death was recovered by co-treatment of ferric citrate, which is indicating iron homeostasis associated ferroptosis. Further investigation of deeper mechanism is ongoing.

Conclusion

BaMA1 induced ER stress associated colorectal cancer cell specific ferroptosis.

EACR22-0683

Interaction of the isomers carvacrol and thymol with Doxorubicin against breast cancer cells

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Introduction

The potential for natural products to interact with chemotherapy has been reported in different cancer cell lines that may increase anticancer activity and reduce the effectiveness or exacerbate the toxicity of conventional drugs. Herein, we will extend the previous line of research by studying the chemo-modulatory effect of the isomers carvacrol and thymol on the toxicity of Doxorubicin in breast cancer cell lines.

Material and Methods

MTT assay was used to evaluate the effect of carvacrol and thymol on the cytotoxic profile of Doxorubicin in breast cancer cell lines (MCF-7 and MDA-MB231). cell death was further assessed by using annexin-V/FITC staining to evaluate the apoptotic and necrotic cells and Acridine orange staining coupled with flow cytometry was used to determine the autophagic cell death.

Results and Discussions

After 72 h of treatment, carvacrol and thymol showed cytotoxic effects against MCF-7 with IC₅₀'s of 124.3±15.1 µM and 112.2±1.2 µM respectively. Combining carvacrol and thymol with DOX increased its IC₅₀ from 1.18±0.7 to 2.8±0.4 µM in combination with thymol and to 3.5±0.5 µM in combination with carvacrol. Similarly, in MDA-MB231 cells, the combination of carvacrol and thymol with DOX resulted in a slight increase in its IC₅₀ (1±0.03 to 1.4±0.5 and 0.1.3±0.2 µM).

However, using annexin-V/FITC staining, carvacrol and thymol significantly increased the percent of apoptotic/necrotic cell death in MDA-MB231 cells after combination with Doxorubicin. we further investigated the effect of the combination of carvacrol and thymol with DOX on the autophagy process within MCF-7 and MDA-MB231 cells using Acridine orange staining coupled with flow cytometry. Similar to what was observed with apoptosis, the combination of carvacrol and thymol with DOX significantly induced autophagic cells higher than DOX treated cells.

Conclusion

In conclusion, carvacrol and thymol possess potential chemo-modulatory effects to Doxorubicin against breast cancer cells. Despite this promising anticancer activity, a careful characterization is needed to establish a reference point for further analysis of carvacrol and thymol and their potential derivatives, which may provide a novel mechanism targeting clinical treatment and prevention of cancer.

EACR22-0699

Auranofin mediates the killing of mutant p53 non-small cell lung cancer cells through distinct molecular cell death mechanisms and innate immunity

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Introduction

Auranofin (AF) is an FDA-approved antirheumatic drug with anticancer properties that acts as a thioredoxin reductase (TrxR) inhibitor. The exact mechanisms through which AF targets cancer cells remain elusive. To shed light on its mode of action, this study provides an in-depth analysis on the molecular mechanisms and immunogenicity of AF-mediated cytotoxicity in non-small cell lung cancer (NSCLC).

Material and Methods

Protein levels of TrxR and thioredoxin (Trx) were assessed in a panel of 72 NSCLC patient's tumor samples via immunohistochemistry. Sensitizing biomarkers were investigated in a NSCLC cell panel with differing p53 status. The molecular response to AF was studied by transcriptome analysis in the NCI-H1299 NSCLC cell line (p53 Null) and its two isogenic derivatives with mutant p53 R175H or R273H accumulation. Relevant findings regarding (immunogenic) cell death, DNA damage and natural killer (NK) cell-mediated killing were validated at the protein and functional level with appropriate techniques.

Results and Discussions

TrxR was highly expressed in the 72 NSCLC tumor samples, making it a valid druggable target for AF in NSCLC. Mutant p53 overexpression was identified as an important sensitizer for AF in the (isogenic) NSCLC cells. Transcriptome analysis revealed the dysregulation of genes involved in oxidative stress response, DNA damage, granzyme A (Gzma) signaling and ferroptosis after AF treatment. Although functionally AF appeared a potent inhibitor of GPX4 in all NCI-H1299 cell lines, the induction of lipid peroxidation and consequently ferroptosis was limited to the p53 R273H expressing cells. In the p53 R175H cells, AF mainly induced large-scale DNA damage, leading to the induction of caspase-dependent apoptotic cell death rather than ferroptosis. Importantly, all cell death types were immunogenic since the release of danger signals (ecto-calreticulin, ATP and HMGB1) and dendritic cell (DC) maturation occurred irrespective of (mutant) p53 expression. Finally, we show that AF primed cancer cells to caspase-independent NK cell-mediated killing by downregulation of several key targets of Gzma.

Conclusion

These novel data demonstrate the therapeutic potential of AF as a versatile, clinically available and off-patent cancer drug that kills mutant p53 expressing NSCLC cells via distinct cell death mechanisms (apoptosis and ferroptosis). In addition, AF improves the innate immune response at both cytostatic (NK cell-mediated killing) and cytotoxic concentrations (DC maturation).

EACR22-0839

Identification and validation of a specific direct-binding survivin inhibitor that induces in vitro and in vivo anticancer effects

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Introduction

Evasion of apoptosis is one of the most predominant mechanisms of treatment resistance in human cancers. In this regard, overexpression of antiapoptotic proteins, such as survivin, has been described in several tumors, contributing to this clinical problem. Survivin has a dual role in key cellular functions, either inducing cell cycle progression and inhibiting apoptosis, which makes survivin a good target for cancer therapy. Hence, we focused on identifying and validating a novel specific direct-binding survivin inhibitor for cancer treatment and tumor sensitization to conventional proapoptotic therapies.

Material and Methods

We conducted a structure-based high-throughput virtual screening (HTVS) at the survivin homodimerization domain. We evaluated the survivin inhibitor effects on cancer cells by MTT cell viability assays. Surface plasmon resonance (SPR) assays were conducted to validate the interaction between survivin and the identified inhibitor. Survivin protein inhibition was evaluated by western blot in lung cancer cells. To assess the safety profile in vivo, multiple doses of the inhibitor were intraperitoneally administered to C57BL/6j mice. In vivo efficacy was assessed in an ectopic mice model, where LLC1 lung cancer cells were inoculated to induce subcutaneous tumors. Survivin inhibitor was evaluated in combination with conventional chemotherapy in lung cancer cells by viability assays and the synergistic effect was evaluated by Compusyn software®.

Results and Discussions

Asenapine Maleate (AM), an approved drug for central nervous system diseases, was identified as a direct binder at the survivin homodimerization domain. AM treatment significantly affected cell viability of lung, colon and brain cancer cell lines. Direct interaction of AM to survivin protein was corroborated by SPR and specific survivin protein decrease was observed in cancer cells, compared to other inhibitor of apoptosis proteins. No severe toxicity was detected at the assayed doses and therapeutic in vivo analyses showed an impairment in tumor growth of AM treated mice. Finally, a synergistic anticancer effect was detected in vitro when combined with some conventional chemotherapies.

Conclusion

Altogether, our results identify AM as a specific direct binder of survivin, which shows anticancer properties in vitro and in vivo and synergistic effect in combined therapies, opening the possibility of its repositioning for cancer treatment.

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EACR22-1041

The synergistic interaction of the BET antagonist GS-626510 with the PI3Kδ inhibitor idelalisib in diffuse large B-cell lymphoma involves the blockade of the tumor-macrophage crosstalk

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Introduction

The presence of intratumoral M2-macrophages have recently been shown to define a subgroup of diffuse large B-cell lymphoma (DLBCL) patients with worst prognosis. The germinal center (GCB) subtype of this disease is considered to rely partially on the activity of the proto-oncogene c-Myc and its regulation by bromodomain and extra-terminal (BET)

proteins, leading to extensive preclinical evaluation of BET inhibitors in GCB-DLBCL. As these agents have also demonstrated ability to modulate PI3K/AKT signaling, and given that about 55% of GCB-DLBCL cases harbor a constitutive activation of the PI3K pathway, we investigated the hitherto combination of the first-in-class PI3K δ i, idelalisib, with the novel BETi, GS-626510, in preclinical models of GCB-DLBCL.

Material and Methods

Eight GCB-DLBCL cell lines and primary samples were cultured with M2-polarized macrophages and subjected to gene expression profiling (GEP). An ELISA-based assay was used to quantify a set of 174 cytokines in co-culture supernatants. Idelalisib-GS-626510 drug interaction was evaluated in vitro by MTT assay and combination index determination, followed by GEP analyses and cytofluorimetric and western blot analysis of apoptotic machinery. Drug safety and efficacy were evaluated in vivo in a Pfeiffer-GFP+Luc+ xenotransplant model of DLBCL.

Results and Discussions

Idelalisib induced a 20%–30% antitumoral effect at 72h in GCB-DLBCL cultures regardless the presence of M2-macrophages, and despite the transcriptional activation of genes related to tumor survival and cytokine-mediated signaling in tumor B cells, and a >2-fold increase in the secretion of 36 soluble cytokines by M2/GCB-DLBCL co-cultures. Exposure to idelalisib/GS-626510 combo counteracted this phenomenon, impairing the production of CCL13, PPBP, IL-1RN, IL-10, and CCL15 by CD20+ cells. This effect was associated with the downregulation of the antiapoptotic proteins BCL-2, BCL-XL, MCL-1 and BFL-1, and with a synergistic triggering of apoptosis even in M2-sustained GCB-DLBCL cultures. In vivo, when compared to idelalisib and GS-626510 single agents, the combo treatment arm underwent a 2-3 fold increase in relative tumor growth inhibition, reaching 85% after two weeks of daily dosing of the animals, with no detectable toxicity.

Conclusion

The combination of idelalisib and GS-626510 is able to disrupt GCB-DLBCL crosstalk with M2-macrophages, thereby modulating the secretion of several inflammatory cytokines involved in the regulation of tumor B cell apoptotic threshold.

EACR22-1165

EWS/FLI1 interacts with DHX9 promoting R-loop accumulation and drug sensitivity in Ewing Sarcoma

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Introduction

Ewing sarcoma (EwS) is an aggressive mesenchymal neoplasm mainly occurring in children and young adults. Genetically, EwS is characterized by a pathognomonic chromosomal

translocation, most often involving the EWSR1 and FLI1 genes. Although long-term survival rates for patients with localized disease are of 70-80%, patients with metastatic disease still present a dismal prognosis with long-term survival rates of around 30%. In fact, >25% of patients present metastasis at diagnosis. Currently, EwS treatment remains based on conventional regimens, including genotoxic agents (GA) such as Etoposide or Irinotecan combined with radiotherapy and/or surgery. Sadly, therapeutic failure, lack of response or resistance, occurs in 25-50% of the cases. This stresses the need for an in-depth understanding the mechanisms underlying GA-sensitivity.

Material and Methods

EwS and non-EwS models were treated with Irinotecan/SN-38, Etoposide or Cisplatin. A673/TR/shEF cell line (carrying an inducible shRNA against EWS/FLI1), was used to evaluate the role of EWS/FLI1 in sensitivity. The correlation between DHX9 levels and patient outcome was evaluated in a cohort of clinically annotated EwS patients (n=200).

Results and Discussions

Using *in vitro* and *in vivo* models, we observed that, when compared to other sarcomas, EwS present a higher sensitivity to GA resulting from a stronger induction of drug-associated DNA damage. Further analysis on factors modulating induction of DNA lesions, revealed that EwS models accumulate higher levels of R-loops than other sarcomas. In turn, we observed that interaction between EWS/FLI1 and DHX9 (a helicase implicated in R-loop metabolism) is a source of R-loop accumulation in EwS. In addition, our results indicate that EWS/FLI1-DHX9 binding reduces drug-induced R-loop resolution promoting replicative stress, genome instability and GA cytotoxicity. In agreement with this, both DHX9 overexpression and inhibition of EWS/FLI1-DHX9 interaction with YK-4-279 reduces drug-induced DNA damage and promotes drug resistance. Finally, we found a significant correlation between poor patient outcome and higher expression levels of DHX9, further supporting the role of DHX9 in drug sensitivity.

Conclusion

Herein, we describe that EWS/FLI1-DHX9 interaction leads to a defect in the resolution of R-loops, suggesting a novel mechanism of drug sensitivity in EwS and a potential role of DHX9 as a predictive factor.

EACR22-0946

Exploring the Role of Endoglin in the aggressiveness of Ewing Sarcoma and Clear Cell Sarcoma

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Introduction

Ewing Sarcoma (ES) and **Clear Cell Sarcoma** (CCS) present overlapping features, mostly associated to the presence of specific chromosomal translocations involving the gene EWSR1. **Endoglin** (ENG) is a mesenchymal stem cell marker typically expressed by active endothelium. Herein, we analyzed ENG expression in Patients and Patient-Derived

xenografts (PDXs) of ES and CCS. In addition, we studied the functional role of ENG in ES, using genetically engineered experimental models. In order to define new potential targets specific to CCS, we performed **Optical Genome Mapping** (OGM) a new cytogenetic method for the comprehensive characterization of **Structural Variants** (SVs).

Material and Methods

ENG expression was analyzed by Immunohistochemistry and q-RT-PCR in ES, CCS patients and CCS PDXs. Transcriptomic and proteomics analyses were performed in ENG downregulated/upregulated models of ES. Functional assays were carried out to evaluate filopodia formation and cell spreading upon ENG modulation. Additionally, CCS samples were studied by OGM. Ultra-high molecular weight DNA was purified from tissue and genomic DNA was labeled using a Direct Label and Stain DNA Labeling Kit. The Saphyr chip was ran to reach a minimum yield of 1300 Gbp for rare variant analysis. Rare variant pipeline and variant annotation pipeline were executed on Bionano Solve V3.7 and reporting and direct visualization of structural variants was done on Bionano Access v1.7.

Results and Discussions

ENG was heterogeneously expressed in ES and overexpressed in CCS patients and PDX models. An in-depth transcriptomic and proteomic profiling of *in vitro* ES models showed that ENG is relevant for ES cell mechanotransduction. Functional assays confirmed that loss of ENG disrupts actin filaments assembly and filopodia formation along the cytoplasm, affecting cell spreading. Moreover, ENG regulates cell-matrix interaction by focal adhesion signaling. OGM analysis in CCS showed the presence of previously unreported undescribed SVs such as unbalanced translocations, associated with the presence of chromoplexy, which might be relevant in the clinical setting

Conclusion

Overall, our results show that ENG plays a significant role in ES, favoring aggressiveness, and suggest that ENG might also play a relevant role in CCS. Additionally, OGM represents a novel tool for the discovery of new potential targets or biomarkers.

EACR22-0078

Nuclear protein export inhibitor chemotherapy combination broadly impacts pancreatic cancer signaling networks

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Introduction

The majority of pancreatic ductal adenocarcinoma (PDAC) patients experience disease progression after Gemcitabine-nab-Paclitaxel (GemPac) treatment indicating the need for more effective combination therapies for this highly recalcitrant disease. Earlier we showed that nuclear exporter protein exportin 1 (XPO1) is a valid therapeutic target in PDAC and selective inhibitor of nuclear export (SINE) selinexor (Sel), synergistically enhances the efficacy of GemPac in pancreatic cancer cells, spheroids, patient derived tumors and showed evaluable response in PDAC patients in a Phase I study. Here we investigated the mechanisms of this synergy utilizing PDAC cellular models *in vitro* and LSL-Kras G12D/+; Trp53 fl/+; Pdx1-Cre (KPC) mouse model.

Material and Methods

We used single-nuclei RNA sequencing in KPC tumors, and poly A RNA sequencing in PDAC cells post sel-Gem-Pac treatment. Prioritized sn-seq and RNA-seq identified molecules were validated in *in vitro* or in the PDAC patient samples through siRNA mediated silencing, quantitative gene expression, cytotoxicity and related assays.

Results and Discussions

In KPC model, selinexor-GemPac caused statistically significant enhancement in survival compared to controls ($p < 0.05$). Immunohistochemical and molecular analysis of residual KPC tumors showed stromal architecture changes, suppression of proliferation and nuclear retention of tumor suppressors in the treated mice. Additionally, the KPC derived primary tumor cells showed reduced colony formation upon combination treatment. Sn-seq showed reduction in tumor cell heterogeneity (less cell clusters based on gene expression), CDK8, CD44 and Camk1d stem cell and EMT related genes alongside significant alterations in mitochondrial signaling after combination treatment. In cellular models, GO and GSEA analyses of RNA-seq data predicted DNA templated regulation of transcription and DNA replication associated genes in Sel or Sel-GemPac mediated growth inhibition. The top downregulated genes including drug resistant related gene PFN1 and HSP90 identified in Sel or Sel-GemPac treated PDAC cells from RNA-seq data were validated using RNAi and confirmed their role in synergy.

Conclusion

Taken together Selinexor in combination with Gemcitabine-nab-Paclitaxel enhanced the survival of KPC mice through inhibition of well-connected tumor promoting molecules resulting in broad penetration in PDAC supporting signaling.

EACR22-0106

Chromomycin A5 is a novel inhibitor of the oncogenic TBX2 in breast cancer

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Introduction

Breast cancer (BC) is the most common cancer worldwide, with over 2 million new cases diagnosed in 2020 alone. The current breast cancer treatments options are limited by both drug resistance and severe side effects. Therefore, targeted therapies have emerged as an attractive strategy for the treatment of BC. To this end, the transcription factor TBX2, which is commonly overexpressed in BC, has been identified as an attractive therapeutic target. Indeed, TBX2 acts as an oncogene in BC where it drives proliferation and promotes DNA damage repair to suppress cell death and confer drug resistance. We previously identified the marine natural compound, Chromomycin A5 (CA5), to have strong TBX2-binding affinity and here we show that CA5 exhibits anti-BC activity through targeting TBX2 for degradation.

Material and Methods

Short- and long-term cytotoxicity of CA5 were tested in two TBX2-driven estrogen-receptor positive BC cell lines (MCF-7 and T47D) and non-malignant breast epithelial cells (MCF-12A), by MTT and clonogenic assays respectively. The impact of CA5 on BC stem cells and 3D-growth was investigated in mammosphere assays and spheroids. To determine the effect of CA5 on TBX2 levels the proteasome-26s pathway was inhibited using MG-132 and western blotting was performed. qRT-PCR was carried out to investigate if CA5 blocks the ability of TBX2 to transcriptionally repress its tumour suppressor target genes. To determine the mechanism by which CA5 exhibits anti-BC activity, the effect of CA5 on DNA damage,

cell cycle progression, and apoptosis was assessed by immunofluorescence, western blotting, and flow cytometry. Finally, to assess whether the anti-BC activity of CA5 involved its ability to target TBX2, we altered the levels of TBX2 using shRNA and TBX2 expression constructs.

Results and Discussions

We show that CA5 exhibits potent and selective short and long-term cytotoxicity in BC cells in both 2D and 3D models and that this is dependent on TBX2 levels. Indeed, depleting TBX2 de-sensitized BC cells to CA5 and over-expressing TBX2 enhanced their sensitivity to CA5. Importantly, we found that CA5 targets TBX2 for degradation through the proteasome-26S pathway resulting in the de-repression of several TBX2 tumour suppressor target genes (p21, PTEN, CST6 and NDRG1). Mechanistically, we demonstrate that CA5 induces DNA damage (γH2AX), cell cycle arrests and both intrinsic and extrinsic apoptotic pathways (caspase and PARP cleavage).

Conclusion

C-A5 is promising for the treatment of TBX2-driven breast cancers.

EACR22-0234

Validation of ERK inhibitor-based drug combinations active on LKB1-mutated NSCLC

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Introduction

Non-small cell lung cancer (NSCLC) is the leading cause of cancer-related death worldwide. The advent of targeted therapies and immunotherapy have changed the management of lung cancer and have improved the overall survival of patient harboring this disease. However, LKB1-mutated NSCLCs do not take advance from these therapies, so patients with this tumor continue to have a poor prognosis. Previous studies in our laboratory demonstrated the sensitivity of LKB1-mutated tumour models to the pharmacological inhibition of ERK. However, the presence of co-mutation renders the LKB1-mutated cancer resistant also to this therapy. In this scenario, the aim of this project is to find ERK inhibitor synthetic lethal partners in order to enlarge NSCLC patient population amenable to this therapy.

Material and Methods

We performed high-throughput screenings of a FDA-approved drug library on two isogenic systems composed by the LKB1 wild-type parental cell line and the CRISPR-CAS9-derived LKB1 deleted clone. We treated the cell lines with the FDA-approved library alone or in combination with a subtoxic concentration of ERK inhibitor. Following the analysis of the data, we validate the hit-combinations with independent cell viability assay.

Results and Discussions

From the screenings, we highlighted as 'hit' those combinations active just on LKB1-deleted clones. Seventy-two hit combinations were highlighted from the two isogenic system, with some compounds in common. Among those hits, the most common class of FDA-approved compounds were the PI3K/Akt/mTOR inhibitors. Moreover, from the subsequent cell viability assays, we validate the screening results of these PI3K/Akt/mTOR and ERK inhibitors combinations and other four hit combinations with FDA-compounds belonging to different classes of drug, as tyrosine kinase receptor inhibitors, MAPK inhibitors and compounds involved in anti-inflammatory pathways, metabolism and DNA damage. These combinations have been validated in four different isogenic systems and we are now performing

validations on a panel of NSCLC cell lines and organoids. We are studying also the mechanism of action of these combinations.

Conclusion

We highlighted different ERK inhibitor-based combinations worthy to be studied. Further analyses are ongoing to validate these hit combinations also on *ex vivo* and *in vivo* systems. Moreover, we are deeply understanding the mechanism of action of them. Positive results could give the chance to develop effective and specific therapies for patient with LKB1-mutated NSCLCs.

EACR22-0360

Antibody-Peptide Conjugates (APeC) blocking cysteine cathepsins activity as a new therapy for B-cell lymphoma and solid tumors

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Introduction

Cathepsin proteases expression and activity are frequently altered in multiple cancers. For example, cathepsin B overexpression is associated with metastasis formation in breast cancer, while cathepsin S plays a role in antigen processing and development of an anti-tumor immune response in lymphoma. Therefore, inhibiting cathepsins activity might reduce tumor metastasis or increase tumour immunogenicity.

Material and Methods

To efficiently block cathepsin activity, we developed non-natural peptide inhibitors (NPIs) by embedding a Michael acceptor in the sequence of a natural substrate to covalently bind and block specific cathepsin proteases. To overcome short half-life and poor membrane permeability of peptides, we conjugated NPIs with antibodies, thereby creating antibody-peptide conjugates (APeCs) for the specific targeting of tumor cells. Using multiple tumor cell lines and semi-humanized mouse models we assessed APeCs therapeutic efficacy *in vitro* and *in vivo*.

Results and Discussions

Initial biochemical assays showed that NPIs efficiently inhibit specific cathepsins, and selected structural changes allow to improve their potency or change their specificity towards a different cathepsin. Selecting multiple antibodies, we observed that APeCs retain good binding efficiency towards surface antigens, they are internalized and able to block intracellular and extracellular cathepsins activity in lymphoma, breast and bladder cancer. In particular, inhibition of cathepsin S using APeC targeting lymphoma cells alters MHC-II maturation, resulting in accumulation of uncleaved MHC-II chaperon protein CD74. In addition, specific inhibition of cathepsin B using APeC targeting breast cancer cells hinders

cell migration and invasion. Next, we used semi-humanized mouse models to assess the therapeutic efficacy of APeCs *in vivo*. Importantly, we observed tumor regression in animals treated with an APeC blocking the activity of cathepsin S, and this was associated with expansion of CD8⁺ T-cells, suggesting increased tumor immunogenicity.

Conclusion

We have developed an innovative strategy to design and deliver to tumor cells potent and specific non-natural peptide inhibitors (NPIs) of cysteine proteases. Notably, inhibition of cathepsin B or cathepsin S in tumor cells using antibodies for precise cellular delivery hinders tumor invasion and induces tumor regression. Our results support the implementation of this innovative drug design in multiple contexts.

EACR22-0388

The novel nonnucleoside DNMT inhibitor (DNMTi) MC3343 induces depletion of DNMT1 and synergizes with DNA damaging drugs in Ewing sarcoma cells

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Introduction

DNA methylation was demonstrated to have an important role in Ewing sarcoma (EWS) tumorigenesis and progression. This encouraged us to study the therapeutic potential of DNMT inhibitors in EWS. Unlike genetic alterations, DNA methylation is reversible, which makes it extremely interesting for therapeutic approaches.. In this study, we evaluate the efficacy of the compound MC3343, a new quinoline-based DNMTi with high activity against cancer cells, including osteosarcoma

Material and Methods

A panel of EWS human cell lines were treated with MC3343 ,5-Aza-2'-deoxycytidine (5azadC) alone or in association with Doxorubicin and/or Talazoparib for up to 72h. DNMT1 expression was detected by western blotting while DNMT activity was quantified with an EpiQuick DNA Methyltransferase Activity/Inhibition Assay. The DNA methylation profiles of the samples were obtained using the Infinium Methylation EPIC Array (850 K). H2AX spots and micronuclei were detected by fluorescent immunostaining

Results and Discussions

MC3343 is reported to bind to DNA and to destabilize the DNMT1–AdoMet DNA complex. Here, we demonstrated that MC3343 specifically inhibits DNMT1 expression and activity in EWS cells, but it does not affect the methylation status of EWS cells. Disruption of DNMT1 induced by MC3343 resulted in the loss of cell proliferation, caused stalled forks and activation of the DNA damage followed by progressive DNA instability and cell death. Accordingly, we observed cells arrest in the G1 phase, formation of γH2AX foci and of micronuclei. In cells with p53wt, the common condition of EWS, inactivation of DNMT1 by MC3343 induced phosphorylation of p53 on Ser15, a residue that is targeted by ATM and whose phosphorylation has been correlated with accumulation of p53 and activation of apoptosis. The pretreatment of cells with the MC3343 increased the cytotoxicity of DNA damaging agents, such as doxorubicin and talazoparib. Combination indexes ranged from

moderate to strong synergism and the formation of micronuclei and of γ -H2AX positive foci was significantly higher in combined treatments.

Conclusion

Overall, we showed that Inactivation of DNMT1 by MC3343, rather than effects on DNA methylation, results in a G1 phase arrest and triggers cell death. The demonstration that MC3343 treatment increases the efficacy of doxorubicin and PARPi in killing EWS cells offers the possibility of effective combined treatments

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EACR22-0530

Real-World Evidence of Anlotinib in Patients with Advanced Hepatocellular Carcinoma and Clinical Role of α -fetoprotein

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Introduction

Anlotinib, a novel multi-targeting tyrosine kinase inhibitor (TKI), has been investigated in a variety of malignant tumors and has shown efficacy in clinical trials for hepatocellular carcinoma (HCC). This study was designed to compare the efficacy of anlotinib versus some classic protein kinase inhibitors (PKIs) as a first- or second-line therapy for advanced or metastatic HCC, and to identify the early predictive biomarkers for treatment response.

Material and Methods

Between August 6, 2013, and October 18, 2021, 158 patients with locally advanced or metastatic HCC receiving PKIs agents were enrolled in this study. 54 were assigned to anlotinib group and 104 were assigned to other PKIs (sorafenib, lenvatinib, regorafenib, apatinib) group. 24 or 30 patients were treated with anlotinib as first- or second-line therapy. The number of patients treated with other PKIs as first- or second-line therapy was 51 or 53. Radiological imaging was done for all patients every 6 weeks after the first intervention dose. Laboratory test results were collected from the time of drug assignment to 30 days following treatment discontinuation. The efficacy of the treatment was assessed by the tumor response and survival.

Results and Discussions

Median OS for anlotinib group was 14.8 months versus 11.7 months for other PKIs group ($P=0.005$). The median PFS was 5.7 months versus 5.1 months ($P=0.100$). Subgroup comparisons revealed baseline AFP concentration was the only factor that would affect treatment options. Among patients with a baseline AFP concentration of 400 ng/mL or greater, median PFS was 6.6 months for the anlotinib group versus 3.8 months for the other PKIs group ($p=0.002$). Anlotinib only exhibited superior OS in second-line therapy. The objective response rate (ORR) was 10.0% versus 9.4% ($P=0.933$), and the disease control rate (DCR) was 66.7% versus 56.6% ($P=0.368$). A multivariate analysis identified AFP response (a reduction in AFP of $\geq 40\%$ from baseline) as an independent predictor of disease control in patients undergoing anlotinib treatment ($P=0.001$).

Conclusion

Anlotinib showed promising efficacy as a potential second-line monotherapy for HCC. AFP response was a early predictor of disease control in patients receiving anlotinib.

EACR22-0714

Targeting tumor-stroma communication by blocking endothelin-1 receptor sensitizes high-grade serous ovarian cancer to PARP inhibitors

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Introduction

The continuous signal exchange between tumor cells and the tumor microenvironment (TME) shapes their malignant phenotype and limits poly (ADP-Ribose) polymerase (PARP) inhibitor (PARPi) therapy response in high-grade serous ovarian cancer (HG-SOC) with high rate of TP53 mutations. In HG-SOC, the alliance between the ET-1 receptor (ET-1R)/ β -arrestin1 adopts a chemotherapy drug escaping strategy by sustaining the YAP/mutant p53 (mutp53) transcriptional integration. Therefore, a deeper understating of the mechanisms instructed by ET-1R acting both in the tumoral and in the TME contexts may elucidate the mechanisms of PARPi sensitivity.

Material and Methods

RNA-seq and TCGA dataset were used for the analysis of the transcriptome of patient-derived (PD) HG-SOC cells (PMOV10) and for the establishment of survival analysis in HG-SOC patients. By loss-of-functions approaches, PMOV10 cells and HG-SOC cell lines were analysed for cell viability, DNA-damage effects and response to pharmacological treatments. Co-cultures of HG-SOC/Endothelial cells (EC) and HG-SOC/fibroblasts and spheroid sprouting assay were used to study HG-SOC/TME communication. *In vivo* efficacy of combination therapies was evaluated in PD xenograft model.

Results and Discussions

RNA-seq analysis in HG-SOC cells revealed that the most significant differentially expressed signaling in response to ET-1R activation belonged to hypoxia-related pathways. Mechanistically, the simultaneous ET-1 axis activation in HG-SOC cells, EC and activated fibroblasts instructs the formation of a transcriptional complex composed by p53/YAP/hypoxia inducible factor-1 α (HIF-1 α) that leads to the enhanced release of a wide array of molecules, including ET-1 and VEGF, contributing to HG-SOC metastatization, TME reprogramming and PARPi evasion. The ET-1R antagonist macitentan, dismantling the ET-1R-mediated p53/YAP/HIF-1 α network, interferes with HG-SOC/stroma interactions and inhibits the acquisition of malignant traits and metastatic progression, enhancing both *in vitro* and *in vivo* the survival benefit of the PARPi olaparib.

Conclusion

These findings recognize in the networking between YAP/p53/HIF-1 α guided by ET-1 axis a tumor/TME shared escaping strategy from DNA damaging agents. The combinatorial use of

macitentan with PARPi, interfering with such signaling network, provides a novel and promising therapy for HG-SOC.

EACR22-0758

ON203: A new antibody targeting the oxidized form of macrophage migration inhibitory factor demonstrates antitumorigenic activity in preclinical models

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Introduction

Macrophage migration inhibitory factor (MIF) is a pleiotropic cytokine whose overexpression is associated with tumor aggressiveness, metastasis, and disease progression. Due to its ubiquitous nature, MIF is considered an unsuitable target for therapeutic intervention. However, a disease-related isoform of MIF, oxidized MIF (oxMIF), is present in solid tumors but not in normal tissues. A 1st-generation anti-oxMIF monoclonal antibody (mAb) demonstrated an acceptable safety and efficacy in a Ph 1 trial in patients with malignant solid tumors. We are developing ON203, a bioengineered 2nd-generation anti-oxMIF mAb with highly improved biophysicochemical and biological properties, designed to exert better tumor retention and effector functions resulting in higher efficacy.

Material and Methods

Cell-based assays were performed to evaluate antibody-dependent cellular cytotoxicity and antibody-dependent cellular phagocytosis in the presence of oxMIF-displaying target cells. Tumor penetration was assessed by infrared-labeled ON203 injected in tumor-bearing mice. Human cancer cell-line xenografted mice were treated with different concentrations of ON203, and efficacy was assessed by tumor volume measurements and by immunohistochemical analysis.

Results and Discussions

The 2nd-generation oxMIF antibody ON203 demonstrated strong immune cell effector functions in reporter- and PBMC-mediated tumor cell killing assays. In tumor-bearing mice, ON203 accumulated in the tumor tissue and was retained for at least 7 days. ON203 significantly reduced tumor volumes in therapeutic and prophylactic experimental treatment of xenografted mice. Compared to the isotype control antibody, Ki67 proliferation marker was substantially decreased in ON203-treated tumors. Furthermore, tumor cell intravasation was suppressed by ON203, which may be reflected in reduced metastatic potential. Ongoing investigations of alterations in tumor cell-intrinsic signaling (e.g., pAKT) and characterization of infiltrating immune cells will further widen the understanding of the anti-tumorigenic mechanisms of ON203. Our results substantiate a Ph 1 trial evaluating safety, tolerability, pharmacokinetics, and pharmacodynamics in patients with solid tumors.

Conclusion

ON203 has a high potential to become a new treatment option for patients with solid tumors as a standalone therapy or in combination with checkpoint inhibitors, chemotherapeutics, anti-angiogenic drugs, or kinase inhibitors.

EACR22-1000

Actionable genetic screens unveil the dual targeting of AURKA and MEK as an alternative therapeutic approach for melanoma treatment

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Introduction

The introduction of systemic targeted therapies (BRAF and MEK inhibitors) and immunotherapy (CTLA-4 and PDL-1 immune checkpoint inhibitors) dramatically improved melanoma patients overall survival rates. Nevertheless, the majority of patients commonly experience recurrences and primary/ acquired resistances, as well as severe immune-related adverse events due to immunotherapeutic regimes. Therefore, more predictive preclinical assays are needed to further support preclinical and translational research, identifying novel druggable vulnerabilities to design more effective therapeutic strategies.

Material and Methods

Making use of our cohort of metastatic melanoma patient-derived xenografts (PDXs), we performed parallel *in vivo* and *ex vivo* drop-out genetic screens by means of a shRNA library targeting genes, for which drugs are already available and could be repurposed for melanoma treatment. We rationally combined targeted therapy agents to reach additive effect and we compare that to the standard-of-care treatment.

Results and Discussions

We proved that our *ex vivo* migration-based genetic screen is a feasible approach to isolate novel melanoma actionable vulnerabilities, predictive of drug efficacy. Our *ex vivo* system emerged as a reliable preclinical platform to define patients drug sensitivity profiles and identify alternative therapeutic strategies. In this setting, we proved that the dual targeting of Aurora kinase A (alisertib) and Mitogen-activated protein kinase MEK (trametinib) (AT) is highly effective in a wide panel of metastatic PDX, regardless their heterogeneous genetic background. Moreover, AT combination showed to outperformed the standard-of-care therapy (in BRAF-mutant PDXs) and to overcome resistance in therapy-resistant models. In two metastatic melanoma PDXs, AT combination dramatically inhibited *in vivo* tumor growth and significantly prolonged mice overall survival. AT revealed additive effect of pathways modulation, transcriptionally regulating a wider set of genes, compared to both monotherapies and unveiling novel points of intervention for a triple combination.

Conclusion

Ex vivo migration assay is a reliable tool to screen for drug sensitivity in highly translational valuable models. Alisertib trametinib combination is a promising alternative therapeutic approach that may be particularly beneficial for therapy-resistant patients.

EACR22-1008

The second generation histone deacetylase inhibitor quisinostat synergises with cisplatin and the PARP inhibitor talazoparib in urothelial cancer

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Introduction

Epigenetic regulators, such as class I histone deacetylases (HDAC1, 2, 3, 8) are frequently dysregulated in urothelial cancers (UC) and are consequently interesting targets for new

therapies. The second generation histone deacetylase inhibitor (HDACi) quisinostat, which targets HDAC1, was shown to have moderate effects on cell cycle distribution and apoptosis induction in urothelial carcinoma cell lines (UCC) and to be tolerated by the normal uroepithelial HBLAK at higher doses compared to other HDACi such as romidepsin.

Interestingly, quisinostat leads to an increase in the DNA double strand break marker γ H2AX in UCC, indicating that it might be a suitable combination partner for DNA damage inducing agents and DNA repair inhibitors.

In this study, we tested the effect of combining quisinostat either with cisplatin or with the PARP inhibitor talazoparib.

Material and Methods

Dose-response curves for single and combination treatments were done with normal HBLAK cells and 3 UCC (VM-CUB1, UM-UC-3, J82) after 72h of treatment. Effects of the combination on cell cycle, apoptosis, proliferation and DNA damage were analysed by flow cytometry, caspase 3/7 assay, western blots and clonogenic assay. The Chou-Talalay method was used to assess synergism between quisinostat and cisplatin and quisinostat and talazoparib.

Results and Discussions

Combination with cisplatin or talazoparib led to decreased cell viability compared to single treatment. Chou Talalay analysis indicated synergism between quisinostat and cisplatin and quisinostat and talazoparib in UM-UC3 and J82 at low dose ratios; the effect was milder in VM-CUB1. Cell cycle analyses revealed that both combinations led cell cycle alterations and a decreased long term proliferation capacity. An increase in cell death could be determined by annexin V staining, PARP cleavage and detection of caspase3/7 activity for both combinations. Additionally, γ H2AX increased upon combined treatment. In HBLAK, the combinations did not lead to a marked increase in normal cell toxicity.

Conclusion

Quisinostat is suitable for a combination with cisplatin and talazoparib at reduced dosage with low normal cell toxicity. Further analyses including RNA-Seq analysis and mice xenografts are ongoing to understand the underlying mechanisms of these promising combinations.

EACR22-1160

Analyzing the antitumor properties of a new adjuvant therapy for the treatment of androgen-dependent prostate cancer.

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Introduction

Prostate cancer is one of the most common cancers in Europe. Although there are procedures like resection or radiotherapy for primary tumors, some malignant cells cause metastasis. In this scenario, androgen deprivation therapy is used, however, some patients develop resistance to this and also to additional drugs used to treat prostate cancer. In this regard, bioactive compounds are being used as adjuvant elements to improve current therapies. Thus, we studied whether the natural compound Ocoxin® could be administered as a complement to Docetaxel and Enzalutamide in patients who suffer from androgen-dependent prostate cancer.

Material and Methods

Human 22Rv1 and LNCaP prostate cancer cells were cultured in the presence of different concentrations of Docetaxel, Enzalutamide and Ocoxin® and cell viability was measured by using PrestoBlue. Later, the most effective dose of each drug was combined with Ocoxin® and also apoptosis and cell cycle were analyzed by flow cytometry after incubating cells with Annexin V/PI or PI alone respectively. Then, the effect of Docetaxel and Ocoxin® was studied on murine RM-1 prostate cancer cells as described before.

Finally, an *in vivo* assay was developed in order to evaluate the antitumor capacity of the combined therapy. RM-1 cells were subcutaneously injected to C57/B6 mice and they were treated with a vehicle solution, with oral Ocoxin, with intraperitoneal Docetaxel or with the combination of both compounds daily. Mice were sacrificed 12 days later and tumors were collected.

Results and Discussions

LNCaP and 22Rv1 prostate cancer cells were sensitive to Docetaxel but resistant to Enzalutamide and RM-1 cells did not respond to Docetaxel *in vitro*. However, all the cell lines were sensitive to Ocoxin® and the combined treatments further decreased cell viability compared to that of the drugs alone. Moreover, Ocoxin® induced apoptosis in human prostate cancer cells, increased the apoptotic effect of Docetaxel and provoked cell cycle arrest, which could explain the lower viability rate of cells treated with Ocoxin®. Furthermore, the combined treatment reduced the weight and volume of tumors *in vivo* compared to those obtained from the rest of the treatment groups. These results are in line with those where Ocoxin® was used as an adjuvant to treat several cancers showing that it was able to increase the effect of first line therapies and to reduce tumor size.

Conclusion

Docetaxel complemented with Ocoxin® could be a potential treatment for patients with hormone-dependent prostate cancer.

EACR22-1170

Comparative Molecular Analysis of the Histone Deacetylase Inhibitor Belinostat and its Novel Prodrug Copper-Bis-Belinostat in Colorectal Cancer

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Introduction

To date, the clinical use of the histone deacetylase inhibitor (HDACi), belinostat, is limited to haematological malignancies. This is primarily due to the susceptibility of the hydroxamate moiety of belinostat to rapid metabolic inactivation, which would explain its limited effect in clinical trials for solid tumours such as colorectal cancer (CRC). To address this, we developed a novel belinostat prodrug, known as Copper-Bis-Belinostat (cubisbel), which consists of two belinostat molecules coordinated to one copper(II) center. It is hypothesized that complexation of copper(II) to the hydroxamate moiety of belinostat can protect this region from rapid metabolic degradation, thus overcoming the pharmacokinetic challenges of belinostat.

Material and Methods

The phenotypic impact of belinostat and cubisbel was assessed in regards to growth, HDAC activity, apoptosis and cell cycle across three CRC cell lines (SW480, SW620 and CACO-2). Hence, *in vitro* metabolism of each HDACi was evaluated in human liver microsomes (HLMs) using Mass Spectrometry. Finally, gene expression alterations induced by both HDACi were determined using mRNAseq, followed by *in silico* analysis to identify master regulators (MTRs) of differentially expressed genes (DEGs)

Results and Discussions

Belinostat and cubisbel significantly reduced cell viability in a cell line-specific manner, mediated primarily through inhibition of HDAC activity and induction of apoptosis. Interestingly, the estimated *in vitro* half-life of cubisbel was 35 minutes longer than belinostat (59.67 and 23.92 minutes respectively) with intrinsic clearance reduced by ~2.5-fold, indicating enhanced metabolic activity of cubisbel. Gene ontology analysis revealed belinostat and cubisbel commonly dysregulated numerous signalling pathways and metabolic processes in SW480 and SW620 cells. Uniquely, cubisbel downregulated pathways involved in vasculature development, Rho protein signalling and immune response in SW620 cells. Furthermore, genes downregulated by cubisbel were uniquely controlled by MTRs such as *VEGFA*, *ERBB2* and *LCK* that perturb cancer-associated genes.

Conclusion

In conclusion, complexation of belinostat and copper(II) does not change the HDAC activity of the molecule, but instead potentially prevents metabolic inactivation *in vitro* and selectively targets anti-cancer pathways in CRC. This study thus provides clear rationale for further comparative analysis of belinostat and cubisbel *in vivo*, which will be pivotal in confirming the pharmacokinetics of the prodrug.

EACR22-1274

A PDX-derived cell line platform for pharmacological screening and functional studies

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Introduction

Despite considerable progress in understanding the biology and genetics of cancer, the development of effective therapies is hampered by the lack of sufficient experimental models that recapitulate the genetic diversity of this disease. The recourse to patient-derived xenograft (PDX) for the evaluation of new candidate anticancer drugs is becoming the gold standard in preclinical oncology. The faithful reproduction of patients' cancer features, and the possibility to generate a large number of models that recapitulate patient population genetic heterogeneity, confer PDXs a critical added value in the evaluation of new candidate drugs. These improved models will hopefully contribute to decrease the attrition rate observed in clinical trials, thus far unacceptably high. Over the last 15 years, we have generated and characterized a collection of 200+ PDXs from different solid tumors that accurately reproduce the histological and molecular heterogeneity of the tumors of origin. Although being an indispensable tool to complete preclinical studies, the use of PDX *in vivo* systems for large-scale screening during early drug discovery is hampered by ethical, economical and throughput burdens limiting the number of test articles being tested.

Material and Methods

To address this problem, we developed a panel of PDX-derived cell lines (PDXDCs). These cell lines have been generated by PDX dissociation and culturing in presence of Rock Kinase Inhibitor. To date, 50+ PDXDCs from various indications such as breast, lung,

prostate and many others have been generated and tested for their response *in vitro* towards standards of care and targeted anti-cancer agents matching patient clinical management. Differently from standard cell line establishment, which is obtained by expansion of a cell clone that survives *in vitro* plating, our cell line development technology allows for maintenance of tumor cell population heterogeneity.

Results and Discussions

PDXDCs RNA and exome sequencing data faithfully match the parental PDX features, and by modulating experimental parameters, such as 2D or 3D growth conditions, drug exposure duration and endpoint read-outs, we could phenocopy *in vitro* the corresponding PDXs' sensitivities to chemotherapies.

Conclusion

These results show our PDXDCs panel is a valuable *in vitro* platform for drug screening to help selecting drug candidates for further validation in parental PDX models *in vivo*.

EACR22-0155

Novel dual CXCR4/ACKR3(CXCR7) targeting agent in combination with immune checkpoint therapy: a promising approach against aggressive breast cancer

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Introduction

The CXCR4/ACKR3(CXCR7)-CXCL12 chemokine axis plays a pivotal role in promoting cancer growth, survival, therapy evasion and metastasis. More recently, the CXCR4/ACKR3(CXCR7)-CXCL12 chemokine axis has been shown to drive resistance to immune checkpoint therapy (ICT) in cancers including pancreatic adenocarcinoma, aggressive breast and ovarian cancer.

Cooperativity is recognised between the CXCR4 and ACKR3 chemokine receptors that governs the precise biological response to CXCL12 stimulation, potentially limiting the effectiveness of targeting either receptor independently.

A dual-targeting approach in combination with ICT was investigated as a potential novel therapy to treat cancers of high-unmet need.

Material and Methods

A panel of CXCR4 binding molecules were screened and validated (*in-vitro*) for dual CXCR4 and ACKR3 binding. The lead dual-targeting candidates were analysed for cardiotoxicity (hERG assay) and cytochrome p450 inhibition to establish the safety profile followed by *in vivo* pharmacodynamic studies.

Orthotopic 4T1 breast tumours were established and then treated with a lead dual CXCR4/ACKR3 inhibitor (1 mg/kg/daily) in combination with an anti-PDL1 (5 mg/kg x 3 doses) monoclonal antibody. Tumour growth and lung metastasis (clonogenic assay) were

analysed. Tumour samples were disaggregated following treatment and single cell profiling conducted using flow cytometry.

Results and Discussions

In vivo pharmacodynamic and tolerability studies have demonstrated “on-target” effects from dose levels of 0.5 mg/kg with repeated dose tolerability confirmed up to 2.5 mg/kg/daily for 28 days.

Dual CXCR4/ACKR3 inhibitor (alone or in combination with anti-PDL1) inhibited breast tumour growth significantly ($p<0.01$) more than anti-PDL1 alone. Combination treatment reduced metastasis to the lung by ~50% and tumour vascularity by 36 %.

Dual CXCR4/ACKR3 inhibitor (alone or in combination with anti-PDL1) reduces levels of intra-tumour T-regulatory cells by >70 %.

Combination therapy exerts superior pleiotropic anti-cancer effects whilst modulating immune response influencing intra-tumour T-regulatory cells.

Conclusion

A novel dual CXCR4/ACKR3 inhibitor in combination with ICT represents a potential new therapy for aggressive breast cancers.

EACR22-0163

Evaluation of Tazemetostat as a therapeutically relevant substance in biliary tract cancer

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Introduction

Tazemetostat is an FDA-approved inhibitor of EZH2, a histone-lysine N-methyltransferase enzyme, that is known to be involved in cancer progression. Biliary tract cancer (BTC) is a malignancy with a poor overall survival and limited treatment option. Currently, there is no pre-clinical data showing a potential use of Tazemetostat as an anti-BTC agent.

Material and Methods

The clonogenic assay as well as a resazurin assay and IC₅₀ calculation evaluate the anti-tumor effect of Tazemetostat on different human BTC cell lines *in vitro*. An extended time-resolved cytotoxic analysis was used to investigate long-term cytotoxic effects. The epigenetic effects of Tazemetostat were evaluated using Western Blot (H3K27me3 levels) and mRNA expression analysis of potential EZH2 target genes. Point-mutation analyses of EZH2 (Y641) in BTC cells were finally investigated via sequencing.

Results and Discussions

Tazemetostat reduced clonogenic growth of BTC cells in a cell line-dependent manner. We observed a latency of the cytotoxic effect of Tazemetostat, as treatment of BTC cells with Tazemetostat for 72h did not result in a decline of cell viability, whereas treatment for up to

15 days did - a phenomenon that was already described in other tumor entities. Interestingly, the epigenetic effect following Tazemetostat treatment (= lower H3K27me3 levels) was already observed 72h after treatment with low concentrations. Real time-PCR unveiled a significant upregulation of the potential tumor suppressor gene, Fructose 1,6 bisphosphatase 1 (FBP1), whereas its protein levels did not change, after drug treatment. Previous studies indicate, that Tazemetostat is more effective in tumors with gain-of-function EZH2 Y641 mutation, therefore sanger sequencing revealed, that several BTC cell lines harbor this mutation.

Conclusion

In this preliminary *in vitro* study, we can show that the FDA-approved EZH2 inhibitor Tazemetostat has potential anti-tumor effects in BTC cells. Notably, despite the late cytotoxic effect of Tazemetostat, its epigenetic effects occur at low concentrations and independent of the cytotoxic effects.

EACR22-0221

AR and mTOR signaling regulates tumor growth and survival in vivo in advanced prostate cancer models

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Introduction

Alterations in the androgen receptor (AR) and mTOR pathways are a common feature of advanced prostate cancer (PCa), often arising in response to androgen deprivation therapy (ADT). In a subset of patients moreover, disease will progress to castration-resistance PCa (CRPC), an outcome associated with prolonged ADT, resulting in poorer prognosis and requiring more aggressive therapeutic lines. Understanding the interdependence of AR and mTOR pathways at the nexus of PCa progression to CRPC may therefore facilitate the development of improved treatments for CRPC patients. We used two patient-derived xenografts (PDX) models of bone-metastatic PCa, BM18 and LAPC9, respectively castration sensitive and resistant. We treated both models *in vivo* and *in vitro* with combinations of ADT and Rapalink-1, a new generation mTOR inhibitor, to evaluate the effect of combined AR and mTOR signaling blockade on PCa tumor growth and cytological characteristics.

Material and Methods

LAPC9 and BM18 PDX were maintained in BALB/c CB17^{scid} mice and, for the ADT cohorts, bilateral orchiectomy was performed; mice were randomized by weight and treated with either vehicle (20% DMSO, 40% PEG-300 and 40% PBS) or Rapalink-1 (1.5 mg/Kg) every 5 days. FFPE tumor tissues were sectioned and stained for the expression of AR, Ki67, CD44 and alpha smooth muscle actin (aSMA). Digested tumor tissues were stained for CD44, DAPI and ALDEFluor and analyzed by flowcytometry.

Results and Discussions

Treatment of BM18 PDX with Rapalink-1 alone was sufficient to stop tumor growth. As expected, treatment of BM18 PDX with ADT induced a dramatic but not curative tumor regression. Co-administration of ADT and Rapalink-1 could not prevent tumor regrowth upon exogenous testosterone administration. While treatment with Rapalink-1 alone significantly reduced the growth rate of LAPC9 PDX, co-treatment with ADT and Rapalink-1 resulted in regression in this CRPC model, a result not achievable by either treatment alone. PDX treated with Rapalink-1 showed reduced Ki67 staining and a reduced nuclear localization of

AR. Compared to untreated PDX, ADT reduced the expression of both CD44 and ALDEFluor markers and treatment with Rapalink-1 did not significantly modulate this downregulation.

Conclusion

While the hormone-sensitive BM18 model requires both AR and mTOR signaling for growing, growth of the CRPC model LAPC9 requires only one of the two pathways to be active. mTOR blockade moreover had an impact on AR signaling, as evidenced by altered AR localization in treated samples.

EACR22-0233

Identification and characterisation of multiple strategies to enhance cancer cell death in preclinical models of head and neck cancer

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Introduction

Squamous cell carcinoma of the head and neck (SCCHN) is the sixth most common cancer worldwide, with overall survival of less than 50%. Current therapeutic strategies involving a combination of surgery, radiation, and/or chemotherapy are associated with debilitating side effects, highlighting the need for more specific and efficacious therapies. In this study, we report three different strategies, targeting either the glutamine metabolic pathway, pyrimidine biosynthesis, or the bromodomain and extra-terminal domain family of proteins, as potential therapeutic regimens in SCCHN.

Material and Methods

Expression analysis of the relevant proteins, Glutaminase (GLS), Dihydroorotate Dehydrogenase (DHODH) and Bromodomain-containing protein 4 (BRD4) were performed in several SCCHN-derived cell lines and tissue microarrays from a large cohort of SCCHN patients. RNA interference and small molecule inhibitors, specifically designed to target these proteins, were used in several clonogenicity and apoptosis assays to characterise the functional roles of these proteins. Similar studies were also performed using the *ex vivo* culture of tumour tissue, resected from SCCHN patients, to increase the translational relevance of our findings.

Results and Discussions

Expression Analyses: All three targets, GLS, DHODH and BRD4, are widely expressed in several SCCHN-derived cell lines, and highly expressed both in the normal and tumour tissue of SCCHN, thus presenting the first obstacle to therapy (lack of tumour-specificity). This could be overcome by co-administration of specific BH3 mimetics (inhibitors of the antiapoptotic BCL-2 family of proteins), as these proteins are more highly expressed in SCCHN tumours than normal tissues.

Functional Assays: RNA interference and inhibitors that selectively target GLS (CB-839), DHODH (Teriflunomide, Leflunomide, Vidofludimus, Brequinar and BAY 2402234) or BRD4 (MZ-1, JQ1, AZD5153, Mivebresib and Birabresib) potently and specifically diminish the clonogenic potential of several SCCHN cell lines. Whilst inhibition of GLS or BRD4 synergises with specific BH3 mimetics to induce apoptosis, BAY 2402234 works as a single agent to induce extensive apoptosis in SCCHN cell lines. However, none of these treatments induce comparable apoptosis in the *ex vivo* SCCHN tumour tissue.

Conclusion

The challenges presented by the tumour microenvironment in diminishing the potential of several promising therapies will have to be characterised further, which is the primary focus of our current studies.

EACR22-0265

Novel antibodies for ion channel and transporter targeting: a new paradigm in the treatment of colorectal and renal carcinoma

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Introduction

Ion channels are emerging as potential biomarkers during the metastatic process of several types of cancer. In this scenario, a relevant role is exerted by ion transporters, which contribute to determine several aspects of tumor microenvironment. It is known that among the main transporters found to have an increased activity in cancer, NHE1 has a central role. More recently, the role of different ion channels and transporters have been elucidated, including Na⁺/H⁺ exchanger 1 (NHE1) and Kv11.1 ion channel in complex with β 1 integrin. The latter play a critical role in cancer establishment and metastatic spreading, along with CA-IX carbonic anhydrase, acting as a joint complex in regulating tumor pH, downstream signaling and hypoxia conditions in tumors such as colorectal cancer (CRC) and clear-cell renal carcinoma (ccRCC).

Material and Methods

Human clear cell renal carcinoma (ccRCC) and colorectal cancer (CRC) cells were used to perform in vitro (co-immunoprecipitations and western blot, immunofluorescence, lateral motility and invasiveness) analysis using novel antibodies, we have developed, directed against ion channels and transporters centered macromolecular complexes. Moreover, CAIX based antibodies, have been test in immunohistochemistry on a cohort of ccRCC patients. 30 ccRCC cases have been collected thanks to the collaboration with the Urology Unit. In parallel, NHE1-Kv11.1/ β 1 antibodies were tested on a cohort of 80 metastatic CRC patients.

Results and Discussions

We hypothesize that the Kv11.1-centered complexes formed with NHE1 and CAIX can be exploited for *targeting* in CRC patients and ccRCC patients especially in the metastatic setting. In fact, we have demonstrated the formation of a complex **(1)** between Kv11.1, the β 1 integrin and NHE1 in CRC cells, **(2)** between Kv11.1, β 1 integrin and CAIX in human ccRCC samples. Notably, **(3)** that the above mentioned complexes regulate intracellular signaling, ensuing the increase in cell motility and metastatic spread in CRC and ccRCC cells. The developed antibodies, tested on ccRCC and CRC human samples, have shown to be promising bimolecular tools for patients' stratification.

Conclusion

The novel immuno-therapeutic strategy we have developed, using the Kv11.1- β 1 centered antibodies could be translated to clinical studies in the near future. Overall, we have shown that *targeting* such central functional hubs, as Kv11.1- β 1-CAIX and Kv11.1- β 1-NHE1, with antibodies, could represent a valuable diagnostic and therapeutic strategy.

EACR22-0315

Therapeutic potential of Fingolimod in triple negative breast cancer preclinical models

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Introduction

Surgery followed by a chemotherapy agent is the first-line treatment for breast cancer patients. Nevertheless, new targets are required for women with triple-negative breast cancer (TNBC) in order to improve the treatment of this aggressive cancer subtype. Multiple pro-inflammatory molecules including lipid-based substances such as sphingosine-1-phosphate (S1P) promote cancer progression. In this preclinical study, we aim to investigate the efficacy of Fingolimod, an inhibitor of S1P / S1P receptors axis, already approved as an immunomodulator in multiple sclerosis.

Material and Methods

The impact of Fingolimod was analyzed using in vitro 2D and 3D cell survival analysis and in vivo orthotopic graft models, using mouse and human TNBC cells implanted in immunocompetent or immunodeficient mice, respectively. Resection of the tumor primary mass was also performed to mimic the clinical standard of care.

Results and Discussions

We demonstrated that Fingolimod repressed tumor cell survival in vitro. We also showed in preclinical mouse TNBC models that Fingolimod repressed tumor progression and liver and spleen metastases without apparent adverse effects on the animals. Our data indicate that Fingolimod induces tumor cells apoptosis and thereby represses tumor progression.

Conclusion

Globally, our data suggest that Fingolimod merits further evaluation as a potential therapeutic opportunity for TNBC.

EACR22-0329

WPD101 as a new drug candidate for targeted therapy of glioblastoma multiforme

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Introduction

Glioblastoma multiform (GBM) is the most common type of malignant brain tumor among adults and it is usually very aggressive. It can grow fast and spread quickly. The estimated median survival in cases with GBM is about 12–16 months. The standard of care treatment includes the surgery, in order to remove as much of the tumor as possible, followed by radiation, to eliminate remaining tumor cells. Chemotherapy (mainly Temozolomide) is also applied however its clinical efficacy is limited. Thus, the more effective GBM therapies are needed. WPD101 is a new drug candidate for targeted therapy of GBM that is an extensive development in preclinical stage. WPD101 is designed as a unique drug cocktail composed of two immunotoxins targeting simultaneously tumor associated IL-13RA2 and EphA2 receptors. Both of receptors are expressed in brain cancer cell lines but not present in normal brain cells.

Material and Methods

Two proteins: IL13-DT390 and PE38QQR-EFNA1 form WPD101 drug cocktail. IL13-DT390 (WPD101a) was expressed as a fusion protein in bacteria, purified in two chromatography

steps (IEX and SEC) and ultrafiltrated to the concentration of 1.2 mg/ml. PE38QQR-EFNA1 (WPD101b) was synthesized in chemical conjugation reaction, separated using size exclusion chromatography (SEC) and the concentration of the 1:1 stoichiometric product (a monomer of PE38QQR to EFNA1 monomer) equals 0.199 mg/ml. The ELISA assay was used to check the binding of synthesized WPD101a and WPD101b to receptors IL-13RA2 and EphA2, respectively.

Results and Discussions

Two WPD101 proteins were synthesized separately and purified with the average yield of 2 mg/l for IL13-DT390 and 27% (189 µg) for PE38QQR-EFNA1 (1:1 stoichiometric product, calculated for the EFNA1 substrate) and stored at -80°C in PBS and both proteins are stable in these conditions. IL13-DT390 binds to IL-13RA2 with $EC_{50} = 5.52 \text{ nM} \pm 1.297$ and PE38QQR-EFNA1 binds to EphA2 with $EC_{50} = 0.351 \text{ nM} \pm 0.0544$.

Conclusion

The binding of the WPD101 proteins to those receptors was confirmed in ELISA assay. Moreover, the cytotoxic activity of WPD101 towards numerous brain cancer cell lines and cells derived from patients was confirmed in WPD lab. The strategy to cover both of receptors expressed on cancer brain cells guarantees specific drug administration to the majority of GBM cells in tumor. In order to overcome blood brain barrier difficulties, the CED of WPD101 directly to the brain tumor will be applied.

Financing

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EACR22-0391

New targets to increase CDK4/6 inhibitors efficacy in breast cancer

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Introduction

Drug resistance is a major problem involved in the effective response to targeted cancer therapies such as CDK4/6 inhibitors. Cellular senescence has been described as a barrier against tumour progression. Although cancer cells naturally avoid senescence, in the last years CDK4/6 inhibitors have been proposed to induce cell cycle arrest in tumour cells, for this reason the development of pro-senescence therapies such as CDK4/6 inhibitors has become an attractive strategy. On the other hand, connexins are channel-forming proteins involved in cell communication. These channel proteins modulate the tumour microenvironment and the response to therapies via channel-dependent or channel-independent functions. Connexin43 (Cx43) has been reported to enhance senescence and to be dysregulated in breast cancer.

Material and Methods

Human ER+/HER2- breast cancer cell lines were used in this study. Lentiviral vectors and extracellular vesicles (sEVs)-enriched in Cx43, were used to deliver transmembrane proteins and to restore Cx43 in breast cancer cells. Standard methods were used to study the effect of Cx43 or sEVs-enriched in Cx43 in combination with CDK4/6 inhibitors in cellular senescence and apoptosis. The CDK4/6 inhibitors (Palbociclib, Abemaciclib, Ribociclib) were obtained from Selleckchem

Results and Discussions

Here, we have demonstrated that the restoration of Cx43 in ER+/HER2- breast cancer cells using lentiviral vectors enhances senescence and sensitizes breast cancer cells to cell death by apoptosis increasing the efficacy of the CDK4/6 inhibitors (Pabociclib, Abemaciclib, Ribociclib). We have obtained similar results using sEVs-enriched in Cx43, in combination with CDK4/6 inhibitors, which significantly increases CDK4/6 inhibitors efficacy in 2D and 3D models. Further, the combination of Cx43, CDK4/6 inhibitors and senolytic drugs, such as Navitoclax, has been assayed and this combination resulted in the best strategy to enhance senescence, reduce the proliferation of breast cancer cells and increase cell death increasing efficacy and preventing drug resistance.

Conclusion

We propose a new and effective drug combination strategy based on the use of sEVs enriched in Cx43 to reduce CDK4/6 inhibitors resistance by improving their efficacy in breast cancer models. The results obtained could impact in the manage and treatment of tumours that respond to CDK4/6 inhibitors treatments and with a potential clinical benefit in patients. The results presented here has been protected in an EU patent application.

EACR22-0447

Multi-omics analysis of the effects of HDAC6 and proteasome inhibition in breast and ovarian cancer cells: a promising therapeutic strategy

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Introduction

The role of epigenetic changes in cancer initiation and progression is well documented. Several protein families are involved in chromatin remodeling and, among others, histone deacetylases (HDACs) are attracting attention as a potential therapy target. In particular, the use of an inhibitor selective for HDAC, such as HDAC6, would be preferred to avoid side effects. Moreover, a recent study of our laboratory demonstrated that HDAC6 inhibition causes a reduction of NOTCH3 protein levels and pathway activation in T-ALL cells. Our aim was to test effects of a potentially novel therapeutic approach with ACY-1215, a selective HDAC6 inhibitor, on High Grade Serous Ovarian Cancer (HGSOC) and Triple Negative Breast Cancer (TNBC), two common and lethal female tumors.

Material and Methods

HGSOC and TNBC cancer cells and tumor samples were screened for *NOTCH3* genomic amplification and expression levels. Cellular and molecular changes mediated by *in vitro* treatment with the HDAC6-inhibitor ACY-1215 in TNBC cells were evaluated by standard methods (cell viability, apoptosis assays, WB analysis, real-time PCR). Finally, ACY-1215 anti-tumor properties in combination with proteasome inhibitor Bortezomib (BTZ) were investigated by a multi-omics approach.

Results and Discussions

We did not find substantial genomic amplification of *NOTCH3* in HGSOC samples, nor any correlation between *NOTCH3* copy number and *NOTCH3* expression levels. Although breast and ovarian cancer cells overexpressing *NOTCH3* and treated with ACY-1215 showed a significant reduction of *NOTCH3* protein, *NOTCH* signaling was not affected.

Transcriptomic analyses pointed out that ACY-1215 mediated multiple nonspecific side effects, leading us to hypothesize that *NOTCH3* plays only a marginal role in these models, at variance with what we previously reported in T-ALL cells. Next, we investigated combination of ACY-1215 with BTZ. Interestingly, antiproliferative and proapoptotic effects mediated by ACY-1215 were boosted by co-administration of BTZ in several HGSOC and TNBC cell lines. Transcriptomic analysis showed the efficacy of the combination to induce cellular stress response, in line with observed anti-tumor effects. Finally, proteomic analysis of MDAMB468 cells highlighted activation of the Unfolded Protein Response.

Conclusion

In HGSOC and TNBC cell lines combinatorial therapy with ACY-1215 and BTZ can be exploited as a potential therapeutic strategy. In the follow-up of our study, we will evaluate this approach through *in vivo* experiments.

EACR22-0461

Specific combination therapy for histone-H3 mutant pediatric high-grade glioma

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Introduction

Pediatric high-grade gliomas (pHGGs) are the leading cause of cancer related death in children. Histone-H3 K27M mutant pHGGs fall into the class of diffuse midline gliomas (DMGs), which are surgically inaccessible and have worse prognosis. H3K27M mutations alter the methylation profile of epigenome, which affects gene expression and reduces genomic stability. Thus, a large fraction of pHGGs is considered to be an epigenetic disease. In consequence, epigenetic therapy has been proposed as a promising strategy for pHGGs. We aim to develop a comprehensive preclinical data package with a H3K27M-selective treatment approach through epigenetic drug library and compound combination screens to initiate a phase I/II clinical trial.

Material and Methods

Epigenetic drug library and compound combination screening were performed as follows: drugs were printed on 384-well round bottom plates in 10-fold dilutions at five different concentrations in duplicates. Combination partners were used in one fixed concentration. pHGG cell models with distinct histone-H3 mutation status were seeded on these plates and grown as spheroids. Upon three-day incubation, cell viability was measured in a metabolic activity assay with CellTiter-Glo® (CTG). The raw data was processed with R-software and the drug effect was calculated as a drug sensitivity score. The synergy screen was performed with a multiple-ray design using a set of fixed ratios and the results were analyzed for drug interactions based on the Loewe additivity model.

Results and Discussions

Epigenetic drug screens of nine histone-H3 K27M, two G34R/V, three wildtype pHGG and three human healthy control cell models displayed hits with H3K27M-selective potency. These top hits were used in combination screens and the most potent combination partners were identified to be targeting the epigenetic/RNA polymerase II complex as a specific vulnerability in H3K27M mutant pHGGs. Synergy and mutation specificity were confirmed by the multiple-ray design screen. The combinations will be further validated *in vitro* with high-content microscopy and *in vivo* in zebrafish and mice xenograft models.

Conclusion

The most effective combination therapy shows substantial potential for the selective treatment of H3K27M mutant pHGGs and could initiate a phase I/II clinical trial.

EACR22-0477

PARP and CHK1 inhibitors synergize in killing MYCN amplified neuroblastoma via an ATM-dependent pathway

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Introduction

MYCN deregulation is a cancer driver and associates with aggressive behavior and refractoriness to therapy, in several neoplasia. Since MYCN targeting isn't achievable in patients, defining new vulnerabilities of MYCN-driven tumors appears mandatory. Control of the replication stress induced by oncogenic pathways is required for tumor development and is being exploited for cancer therapy. We studied a combination of PARP and CHK1 inhibitors (PARPi+CHK1i) as a new therapeutic approach for MYCN-driver tumor such as MNA neuroblastoma (NB) and Sonic-Hedgehog medulloblastoma (SHH-MB).

Material and Methods

A panel of non-MNA and MNA NB cell lines and MB neurosphere cultures were used. MTS, trypan blue and comet assays were performed to measure cell growth, cell survival and DNA damage accumulation. Mice models for NB and MB were treated with vehicle, PARPi olaparib (50 mg/kg), CHK1i MK-8776 (25 mg/kg) or their combination and tumor growth was measured by caliper or bio-luminescent imaging. ATM genetic modification in IMR32 cells was obtained by CRISPR/Cas9.

Results and Discussions

We demonstrated that the cotreatment with olaparib+MK-8776 led to accumulation of DNA damage and cell death in a synergistic and MYCN-dependent manner, *in vitro*. Consistently, olaparib+MK-8776 significantly reduced the growth of MNA NB subcutaneous and orthotopic xenografts, and the growth of a SHH-MB model with no major toxicities.

Since ATM loss seems to sensitize to PARPi and CHK1i in 11-q deleted NBs and IMR32, the most responsive cell line, bears a putatively pathogenic ATM mutation, we asked whether ATM was involved in the responses to PARPi+CHK1i. Not only genetic correction showed that ATM mutation did not fully abolish ATM kinase function and was irrelevant for the biological responses to PARPi+CHK1i; rather, ATM knock-out revealed that ATM is required for the proapoptotic effect of PARPi+CHK1i in MNA NB cells. This is unexpected since literature data and our analysis performed in non-MNA cells indicate that loss of ATM function has opposite effects

Conclusion

In conclusion, our data highlight a new potential chemo-free strategy to treat MNA NB. Furthermore, emerging evidence indicates that enhanced RS plays a central role in the activation of immune response (IR) and could serve as a predictive marker for immunotherapy. Since PARPi have been linked to modulation of IR, it is possible that additional benefits may be obtained by PARPi+CHK1i in immunocompetent mouse models.

EACR22-0535

Microbubble Sonoporation Orchestrates Tumor Microenvironment to Strengthen Chemotherapy against Pancreatic Tumors

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Introduction

Pancreatic adenocarcinoma (PDAC) is one of the lethal cancers. Nearly all newly diagnosed patients already have progressive unresectable tumors due to difficulties in diagnosis at the early stages. Novel theragnostic intervention is needed to target the PDAC tumor microenvironment (TME). Contrast-enhanced ultrasound (CEUS) has been promising for better tumor image. The combination of ultrasound and microbubble, usually called sonoporation, has encouraged diagnosis and enhanced drug delivery. We have previously demonstrated that CEUS could detect early PDAC lesions and enhance the therapeutic efficacy of doxorubicin. However, the detailed mechanism wasn't addressed, and whether it could benefit the first-line medication, gemcitabine, for PDAC patients is not known. In this study, we sought to explore the tumor microenvironment changing with time after the treatment and test the feasibility of CEUS to combine gemcitabine to strengthen chemotherapy in a pre-clinical pancreatic tumor model.

Material and Methods

An orthotopic PDAC murine model, UN-KC-6141, was used. CEUS was performed with a clinical ultrasound apparatus via TBS-002 (Contrast-enhanced microbubbles, Phase I/II clinical trial NCT04185246 for contrast echocardiography, TRUST BIO SONICS, Taiwan). Different ultrasound settings for sonoporation was tested. The hypoxia probe, pimonidazole (PIMO), and CD31 were used to track the tumors' hypoxic status and mean vessel density. T-cell infiltration was also examined one day and six days after the treatments.

Results and Discussions

The results showed that the doppler-based sonoporation prolonged the median survival of the doxorubicin (Dox)-treated mice for 10.5 days and increased gemcitabine-treated mice

from 34.0 days to 43.0 days compared to the drug-only group. The immunofluorescence staining revealed a 90% decrease in hypoxic tumor area and increased mean vessel density one day after the sonoporation. Five-fold more T-cell infiltration was seen six days after the combination treatment. These could be the primary mechanisms of doppler-based sonoporation treatment to enhance chemotherapy.

Conclusion

We demonstrate that sonoporation under diagnostic ultrasound insonation could strengthen the efficacy of different chemo-drugs by relieving tumor hypoxia and increasing mean vessel density, increasing the T-cell flux afterward. This provides a rationale for designing a new adjuvant therapeutic protocol to include sonoporation with diagnostic ultrasound for not only diagnosing but treating the pancreatic tumor.

EACR22-0635

Therapeutic potential of Fingolimod and Dimethyl Fumarate in non-small cell lung cancer preclinical models

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Introduction

New therapies are required for patients with non-small cell lung cancer (NSCLC) for which the current standards of care poorly affect the patient prognosis of this aggressive cancer subtype. In this preclinical study, we aim to investigate the efficacy of Fingolimod, an inhibitor of S1P / S1P receptors axis, and Dimethyl Fumarate (DMF), a methyl ester of fumaric acid, both already approved as immunomodulators in auto-immune disease.

Material and Methods

The impact of both drugs was analyzed with in vitro cell survival analysis and in vivo graft models using mouse and human NSCLC cells implanted in immunocompetent or immunodeficient mice respectively.

Results and Discussions

We demonstrated that Fingolimod and DMF repressed tumor progression without apparent adverse effects in vivo on the preclinical mouse NSCLC models. In vitro, Fingolimod did not affect either the tumor proliferation or cytotoxicity, although DMF reduced tumor cell proliferation.

Conclusion

These results suggest that Fingolimod and DMF affected tumor progression through different cellular mechanisms within the tumor microenvironment. Fingolimod and DMF might uncover potential therapeutic opportunities in NSCLC.

EACR22-0695

APPLICATION OF OMICS TECHNOLOGIES FOR EFFICIENT IMPLEMENTATION OF NATURAL PRODUCTS IN CANCER THERAPY

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Introduction

Integration of omics technologies in new natural products with anti-cancer potential by investigating the molecular basis of therapeutic efficacy through associated proteomic signatures.

Material and Methods

Biopharmaceuticals have been developed and evaluated by targeting proteomic processes / mechanisms, exploring new molecular structures and delivery systems at the cellular and intracellular level.

Results and Discussions

The use of new proteomic technologies - xMAP, protein microarray, xCELLigence, multiple ELISA etc. in determinations of efficacy, toxicity of natural products, completes the identification of the molecular signature and the decipherment of the molecular mechanisms of cancer, representing an important tool in preclinical testing, through “mechanistic toxicology” analysis and bioinformatics application, in predictive toxicoproteomics.

These technologies have been applied on a wide range of bioproducts: collagen, combinations of phytocompounds, complex protein extracts, collagen combinations and bionanostructures. The obtained results showed a plethora of effects of various bioproducts, such as regenerative effects, hormonal therapeutic effects, immunotherapy effects (for autoimmune pathologies), reduction of effects after heavy metal poisoning, as well as anti-inflammatory or anti-tumor effects.

Conclusion

Implementation of omics biotechnologies on natural products evaluation provided relevant data on mechanisms of action, interactions with other compounds, etc. in order to develop new methodologies and skills, in pharmacological performance research for the optimization of new bio-products in cancer therapy.

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EACR22-0711

From bench to bedside and back to bench: identification of potential resistance markers for the SHERPA (SHP2 and ERK inhibition in pancreatic cancer) trial.

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Introduction

Over 90% of pancreatic tumors carry a driving mutation in the KRAS oncogene, which is also altered in 30-40% of lung and colorectal cancers, thus representing an ideal target for precision oncology. Nevertheless, despite decades of research and the recent emergence of isoform-specific KRASG12C-inhibitors, most mutant KRAS isoforms, including the ones frequently associated with pancreatic ductal adenocarcinoma (PDAC), cannot be targeted directly. We previously discovered, using experimental mouse models, that tumors carrying

activating KRAS mutations are sensitive to the inhibition of SHP2, a phosphatase helping the transmission of the growth-promoting signals from the cell surface receptors to RAS. Moreover, SHP2 inhibitors cooperate with inhibitors of the RAS downstream effectors MEK and ERK to achieve superior disruption of the MAPK pathway, increased apoptosis and better tumor growth control, in the absence of *in vivo* toxicity.

Material and Methods

Based on those compelling results, we begun to investigate the combination of the SHP2 inhibitor RMC4360 with the ERK inhibitor LY3214996, in a Phase I/Ib clinical trial (SHERPA, **SHP2** and **ERK** inhibition in **pancreatic cancer**, NCT04916236). Furthermore, in order to identify dynamic biomarkers of resistance, we performed a series of unbiased CRISPR-based resistance screenings, and generated spontaneously resistant PDAC cell lines.

Results and Discussions

Our resistance screenings led to the identification of PTEN downregulation and c-JUN hyperactivation as two mechanisms allowing pancreatic cancer cells to overcome the complete suppression of the MAPK pathway induced by the SHP2+ERK inhibition. In parallel, spontaneous resister cell lines also demonstrated increased PI3K/AKT/mTOR pathway activation and sensitivity to mTOR inhibitors.

Conclusion

We conclude that alteration of nodes in the PI3K/AKT/mTOR and in the c-JUN pathway may serve as markers for sensitivity/resistance to the drug combination investigated in the SHERPA trial.

EACR22-0937

Evaluating a splicing inhibitor against MYCN-amplified neuroblastoma.

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Introduction

Neuroblastoma (NB) is one of the deadliest childhood tumours, responsible for 15% of childhood cancer mortality.

Children classified as high-risk for disease relapse have a long-term survival of less than 50%. The MYCN gene is amplified in half of the high-risk cases. The therapy for high-risk patients includes, among others, genotoxic combination chemotherapy with long-term side effects which severely impact survivors' lives. Therefore, less toxic and more effective treatments are desperately needed.

Previous data from our lab suggests that targeting splicing is effective against MYCN-amplified NB. Therefore, our aim was to test the effects of a splicing inhibitor in MYCN-amplified NB cells and to investigate if it synergizes with a currently used chemotherapeutic drug, vincristine.

Material and Methods

Four NB cells with different MYCN status were used in this project, including the MYCN-amplified: Be2C, IMR-32, and non-amplified NBL5, and SH-SY5Y cells.

Cells were treated with different drug concentrations of a splicing inhibitor and/or vincristine. After 72 hours treatment, cell viability was measured using CellTiter-Glo® Luminescent Cell Viability Assay. From the combinatorial treatments, synergy scores were calculated using the zero interaction potency model. Mass spectrometry-based proteomics was used to

reveal the mechanisms of action of the splicing inhibitor and mechanisms behind synergistic effects of the combined treatment.

Results and Discussions

EC50 (half maximal effective concentration) was determined following treatment with vincristine or the splicing inhibitor for each cell lines tested. IMR-32 and NBLS were the most sensitive to vincristine followed by SH-SY5Y and Be2C. The EC50 of the splicing inhibitor is almost the same for all cell lines tested. The cell viability data were used for the synergy analysis.

We found strong synergy for all the four cell lines, in particular, for Be2C and SH-SY5Y. Proteomics revealed potential mechanisms explaining synergy between the splicing inhibitor and vincristine.

Conclusion

Single-agent therapies tend to fail in clinical trials. When combining drugs, a synergistic interaction is desired that increases the effects of the treatment but reduces the dosage, therefore, the associated toxicities. Here, we identified synergy between a splicing inhibitor and vincristine and this drug combination should be further evaluated *in vivo* in pre-clinical animal model. This combination might be incorporated in the therapy of high-risk NB.

EACR22-1157

Unveiling a new spliceosome-inhibitor drug with antitumor activity on soft tissue sarcoma patient-derived primary cultures

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Introduction

Soft tissue sarcomas (STS) are a group of more than 50 histological subtypes of malignant mesenchymal tumors which represent less than 1% of all solid adult cancers. The poor prognosis of advanced STS and the lack of efficient treatments makes the development of new targeted therapies a capital need. Our previous published data point to the Wnt signalling pathway inhibition as a therapeutic strategy that could complement current STS treatments. The aim of this study was to test the antitumor activity of different Wnt inhibitors that are currently in clinical trials in patients with diverse solid tumors in Wnt/ β -catenin signalling activated STS patient-derived primary cultures.

Material and Methods

Fresh resected STS tumor samples from Son Espases University Hospital patients were minced into small fragments and underwent mechanical and enzymatical disaggregation with the use of gentleMACS™ dissociator and collagenase incubation. Cell suspensions were expanded in vitro to obtain primary cultures. Wnt pathway molecular alterations were analyzed by Western blotting, immunofluorescence and RT-qPCR. Tested Wnt inhibitors

were LGK974 (Wnt ligands production inhibitor), PRI-724 (CBP/ β -catenin interaction inhibitor) and SM08502 (CDC-like kinase inhibitor). Cell viability was assessed using CellTiter 96® AQueous One Solution (Promega).

Results and Discussions

Canonical Wnt signalling activation was analyzed in successfully established patient-derived primary cultures proceeding from different STS subtypes. All primary cultures presented the active form of the Wnt signalling key effector β -catenin (Ser552 phosphorylation) and nuclear β -catenin accumulation, but only 2 out of 8 had elevated Wnt target genes expression such as *CCND1*, *c-MYC*, *CUL4A* and *CDC25A*. STS primary cultures had no response to the Wnt inhibitor LGK974 (acting upstream of β -catenin) and a moderate response to PRI-724 (acting downstream of β -catenin) at 72 hours of treatment. Conversely, SM08502 inhibited viability of all STS primary cultures with IC₅₀ values ranging from 0.5 to 10 μ M.

Conclusion

All studied STS-derived primary cultures possess nuclear β -catenin accumulation. Therefore, inhibiting Wnt signalling pathway could represent a promising therapeutic strategy in STS. Among the tested Wnt inhibitors, SM08502 effectively inhibited the proliferation of STS primary cells. SM08502 antitumoral effects and its implication in Wnt signalling pathway molecular changes will be better characterized to propose novel therapeutic opportunities for STS patients.

EACR22-1205

Design and development of new, highly potent TAM family inhibitor as a potential treatment for cancers

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Introduction

The TAM family includes 3 proteins – TYRO3, AXL, and MER. In cancer diseases, overexpression or ectopic expression of TAMs and their ligands are often observed. In addition to tumor cells, TAM proteins are also expressed in the tumor microenvironment. Inhibition of TAM proteins, both in tumor cells and in the tumor microenvironment, can bring many benefits, besides inhibiting tumor growth or metastasis reduction - stimulation of anti-tumor immunity. Inhibition of the TAM family of tyrosine kinases is currently a very interesting target for developing anti-cancer therapies. The development of a highly active TAM inhibitor with oral bioavailability, good pharmacokinetic profile, and low toxicities is highly desirable.

Material and Methods

CPL423 compound was designed and developed as a TYRO3, AXL, and MER small molecule inhibitor. The activity of the newly synthesized compound was tested against the kinases that belong to the TAM family (ADP-Glo™ Kinase Assay). The biological potency of compounds was evaluated in a number of cancer cell-based models using i.a. Western Blot, cell viability assay (ATPlite™), and colony formation assay. Drug-like properties (solubility, lipophilicity, permeability, microsomal stability) were also determined using *in vitro* approach. A pharmacokinetic study in BALB/c was conducted to study the PK profile of the compound. The efficacy of CPL423 and its accumulation in the tumor was analyzed in xenograft mouse models (melanoma, NSCLC, and AML).

Results and Discussions

CPL423 has subnanomolar activity against MER kinase (0,4nM) and nanomolar activity against AXL and TYRO3 kinases (2,16nM and 6,73nM, respectively). The compound shows a decrease in pathway protein phosphorylation, inhibition of colony formation, and an IC₅₀ of viability at the submicromolar level in the cancer cell models. CPL423 has drug-like properties (i.a. high solubility, good human and mice microsomal and hepatocyte stability). A pharmacokinetic study in mice showed a favorable PK profile of the compound and its high bioavailability (73%). The efficacy and high accumulation of the compound in the tumor were confirmed by the analyzes performed on xenograft samples.

Conclusion

We have designed a potent TAM family inhibitor with promising *in vitro* results and favorable PK profile and efficiency. Preclinical findings may lead to the development of a novel anticancer therapy for solid tumors.

EACR22-1241

Genetic engineering of Mesenchymal Stromal Cells to express anti-cancer proteins

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Introduction

The success of conventional anti-cancer treatments is often constrained by their lack of specificity, which is accompanied by severe side effects and lack of therapeutic efficacy. As such, the development of new tumor-targeted strategies has been one of the focal points of cancer research. Novel approaches focus on selective tumor targeting by applying customized drug delivery systems and, recently, cell-based therapies have been explored as living tools for the delivery of anti-tumor therapeutics. In this perspective, mesenchymal stromal cells (MSC) hold a promising future, due to their unique tumor-specific migratory potential.

Material and Methods

Here, nonviral, serum/ xeno-free, strategies for the engineering of MSC towards the expression and secretion of the potent anti-tumoral protein azurin (hazu) were developed. In a first line of research, a transient gene delivery method was developed as a proof-of-concept to attest the biomedical potential of *hazu*-expressing MSC's conditioned media (CM) in anti-cancer treatment. In a second line of research, the establishment of a stable transgene-expressing MSC line was developed, through the CRISPR/Cas9 system.

Results and Discussions

Engineered *hazu*-MSC were shown to preserve tumor tropism toward breast and lung cancer cell lines, comparable to non-modified MSC. Moreover, *hazu* was detected in the CM of engineered cells and, upon treatment with *hazu*-MSC-CM, a decrease in cancer cell proliferation, migration, and invasion was observed, as well as an increase in cell death for both cancer cell lines. Additionally, the *AAVS1* locus, a genomic safe harbor (GSH) in the human MSC genome, was targeted by CRISPR/Cas9 achieving 11.6% transgene knock-in efficiency.

Conclusion

By stably inserting the *hazu* gene into MSC genome, MSC could be employed as a cellular therapy, taking advantage of their innate tumor tropism and secretory potential; in addition, it could be disclosed a streamline for the production of a bioactive anti-tumoral CM product, by

employing *hazu*-MSC as a continuous living factory. Importantly, using the system established in this work, other therapeutic transgenes could potentially be targeted, aiming at different disease or biological contexts other than cancer therapy. Thus, it is anticipated that the work here developed will boost precise MSC genome editing, under conditions amenable to GMP compliance, envisioning a clinical application scenario.

EACR22-1273

Molecular and pharmacological profiling of a novel prostate cancer-derived xenograft panel to identify resistance mechanisms and new therapeutic options

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Introduction

Prostate Cancer (PCa) is the most frequently diagnosed cancer in men and remains one of the leading causes of cancer death worldwide. It is a highly heterogeneous and complex disease, presenting serious challenges to preclinical drug development and biomedical research. Prostate normal development, growth and function, as well as most of its pathological drifts, are associated to androgen receptor (AR) pathway regulation. Most patients subjected to androgen deprivation therapy (ADT) respond well to the treatment, however they usually progress into intractable castration-resistant PCa (CRPC) within 2 to 3 years. Therefore, understanding the mechanisms of castration resistance underlying PCa progression is key to develop future therapies. To fill this gap, XenTech and Gustave Roussy Institute are collaborating to develop a panel of PCa PDX models.

Material and Methods

In the framework of the MATCH-R clinical trial (NCT02517892), in which patients with disease progression under treatment are enrolled and switched to new targeted therapy based on the genetic alterations identified in biopsies, 8 PCa PDXs have been developed from metastatic lesions biopsy. In addition, we developed a PCa PDX model from circulating tumor cells (CTCs) obtained by leukapheresis at diagnosis.

Results and Discussions

These 9 models, plus 4 PCa PDXs previously developed at XenTech, increases our PCa PDX panel to 13 models. All models were characterized at molecular level by whole exome and RNA sequencing and pharmacologically for response to standards of care, physical castration and response to the androgen receptor inhibitor enzalutamide. Patient-Derived Xenografts (PDX) are mouse models that recapitulate the disease more faithfully than any other in vivo model in terms of histopathologic, genomic and metastatic features, facilitating the translation of preclinical results in the clinical setting. PCa PDXs are challenging to develop, and only few are available to the scientific community. This lack of relevant preclinical models is a major limitation in PCa research. In particular, the development of

PCa PDX models from castration-resistant tumors to explore new treatments against CRPC is a requirement.

Conclusion

This PDX collection is a valuable preclinical tool to identify pivotal mechanisms underlying acquired resistance to current therapies and develop novel treatment strategies against PCa and CRPC.

EACR22-0210

Streamlining preclinical in vivo treatment trials by multiplexing genetically labelled PDX models of several patients in a single mouse

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Introduction

Better treatment options are intensively needed for acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML). Before application in clinical trials, novel therapies require preclinical testing which is resource intensive. In previous work, we had shown that multiplexing several PDX cell populations within a single mouse in competitive *in vivo* experiments gives identical results to studying each population in separate mice (Liu et al., Biomarker Research 2020).

Material and Methods

From primary patient material, we had established patient derived xenograft (PDX) mouse models of ALL and AML, which allow serial transplantation in immunodeficient NSG mice. We labelled 5 individual samples with both a unique fluorochrome and a unique genetic barcode, for later detection by flow cytometry or next generation sequencing, respectively. In addition, luciferase was recombinantly expressed in all samples to allow repetitive monitoring of leukemia growth using bioluminescence *in vivo* imaging (BLI). Up to 5 PDX samples, either ALL or AML, were multiplexed and aliquots injected into groups of mice (n=4-6). For post mortem analysis, PDX cells were reisolated from murine bone marrow and spleen and absolute tumor burden was quantified by flow cytometry. Determining fluorochrome composition allowed quantifying the proportion that each of the 5 samples contributed to the entire tumor burden.

Results and Discussions

Mice containing multiplexed PDX cells from 5 different patients with ALL were treated for 2 weeks with either verum treatment or with solvent as control. In a study using the BCL-2 inhibitor Venetoclax in PDX ALL models, we were able to distinguish between sensitive and resistant PDX samples. While two samples showed a drastic decrease in tumor burden, three samples showed no or only a mild response. This effect could be observed both in cells isolated from bone marrow as well as spleen. Variances between mice were rather small, with few exceptions for some treated mice. With this approach, we were able to define the effect of a single drug on up to five distinct samples in parallel, reducing resources by factor 5.

Conclusion

Taken together, we established a multiplex protocol for *in vivo* therapy trials that allows simultaneous testing of up to 5 PDX samples in competitive *in vivo* trials. The approach reduced the required number of experimental mice by a factor 5, in line with the 3R concept. In the future, our approach might rationalize *in vivo* drug trials.

EACR22-1120

Inhibiting Eukaryotic Initiation Factor 4E (eIF4E) in Non-Small Cell Lung Cancer

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Introduction

The mTOR pathway is highly deregulated in several neoplasms including non-small cell lung cancer (NSCLC); a malignancy which exhibits one of the highest mortality rates. The downstream effects result in high levels of eukaryotic initiation factor 4E (eIF4E), which promote key oncogenic networks driving proliferation, metastasis and cell survival.

The aim was to study the dose-response profiles of direct eIF4E inhibitors on NSCLC cell lines, in order to identify concentrations that produce a 30% loss in cell viability. In the longer term, these compounds will be independently studied in combination with other eIF4E-targeting approaches.

Material and Methods

H460 (large cell carcinoma), H520 (squamous cell carcinoma), and A549 (adenocarcinoma) cells were treated with 0.1 to 0.5 μ M (in 0.1 μ M increments) of Briciclib® and 4E1RCat® separately. Experiments were effected using culture media supplemented with both 10% fetal bovine serum (FBS) and 1% FBS in order to investigate the effect of drug-protein binding on the outcomes. A PrestoBlue® cell viability assay was carried out after 24, 48 and 72 hour drug exposure for each cell line upon treatment with each drug.

Results and Discussions

Results using media supplemented with 1% FBS showed higher reproducibility and caused a more pronounced decrease in cell viability than 10% FBS supplemented media. Initial drug-free studies indicated that cell viability was not altered by low FBS for 72 hours. Briciclib® resulted in a dose and time-dependent decrease in cell viability, with 30% loss confirmed at 0.3 μ M for H460 and H520 cells. The full effect of the drug was observed after 48 hours and there was no further decrease in cell viability at 72 hours.

A concentration of 0.1 μ M 4E1RCat® reduced the viability of H460 cells by 50% in a time-independent manner. H520 cell viability decreased by 30% after 72 hours with 0.4 μ M 4E1RCat®. Currently, these experiments are also being effected on A549 cells.

Conclusion

A significant decrease in viability occurred using two independent eIF4E inhibitors, emphasizing the importance of this factor as a drug target for the inhibition of NSCLC proliferation. These will later be independently studied in combination with gene knockdown approaches on NSCLC and healthy cell controls, with the aim of obtaining a synergistic effect which exhibits enhanced anti-proliferative action with potentially lower toxicity to healthy cells. Data will be further evaluated through protein studies on suitable drug target endpoints.

EACR22-1176

Olaparib plus NOV202: Combination of PARP inhibitor olaparib with a novel vascular disrupting agent, NOV202, reduces tumor growth in BRCA1/2 mutated prostate cancer xenografts.

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Introduction

Despite the rapidly changing therapeutic landscape in prostate cancer, metastatic castration-resistant prostate cancer (mCRPC) remains an incurable malignancy with poor clinical outcome and poor prognosis. Up to 25% of mCRPC patients harbor homologous recombination repair (HRR) defects, with *BRCA1/2* mutations representing the most frequent events. Recent clinical trials have shown that mCRPC patients with HRR deficiencies responded well to poly (ADP-ribose) polymerase inhibitors (PARPi). PARPi olaparib significantly improved survival in patients with *BRCA1/2* mutated mCRPC and recently the FDA approved olaparib for mCRPC patients harboring HRR alterations. Although robust data exist for PARPi as monotherapy in selected patients, there is a clinical need for combinational therapeutic approaches to improve the efficacy of PARPi and overcome or prevent PARPi resistance. NOV202 is a novel vascular disrupting agent (VDA) with strong anti-proliferative effect in a large panel of cancer cells. This study aimed to evaluate if NOV202 could improve the efficacy of olaparib in prostate cancer cells with *BRCA1/2* mutations *in vivo*.

Material and Methods

Prostate cancer-based xenografts were developed from human DU145 (*BRCA1/2* mutated) cell line in male NMRI nude mice. Animals were treated with olaparib (100 mg/kg), NOV202 (30 mg/kg), or their combination for 21 days, followed by either 1 or 2 weeks of no treatment. Tumor growth and response to therapy were monitored by non-invasive bioluminescence optical imaging (BLI) and tumor size was also measured by caliper. During the experiment, a 25-parameter blood analysis, including hemoglobin concentration, red and white blood cells, and neutrophil granulocytes was performed weekly.

Results and Discussions

Olaparib and NOV202, as single drug treatments, induced tumor growth inhibition in *BRCA1/2* mutated xenografts. Compared to monotherapy, the combination of olaparib and NOV202 exerted significant synergistic effect. The combinational effect continued for 2 weeks post-treatment period. Changes in the mean values of hematological parameters were not statistically significant in combinational versus monotherapy groups.

Conclusion

Single agent treatment with the VDA agent NOV202 and with olaparib reduced tumor growth in prostate cancer xenografts with *BRCA1/2* mutations *in vivo*. The anticancer efficacy of olaparib was significantly increased when combined with NOV202 which represents an option to increase the efficacy of PARPi. A clinical trial of this combination is planned.

EACR22-1256

Mesotheliomas exhibit conserved DNA damage repair genotype-attributable sensitivity to PARP inhibition

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Introduction

Mesothelioma is an aggressive cancer caused by asbestos. Therapeutic intent is always palliative and precision medicines are still lacking. We recently reported a Phase II clinical trial confirming poly(ADP-ribose) polymerase (PARP) inhibitor-induced tumour responses were attainable in the clinic. We hypothesized that mesotheliomas harbour conserved somatic DNA damage repair (DDR) alterations conferring homologous recombination deficiency that underpins sensitivity to PARP inhibition *in vitro* and *ex vivo*.

Material and Methods

To explore PARP inhibitor sensitivity, a panel of 19 primary mesothelioma cell lines were whole exome sequenced. In addition, haploid crispR-cas9 mediated BAP1 knockout and wild type isogenic expressing cell lines were used as a BAP1 specific model. Four PARP inhibitors were tested, (rucaparib, olaparib, talazoparib, and niraparib) using a high-throughput screening platform assaying DNA damage foci (γH2AX) and cell death. Orthogonal drug sensitivity was assayed by COMET for rucaparib on 11 of these 19 exome-sequenced cell lines, and using the public genomics of drug sensitivity of cancer (GDSC) exome sequenced mesothelioma panel. Further validation employed rucaparib treated primary mesothelioma explants sequenced in the MEDUSA study, assaying γH2AX. Interpretative machine learning (extreme gradient boosting) was used to quantify the contribution of DDR somatic alterations and PARP inhibitor sensitivity (using Z-scale transformation to normalise responses). Explanatory Shapely variables (SHAP) were correlated between models to assess concordance, using regression and 99% confidence limits to filter DDR genes of interest.

Results and Discussions

Drug screens revealed marked response heterogeneity with conserved resistance and sensitivity profiles for γH2AX, COMET, cell death, clonogenicity and apoptosis. SHAP values were highly correlated between PARP inhibitors (rucaparib versus all others), and between high-throughput γH2AX versus COMET drug screens. SHAP values were also strongly correlated between cell lines and explants.

Conclusion

In summary, mesotheliomas harbour heterogeneous DDR mutations which strongly correlate with sensitivity to PARP inhibition. Our results, suggest that PARP inhibitor efficacy in mesothelioma may be predicted, and this is being explored in the MIST1 trial cohort, with further validation planned in an upcoming randomised Phase II trial called NERO.

EACR22-0185

Antitumor activity of the SARM RAD140 in hormone-independent estrogen receptor-

positive breast cancer patient-derived xenografts

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Introduction

Up to 90% of estrogen receptor-positive (ER+) breast cancers (BC) also express the androgen receptor (AR). Selective modulators of the AR (SARMs), such as RAD140, have shown clinical activity in endocrine therapy (ET)-resistance. However, the mechanism by which AR agonists block ER is not clearly understood. Moreover, the combination of a SARM plus CDK4/6-inhibitors (CDK4/6i) could be a novel therapeutic strategy for ER+/HER2- patients who do not tolerate ET. Here, we aimed to study the antitumor activity of RAD140 in a cohort of ER+/HER2- patient-derived xenografts (PDX) to identify biomarkers of response.

Material and Methods

Hormone-dependence was evaluated in 30 PDXs using ovariectomized (OVX) vs non OVX mice. Alterations in *ESR1* and *PIK3CA* genes were determined with the MSK-IMPACT™ targeted exome panel and RNA-sequencing analysis. Pharmacodynamic experiments (PD) were conducted in 35 PDX models after 5-days treatment with the selective ER degrader fulvestrant, RAD140 or dihydrotestosterone (DHT). Protein levels of AR, ER and Ki67 were assessed by immunohistochemistry. Modulation of cell-cycle proteins by AR agonists was evaluated by Western blot. *In vivo* antitumor activity of RAD140 alone or in combination with palbociclib was tested in comparison to fulvestrant plus palbociclib.

Results and Discussions

Ten out of thirty ER+/HER2- PDXs harbored *ESR1*-mutations or fusions and were hormone-independent *in vivo*. PD experiments showed a Ki67-antiproliferative response with both RAD140 and DHT in 10 out of 35 models. We confirmed sensitivity to RAD140 monotherapy in 4 out of 7 Ki67-responsive models tested. Interestingly, AR agonists decreased E2F1, cyclin A/E2 protein levels in RAD140-sensitive but not resistant-PDX. RAD140 showed equal antitumor activity than fulvestrant when combined with palbociclib in 7 out of 8 models tested and superior activity in an *ESR1*-mutant model.

Conclusion

RAD140 exhibits antitumor activity in hormone-independent and *ESR1*-altered ER+/HER2- PDX models as single agent or in combination with CDK4/6i. Mechanistically, AR agonists decrease proliferation as shown by a decrease in Ki67 and S/G2-cell cycle cyclin levels.

Targeting DNA damage response and replication stress as a new therapeutic opportunity in colorectal cancer

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Introduction

Genomic instability is a hallmark of cancer and targeting the DNA damaging response (DDR) is emerging as a promising therapeutic strategy in different solid tumors. Systematic DDR analysis and targeting has not been extensively explored in colorectal cancer (CRC) yet. The identification of novel vulnerabilities to overcome resistance to commonly used therapies represents indeed a key finding for CRC treatment.

Material and Methods

We screened 112 CRC cell models recapitulating the genomic landscape of metastatic CRC with ATM, ATR, CHK1, WEE1, and DNA-PK inhibitors, currently undergoing clinical testing in phase I-III trials. In parallel we tested chemotherapeutic agents (oxaliplatin, irinotecan, 5-fluorouracil) to pinpoint response correlations of clinical utility. We focused on ATR inhibitors (ATRi) that are in advanced clinical development and have shown effectiveness also as monotherapy. In order to identify putative biomarkers of response and resistance to ATRi, we performed our multilevel analysis on CRC models highly sensitive or resistant to this inhibitor. Further bioinformatic investigation has been performed and validation on patient-derived organoids is provided.

Results and Discussions

We found that around 30% of CRC are sensitive to DDR inhibitors including those carrying KRAS and BRAF alterations and intractable with targeted agents. Sensitivity to ATRi correlated with response to chemotherapeutic agents. ATRi-sensitive cells displayed reduced phospho-RPA32 foci at basal level, while ATRi-resistant cells showed increased RAD51 foci formation after hydroxyurea treatment. Lack of ATM expression was associated with ATRi sensitivity. Analysis of mutational signatures and HRDetect score identified a subgroup of ATRi-sensitive models. Patient-derived organoids recapitulated findings obtained in cell lines.

Conclusion

In conclusion, a subset of CRC refractory to current therapies could benefit from inhibitors of DDR pathways and replication stress. A composite biomarker involving analysis of multiple biochemical and genomic features might identify CRC patients likely to respond to ATRi.

PARP1 expression and PBRM1 mutation as potential biomarkers for predicting the response to atezolizumab plus bevacizumab or to sunitinib in clear cell renal cell carcinoma patients

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Introduction

The survival of metastatic renal cell carcinoma patients has largely improved in the last decades with the development of antiangiogenic drugs and more recently with the approval of immune checkpoint inhibitors (ICIs). However, there is a large variability in treatment response that cannot be predicted. In clear cell renal cell carcinoma (ccRCC), *PBRM1* mutations have been shown to augment tumor angiogenesis and improve antiangiogenic response, and a recent study suggested an interaction between *PARP1* expression and *PBRM1* mutation predictive of ICI response (1). This study aimed to further explore the interplay between these potential predictive biomarkers.

Material and Methods

IMmotion151 trial, which includes advanced renal cancer patients treated with either atezolizumab plus bevacizumab (ICI plus antiangiogenic) or sunitinib (antiangiogenic) (n=794) (2), was used to extract genomic, transcriptomic (EGAS00001004353), molecular expression clusters and clinical data. Progression Free Survival (PFS) and molecular clusters were compared among ccRCC cases after *PARP1/PBRM1* stratification.

Results and Discussions

PFS was shorter in the *PARP1*-high expression group in atezolizumab plus bevacizumab arm and in sunitinib arm ($p < 0.005$). Poor/intermediate MSKCC prognostic risk groups were over-represented in *PARP1*-high patients, however, *PARP1* association with PFS remained statistically significant after adjusting for prognostic classification. When *PBRM1* mutation was used to further stratify *PARP1* groups, in sunitinib arm *PARP1*-high/*PBRM1*-wildtype patients exhibited a significantly shorter PFS compared to the rest of the groups ($p < 0.0005$). In addition to *PBRM1* mutations, *PARP1* expression also had an effect on tumor angiogenesis ($p < 10^{-5}$), with *PARP1*-high/*PBRM1*-wildtype cases having the worst angiogenesis scores. Interestingly, the highest proportion of T-effector/proliferative and Proliferative clusters corresponded to this same group. Unfortunately, IMmotion151 lacked an ICI alone arm, thus limiting our ability to investigate *PARP1/PBRM1* role in this context.

Conclusion

We show for the first time that *PARP1* expression status appears to be a predictor of PFS in ICI plus antiangiogenic and antiangiogenic alone treatments, and potentially a predictor of PFS in *PBRM1*-wildtype patients treated with antiangiogenics. Further studies analyzing the interplay between *PARP1* and *PBRM1* should be performed in ICI treated ccRCC patients, and extended to antiangiogenic treatment.

1. Hagiwara, *European Urology* 2021

2. Motzer, *Cancer Cell* 2020

Pharmacokinetic characterization of the DDAH1 inhibitors ZST316 and ZST152 in mice *T. Ceruti¹*

Introduction

The inhibition of the isoform 1 of the enzyme dimethylarginine dimethylaminohydrolase (DDAH1) is emerging as an attractive therapeutic strategy for tumors characterized by neo-vascularization driven by excessive nitric oxide (NO) synthesis. DDAH1 has a critical role in the metabolism of asymmetric (ADMA) and N-monomethyl (LNMA) arginine, endogenous inhibitors of NO synthase (NOS). Therefore, DDAH1 regulation strongly influences NO synthesis. The DDAH1 inhibitors ZST316 and ZST152 have been shown to suppress vasculogenic mimicry *in vitro*, an alternative neo-vascularization pathway driven by iNOS. After developing efficient HPLC-MS/MS methods, we determined for the first time their pharmacokinetics and tolerability to establish the best route of administration for activity studies.

Material and Methods

The pharmacokinetics and urinary excretion of ZST316 and ZST152 were investigated by HPLC-MS/MS. Four-week-old female FVB mice received 30 mg/Kg of ZST316 and ZST152 by intravenous bolus (iv) or 60 mg/Kg by oral (po) gavage and blood and urine were sampled for up to 24h after dosing. Then, acute and chronic intraperitoneal (ip) treatment with the more potent inhibitor, ZST316, 30 mg/Kg, were compared. Further studies on the main metabolites in urine and plasma are ongoing and will be presented at the conference.

Results and Discussions

The method resulted specific, sensitive, precise, and accurate for compounds quantitation in plasma and urine. After po and iv treatment, ZST316 achieved C_{max} of 1.02 and 67.4 µg/mL, respectively, rapidly distributed in the body, and was eliminated with half-life (HL) of 6h. ZST152 achieved C_{max} of 24.9 and 1.65 µg/mL after iv and po, respectively, and cleared with shorter HL of 1.2h. The urinary excretion of ZST152 and ZST316 amounted to 12.5-22.2% and 2.3-7.5%, respectively. After acute and chronic ip administration, ZST316 reached C_{max} of 40.2 and 33.6 µg/mL and was eliminated with HL of 5.7 and 8.4h, respectively. Bioavailability was 59% and no accumulation was observed. Chronic administration was well tolerated with no signs of weight loss, clinical toxicity, or distress.

Conclusion

The developed methods proved well suitable to conduct the pharmacokinetic study. ZST316 exhibited a more favorable pharmacokinetic profile than ZST152 and was well tolerated during chronic ip treatment. The results of this study will be useful to adequately design *in vivo* studies to determine whether DDAH1 inhibition is an important target for antitumor activity.

EACR22-0068

Deciphering the intra-tumor and inter-patient signaling heterogeneity in cancer for the rational design of patient specific drug cocktails

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Introduction

Large-scale cancer datasets have been rapidly accumulating over the past years. The challenge has now become to find a way to obtain useful information from these masses of

data that will allow to determine which combination of approved drugs is best suited to treat the specific tumor.

Material and Methods

We have recently developed a computational, thermodynamic-based strategy allowing to accurately predict the response of cells to drug treatments or to rationally design anti-cancer personalized drug combinations. While conventional approaches classify cancers based on characteristic biomarkers, we identify patient-specific, central protein nodes *representing* a patient-specific signaling signature (PaSSS), consisting of several distinct subnetworks, or unbalanced processes (i.e. a set of altered co-expressed, protein-protein subnetworks deviating from the steady state in each tumor).

Results and Discussions

Our recent in-vivo results in melanoma, breast, lung and oral cancers indeed show that PaSSSs successfully dictate the design of patient-specific drug cocktails. We demonstrate that simultaneous inhibition of central protein targets from the entire set of *distinct* unbalanced processes blocks the patient-specific altered signaling flux and is more effective than the treatments used in clinics.

Lately we took a step forward and extended the approach to single cells. This development allows quantifying a set of unbalanced processes in each individual cell and then mapping distinct subpopulations in every tumor without a need for large datasets, usually including multiple tissues from different patients. A high number of measured cells (>500,000) from *one* tumor are compared instead of many patients, thereby providing statistically significant information about different unbalanced processes within *each* sample. We demonstrate how this strategy can be used in order to sensitize aggressive Triple Negative Breast Cancer (TNBC) to radiotherapy (RT). Using mouse models and patient-derived TNBC tumors we show that two distinct subpopulations expanded in response to RT. We demonstrate that simultaneous targeting of central protein nodes representing those subpopulations, Her2 and cMet, was essential in order to sensitize TNBC to RT and stop its growth.

Conclusion

We show how the information from multiple patients/ cells can be transformed into a low-dimensional space to provide patient-specific, unbalanced network structures. These structures can be used to rationally design patient-specific drug therapies.

EACR22-0242

A CTC-Derived eXplant (CDX) model of a Merkel cell carcinoma to inform treatment opportunities

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Introduction

There is an unmet need for preclinical models and biomarkers to inform treatment and improve outcomes of patients with Extra-Pulmonary (EP) NeuroEndocrine Carcinoma (NEC); a rare, yet lethal diagnosis. In the pulmonary NEC counterpart, Small Cell Lung Cancer (SCLC), patient-relevant mouse models can be generated from circulating tumour cells (CTCs); termed CTC-Derived patient eXplants (CDX).

Material and Methods

A prospective pilot study is on-going to serially enumerate CTCs by CellSearch (CS; epithelial EpCAM-positive CTCs) and assess feasibility of CDX generation in patients with advanced stage EP-NEC who undergo palliative chemotherapy. CDX generation is attempted via implantation of CTCs enriched from 10mL of blood (after reduction of lymphocytes by RosetteSep) into immunodeficient mice.

Results and Discussions

A CDX was generated from a pre-treatment blood sample from a patient with a metastatic NEC of unknown origin who responded to platinum/etoposide (P/E). Histopathological analysis of this CDX showed a small cell NEC morphology, 90% Ki-67 positive cells, expression of pan-cytokeratins and neuroendocrine (NE) diagnostic markers, concordant with the donor biopsy. Whole-exome sequencing of the CDX and donor's pre-treatment cell-free DNA revealed overlapping genomic features, including an actionable *PIK3CA* mutation. Further immunohistochemistry and RNA sequencing revealed expression of the NE transcription factor atonal homolog 1 (ATOH1), CK20 and Merkel cell polyomavirus transcripts. These characteristics prompted reclassification of the original patient diagnosis to Merkel cell carcinoma (MCC); a NEC usually arising from the skin. With a CS-CTC count in the parallel pre-treatment sample was only 1 CTC in 7.5mL of blood, the CDX was most likely derived from either non-epithelial CTCs (missed by CS) or a mixed epithelial/non-epithelial CTC pool. Mice bearing this CDX responded to P/E, reflecting the chemosensitivity of the donor. *Ex vivo* CDX cultures were successfully established providing a platform supporting therapy testing.

Conclusion

We report the generation of a MCC CDX from donor who is still alive 30 months from diagnosis. The CDX facilitated expansion of the donor's tumour allowing comprehensive molecular characterisation, a vehicle for drug testing with potential to define new therapeutic opportunities including a PI3K inhibitor.

EACR22-0332

The mTOR pathway as a potential therapeutic target in sinonasal intestinal-type adenocarcinoma

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Introduction

Sinonasal intestinal-type adenocarcinoma (ITAC) is a rare tumor etiologically related to professional exposure to wood dust and leather. The overall prognosis is poor due to the difficulty to resect the tumor completely in this anatomically complex region. Therefore, there is great need for alternative treatments. Previous studies have shown mutations in genes involved in the mTOR pathway. Our aim was to evaluate mTOR expression in a series of primary ITAC and to preclinically test mTOR as a target for treatment with specific inhibitors.

Material and Methods

Phospho-mTOR expression was analyzed in a cohort of ITAC by immunohistochemistry. The usefulness of tumor cell line ITAC-3 (Pérez-Escuredo et al, Cell Oncol 2011) with regard

to mTOR pathway alteration was confirmed by next generation sequencing, PCR, qPCR and Western blot. The effects of mTOR inhibitors rapamycin and everolimus on cell proliferation and mTOR pathway signaling activity were investigated.

Results and Discussions

Until the present moment, 50 ITAC tumors have been analyzed of which 44 (88%) demonstrated immunohistochemical staining of phospho-mTOR, 18 cases scored as 1+, 16 cases as 2+, and 6 cases as 3+. These results confirm the relevance of the mTOR pathway in ITAC. Also, the ITAC-3 cell line presented an elevated expression of phospho-mTOR, possibly caused by an inactivating mutation in IRS4 and overexpression of TSC1. This makes ITAC-3 a good model for preclinical testing of specific mTOR inhibitors. We found a growth inhibitory effect of rapamycin and everolimus inhibitors associated with a decrease in phospho-mTOR and an increase in phospho-AKT expression level.

Conclusion

The mTOR pathway is frequently overactivated in ITAC and could be used in the clinic as a molecular target for personalized treatment. Using an in vitro model we demonstrate the growth inhibitory efficacy of two different mTOR inhibitors.

EACR22-0616

Personalised medicine approach in pancreatic cancer via FAK priming improves response to Gemcitabine and Abraxane guided by intravital imaging

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Introduction

A key factor that limits the efficacy of drug treatment in cancer is extensive deposition of extracellular matrix (ECM) or fibrosis, which provides positive biomechanical feedback signalling to cancer cells to enhance tumour progression and treatment resistance. Pancreatic ductal adenocarcinoma (PDAC) is a highly fibrotic and metastatic malignancy with its' dense, desmoplastic ECM known to both promote and prevent PDAC development. The multifunctional protein, Focal Adhesion Kinase (FAK), is at the intersection of various pathways often hijacked in cancer and is a known regulator of cancer fibrosis as well as ECM stiffness and mechano-signalling. Here, we aim to deconstruct stromal *versus* epithelial targeting in a stratified subset of human PDAC patients to determine which patients will best benefit from FAK inhibition (or 'priming') prior to chemotherapy.

Material and Methods

Intravital (*in vivo*) imaging provides a dynamic real-time readout of cancer cell response to drug therapy in their native environment. Here, we used a biosensor of FAK activity in combination with Second Harmonic Generation (SHG) imaging of collagen fibres to quantify the anti-fibrotic efficacy of FAK priming in mouse and patient-derived PDAC models. Subsequent assessment of the Fucci cell cycle reporter allowed us to track PDAC cell response to standard-of-care chemotherapy after anti-fibrotic FAK priming in live primary and secondary PDAC sites, which we further evaluated using pillar plates and hydrogels. Shear stress assays and organotypic matrices were used to assess how FAK priming reduces metastasis *in vivo*.

Results and Discussions

Our analysis of ECM ultrastructure revealed that FAK priming reduced collagen crosslinking and ECM stiffness, resulting in altered mechano-reciprocity and tumour-stroma feedback. This softer environment stalled cancer cells during cell cycle progression rendering them increasingly vulnerable to Gemcitabine/Abraxane chemotherapy at both primary and secondary sites. Long-term survival studies in stratified patient-derived PDAC models demonstrate that FAK priming prior to chemotherapy can reduce metastasis by up to 50% while extending survival. Lastly, we show that companion biomarker assessment of the tumour suppressor gene Merlin may highlight, which PDAC patients respond well to our combination therapy approach.

Conclusion

Shifting the biomechanics of the PDAC stroma *via* FAK priming may allow us to maximise response to standard-of-care chemotherapy while profoundly reducing metastasis in PDAC patients.

EACR22-0628

THREE DIMENSIONAL MODELS TO FIND EFFECTIVE TREATMENTS IN UNDRUGGABLE LKB1 MUTATED NSCLC

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Introduction

In the recent years, we assisted to a reduction in mortality among patients affected by non-small-cell lung cancer (NSCLC). However, in the heterogeneous landscape of NSCLC there are specific subgroups resistant to immune checkpoint inhibitors (ICIs) and tyrosine kinase inhibitors, as is the case of LKB1 mutated tumors. Inactivating mutations in LKB1, which

occur in about 30% of NSCLCs, have been recently described as negative determinant of ICIs response and are mutually exclusive with targetable alterations of EGFR, ALK and ROS1 genes. We and others identified some treatments specifically active on LKB1 mutated preclinical models. However, there is cumulating evidence that also in the molecularly defined subgroups of LKB1 NSCLCs, intratumor heterogeneity exists and the co-occurring alterations could be impactful on drug sensitivity/resistance. Therefore, cell-based 2D models could limit the clinical translation of the results. Patients-derived organoids, compared to 2D cell cultures, constitute near physiological models mimicking the structure and the environment of the tissue and better recapitulate tumor heterogeneity. Thanks to the already established collaboration with the Fondazione Ca' Granda Ospedale Maggiore Policlinico in Milan in providing fresh tumor specimens, we generated PDX-derived organoids (PDXOs), with the aim to test the findings obtained in cell lines-derived 3D models.

Material and Methods

We generated PDXOs with procedures optimized in the lab, starting from protocols in the literature. Cytotoxicity experiments were carried out by seeding PDXOs with or without Basement Membrane Extracts and Cell-Titer Glo assay was performed at the end of the treatments. Western Blot, immunofluorescence and immunohistochemistry analyses were performed by standard procedures.

Results and Discussions

At present, we have successfully obtained 3D cultures from different PDXs, which grew in culture for several passages. Histologically, they expressed primary lung cancer markers, and maintained the molecular alterations found in both patients' and PDXs samples. Preliminary pharmacological studies confirmed the feasibility on PDXOs for the screen of potential new therapies.

Conclusion

PDXOs better mimicked the heterogeneity typical of NSCLCs and represented valid tools to validate the results obtained in canonical in vitro models and test new treatment strategies.

EACR22-0846

Peptide-guided macrophage photoimmunotherapy

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Introduction

Anti-inflammatory tumour-associated macrophages (M2 TAMs) exert various protumoural actions, are highly overrepresented in tumour microenvironment and can be specifically targeted through CD206. Here, with the aim of developing a rigid, stable, easy to synthesise TAM-targeting ligand, we designed a new peptide, a hybrid between a short linear CD206-

targeting peptide that we previously identified (mUNO) and the Sunflower Trypsin Inhibitor-I and named it “TrimUNO”. We then coupled TrimUNO to an FDA-approved photosensitizer, Verteporfin, designated it “Vert-TrimUNO”, and used it to perform macrophage photoimmunotherapy (PIT). PIT is a potential treatment option for solid tumours by eliciting immunogenic cell death. The aim of our study was to evaluate the therapeutic potential of Vert-TrimUNO.

Material and Methods

Liquid chromatography-mass spectrometry (LC-MS) was used to determine the stability of peptides in tumour lysates. Human macrophages from peripheral blood mononuclear cells, stimulated to become M1 or M2, were used to analyse the binding of fluorescein-labelled (FAM) TrimUNO to CD206, and to determine the cytotoxicity of Vert-TrimUNO PIT. In vivo biodistribution studies in orthotopic triple negative breast cancer (4T1) model were used to assess the targeting of FAM-TrimUNO to M2 TAMs.

Results and Discussions

Computational modelling showed a strong interaction of TrimUNO with the interlectin domain of CD206 and a high degree of conformational stability. LC-MS revealed a 5.5-fold increased stability of TrimUNO in tumour lysate respect to parental mUNO. In vitro binding assay of FAM-TrimUNO revealed higher binding to CD206 compared to FAM-mUNO. Biodistribution studies of intravenously administered FAM-TrimUNO in mice bearing 4T1 breast tumours showed higher FAM/CD206 colocalisation than FAM-mUNO, and intraperitoneally administered FAM-TrimUNO revealed 9x more FAM-TrimUNO signal inside the targeted cells compared to FAM-mUNO. Additionally, FAM-TrimUNO was able to target M2 TAMs through oral administration. Vert-TrimUNO PIT was highly and selectively toxic to human M2 macrophages in vitro, being 2x and 3x more toxic than doxorubicin (Adriamycin®) and Vert-mUNO PIT, respectively. Importantly, the toxicity of Vert-TrimUNO PIT was abolished when blocking CD206, indicating that the toxicity of Vert-TrimUNO PIT requires CD206/TrimUNO interaction.

Conclusion

TrimUNO is a new and improved M2 TAMs targeting peptide that in combination with Verteporfin could be used to treat solid tumours using PIT.

EACR22-0896

Ovarian cancer Zebrafish Avatars for personalized multiple drug testing

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Introduction

Ovarian Cancer is the 1st cause of death amongst gynecological cancers with a relapse rate of 70-80%, with chemo-resistant metastases. Second line therapeutic alternatives are chosen based on trial-and-error approaches, exposing patients to unnecessary treatments. Until now, there is no personalized test to assess the best option for each patient. We have therefore developed the zebrafish Avatar model (zAvatars) to be used as a personalized test to find the best treatment for each patient.

Material and Methods

Cells were isolated from ovarian cancer patients, after obtaining their informed consent. zAvatars were generated by injecting cells in the perivitelline space of 2 days post

fertilization zebrafish embryos. Characterization was performed by live imaging and immunofluorescence confocal microscopy to assess tumor size, mitochondrial morphology, apoptosis, angiogenic potential and metastatic spread. zAvatars were challenged with the 1st and 2nd line therapeutic options, to evaluate drug sensitivity.

Results and Discussions

We observed a heterogeneity of responses: while some zAvatars showed high sensibility to Paclitaxel+Carboplatin (PC), others did not respond. In one case, zAvatars were generated from cells isolated from the ascitic fluid of a patient undergoing neoadjuvant PC chemotherapy. Tumor cells showed no sensitivity to PC nor to 2nd line doxorubicin or bevacizumab contrasting with the high sensibility to combinations of bevacizumab+paclitaxel and bevacizumab+topotecan - increased cytotoxicity and reduced tumor implantation in both cases. The absence of response to PC matched the patient clinical response, where only a mild effect of PC was observed. Nonetheless, in other cases, tumor cells showed sensitivity to PC, matching the patient clinical response. In all cases, treatment with topotecan showed the highest cytotoxicity.

In all cases tumors showed a high metastatic and angiogenic potential. Live imaging showed macrophage infiltration and endothelial cell recruitment within the first 10 hours after injection.

Conclusion

Ovarian cancer zAvatars allowed us to assess in a short-term assay drug sensitivity, and to evaluate the impact of these therapies on tumor-induced angiogenesis and metastatic spread.

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EACR22-0984

Zebrafish Avatar model towards personalised Breast Cancer therapy

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Introduction

Triple Negative Breast Cancer (TNBC) is a biologically heterogeneous disease which lacks effective targeted therapy options. Thus, chemotherapy (CT) was until recently the only available systemic treatment for TNBC patients. There are no predictive markers of response to different types of cytotoxic agents and no optimal sequence of therapy in metastatic breast cancer. In early TNBC, the mainstay is neoadjuvant CT with sequential use of anthracyclines and taxanes, irrespective of the individual tumour response. Our main aim is to develop an assay to predict tumour's response to CT agents to tailor therapy, avoiding unnecessary toxicities using the zebrafish patient derived xenografts model (zPDXs or zAvatars).

Material and Methods

Zebrafish xenografts were generated by injecting fluorescently labelled TNBC cell lines to examine differences in response to taxanes and anthracyclines. Zebrafish Patient-derived xenografts (zAvatars) were generated from breast surgery tumours, fine core needle

biopsies and ascites. zAvatars were exposed to the same CT as its matching Patient. After 4 days of chemotherapy treatment, proliferation, apoptosis, and tumour size were analysed by single-cell confocal microscopy. Patient's response to treatment was compared to its matching zAvatar.

Results and Discussions

We show that TNBC zebrafish xenografts have resolution to distinguish responses to compounds of the same family, such as taxanes and anthracyclines. Additionally, we were able to generate zAvatars from breast surgery tumours and ascites effusions and show that these recapitulate patient's treatment response. Most importantly, we demonstrate the successful engraftment of core needle BC biopsies and its ability to reproduce patients' clinical response, both in early and advanced disease.

Conclusion

Altogether, our results suggest that zAvatar model constitutes a promising *in vivo* assay for screening chemotherapy in breast cancer and a valuable tool towards personalized cancer treatment in early and metastatic BC.

EACR22-0996

Co-targeting the AR and FGFR signalling to inhibit growth and progression of prostate cancer

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Introduction

Androgen receptor (AR) plays a key role in progression of prostate cancer (PCa) and is the main therapeutic target of PCa. PCa that advances irrespective of androgen deprivation can progress to castration resistant PCa via bypass signaling, activating pathways like fibroblast growth factor receptor (FGFR) signaling. In this study, we tested if next generation, covalent-binding and irreversible FGFR inhibitors in combination with clinically approved AR-antagonists such as enzalutamide and darolutamide were more effective against advanced PCa. We used physiologically representative *in vitro* 2D and 3D mono and co-culture models in order to include the role of extra cellular matrix (ECM) in facilitating the tumor-stroma interactions.

Material and Methods

LNCaP, CWR-R1 and VCaP cell lines in 2D and 3D mono- and co-culture with cancer-associated fibroblasts (CAFs) were treated with AR inhibitors (enzalutamide, darolutamide) and FGFR inhibitors (FIIN1, FIIN2) in single and combination format. The effects of treatments on proliferation, viability and downstream proteins were monitored by imaging and immunoblotting. The morphology of 3D organoids were analyzed using confocal microscopy and quantitative image analysis.

Results and Discussions

The single and combination treatments were around 40% more effective in 3D co-culture conditions in which tumor cells and CAFs engage in paracrine and autocrine AR and FGFR signaling, resulting in mutual stimulation and support. FGFR inhibitors alone and in combination with both the AR inhibitors led to a loss of tumor-stromal cell interactions and a reduction in the viability of tumor cells. In the combination treatment, certain concentrations showed an additive effect of the inhibitors on the cells. At a molecular level, AR inhibitors decreased the expression of the AR-regulated proteins such as prostate specific antigen

(PSA) in a concentration dependent manner. The FGFR inhibitors decreased the downstream FGFR pathway such as p-ERK42/44, explaining the reduction in cell viability.

Conclusion

The next-generation inhibitors like FIIN1 and FIIN2 by themselves and in combination with AR inhibitors effectively reduced the viability of FGFR-dependent PCa cells by inhibiting the respective signaling pathways. Moreover, using model systems like 3D co-culture containing an ECM with active tumor-stroma interactions provide better insights on the effects of inhibitors on tumor cells.

EACR22-0283

CAE606: A NOVEL EXTERNAL AUDITORY CANAL SQUAMOUS CELL CARCINOMA CELL LINE SENSITIVE TO CDK4/6 INHIBITION

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Introduction

The External Auditory Canal Squamous Cell Carcinoma (EACSCC) is a rare and aggressive cutaneous malignance that represents 0.2% of head and neck tumours. Diagnosis is often delayed and most of cases are detected in advanced stages with a 5-year survival of 10%. The main treatment option is surgery with adjuvant radiotherapy. Previous studies have reported frequent alterations in cell cycle regulating genes as TP53, RB1, CDKN2A and CCND1, similar to cutaneous and head and neck SCC. We established tumour cell line CAE606 as a model to investigate new therapeutic approaches for EACSCC.

Material and Methods

CAE606 cell line was derived from a 62-year-old man with a previously untreated, primary moderately differentiated T2N0M0 EACSCC. Genomic analyses included NGS, PCR Sanger sequencing and SNP array. Growth rate was determined and drug sensitivity was deduced from EdU incorporation and a time-lapse wound-healing assay. Protein expression analysis was performed by western blot and immunofluorescence techniques.

Results and Discussions

CAE606 cells grew with a population doubling time of 26 hours. Cells showed strong positivity for cytokeratin 34βE12 and p63 confirming the diagnosis of SCC. Vimentin expression was observed in 50% of cells, possibly reflecting the invasive behaviour of the primary tumour. NGS revealed somatic inactivating mutations in CDKN2A and TP53 that caused complete absence of p16 and p53 expression. NGS and SNP array also revealed high level amplification of CCND1 and EGFR. These genetic alterations affect the G1-S checkpoint causing unscheduled DNA replication and uncontrolled cell proliferation, making CAE606 a good model for testing cell cycle inhibitors. Exposure to CDK4/6 inhibitor palbociclib caused a strong decrease of phospho-Rb expression and growth inhibition with an IC50 of 0.46 μM. However, we found that the cytostatic effect of palbociclib is overcome after 72 hours exposure.

Conclusion

We established and characterised a new EACSCC cell line. Our results show CAE606 to be sensitive to CDK4/6 inhibition through phospho-Rb downregulation, although the effect is transient. To achieve a durable response we are currently investigating combinatory

treatments schemes with chemotherapy (including cisplatin), radiotherapy as well as PARP inhibitors, that may be applied in treating EACSCC patients in the clinic.

EACR22-0619

Developing a robust methodology and metric to study anticancer drug responses on PDX-Derived Tumour cells

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Introduction

Preclinical models like patient derived tumour xenografts (PDX) that recapitulate the heterogeneity of human tumours enhance clinical predictive power compared to cell lines. PDX-derive tumour cells PDTCs in short term culture provide a cost-effective alternative in high-throughput drug response analysis. CellTiter-Glo (CTG) is the commonly used endpoint method but it is a lytic assay, and therefore does not allow dynamic analysis. The need for real time measurement of cell proliferation is essential. To improve the clinical predictive value of the PDTC biobank, we develop a dynamic and tailored experimental pipeline supported by improved computational methods.

Material and Methods

The frozen tissues of 40 PDXs were dissociated into single-cell suspensions with mechanical and enzymatic methods and cultured in 384-well plates. Two different bioassays were used to profile cell growth and to quantify drug responses: the CTG assay, used for measuring cell viability at specific end points, and the RealTimeGlo MT Cell assay (RTG), that allows real-time monitoring of cell viability. Reading intensities were obtained at days 0, 1, 2, 3, 6 and 7.

Results and Discussions

Capturing the dynamics of cell growth in PDTCs is essential for accurate drug sensitivity estimation. We compare growth rates using both CTG and RTG and demonstrate the advantages of using the RTG protocol, allowing us to track the same group of cells over time. We compare biological and technical reproducibility and show how RTG allows us to reduce the inherent noise in high-throughput drug sensitivity studies. We derive AUC/IC50 based metrics for RTG experiments and present detailed comparisons at different time points that highlight differences in drug effects over time and across models, demonstrating the utility of our drug testing platform. Finally, we present additional advantages of RTG, such as the reduction in the required number of cells, compounds and consumables in high-throughput testing experiments.

Conclusion

We have developed and optimised an RTG-based bioassay to estimate dose-response effects on PDTCs in high-throughput drug screenings accurately. Its robustness is the result of measuring individual dose responses over time by real-time monitoring of growth rate inhibition.

Synergic activity of FGFR2 and MEK inhibitors in the treatment of FGFR2-amplified cancers of unknown primary

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Introduction

Patients with cancer of unknown primary (CUP) carry the double burden of an aggressive disease and reduced access to therapies, which are typically tumor-type oriented. Experimental *in vitro* and *in vivo* models summing up CUP features and heterogeneity are currently unavailable.

Material and Methods

We derived and expanded two CUP cell lines (CUP#96 and CUP#55), and corresponding patient-derived xenografts (PDXs), from ascites tumor cells. CUP cell lines and PDXs were subjected to histological, immune-phenotypical, molecular and genomic characterization to show their ability to recapitulate the original tumor. MicroRNA profile, target sequencing and low-pass Whole Genome Sequencing were applied to identify the possible sites-of-origin and therapeutic targets. FGFR2 gene amplification in both cell lines provided the rationale for evaluating the potential of targeting this receptor for therapeutic intervention. To target FGFR2 we first tested a panel of FGFR2 inhibitors, then we selected the most effective and combined this drug with a MEK inhibitor and compared with the standard therapy received by the patients.

Results and Discussions

The predicted primary sites were gastrointestinal for CUP#96 and cholangiocarcinoma for CUP#55 model. Genetic testing and FISH analysis identified FGFR2 amplification as druggable genetic alteration in both patients, which was also validated in cellular and animal models. Drug-screening assays were performed *in vitro* to test the activity of FGFR2 targeting drugs on cancer cell proliferation and viability. The permanence of MAPK signaling activity upon FGFR2 targeting prompted the combination treatment with MEK inhibitor, trametinib, which proved to be synergic and exceptionally active.

Conclusion

This study brings personalized therapy closer to CUP patients and paves the way to future applications of personalized medicine for metastatic patients with adverse prognosis.

Inhibition of nuclear export can overcome resistance to tyrosine kinase inhibitors in ALK-rearranged lung cancer

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Introduction

ALK rearrangements are found in 3–7% of Non-Small Cell Lung Cancers (NSCLCs), resulting in constitutive activation of the ALK-kinase and subsequent onset of malignant transformation. EML4-ALK can be specifically targeted by ALK tyrosine-kinase inhibitors (TKIs) but treatment is frequently associated with the development of acquired resistance and a few therapeutic options are available for refractory and/or resistant ALK+ NSCLC patients. Selective inhibitors of nuclear export (SINE) compounds have been developed and used as novel therapeutic strategies to block tumor development or relapse, alone or together with standard therapies. We then investigated the efficacy of the XPO1 inhibitor selinexor in combination with ALK TKIs in both ALK+ NSCLC cells and pre-clinical models of ALK-driven NSCLC.

Material and Methods

ALK+ NSCLC cell lines, patient derived crizotinib-resistant ALK+ NSCLC cell lines and a patient-derived organoid (PDO) from ALK+ NSCLC patient resistant to lorlatinib were treated *in vitro* with selinexor alone and in combination with ALK TKIs. Cell viability and cell death were measured and IC50 curves were determined. EML4-ALK transgenic (Tg) mice were used to test the efficacy of the combination *in vivo*. Mice were stratified based on tumor volume measured by Magnetic Resonance Imaging.

Results and Discussions

We tested selinexor *in vitro* alone or in combination with crizotinib in different ALK+ NSCLC cells and observed that it significantly affected cell viability and induced cell death as single agent; and potentiated the antiproliferative effect of crizotinib when combined. We found that upon XPO1 inhibition, expression levels of anti-apoptotic proteins, such as BCL2 and MCL1, decreased proportionally. Consistently with *in vitro* data, we observed that the combination of crizotinib with selinexor impaired tumor growth more efficiently than the single agents also in EML4ALK Tg mice. In addition, we demonstrated that cell viability was greatly reduced by selinexor alone or in combination with ALK-TKIs when tested in cell lines derived from crizotinib resistant patients and in a patient-derived organoid (PDO) from ALK+ NSCLC patient resistant to lorlatinib.

Conclusion

Overall, these data support the therapeutic value of selinexor as a single agent or in combination with ALK TKIs for the treatment of ALK+ NSCLC, including ALK TKI resistant NSCLC.

EACR22-1225

Multi-omics approach to ovarian cancer research

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Introduction

Ovarian cancer remains one of the deadliest of all gynecologic cancers. The recent addition of PARP inhibitors to its treatment options has caused a paradigm shift in the management of this disease. The studies that led to the approval of such drugs, initially for recurrent ovarian cancer patients with *BRCA1/2* mutations, call for a precision medicine approach based on biomarkers. Other than the presence of these mutations and several clinical factors, such as platinum sensitivity, no other biomarkers are currently used in the clinic and new therapeutic approaches in the resistance settings are urgently needed. To tackle these problems, we are studying multiple features of ovarian tumors before and after treatment (both at a cellular and molecular level) to find drug response biomarkers and potential novel treatments to be tested, both *in vitro* and *in vivo*.

Material and Methods

Fresh tumor samples from the surgery of ovarian cancer patients are collected to generate patient-derived organoids (PDOs) that are subsequently characterized. Simultaneously, we are analyzing pre- and post-treatment paired samples from patients who progress after PARP inhibitors by 10X Visium Spatial Transcriptomics (ST).

Results and Discussions

ST experiments provide a high-resolution cellular map with information on the physical location of the various cell types in the tumor and their gene expression profiles. These spatial transcriptomic profiles, or gene expression maps, can help to better understand the biology of cancer and elucidate resistance mechanisms to PARP inhibitors. PDOs represent a superior preclinical model system than previous models through their inherent heterogeneity, long-term stability, and applicability for high throughput screens, making them extremely useful in the evaluation of resistance biomarkers.

Conclusion

Our results could positively impact an unmet need for one of the deadliest cancer types in women, suggesting new biomarkers to better use PARPi and new treatment approaches in the resistance settings. Finally, our multi-omics approach could be extended to other cancer types in which this type of treatment is currently being used or explored.

EACR22-0324

Single-Cell Signalling Analysis of Engineered $\gamma\delta$ T cell Biotherapeutics for the Treatment of Patient-Derived Colorectal Cancer Organoids

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Introduction

Colorectal cancer (CRC) is a devastating disease that kills ~700,000 people worldwide annually. There is an unmet clinical need for late-stage patients, with immunotherapies struggling against the immunosuppressive CRC tumour microenvironment (TME). $\gamma\delta$ T cells comprise 1-10% of CD3⁺ T cells in human blood and perform both innate and adaptive immune processes. Tumour infiltrating $\gamma\delta$ T cells (specifically V γ 9V δ 2) confer a prognostic benefit to CRC patients and can kill cancer via multiple mechanisms - including antibody-dependent cellular cytotoxicity (ADCC) against tumour antigens. We hypothesise that $\gamma\delta$ T cells can be exploited as an anti-CRC biotherapeutic but their complex interactions within the CRC TME need to be elucidated.

Material and Methods

We performed 3D TME co-cultures of CRC patient-derived organoids (PDOs) with CRC cancer-associated fibroblasts (CAFs) and human $\gamma\delta$ T cells engineered to secrete a modified IL15 cytokine to augment $\gamma\delta$ T cell proliferation and cytotoxicity. Following the addition of anti-B7-H3 IgG to the cultures, we analysed anti-PDO ADCC and TME signalling interactions using 126-plex Thiol Organoid Barcoding *in situ* (TOBis) mass cytometry (MC) (Qin *et al.*, Nature Methods, 2020) (Sufi and Qin *et al.*, Nature Protocols, 2021). TOBis MC enabled us to study cell-type specific post-translational modification (PTM) signalling responses in PDOs, fibroblasts, and $\gamma\delta$ T cells across 126 organoid co-cultures via Earth Mover's Distance (EMD) and Potential of Heat-diffusion for Affinity-based Trajectory Embedding (PHATE).

Results and Discussions

Engineered $\gamma\delta$ T cells exhibit superior proliferation, purity, cytotoxicity, and viability, with $\gamma\delta$ donor-specific responses to cytokine engineering (¹²⁷IdU⁺ and pSTAT5 [Y694]⁺) and PDO co-culture (CD27⁺ and CD69⁺). Engineered $\gamma\delta$ T cells demonstrate a substantial anti-B7-H3 ADCC killing phenotype against multiple CRC PDOs (cCaspase3 [D175]⁺ and cPARP [D214]⁺). We detect the transfer of ADCC effector proteins such as Granzyme B from $\gamma\delta$ T cells to PDOs, offering mechanistic insight into PDO apoptosis. ADCC performance varies between $\gamma\delta$ donors and is associated with Fc γ R CD16 expression. CAFs can protect PDOs from $\gamma\delta$ ADCC and work is continuing to understand the mechanistic basis of this.

Conclusion

TOBis MC enables highly multiplexed single-cell experiments to investigate anti-PDO ADCC by engineered $\gamma\delta$ T cells in a CRC TME model with CAFs. We performed multivariate cell-type specific analysis to understand changes in phenotype, cell-state and PTM signalling.

EACR22-0303

Mutant RUNX1 as a target for immunotherapy in acute myeloid leukemia

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Introduction

About 10% of patients with acute myeloid leukemia (AML) present with mutations in Runt-related transcription factor 1 (RUNX1). RUNX1 is an important transcription factor implicated in hematopoietic differentiation and has been proposed to function as tumor suppressor in the process of apoptosis. Mutations in RUNX1 predict for a poor prognosis in AML.

RUNX1 mutations occur throughout the entire gene, but insertions and deletions in the 3' half of the gene often create mutant RUNX1 proteins with C-terminal amino acids translated

in the same alternative reading frame. Here, we investigated whether this alternative reading frame is a relevant target for immunotherapy.

Material and Methods

A construct with the c.883dupT-mutated RUNX1 gene encoding p.S295FfsTer305 was introduced into 4 different EBV-B cells expressing common HLA class I alleles. Peptides presented in HLA class I were eluted and identified by mass spectrometry. Synthetic peptides and pMHC-tetramers were generated to isolate RUNX1-neopeptide specific CD8⁺ T cell clones from blood from healthy donors. T cell recognition was determined by coculture with relevant AML cell lines and subsequent IFN γ measurements by enzyme-linked immunosorbent assay (ELISA).

Results and Discussions

A total of 13 mutant RUNX1 peptides were identified including 9 peptides presented in HLA-B*07:02. For the 4 most C-terminal peptides, healthy individuals were screened for specific T cells. A total of 9 pMHC tetramer-positive CD8 T cell clones were isolated. The T cell clones recognized 2 out of 4 peptides upon exogenous peptide-loading, as well as HLA-B*07:02-positive AML cell lines transduced with the full-length RUNX1 gene with the c.883dupT mutation. Additionally, we created two AML cell lines (K562 and SIGM5) with an endogenous RUNX1 frameshift mutation by CRISPR/Cas9. The cell lines expressed the HLA-B*07:02 restriction allele endogenously (SIGM5) or after retroviral transduction (K562). The T cell clones also reacted against the AML cell lines with the endogenous RUNX1 mutations, whereas the parental cell lines with the wildtype genes were not recognized.

Conclusion

In conclusion, we identified two HLA-B*07:02 neoantigens in an alternative reading frame created by frameshift mutations in RUNX1 that may be relevant targets for immunotherapy. Currently we are sequencing and cloning the T cell receptors (TCRs) of the T cell clones to investigate whether RUNX1 neoantigens in AML can be targeted by TCR gene therapy.

Immunotherapy

EACR22-0747

Isolation and characterization of RNF43 neo-epitope specific TCRs for T cell therapy in pancreatic cancer

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Introduction

Adoptive transfer of patient-derived autologous T cells modified to express tumor-specific T cell receptors (TCRs) represents an attractive treatment approach for cancer, including PDAC. This is particularly true if antigens with exclusive expression in the tumor, such as mutation-derived neoantigens, are targeted, as on-target/off-tumor toxicity would be minimized and highly functional TCRs are more likely to be identifiable from healthy donor TCR repertoires.

Here, we focus on neo-epitopes derived from the tumor suppressor gene *RNF43*, which bears a high number of frame-shift mutations giving rise to novel open reading frames and diverse antigenic peptides. *RNF43* is found to be mutated in 7 to 10% of pancreatic tumors. We have observed that loss of *RNF43* function has an effect on pancreatic cell proliferation

and migration. Moreover, mice bearing mutations in *RNF43* have an increase in fibrosis, acinar cell proliferation, and develop acinar to ductal metaplasia under inflammatory conditions.

Material and Methods

Peptide candidates were selected by analyzing all possible *RNF43* neo-open reading frames and their predicted binding for the most common Human Leucocyte Antigen (HLA) class I molecules. Epitope-specific TCRs were isolated and characterized by their specificity, avidity, and peptide sensitivity. TCR cytotoxicity was further assessed by co-culture with pancreatic cancer cell lines bearing *RNF43* mutations.

Results and Discussions

We have identified HLA-A0201 and HLA-B0702 restricted epitopes from *RNF43* frame shifts by *in silico* predictions and *in vitro* proteasomal processing assays. For promising epitope candidates, we have isolated the extremely rare epitope-specific T cell populations from peripheral blood of a healthy donor via single cell sorting on combinatorial MHC multimer stained CD8+ naive T cells. Selected TCRs have been re-expressed via orthotopic T cell receptor replacement and functionally characterized in terms of dose dependent cytokine release upon peptide restimulation. More importantly, TCR functionality was proven in *in vitro* killing assays using pancreatic cancer cells expressing mutated *RNF43*.

To this point, we have identified TCRs with high specificity to a selected neo-epitope, dose-dependent cytokine release upon peptide stimulation and killing of neo-epitope expressing target cells.

Conclusion

This study demonstrates the relevance of *RNF43* as a therapeutic target and serves as a proof-of-concept for a platform that can supply neo-epitope reactive TCRs against any target of interest.

EACR22-0921

Microfluidic T Cell Selection by Cellular Avidity

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Introduction

No T cell receptor (TCR) T cell therapies have obtained clinical approval. The lack of strategies capable of selecting and recovering potent T cell candidates may be a contributor to this. Existing protocols for selecting TCR T cell clones for cell therapies such as peptide-MHC multimer methods have provided effective measurements on TCR affinities. However, these methods lack the ability to measure the collective strength of intercellular interactions (i.e. cellular avidity) and markers of T cell activation such as immunological synapse formation. This study describes a novel microfluidic fluid shear stress-based approach to identify and recover highly potent T cell clones based on the cellular avidity between living T cells and tumor cells.

Material and Methods

SUPT1 human T cells, patient-derived transduced T cells and primary mouse T cells were labelled with calcein-AM (5 μ M, 1×10^6 cells/ml) for 1 h at 37°C. High avidity SUPT1 DM β , primary Pmel and patient-derived T cells were stained green; SUPT1 wild-type (WT), primary mouse WT T cells and low avidity human primary T cells were stained red; SUPT1 non-transduced (NT) were stained with blue. T cell variants were fed into the devices at 1:1 ratios and T cells were left to attach to melanoma cells for 10 min under static conditions. T cell detachment and collection was examined under shear-induced flow in the range of 0 – 19.2 Pa using a programmable syringe pump.

Results and Discussions

Our approach is capable of probing approximately up to 10,000 T cell-tumor cell interactions per run and can discriminate and recover highly potent T cells with up to 100% purity from mixed populations of T cells within 30 minutes. The cytotoxic activity of T cell fractions against tumor cell lines is proportional to their ability to resist shear-induced detachment. These results are a demonstration of how microfluidic probing of cellular avidity as a crucial biomarker may fast track the therapeutic T cell selection process.

Conclusion

The device developed in this work was able to analyze thousands of cell-cell interactions in real-time under precisely controlled environments using a cellular avidity approach. This provides a more physiological analysis of T cell – tumor cell interactions that incorporate key immune signaling events such as IS formation. The ability to rapidly identify and recover TCRs of optimal functional activity through cellular avidity may accelerate our expansion of immunotherapeutic strategies and move us closer towards precision cancer medicine.

EACR22-1065

Preclinical evaluation and structural optimization of anti-BCMA CAR to target multiple myeloma

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Introduction

Chimeric antigen receptor (CAR) T-cell based immunotherapy has become a promising treatment mainly for hematological malignancies. Following the major success of CD19-targeted CAR, new potential targets for other malignancies are required. As such, BCMA is an attractive tumor associated antigen to be targeted in multiple myeloma (MM).

Material and Methods

Herein, we aimed at assessing the function and optimal configuration of different BCMA-specific CARs, based on the same targeting moiety but with a different hinge and co-stimulatory domain. We compared their function to that of a previously characterized BCMA-CAR used in clinical trials.

Results and Discussions

All constructs were expressed at high levels by primary human T-cells and could trigger cytokine production and cytotoxicity upon co-culture with multiple myeloma targets.

Nonetheless, critical differences were observed in off-target activation, exhaustion, and activation markers expression and *in vivo* antitumoral activity mediated by these different constructs. Interestingly, we noted that CD8-based hinged combined with a 4-1BB intracellular domain proved superior compared to IgG4 connecting regions, and/or a CD28 signaling moiety respectively.

Conclusion

Overall, this study emphasizes the influence of CAR primary structure on its function and led to the identification of a highly efficient BCMA-specific CAR, namely H8BB, which displayed superior anti-tumor activity both *in vitro* and long-term *in vivo* efficacy. This novel CAR is now being evaluated in a Phase I/II clinical trial to treat patients with multiple myeloma and other related diseases.

EACR22-1279

ORAL PRESENTATION

Restoring Immune Control of Bone-Metastatic Breast Cancer With Type I Interferon Modulators

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Introduction

Bone metastasis (BM) is a frequent and fatal complication of breast cancer (BC), representing over 50% of first distant relapse diagnoses. To date, conventional therapies only confer palliative care once progression to bone has occurred, and immunotherapeutics have yielded little long-term benefit in the bone-metastatic setting. The failure of such therapies has been linked to the immunosuppressive microenvironment of the bone, and several immune evasion mechanisms that hinder therapeutic effectiveness. Type I interferons (IFNs) are cytokines that have anti-tumour and immune activities and play a key role in dictating therapeutic efficacy in the cancer context. Our laboratory has identified tumour-intrinsic type I IFN loss as key driver of immunoevasion in BC and prostate cancer (PC). As evidence points toward a stable-epigenetic loss and restoration via epigenetic agents reprogramming in PC, the use of epigenetic compounds in BC has been regarded as a potential strategy to improve tumour immunogenicity and promote the elimination of BC cells in bone. Here we aim to derive and characterise bone-metastatic BC models to promote type I IFN responses through novel immune-activating agents.

Material and Methods

We have designed a high-throughput screening (HTS) pipeline for the screening of an epigenetic compound library against stably transfected metastatic mouse and human BC cell lines expressing a GFP and Luc2-type I IFN stimulated response element (ISRE) reporter system. Hit selections were validated via enhanced bioluminescent activity in bone-derived cell lines and subsequent impact on immunogenicity was assessed via T cell functional assays. Further assessment of selected agents was evaluated in preclinical models of experimental metastasis.

Results and Discussions

Using the HTS pipeline, we have identified epigenetic agents, including a DNA methyltransferase inhibitor, that restore type I IFN signalling and immunogenicity in bone metastatic BC cell lines. Preliminary *in vivo* assays showed enhanced metastasis-free survival and immune activities of peripheral T and NK cells whilst reducing bone metastasis in treated mice. Combination therapeutics with immune checkpoint inhibitors will now be assessed.

Conclusion

Restoration of tumour-intrinsic type I IFN may be critical to prevent bone-metastatic disease in BC. Preliminary data suggests the utilisation of the HTS pipeline in BC cells underpins the development of novel therapeutic approaches that can improve immunotherapeutic efficacy and reduce patient mortality.

EACR22-0226

Galectin 3 in exosomes derived from triple negative breast cancer cells, induce immunosuppression through the CD45 molecular pathway

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Introduction

Malignant cell escape mechanisms include the up-regulation of immunosuppressive checkpoint pathways affecting the anti-tumor response of T cells. Therefore, clinical trials for Triple Negative Breast Cancer (TNBC) employing immunotherapy were based on chemotherapy skeleton with checkpoint blockade. However, these regimens showed limited benefit both in the early stage and the metastatic disease. New effective immunotherapy strategies are required to evoke durable clinical responses, with minimal toxicity. We have recently published a novel tumor escape mechanism via which TNBC cells suppress immune cells through a cascade of the Src family of tyrosine kinases in the CD45 intracellular signals (Oncoimmunology 2021). One of the immunosuppressive proteins which binds several receptors including CD45 is Galectin 3 (Gal 3). Gal 3 was found in TNBC derived exosomes (TEX). TEX are known to carry immunosuppressive factors, like Gal 3, affecting the anti-tumor activities of immune cells.

Our aim was to decipher the mechanism implemented by exosomal Gal 3 derived from TNBC cells to induce immunosuppression through the CD45 pathway in order to find new strategies for TNBC immunotherapy.

Material and Methods

Exosomes were isolated from MDA-MB-231(TNBC) and MCF-10A (normal breast, as control) cell lines. Exosomes were characterized by nanoparticle tracking analysis, by FACS analysis using CD9 and CD81 antibodies, and by transmission electron microscopy.

Results and Discussions

We first studied the transcriptomes profiling RNA-Seq in leukocytes of TNBC patients compared to leukocytes from healthy donors. Results revealed over expressed genes related to exosomes secreted by tumors, like CD9.

Addition of TEX to normal leukocytes inhibited significantly the Src tyrosine kinase Lck, ZAP-70 and VAV-1 phosphorylation resulting in a significant decrease in INF γ secretion ($p < 0.001$). Silencing (shRNA) or blocking the exosomal Gal 3 cancelled the immunosuppression exerted by TEX through the CD45 pathway. TEX expressing Gal 3 bound CD45 on CD4 $^{+}$ and CD8 $^{+}$ cells (observed by immunofluorescence in a confocal microscope). Blockade of CD45 in CD4 $^{+}$ and CD8 $^{+}$ cells cancelled the TEX expressing Gal 3 induced immunosuppression through CD45 signaling.

Conclusion

This is the first study demonstrating that TNBC derived exosomes, which contain or express Gal 3, target CD45 signaling, suppressing the anti-tumor immune response. This study suggests CD45 as a new target for TNBC immunotherapy.

EACR22-1006

Oncolytic virotherapy as an immunotherapeutic strategy for the treatment of non-small cell lung cancer and other solid tumors

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Introduction

Non-small cell lung cancer (NSCLC) is one of the main cause of cancer-related death. Despite some improvements in overall survival have been achieved in advanced NSCLC patients treated with checkpoint inhibitors, the development of novel effective strategies is an urgent need to overcome significant immune-related adverse effects, resistance mechanisms and to effectively prolong patients' survival. Oncolytic virotherapy has been proposed as a new promising immunotherapy against solid tumors, including NSCLC. The aim of this work was to test the oncolytic activity of a bovine herpesvirus 4 (BoHV-4), because of its favourable properties, in NSCLC.

Material and Methods

Murine and human NSCLC cells were infected *in vitro* with BoHV-4 and heat-inactivated BoHV-4, as control. *In vivo* the transplantable and the spontaneous NSCLC mouse models, represented by K-Ras^{G12D} transgenic mice, were administered intra-tumor and intra-trachea respectively, with BoHV-4 or heat-inactivated BoHV-4. The immunogenicity induced by BoHV-4 was analysed by flow cytometry, *in vitro* antibody-dependent cellular cytotoxicity (ADCC) and cellular cytotoxicity.

Results and Discussions

BoHV-4 efficiently infects and kills NSCLC cell lines *in vitro*, likely through the induction of an immunogenic cell death. Intra-tumor delivery of BoHV-4 hampers the growth of murine NSCLC tumors injected subcutaneously and significantly prolongs the survival. In some cases, a complete tumor regression was observed and all these mice rejected a second lethal NSCLC tumor challenge, suggesting the establishment of an immune memory. Intra-tracheal administration of BoHV-4 in K-Ras^{G12D} transgenic mice significantly reduces the tumor burden. Interestingly, the BoHV-4 oncolytic activity observed *in vivo* is associated with the stimulation of both a humoral and cellular anti-tumor immune response and with the induction of an effective immune cell infiltration in tumors, in both NSCLC mouse models.

Conclusion

Despite further investigations will be needed to better define the underlying mechanisms, here it was demonstrated the potential “clinical” efficacy of BoHV-4 treatment in valuable pre-clinical NSCLC models. To extend its application to other solid tumors, the BoHV-4 oncolytic activity was tested in Her2-positive breast cancer *in vitro* and *in vivo*, and the preliminary data obtained are in line with what was observed in NSCLC model.

EACR22-1142

Delineating early immunological changes in tumor and peripheral blood upon neoadjuvant aPD1 with or without aCTLA4 immunotherapy in Cutaneous SCC; The MATISSE trial

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Introduction

Immune checkpoint blockade (ICB) has revolutionised cancer treatment. However, ICB efficacy is hampered by the lack of knowledge on the mechanisms underlying response, which makes it difficult to 1. predict the response and stratify patients, and 2. define novel targets in non-responders. Most studies in the field include patient samples acquired 3 weeks to 2 months after treatment, lacking temporal resolution of early immunological changes, which are critical to gain insight into the immune dynamics upon ICB.

The MATISSE trial (NCT04620200), currently ongoing at the Netherlands Cancer Institute Amsterdam and University Medical Center Utrecht, is a phase II trial exploring the efficacy of Nivolumab and Ipilimumab versus Nivolumab in patients with cutaneous squamous cell carcinoma (CSCC) prior to extensive surgery. It provides a unique sequential patient sample collection for extensive translational research in order to unravel the early dynamics upon ICB in tumor and blood, key for identifying novel therapeutic targets and biomarkers of response. **Material and Methods**

Matched blood samples and tumor biopsies are acquired at baseline, during ICB (weeks 1 and 2) and at time of surgery (week 4). PBMCs and leukocytes are isolated from blood samples. Tumor biopsies are processed into patient-derived tumor fragments (PDTFs) for both steady-state profiling and ex vivo culture. A comprehensive profiling of blood and tissue samples is performed by using high-dimensional flow cytometry, cytokine assays, immunohistochemistry, functional ex vivo cultures, single-cell RNA and TCR sequencing.

Results and Discussions

~90% of samples have been successfully collected for 25 patients at given time-points, creating a unique set of samples for a broad definition of early ICB-induced changes. Standardised immunoassays to assess immune composition, immune activation and T cell clonal dynamics have been established using the methods described above, and profiling of the acquired longitudinal samples is ongoing. Data describing the feasibility of the approach and dynamics of the first few patients will be presented.

Conclusion

The MATISSE trial generates a unique sequential tumor and blood sample collection. A combination of different high-dimensional and functional technologies will allow to create an extensive atlas of very early response dynamics induced by ICB in patients with CSCC treated with Nivolumab with or without Ipilimumab. This dataset can be exploited for identification of predictive biomarkers and novel therapeutic targets.

EACR22-0617

PD-L1 expression, BRAF mutation and IL-6 as predictive biomarkers for immunotherapy response in advanced melanoma patients

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Introduction

Despite having a low relative incidence, melanoma presents substantial risk of metastasis and death. Increasing advances in the treatment of advanced melanomas have been achieved with the use of monoclonal antibodies addressed to inhibit immune checkpoints. These molecules stimulate antitumor acquired immune activity. However, this therapy exhibits high cost, may present adverse effects and be ineffective in an expressive number of patients. Thus, the aim of this study was to investigate molecular markers capable of predicting the therapeutic response to the use of immune checkpoint inhibitors in patients with advanced melanoma.

Material and Methods

Data from 87 patients with advanced melanoma who underwent anti-PD-1 or anti-CTLA-4 immunotherapy at Barretos Cancer Hospital were collected from medical records. The expression of tumor PD-L1 by immunohistochemistry and the release of immunomediators into plasma by Cytometric Bead Array using the Human Th1/Th2/Th17 Cytokine Kit were accessed. Patients with tumor progression and stable disease were classified as non-responders and patients with partial or complete response were classified as responders.

Results and Discussions

Patients mean age was 54 years. Primary lesions main site was lower limbs (26.4%), followed by trunk (25.3%), and head and neck (25.3%). The nodular melanoma was the most prevalent histological subtype (37.9%), followed by superficial spreading (27.6%). Among the patients, 89.3% used anti-PD-1 immunotherapy and 59.2% of them presented disease progression after the treatment. Association analysis showed that 46.7% of the patients with partial or complete responses had superficial spreading tumors, while most of the non-responders had other tumor subtypes ($p=0.023$). For *BRAF* alterations, 93.8% of non-responders were wild-type, whereas 40.9% of responders presented V600 mutations ($p=0.004$). For PD-L1, 84.6% of non-responders had low expression (Tumor Proportion Score (TPS) <1%), while 43.8% of responders presented high expression (TPS >5%, $p=0.034$). Finally, the concentration of interleukin-6 (IL-6) showed a trend towards response, where, among the non-responders, 87.8% had higher concentrations of this cytokine in plasma (>2.83 pg/mL; $p=0.06$).

Conclusion

Histological subtype, PD-L1 tumor expression, *BRAF* mutation, and concentration of IL-6 were identified as predictors of response to anti-PD-1 and/or anti-CTLA-4 immunotherapies and could be used to help identify patients who will benefit from this treatment.

EACR22-0613

Screening for drugs promoting activation of immunogenic interferon responses

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Introduction

Inhibitors of cyclin-dependent kinases 4/6 (CDK4/6) have recently been discovered to activate anti-tumoral interferon responses in addition to their cytostatic properties, prompting studies on their utility as combination therapies alongside checkpoint inhibitors. However, resistance to CDK4/6 inhibitors is a well-documented phenomenon which is still not fully understood, significantly reducing the clinical benefit proffered by these drugs. Previous work has found that CDK4/6 inhibitor-mediated interferon production can be enhanced by inhibiting degradation of intermediary signalling complexes, such as TANK-binding kinase 1 (TBK1). Hence, we sought to design novel strategies to enhance the anti-tumour effects of CDK4/6 inhibitors by targeting downstream immune signalling.

Material and Methods

We screened 1620 FDA/EMA-approved compounds for enhanced immune signalling alongside the CDK4/6 inhibitor palbociclib. Human lung (A549) and breast (MCF7) carcinoma lines were treated for 24 hours with either palbociclib, candidate compounds, or both. Activation of TBK1 was detected by immunofluorescence and quantified as a reporter for activation of immune signalling. Hit compounds were validated for expression of downstream interferon-stimulated genes (ISGs) by western blot.

Results and Discussions

29 compounds were identified as hits in combination with palbociclib. Following further validation, 4 compounds (disulfiram, reserpine, fenbendazole, niclosamide) were selected as potent inducers of interferons in combination with Palbociclib. Further *in vivo* work in relevant models of cancer will be crucial to understanding the efficacy of these drug combinations in activating host anti-tumour immunity.

Conclusion

We adopted a phenotypic screening approach to identify drugs that enhance interferon responses by CDK4/6 inhibitor treatment. Confirmation of downstream ISG upregulation provides further confidence in the validity of our approach. The FDA/EMA approved status of the screened compounds will also have the added benefit of greater translatability into clinical settings.

EACR22-0627

Synergistic antitumor efficiency of RIG-I and TLR-3 dual immune pathway activation by double-stranded RNA transfection induced neutrophil mediated antitumor effect.

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Introduction

Cancer immunotherapy is dependent on immunostimulation to reorganize the tumor microenvironment (TME) and increase anti-tumor effect of immune cells. There are currently many immunostimulants applicable to cancer therapy, such as antibodies, molecular drugs and PAMP activators. Not only is immunostimulant choice important, method of delivery of treatment is equally as important as different modes may stimulate different immune pathways. Polyinosinic:polycytidylic acid (poly I:C) is known to interact with toll-like receptor 3 (TLR3) when taken up by endocytosis, but when directly transfected into cells, poly I:C stimulates retinoic acid-inducible gene I (RIG-I) instead. Here, we evaluate whether poly I:C treatment via electroporation would induce stronger anti-tumor effects than conventional syringe method in the 4T1 mouse mammary cancer model.

Material and Methods

4T1 mammary tumor-bearing mice were treated with poly I:C by electroporation or syringe injection (SI) and monitored for tumor growth. To analyze TME immunity in vivo, poly I:C treated tumor tissue were collected and analyzed using cytokine array, quantitative real-time PCR and flow cytometry. Inhibitors for RIG-I and TLR3 were used to determine immune pathway activation. Neutrophil depletion was performed using Ly6G antibody. Due to known PD-1 resistance in 4T1 model, PE was used in conjunction to test whether it can improve PD-1 antibody treatment in 4T1 tumor model.

Results and Discussions

Our results showed that poly I:C electroporation (PE) induced stronger anti-tumor effect than syringe injection (SI) and that this anti-tumor effect could be suppressed by RIG-I and TLR3 pathway inhibitors, but not in SI. Flow cytometry analysis of tumor tissue showed higher neutrophil infiltration in PE group, in addition to elevated neutrophil anti-tumor N1 marker Fas expression. Neutrophil depletion decreased PE-induced anti-tumor effect, but not in SI, confirming that PE anti-tumor effect is mediated by neutrophils. And even though 4T1 is a PD-1-resistant tumor, PE combined with PD-1 antibody treatment induced stronger anti-tumor effect compared to either treatment alone. This suggests that PE can work in synergy with PD-1 antibody treatment in 4T1 tumor model.

Conclusion

poly I:C electroporation (PE) stimulated both RIG-I and TLR3 dual pathway activation and induced stronger anti-tumor effect than conventional syringe injection (SI). This PE-induced anti-tumor effect is mediated by neutrophils and can enhance PD-1 antibody treatment in 4T1 tumor model.

EACR22-0661

Development of biomaterials-based immunotherapy: chitosan/poly-(y)-glutamic acid nanoparticles synergize with IFN- γ to induce antitumor immunity

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Introduction

Interferon- γ (IFN- γ) is a key factor for antitumor immunity and immunotherapy response. However, its clinical use remains restricted to chronic granulomatous disease and malignant osteopetrosis. In cancer, most of the clinical trials reported a limited potential of IFN- γ as single therapy, being under clinical evaluation its combination with immunotherapy, like immune checkpoint inhibitors. Here, we addressed the synergistic effects of chitosan/poly(γ -glutamic acid) nanoparticles (Ch/ γ -PGA-NPs) when combined with IFN- γ using a immunocompetent 4T1 orthotopic breast tumor model. We previously showed that Ch/ γ -PGA-NPs are able to reeducate immature/immunosuppressive antigen-presenting cells (APCs) to an immunostimulatory profile, leading to T cell activation and impairing APCs-mediated cancer cell invasion. Recently, we demonstrated Ch/ γ -PGA-NPs as adjuvants to conventional radiotherapy in breast cancer [4]. Thus, these immunomodulatory abilities of Ch/ γ -PGA-NPs point them as appealing adjuvants to IFN- γ -based therapies.

Material and Methods

Briefly, after seven days of 4T1 tumor cell inoculation, when the tumors reached $94.1 \pm 32.1 \text{ mm}^3$, animals were subcutaneously injected, adjacent to the tumor, 6 times for 2 weeks with Ch/ γ -PGA-NPs, IFN- γ or with both (NPs+IFN- γ). Tumor burden, lung metastasis formation and immune cell profile were explored.

Results and Discussions

Regarding *in vivo* studies, treatments had no significant impact on mice weight nor liver or kidney structure, confirming their safety. While non-treated animals had progressive tumor growth ($514.7 \pm 61.2 \%$) and developed lung metastasis, NPs- and IFN- γ -treated animals significantly decreased primary tumor burden ($286.5 \pm 41.6 \%$ and $262.0 \pm 43.4 \%$, respectively). Remarkably, when both treatments were combined, breast tumor growth was blocked ($107.8 \pm 25.5 \%$). This impairment was associated with a reduction in splenomegaly, a decrease in the percentage of splenic myeloid-derived suppressor cells and an increase in antitumoral CD4+IFN- γ + population. Notably, animals from the combinatorial treatment (NPs+IFN- γ) presented lower metastatic burden in the lungs than other groups. Additionally, NPs+IFN- γ -treated animals had lower levels of the systemic pro-tumoral cytokines (IL-3, IL-4, IL-10) in comparison to non-treated animals.

Conclusion

Overall, Ch/ γ -PGA-NPs potentiate and synergize with IFN- γ to reduce tumor progression and systemic immunosuppression, opening new perspectives to be used in anticancer strategies.

EACR22-0857

ATAPEMBRO trial: phase I study combining anti-PD1 checkpoint inhibition with premature termination codon read-through in metastatic mismatch repair deficient colorectal and endometrial cancer.

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Introduction

Mismatch repair deficient (dMMR) tumors harbor many frameshift mutations that lead to increased out of frame, non-self-proteins, with the potential to be degraded and presented by MHC as neoantigens. Checkpoint-inhibition (CI) therapy response rates are higher in such tumors compared to mismatch repair proficient (pMMR) tumors. Neoantigen formation is dependent on the length of the translated frameshifted RNA, which is limited by premature termination codons (PTCs). PTC read-through translation can be facilitated by medication developed for Duchenne dystrophy patients. In this study we investigate the safety of checkpoint inhibition and PTC read-through medication combination therapy in metastatic dMMR and pMMR colorectal carcinoma (CRC) and dMMR endometrial carcinoma (EC).

Material and Methods

This trial is a single-center, open label, phase 1 pilot study with an dose-escalation approach. Study treatment consisted of a fixed dose of 200mg Pembrolizumab i.v. every 3 weeks starting at week 1, and 3 times daily Ataluren orally starting 1 week prior to the first CI infusion. Ataluren dosage for each escalation cohort were: 2,5-2,5-5mg/kg, 5-5-10mg/kg and 10-10-20mg/kg. Dose limiting toxicity (DLT) was determined after 28 days of treatment. Minimal treatment and safety evaluation before start of the next patient was 21 days. Each dose escalation cohort consisted of 3 patients with an additional 3 patients at the determined maximum tolerable dose (MTD).

Results and Discussions

12 patients were included and treated. 9dMMR (8 CRC; 1 EC) and 3pMMR patients (all CRC). Fifty percent of patients were male. Mean age was 70 (dMMR) and 62 (pMMR). dMMR patients had on average 1 prior line of treatment compared to 4 in pMMR. No DLT was observed. The MTD was determined at 10-10-20mg/kg. Adverse events (AE) of at least grade 3 occurred in 2 patients, 1 required hospitalization and was therefore classified as an Serious Adverse Event (SAE). None of these events were related to study-treatment. Six patients experienced grade 2 AEs, most frequently gastro-intestinal complaints (50%). Fifty percent of the patients have shown stable disease or partial response (RECIST criteria) at CT-scan evaluation at 13 weeks.

Conclusion

Our findings demonstrate that combined treatment with PD-1 checkpoint inhibition and PTC read-through medication is safe and tolerable for metastatic dMMR and pMMR CRC and dMMR EC patients. Promising response rates seen at first evaluation warrant further study.

EACR22-0975

"Inhibition of ERAP1 reprogramms the tumor immune microenvironment to overcome immune evasion in high-risk neuroblastoma"

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Introduction

Endoplasmic reticulum (ER) aminopeptidase (ERAP1) is a multifunctional zinc metallopeptidase crucial for the maturation of a wide spectrum of substrates. In the ER, ERAP1 shapes the peptide repertoire presented by MHC class I (MHC I) molecules to CD8+ T cells and NK cells. Defective expression of ERAP1 has been detected in many tumor types, including melanoma, lymphoma, breast, colon and lung cancer. Several studies have highlighted the role of ERAP1 in reprogramming the immunogenicity of tumor cells and enhancing innate and adaptive anti-tumor immune responses. Loss of ERAP1 causes an alteration of the normal repertoire of peptides bound to MHC I molecules able of stimulating potent CD8+ T cell and NK cell responses.

Material and Methods

ERAP1 expression was inhibited in two murine neuroblastoma (NB) cell lines by infection with lentivirus expressing short hairpin RNA specific for ERAP1. Silenced cells were subcutaneously injected into syngeneic C57BL/6 mice and tumor growth was monitored daily. Tumors were sacrificed after 15 days after inoculation and analysed for tumour microenvironment by flow cytometry. Murine-derived organotypic tumour spheroids (MDOTS) were generated from explanted NB masses and used to assess the ability of ERAP1 inhibitors to attract the immune system.

Results and Discussions

We found that genetic inhibition of ERAP1 reduces NB growth and increases host survival. ERAP1 depletion modulates the immune content of the tumor microenvironment by increasing the recruitment of activated NK cells. Accordingly, MDOTS treated with selected ERAP1 inhibitors significantly induce increased NK cells recruitment.

Conclusion

We provided new insight about the role of ERAP1 in NB development, and how its inhibition could be used to strengthen the antitumor immune response. This information depicts ERAP1 as a possible promising therapeutic target in the treatment of high-risk NB cancers and opens a window of opportunity for the potential use of ERAP1 inhibitors to enhance antitumor immunity.

EACR22-0995

Dissecting the immune response elicited by olaparib in homologous recombination repair deficient breast cancer

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Introduction

Tumors with defective DNA repair by the homologous recombination repair (HRR) pathway are sensitive to PARP inhibitors (PARPi). The chromosomal instability (CI) resulting from DNA damage activates the cyclic GMP–AMP synthase (cGAS)/stimulator of interferon genes (STING) pathway, which stimulates the anti-tumor immune response via the Type 1 Interferon (IFN-I) axis. STING pathway suppression has been suggested as an immune escape mechanism; thus, STING agonists (STINGa) have been developed to enhance anti-cancer effects. We studied the impact of STING pathway activation in PARPi antitumor response using patient-derived tumor xenograft (PDX) models.

Material and Methods

We used 36 PDX from breast cancer (BC) patients harboring HRR alterations implanted in two strains with increasing immunodeficiency (NMRI vs. NSG mice), and an immunocompetent Mouse-Derived Xenograft (MDX) derived from a *Brca1*^{f22-24/-} transgenic mouse model. The response to the PARPi olaparib was assessed according to modified

RECIST criteria. CI was evaluated by micronuclei formation, and STING-pathway activation by Western blot and RNA-sequencing.

Results and Discussions

We observed that the anti-tumor activity of olaparib in PDX was superior in NMRI mouse, lacking T cells, compared to NSG, also lacking B, innate lymphoid and NK cells ($p=0.007$), suggesting a cooperation between the mouse innate immunity and the PARPi anti-tumor response. Across models, olaparib sensitivity was associated with an increased formation of micronuclei-positive cells, γ H2AX micronuclei-positive cells, cGAS micronuclei-positive cells and STAT1 phosphorylation, indicating the activation of the STING-IFN pathway after DNA damage in sensitive PDX. RNA sequencing is ongoing to further elucidate the impact of PARPi on immune-related pathways. Interestingly, treatment with a STINGa activated the IFN-I axis in PDX, even in those expressing low STING. *In vivo* studies are ongoing to test if the combination of PARPi and STINGa results in an enhanced and/or prolonged antitumor response.

Conclusion

These results in PDX suggest that endogenous and PARPi-induced DNA damage activates the STING-IFN pathway and contributes to the antitumor efficacy of PARPi. Preliminary results suggest the combination of PARPi and STINGa as a potential novel strategy to improve the antitumor responses in patients.

EACR22-1175

Ultra-sensitive immunophenotyping of TCR and BCR clonotypes

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Introduction

The potential of TCR and BCR repertoire profiling for understanding disease mechanisms and for development of new therapeutics in infectious diseases, autoimmunity and in immuno-oncology is great. However, this potential could be greatly improved by combining information about receptor clonotypes with immunophenotypes of T and B cells. To facilitate these studies, we developed a novel technology for combined profiling of all human TCR and BCR variable regions and phenotypic characterization of immune cells in bulk PBMC and immune cell fraction samples.

Material and Methods

The TCR and BCR Immunophenotyping method developed here involves multiplex RT-PCR amplification and sequencing of CDR3 regions of TCR and BCR genes and a set of the most informative T- and B-cell phenotyping genes. Bioinformatics analysis of the next-generation sequencing (NGS) data allows profiling of TCR and BCR clonotypes, as well as identification of major immune cell subtypes and their activation status.

Results and Discussions

Data will be presented showing how combined TCR/BCR clonotype analysis combined with targeted expression profiling of immune cells can be applied for large-scale discovery of novel cell typing and activation biomarkers in several immune-responsive model systems.

Conclusion

The preliminary data demonstrate that the assay has unparalleled throughput, sensitivity, and improved cost-effectiveness for high-throughput immunity biomarker discovery applications.

Evaluating the anti-tumor and immune effects of anti-CD70 antibody-based therapy as a single agent and in combination with chemotherapy in non-small cell lung cancer *in vitro* and *in vivo*

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Introduction

Constitutive expression of the immune checkpoint molecule CD70 has been reported in a notable subset (31%) of non-small cell lung cancer (NSCLC) patients. In this study, we explored the cytotoxic and immune stimulatory potential of an anti-CD70 antibody-based therapy as a single agent and in a sequential combination strategy with the immunomodulating regimen of docetaxel with cisplatin in NSCLC *in vitro* and *in vivo*.

Material and Methods

Different NSCLC cell lines were used and treated with anti-CD70 antibodies with enhanced effector functions or isotype and/or docetaxel and cisplatin. The expression of CD70 was assessed after chemotherapy via flow cytometry (FC). Natural Killer (NK) cell-mediated NSCLC cell killing (5:1) and cytokine secretion were analyzed using the IncuCyte™ ZOOM system and electrochemiluminescence, respectively. Validation of the treatment effect of chemotherapy, anti-CD70 therapy, and the sequential combination of chemotherapy and anti-CD70 therapy *in vivo* was evaluated by tumor growth kinetics and survival of LLC-bearing mice. Immune cell subsets in tumors and tumor-draining lymph nodes of LLC-bearing mice were characterized using multi-color FC.

Results and Discussions

We observed increased CD70 expression after chemotherapy treatment on NSCLC cell lines *in vitro*, suggesting its ability to broaden the therapeutic window of anti-CD70 therapy. Significant NK cell-mediated killing was observed after treatment of co-cultures with anti-CD70 therapy at varying levels in the NSCLC cell lines, whereby the combination therapy resulted in increased NSCLC cell killing by NK cells *in vitro*. NK cell-mediated NSCLC killing with either anti-CD70 therapy or the combination therapy was accompanied by increased levels of granzyme B, TNF- α , and IFN- γ after treatment. Furthermore, treatment with the sequential combination therapy demonstrated significant improved survival and delayed tumor growth in LLC-bearing mice compared to the single agents. We observed an increase in dendritic cell numbers in the tumor-draining lymph nodes after treatment with chemotherapy, highlighting the immunogenic potential of docetaxel and cisplatin in NSCLC. The sequential combination therapy resulted in enhanced infiltration of both T and NK cells, as well as an increase in the ratio of CD8⁺ T cells over CD4⁺ CD25⁺ FoxP3⁺ regulatory T cells in LLC-bearing mice.

Conclusion

These novel preclinical data demonstrate the potential of combining anti-CD70 therapy with chemotherapy to enhance anti-tumor immune responses in NSCLC.

EACR22-0172

Modulation of tumor immune microenvironment is linked to tumor growth inhibition in response to Met inhibition and PD-L1 blockade in Small Cell Lung Cancer

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Introduction

Small cell lung cancer (SCLC) accounts for 15% of all lung cancers and its 5-year overall survival is below 2% for patients with stage IV disease. The Met/Hepatocyte Growth Factor (HGF) receptor pathway activation has been linked to chemotherapy resistance. Moreover, the enrichment of immunosuppressant Met⁺ immune cells in the tumor microenvironment has been associated with resistance to anti-programmed death-ligand 1 (PD-L1) agents. Here, we explored the antitumoral effect of Met tyrosine kinase inhibition (savolitinib) added to an anti-PD-L1 agent as a potential therapeutic strategy in SCLC.

Material and Methods

KP1 mouse SCLC cell line was subcutaneously injected in immunocompetent B6129SF1/J mice. Mice were allocated in four arms: control (isotype IgG), savolitinib (30 mg/kg daily PO), anti-mouse PD-L1 (10 mg/kg twice a week IP) and the combination of both compounds. Tumor growth was followed-up twice a week until day 18 post-treatment, when mice were sacrificed to obtain cellular suspensions from tumors. Tumor immune infiltrates were assessed by multiparametric spectral flow cytometry.

Results and Discussions

The addition of savolitinib to anti-PD-L1 reduced tumor growth (5 ±1.62-fold change) compared with control (9.1 ±1.87-fold change; p<0.05), savolitinib (7.46 ±3.54-fold change; p>0.05) and anti-PD-L1 (7.78 ±2.28-fold change; p<0.05) groups. Tumors treated with the combinatorial approach exhibited an increase of MHC-I expression on CD45⁺ cells (39.22 ±22.09% vs 8.3 ±4.73%; p<0.05) and an increased infiltration of CD45⁺ cells (12.85 ±5.26% vs 5.48 ±5.12%; p=0.0667) compared with anti-PD-L1 treated tumors. In addition, tumors treated with the combination displayed a significant increased infiltration of effector memory (CD44⁺CD62L⁻) CD4⁺ T cells (14.53 ±4.05% vs 5.32 ±2.53%; p<0.01) and Granzyme B⁺CD8⁺ cytotoxic T cells (3.5 ±2.03% vs 0.77 ±1.09%; p<0.05) compared with control. A decreased infiltration of granulocytic-myeloid-derived suppressor cells (G-MDSC) was also observed in both combinatorial (1.68 ±1.33%) and savolitinib alone (2.92 ±4.34%) arms compared with control (4.39 ±3.92%) and anti-PD-L1 (3.69 ±1.95%) groups (p>0.05).

Conclusion

The addition of Met inhibition to PD-L1 blockade contributes to tumor control in a SCLC mouse model. This antitumoral effect appears to be derived from increased surface expression of MHC class I molecules on CD45⁺ cells and tumor immune microenvironment modulation favoring an effector immune response.

THE COX-2/PGE2 PATHWAY AS AN INDEPENDENT IMMUNE CHECKPOINT

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Introduction

Mechanistic studies to determine the basis for lack of patient response to immune checkpoint inhibitors (ICIs) are required for the development of rational combination therapies to maximise the efficacy of ICIs. Previous work from our lab has uncovered a central role for cancer cell-derived prostaglandin E2 (PGE2) in immune evasion and resistance to ICIs.

Material and Methods

Tumour-bearing wild-type, *Rag-/-*, *Batf3-/-* or *Ifng-/-* mice were treated with either a selective COX-2 inhibitor (COX-2i) or EP2/EP4 receptor antagonists (EPAT) and/or anti-PD-1 to evaluate anti-tumour control. Characterisation of the potential changes in the tumour inflammatory landscape was performed early on-treatment both at a molecular and cellular level via RNA sequencing and multi-colour flow cytometry analysis, respectively.

Results and Discussions

Our data demonstrates that the combination of either COX-2i or EPAT similarly enhance ICI efficacy in independent syngeneic mouse cancer models otherwise poorly responsive to ICI. On-treatment bulk tumour transcriptional profiling revealed that monotherapy with COX2i or EPAT leads to a profound IFN-γ-driven reprogramming of the tumour microenvironment (TME) two days post-treatment, resembling that associated with better clinical outcome in cancer patients receiving ICIs. This intratumoural molecular switch could be detected as early as four hours after a single dose of COX-2i and was further amplified upon daily administration and when combined with anti-PD-1 treatment ultimately resulting in an increased accumulation of effector cytotoxic T cells seven days post-treatment. Specifically, multiparametric immunophenotyping of the tumour infiltrating immune cells showed that ICI + COX-2i treatment promoted a phenotypic change that favoured the accumulation of TCF1+ naïve-like CD8+ T cells with increased cytotoxic activity. Analysis of mice lacking mature T and B cells, type I conventional dendritic cells (cDC1), NK cells, or IFN-γ showed impaired TME remodelling following COX-2 inhibition and loss of tumour control after ICI + COX-2i treatment. Altogether, these data reveal an essential role of IFN-γ signalling and effector NK/cDC1/T cell axis in orchestrating the therapeutic benefit of combining inhibitors of COX-2/PGE2 pathway with ICIs.

Conclusion

PGE2 signalling via EP2/EP4 receptors on tumour infiltrating leucocytes represents a readily targetable independent immune checkpoint to increase the efficacy of currently approved immunotherapies.

Cytotoxicity of Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-modified Natural Killer cells against ovarian cancer.

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Introduction

The global 5yr ovarian cancer (OC) survival rate is <50%¹. Gold standard treatment is cytoreduction surgery and platinum based chemotherapy¹, yet 75% of women experience a platinum resistant relapse >5 years¹. Cell immunotherapy which primes the patient's immune system to fight cancer has promising results clinically. Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) is expressed by immune cells capable of selectively killing tumours with healthy cells unaffected². TRAIL triggers apoptosis by interacting with TRAIL receptors expressed by target cells, Death Receptor (DR)4 or DR5. Increased receptor selectivity and affinity enhances cancer killing efficiency of immune cells³. We are exploring the cytotoxicity of TRAIL-modified Natural Killer (NK) cells against OC.

¹O'Dwyer *Adv. Therap.* **3**, '20

²Khaider *Am. J. Cancer Res.* **2**, '12

³Duiker *Clin. Cancer Res.* **15**, '09

Material and Methods

The KHYG-1 NK cell line was modified with a TRAIL variant targeted to DR5 (TRAILv-KHYG-1). Tag-It Violet labelled OVCAR-3 OC cells (targets) were co-cultured with non-modified or modified NK cells (effectors) at 1:1 or 10:1 effector-to-target (E:T) ratios. After 4 or 16 hrs cytotoxicity of NK cells, samples were analysed by spectral flow cytometry on Cytex Bioscience Northern Lights 3000, using Annexin V-APC Apoptosis Detection Kit.

Results and Discussions

After 4hrs co-culture with TRAILv-KHYG-1s (TRAILv), OVCAR-3 viability was 62% at 1:1 and 43% at 10:1 E:T ratios. After 16hrs this reduced to 37% at 1:1 and 10% at 10:1. After 4hrs co-culture with non-modified KHYG-1s (KHYG-1s), OVCAR-3 viability was 75% at 1:1 to 61% at 10:1 which reduced to 50% at 1:1 and 36% at 10:1 after 16hrs. Regarding apoptosis, co-culture of OVCAR-3 cells with TRAILv for 4hrs resulted in 35% apoptosis at 1:1 and 56% at 10:1, increasing to 60% at 1:1 and 86% at 10:1 at 16hrs. KHYG-1s induced 24% apoptosis at 1:1 and 37% at 10:1, increasing to 47% at 1:1 and 60% at 10:1 after 16hrs. Soluble DR5 ligands produce similar cytotoxicity (40-80%) *in vitro*³. However, short half-life⁴ limits routine use. Our TRAILv-NK cells may overcome these challenges, potentially producing a potent treatment for OC.

⁴Kelley *J. Pharmacol. Exp. Ther.* **299**, '01

Conclusion

TRAILv modified NK cells are more effective at inducing OVCAR-3 cell death than non-modified NK cells. Further assessment is required to confirm findings. Future work includes pre-sensitising cancer cells to chemotherapy and exploring the specificity of TRAIL modified NK cell cytotoxicity to cancer cells.

EACR22-0131

Intra-tumor combination therapy with poly(I:C) and resiquimod free or loaded in polymeric nanocapsules synergistically triggers tumor-associated macrophages for effective anti-tumoral immunity

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Introduction

Intratumoral administration of immunotherapy has emerged as an effective therapeutic approach against solid tumors. In the tumor microenvironment (TME), tumor-associated macrophages (TAMs) play a key immunosuppressive role that limits the ability of the immune system to fight cancer. Here, we have investigated the antitumoral efficacy of combinations of poly(I:C) (TLR3 agonist) with imiquimod or resiquimod (TLR7 and TLR7/8 agonists, respectively). Furthermore, we are working on drug delivery approaches to improve TLR agonist's stability, pharmacokinetic profile and systemic toxicity, thus allowing their application in the clinic.

Material and Methods

TLR agonist treatments were evaluated *in vitro* for toxicity and immunostimulatory activity by Alamar Blue, ELISA and FACS. For *in vivo* experiments, fibrosarcoma (MN/MCA1), lung (i.e. CMT167) and pancreatic (PANC1 or K8484) cancer models were used; tumor-infiltrating leukocytes were evaluated by FACS, RT-qPCR, multispectral immunophenotyping, quantitative proteomic experiments, and protein-protein interaction analysis. Different routes of administration were used. Polymeric nanocapsules (NCs) were developed by the solvent displacement and layer-by-layer methodologies; and characterized by DLS and TEM. Poly(I:C) association efficiency by gel electrophoresis and R848 encapsulation efficiency by HPLC. Biodistribution of NCs functionalized with mannose was evaluated by IVIS.

Results and Discussions

We demonstrate the higher efficacy of poly(I:C) combined with R848, versus single treatments, to polarize macrophages towards M1-like antitumor effectors *in vitro*. *In vivo*, the intratumoral synergistic combination of poly(I:C)+R848 free or in NCs significantly prevented tumor growth and metastasis. Regressing tumors showed increased infiltration of macrophages with a higher M1/M2 ratio, recruitment of CD4+ and CD8+ T cells. The depletion of both CD4+ and CD8+ T cells resulted in complete loss of treatment efficacy in lung and pancreatic cancer models. Proteomic experiments validate the superior activation of innate immunity by poly(I:C)+R848 combination and protein-protein-interaction network analysis reveal the key activation of the STAT1 pathway. Hyaluronic acid nanocapsules functionalized with mannose show a higher accumulation in the tumor versus the non-functionalized ones.

Conclusion

Combination of poly(I:C)+R848 free or loaded in NCs showed the capacity to repolarize TAMs, to reduce tumor progression and prevent metastasis in murine tumors.

Neddylation regulates T cell function and antitumor immunity

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Introduction

Neddylation is a post-translational modification (PTM) by which the ubiquitin-like protein NEDD8 is covalently attached to target proteins. This pathway participates in the regulation of processes such as protein homeostasis, cellular stress pathways and cell survival. Of interest, neddylation is often upregulated in tumor cells, offering therapeutic opportunities for the treatment of cancer (e.g. mln4924). The relevance of neddylation in the regulation of anti-tumor immune responses and T cell function remains largely unexplored.

Material and Methods

Single-cell transcriptomic analyses of tumor-infiltrating T cells (TILs) were performed on datasets from four different tumor types. We generated a conditional knockout mouse lacking NEDD8 Activating Enzyme (NAE1) in T lymphocytes. Viability, proliferation (CFSE) and expression of immune checkpoint receptors were assessed in NAE1-KO CD8⁺ T cells by multi-color flow cytometry after in vitro T cell activation. We also characterised the phenotype of human primary CD8⁺ T cells treated with mln4924, including cytotoxicity in a CAR-T assay.

Results and Discussions

Single cell transcriptomic analyses reveal that NEDD8 expression is enriched in TILs according to their differentiation status. In vitro, both mouse and human activated CD8⁺ T cells upregulate the expression of the neddylation pathway, resulting in an enrichment of neddylated cullins. Mouse NAE1-KO CD8⁺ T cells showed impaired viability and proliferation after activation, and increased expression of immune checkpoint receptors TIM3, LAG3 and PD1, suggesting a potential role of neddylation in the control of T cell function. In this context, we performed an in vitro cytotoxicity assay by co-culturing CD8⁺ CAR-T cells treated with mln4924 with target tumor cell lines, demonstrating that neddylation is required by T cells to achieve their full cytotoxic potential.

Conclusion

Tumor infiltrating lymphocytes are characterised by high expression of NEDD8. In vitro assays demonstrated that neddylation is required for optimal CD8⁺ T cell proliferation and viability. Functional assays revealed that neddylation supports T cell cytotoxic capacity, suggesting that this pathway could be modulated for cancer immunotherapy.

PROTACs as a novel immunotherapeutic tool to boost CTL responses against cancer

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Introduction

A major goal of cancer immunotherapy is to prime cytotoxic T-lymphocytes (CTLs) that kill malignant cells upon recognition of antigens presented on major histocompatibility complex (MHC) class I molecules. Cancer cells often evade CTL recognition by downregulating MHC class I presentation. Despite significant advancements in recent years, there is currently no therapeutic strategy available to specifically promote the MHC class I presentation of cancer-associated antigens. We recently used proteolysis targeting chimeras (PROTACs) – bifunctional molecules that specifically target proteins for ubiquitin/proteasome-mediated degradation – to boost the MHC class I presentation of the H-2K^b-restricted model antigen SIINFEKL (S8L; from chicken ovalbumin) *in vitro*. Here we explore PROTAC treatment as a novel strategy to boost the CTL response in clinically relevant mouse models for melanoma.

Material and Methods

We generated murine B16F10 melanoma cells expressing an S8L-based model antigen that was sensitive to PROTAC-mediated degradation via an FKBP12-based degradation tag (dTAG system). S8L was fused in between click beetle red luciferase (CBR2) and the degradation tag FKBP12 (CBRed-S8L-F12), allowing us to monitor PROTAC-mediated antigen degradation by luciferase imaging *in vitro* and *in vivo*. After *in vitro* treatment with the PROTAC drug dTAG-13, antigen degradation, MHC class I presentation, S8L-specific CTL activation and killing was followed by western blot, luminescence assays and flow cytometry. Next, we established a B16F10-based mouse melanoma model expressing CBRed-S8L-F12 in combination with constitutive click beetle green luciferase (CBG2) to follow antigen degradation and tumor regression in living mice by dual-color bioluminescence imaging on an IVIS spectrum.

Results and Discussions

Our experiments showed that PROTAC-mediated degradation of CBRed-S8L-F12 increased the MHC class I presentation of S8L, which directly translated to enhanced CTL activation and killing of melanoma cells *in vitro*. We could confirm our findings using the endogenous melanoma-related protein gp100 as a second model antigen. In addition, we successfully showed PROTAC-mediated degradation of CBRed-S8L-F12 in living mice having subcutaneous B16F10 tumors.

Conclusion

Together, our results suggest that PROTACs could potentially be used in cancer immunotherapy to increase CTL-mediated killing of cancer cells. Currently, we utilize our established mouse melanoma models to test this hypothesis *in vivo*.

EACR22-0120

The Hu2G10 mAb exploits selective cancer vulnerability through 3D-informed targeting of the Trop-2 activation site

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Introduction

Trop-2 induces activatory calcium signaling and growth-signaling networks centered on Akt, ERK, Cyclin D1, NFκB (1, 2). We discovered that Trop-2 is activated by ADAM10 cleavage in cancer cells, for induction of cancer growth and metastatic diffusion (3, 4). This cleavage causes a spatial rearrangement of the extracellular Trop-2 domain, and exposes previously

inaccessible protein glycosylation sites. We designed a recognition strategy of such sites, for exploiting selective cancer vulnerability in patients.

Material and Methods

Mice were immunized with engineered target proteins, expressing multi-phyla end-glycosylation patterns, for extensive coverage of cancer-specific glycosylation determinants. Cancer-specific mAb were selected by cell-based ELISA, bio-layer interferometry, flow cytometry and living-cell fluorescence microscopy.

Results and Discussions

Most indiscriminating, low efficacy anti-Trop-2 monoclonal antibodies (mAb) recognize a single immunodominant epitope. Hence, we removed this epitope by deletion mutagenesis. mAb were obtained that only bound Trop-2-expressing cancer cells, but not normal cells. The humanized 2G10 mAb shows affinity for recombinant, cancer-specific, cleaved Trop-2 that is several orders of magnitude higher than leading anti-Trop-2 mAb. In vivo Hu2G10 ablated growth of Trop-2-expressing breast, colon, prostate cancers, in the absence of detectable toxicity, for unprecedented therapeutic index.

Conclusion

Naked and ADC Hu2G10 drive high anti-tumor effectiveness, for best-in-class anti-Trop-2 therapy.

- (1) E. Guerra et al., Trop-2, Na⁺/K⁺ ATPase, CD9, PKC α , cofilin assemble a membrane signaling super-complex that drives colorectal cancer growth and invasion. *Oncogene* (2022).
- (2) E. Guerra et al., Trop-2 induces tumor growth through Akt and determines sensitivity to Akt inhibitors. *Clin Cancer Res* 22, 4197-4205 (2016).
- (3) M. Trerotola et al., Trop-2 cleavage by ADAM10 is an activator switch for cancer growth and metastasis. *Neoplasia* 23, 415-428 (2021).
- (4) E. Guerra et al., Trop-2 induces ADAM10-mediated cleavage of E-cadherin and drives EMT-less metastasis in colon cancer. *Neoplasia* 23, 898-911 (2021).

EACR22-0674

Acquired cancer cell resistance to T cell bispecific antibodies and CAR T targeting HER2 through JAK2 down-modulation

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Introduction

Elimination of cancer cells by effector immune cells represents the culmination of a complex cascade of events, and disruption of any of those events may result in resistance. T cell-engaging therapies, such as T cell bispecific antibodies (TCBs) or chimeric antigen receptors (CARs), are raising extraordinary expectations as future treatments for virtually all cancers. Encouraging these expectations, TCBs and CARs have been recently approved to treat some hematologic malignancies. In contrast, TCBs and CARs against solid tumors tested to date, have failed to show clinical efficacy. This failure prompted intense research and the subsequent identification of mechanisms of primary and acquired resistance. All these mechanisms impinge on the ability of T cells to reach cancer cells and/or on the inhibition of T cells. However, little is known about putative intrinsic mechanisms of resistance of cancer

cells. That is, mechanisms deployed by tumor cells to resist killing by fully active and correctly engaged T cells.

Material and Methods

Using HER2⁺ cell lines and patient-derived xenografts (PDXs) and a TCB targeting HER2, we generated *in vitro* and *in vivo* acquired resistant models to HER2-TCB. This resistance was obtained after coculturing *in vitro* with peripheral blood mononuclear cells (PBMCs) or *in vivo* by humanizing mice with CD34⁺ cells obtained from cord blood, both from healthy donors.

Results and Discussions

Here we demonstrate that disruption in interferon-gamma (IFN- γ) signaling in cancer cells is a mechanism of intrinsic resistance to killing by fully active, correctly engaged, T lymphocytes. Importantly, we have identified that the kinase JAK2, which transduces the signal initiated by IFN- γ , is the component preferably disrupted to acquired resistance across all models used.

Conclusion

These results unveil a novel mechanism of resistance to T cell based therapies, and imply the potential use of JAK2 and IFN- γ response as a surrogate biomarker of response to immunotherapies. In addition, they open the avenue for the screening for therapies that can overcome deficient interferon-gamma response or restore JAK2 levels, which are promising potential candidates to increase the benefits of immunotherapies.

EACR22-1098

Therapeutic efficiency of Fc-engineered anti-cathepsin D antibody in mono and combotherapy in triple negative breast cancer

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Introduction

Triple-negative breast cancer (TNBC) defined by the absence of estrogen receptor, progesterone receptor and human epidermal growth factor receptor 2 overexpression and/or amplification, accounts for 15-20% of all breast cancer (BC) cases. Resistance to systemic treatment is common in TNBC so new treatments are required.

The aspartyl protease cathepsin D (cath-D), an independent marker of poor prognosis in BC, is overexpressed and hypersecreted into the tumor microenvironment. Recently our team has shown that cath-D is a tumor-specific extracellular target in TNBC suitable for antibody-based therapy (Ashraf, Mansouri *et al.*, JITC, 2019, 7:29). The objective of this project is to develop a targeted therapy in TNBC using an optimized anti-cath-D antibody.

Material and Methods

Our team has developed the human anti-cath-D IgG1 (F1) by phage display. Its therapeutic efficiency was studied *in vivo* in athymic nude mice (Foxn1^{nu}) xenografted with TNBC cell lines (MDA-MB-231) or PDX (Patient-derived xenografts).

Results and Discussions

F1 antibody significantly reduces tumor growth of TNBC cells and PDX in nude mice and exhibits immunomodulatory activity with natural killer cell activation and myeloid cell tumor depletion (associated with patent WO/2016/188911 and filed patent EP18306735.4). Because the mechanism of action of therapeutic antibodies involves both its binding to the target and immune cell recruitment by its Fc part (crystallizable fragment), glycoengineering was made on F1 to obtain a new F1Fc+ antibody with mutations in its Fc part known to increase ADCC (antibody-dependent cellular cytotoxicity), CDC (complement-dependent cytotoxicity) and ADCP (antibody-dependent cellular phagocytosis). The study of these mechanisms has shown that F1Fc+ optimized antibody activates NK cells, resulting in tumor cell lysis via ADCC mechanism *in vitro*. Moreover the therapeutic efficacy of the F1Fc+ antibody in an *in vivo* TNBC model is better than the one of non-mutated F1 antibody in terms of inhibition of tumor growth but also mouse survival. NK cell activation will now be studied *in vivo*. Besides, combination treatment strategies with this optimized antibody are being studied, particularly with chemotherapy and show promising results.

Conclusion

Based on these encouraging *in vivo* results, we hope that antibody-based targeting of cath-D may represent an attractive avenue for therapeutic applications in TNBC.

EACR22-0869

Phase I/II, multicenter, open-label study of REGN5668 (R5668; mucin [MUC]16 x CD28 bispecific antibody [bsAb]) with cemiplimab or REGN4018 (R4018; MUC16 x CD3 bsAb) in recurrent ovarian cancer (rOVCA)

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Introduction

High unmet need exists in rOVCA treatment. R5668 and R4018 are human IgG4-based bsAbs that bridge ovarian MUC16+ tumour cells to CD28 and CD3, respectively, on T-cells to stimulate cytotoxicity. Cemiplimab is a human monoclonal Ab that blocks programmed death [PD]-1 binding to PD-ligand(L)1 and PD-L2. R5668 + PD-1 inhibition or + R4018 showed greater preclinical anti-tumour activity than each monotherapy.

Material and Methods

This first-in-human study (NCT04590326) will assess safety, tolerability, pharmacokinetics, and preliminary anti-tumour activity of R5668 + cemiplimab (Module 1) or + R4018 (Module 2) in patients (pts) with rOVCA. Inclusion criteria include histologically confirmed diagnosis of advanced epithelial ovarian (except carcinosarcoma), fallopian tube, or primary peritoneal cancer; serum CA-125 level $\geq 2 \times$ upper normal limit; ≥ 1 prior-line of platinum-based therapy; prior treatment with or intolerance to available standard-of-care therapy. Exclusion criteria include recent biologic therapy (< 5 half-lives [$t_{1/2}$] or 28 days, whichever is longer, except $< 3 t_{1/2}$ for bevacizumab or other nonimmunomodulatory Abs with $t_{1/2} > 7$ days); approved conventional therapy (except biologics or immunotherapy) < 3 weeks (wks) or investigational

agents <4 wks prior to first study dose; and anti-PD-L1 therapy <5 T_{1/2} prior to first study dose.

This study includes dose escalation (Bayesian optimal interval design) and expansion phases. In Module 1, ≤84 pts will receive 3–4 wks of R5668 monotherapy lead-in at assigned intravenous (IV) weekly (QW) dose levels, followed by R5668 QW + cemiplimab IV every 3 wks. In Module 2, ≤106 pts will receive 4–5 wks of R4018 QW IV lead-in, followed by R4018 full (or highest tolerated) QW dose + R5668 at initial and full assigned QW doses. In expansion, R5668 + cemiplimab and R5668 + R4018 combinations will each recruit 20 pts in stage 1 and 30 pts in stage 2 using a Simon two-stage design. In escalation, primary endpoints are dose-limiting toxicities, serious and treatment-emergent adverse events (TEAEs), deaths, laboratory abnormalities (Grade ≥3), concentrations of R5668 in serum alone and in each combination regimen; key secondary endpoint is objective response rate (ORR) per RECIST 1.1. In expansion, primary endpoint is ORR by RECIST 1.1 for each combination; key secondary endpoints are TEAEs, serious AEs, deaths. Key exploratory endpoints are correlation between clinical efficacy endpoints and baseline protein expression levels of MUC16 and PD-L1.

Results and Discussions

NA

Conclusion

NA

EACR22-0090

Multifunctional nanovaccine synergizes with OX40 agonist in triple-negative breast cancer

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Introduction

Triple negative breast cancer (TNBC) is the most aggressive subtype of breast cancer. Immunotherapy, notably cancer vaccines and immune checkpoint inhibitors, has emerged as a promising alternative therapy. However, limited efficacy has been obtained for cancer vaccines and severe immune-mediated side effects have been related to immune checkpoint inhibitors under clinical development. This study focused on the development of a multifunctional nanovaccine to re-shape tumor microenvironment (TME), sensitizing TNBC to the immune checkpoint OX40 agonist.

Material and Methods

Polymeric nanoparticles (NP) were designed to target dendritic cells (DC) and the TME by incorporating TNBC-associated antigens, toll-like receptor ligands CpG and Poly(I:C), and regulators of potent immune suppressor molecules within the TME. NP surface was modified to promote DC activation, but also to potentiate their delivery to the TME. NP size, surface charge and morphology were characterized by Dynamic Light Scattering, Laser Doppler Electrophoresis and Atomic Force Microscopy, respectively. The entrapment efficiencies (EE) of each bioactive agent entrapped within NP were fully determined and NP impact on cell viability was assessed by the Alamar Blue assay. NP internalization and DC activation

profile were evaluated *in vivo* by flow cytometry. The immunotherapeutic potential of the nano-vaccines was assessed, isolated and in combination with α OX40, in 4T1 and E0771 mammary carcinoma.

Results and Discussions

NP presented a mean diameter close to 200 nm with low polydispersity index (PDI) values, surface charge close to neutrality, and EE superior to 85%. NP showed no negative effects on DC and tumor viability. Targeted NP were extensively taken up by DC, but also by tumor cells. Nanovaccines induced DC activation and maturation, significantly increasing the expression of CD80, CD86 and MHCII surface markers. 4T1 and E0771 tumor-bearing animals treated with the multifunctional nanovaccine combined with α OX40 showed a noteworthy tumor remission, with a prolonged overall survival. The nanovaccine re-shaped the immune profiling within the TME, which correlated with the overall anti-tumor effect obtained in this combinatorial scheme.

Conclusion

This study reveals synergy between a multi-targeting nanovaccine and α OX40 in TNBC, providing important insights for the establishment of novel combination regimens against this tumor.

EACR22-0656

Investigating the potential of anti-CSPG4 immune-targeting for the treatment of osteosarcoma: a comparative study

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Introduction

Osteosarcoma (OSA) is a pediatric tumor with high frequency of metastasis and resistance to therapies. The chondroitin sulfate proteoglycan (CSPG)4 is overexpressed by several tumors that fail to respond to conventional treatments, including OSA. Due to the rarity of the disease and the young age of patients, the possibility of testing new therapies is limited. Canine OSA is widely recognized as a reliable avatar of the human disease, representing a valuable translational model for testing new treatments. We aimed to investigate CSPG4 role in both human and canine OSA and the consequences of its immune-targeting via a DNA-based vaccine.

Material and Methods

We evaluated CSPG4 overexpression and its significance for human and canine OSA patients' survival. We treated cells with anti-CSPG4 monoclonal antibodies (mAbs), alone or combined with chemotherapy, and evaluated their effects on malignant features *in vitro*. We immunized mice with a chimeric human/dog (HuDo)-CSPG4 DNA vaccine, and the vaccine-induced immune response was analysed. Naturally-occurring OSA-bearing dogs were enrolled in a clinical veterinary trial and adjuvantly treated with HuDo-CSPG4 vaccine combined with electroporation, and the vaccine-induced immune response was evaluated.

Results and Discussions

We found a strong correlation between CSPG4 overexpression and a worse prognosis in both human and canine OSA patients. CSPG4 overexpression conferred resistance to doxorubicin, suggesting that CSPG4 immuno-targeting could sensitize OSA cells to chemotherapy. Anti-CSPG4 mAbs significantly inhibited both human and canine OSA cells pro-tumoral functions *in vitro*, potentiating the effect of chemotherapy. HuDo-CSPG4 vaccine-induced antibodies hampered CSPG4+ OSA cells proliferation *in vitro*. Furthermore, adoptively transferred vaccine-induced CD8+ T cells delayed human OSA tumor growth in NSG mice. In parallel, the adjuvant HuDo-CSPG4 vaccination is demonstrating to be clinically effective in spontaneously occurring CSPG4+ OSA-bearing dogs. Immunized dogs have shown a prolonged overall survival as compared to controls that received standard treatments alone. In vaccinated dogs, the induction of both anti-CSPG4 humoral and cellular response have been observed.

Conclusion

Overall, considering the high predictive value of spontaneous canine tumors, these results will possibly route the translation of this novel immunotherapeutic approach into a human setting, eventually improving life expectancy of OSA patients that cannot benefit from present therapies.

EACR22-0802

VACCINE IMMUNOTHERAPY AGAINST HUMAN ENDOGENOUS RETROVIRUS: A FOCUS ON ANTI-HERV-K ANTIBODIES

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Introduction

Immunotherapies have managed to cover the needs of cancer patients that traditional therapies could not treat. Despite the improvement, broadly acting and highly effective therapies capable of eliminating human cancers without mutated antigens still need to be developed. In this regard, virus-like-vaccine (VLV) technology together with the selection of a cancer specific but ubiquitous antigen can be the key to a successful immunotherapy. VLVs combine the benefits of adenoviral vectors and virus-like-particles (VLPs), leading to the activation of both B- and T-cell responses.

Human endogenous retroviruses (HERV) are remnants of ancient viral infections that got integrated into our genome millions of years ago, comprising now the 8% of it. HERVs are usually silenced in healthy tissues but are overexpressed in various cancer types, making them good antigen candidates. HERV-K is the most widely studied HERV, and evidence of HERV-K expression is particularly strong in breast cancer. There are currently no curative therapies for advanced breast cancer, thus our research has been focused on the putative effect of our cancer vaccine in advanced breast cancer.

Material and Methods

This project seeks to describe the effects of HERV-K specific humoral immunity on breast cancer progression. Expression of HERV-K protein in a wide range of breast cancer cell lines was initially investigated. We optimized proliferation, cell migration and invasion, and colony formation assays to explore the role of HERV-K in the phenotype of breast cancer cells. These are then subjected to treatment with commercial and *in vivo* generated antibodies against HERV-K, protease inhibitors, and agents that block putative HERV-K interaction partners.

Results and Discussions

HERV-K was shown to be present in a wide panel of breast cancer cell lines of various HR, ER, and HER2 status. The project is still ongoing, and I will be analyzing data with this cell lines over the next four months, showing the oncogenic phenotype of HERV-K. Additionally, I will be assessing the effect of our vaccine derived antibodies in tackling the immunosuppressive properties of HERV-K in T-cell and NK, as well as in the involvement of antigen presentation and T-cell activation

Conclusion

This project will reveal whether HERV-K is a valuable target for VLVs in advanced breast cancer.

EACR22-0824

Combining xCT targeting with APR-246: a new strategy against breast cancer

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Introduction

Breast cancer is the most frequent cancer in women and, despite recent clinical advances, new therapeutic approaches are still required. Studies on TP53 have revealed that both wild-type and mutant p53 (mut-p53) can induce the post-transcriptional down-modulation of SLC7A11, encoding for the cystine/glutamate antiporter xCT, a tumor-associated antigen crucial for the maintenance of intracellular redox balance, rendering mutant-p53 tumors susceptible to oxidative damage. On the one hand, the expression of xCT may predict tumor response to the first-in-class reactivator of mut-p53 used in clinical trials, named APR-246, while on the other hand xCT targeting may enhance the efficacy of APR-246 treatment. Here, we propose a combinatorial strategy based on anti-xCT immunotargeting and APR-246 treatment as a new strategy for tackling breast cancer.

Material and Methods

Viability and apoptosis assays have been performed on murine and human breast cancer cell lines, cultured in monolayer or as cancer stem cell (CSC)-enriched tumorspheres, in the presence or absence of the xCT inhibitor sulfasalazine (SAS) and of APR-246. BALB/c mice were orthotopically injected with murine mammary cancer cells prior to receiving APR-246 treatment and/or two anti-xCT DNA vaccination courses.

Results and Discussions

Even though APR-246 is able to restore the wild-type conformation of p53 in mut-p53 tumors, it can up-regulate the expression of xCT, which contributes to CSC self-renewal and therapy resistance. However, our results demonstrated that the combination of SAS and APR-246 treatment significantly reduced breast cancer cell viability *in vitro*, induced apoptosis, and affected CSC self-renewal if compared to the single treatments. Moreover, xCT immunotargeting, in combination with APR-246 treatment *in vivo*, synergistically hindered tumor progression and reduced lung metastasis formation. These effects could be mediated by the production of anti-xCT antibodies, able to induce antibody-dependent cellular cytotoxicity. Based on the so far obtained results, we demonstrated that DNA vaccination against xCT is able to synergize with APR-246 treatment to enhance its therapeutic effect.

Conclusion

Overall, our results suggest that combining xCT immunotargeting with APR-246 treatment could represent a new valuable strategy for the management of breast cancer.

EACR22-0858

Mismatch Repair Deficient Colorectal Cancer Highlights the Limited Importance of Neojunctions as a Source of Neoantigens.

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Introduction

The identification of neoantigens, tumor specific antigens that arise during tumor development, is crucial not only for selecting patients that benefit from checkpoint inhibition therapy, but even more so for the development of novel anti-cancer vaccination strategies. In that regard, it is important to evaluate the contribution of neoantigens derived from alternatively spliced exon-exon junctions, so called neojunctions.

Material and Methods

We have determined the difference in the neojunction landscape between 43 mismatch repair deficient (dMMR) and 214 proficient (pMMR) colorectal cancer (CRC) patients from the Cancer Genome Atlas and compared this to neojunction data from Kahles et al (Cancer Cell 2018). First off all, we analyzed the overlap between mutational hotspots in DNA sequencing data and neojunction events. Secondly we calculated if frameshift mutations could induced skipped regions in the RNA sequencing data as an alternative measurement of neojunction formation.

Results and Discussions

The mean number of neojunctions for dMMR CRC is not significantly increased compared to pMMR CRC ($p = 0.056$). Only 0.1% of neojunctions occur in more than 20% of dMMR CRC patients and this is similar to pMMR CRC. Moreover the overlap between the top 500 most occurring neojunctions and the top 500 most occurring frameshift mutations is only 3.4%. Finally we found no sign of increased RNA skipping counts in frameshift mutated exons (selection threshold: Fold Change ≥ 2 ; p -value ≤ 0.05).

Conclusion

Our data clearly show that the neojunction landscape of dMMR and pMMR CRC is similar and not related to frameshift mutations commonly found in dMMR CRC. As dMMR CRC patients are known to respond to checkpoint inhibition therapy in contrast to pMMR CRC patients, these data highlight the limited importance of neojunction derived neoantigens in dMMR CRC and therefore the targetability of such neoantigens for anti-cancer vaccination strategies is questionable.

EACR22-0952

ONCOLYTIC THERAPY ESTABLISHES AN IMMUNE ANTITUMORAL ENVIRONMENT RESULTING IN GLIOMA ERADICATION

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Introduction

Glioblastoma is the most aggressive form of brain tumor. Median survival time of glioblastoma patients treated with the standard of care protocol is about 14 months. This life expectancy has not substantially changed in the past decades. In this scenario, oncolytic viruses appear as a safe, remarkable, and dynamic alternative to common chemotherapies since they can selectively target, replicate and kill tumor cells. We recently proved the preclinical efficacy of R-115, an oncolytic Herpes Simplex Virus (oHSV) targeting HER2 and armed with IL-12, that eradicated 30% of tumors and established an antitumor immune memory.

Material and Methods

We evaluated the effect of R-115 in our murine model of high-grade glioma comparing HER2 high expressing (>90%) to HER2 low expressing (<30%) tumors. Moreover, we evaluated if doubling the oHSV treatment could increase the therapeutic outcome in terms of modifications of the tumor microenvironment from pro- to anti-tumoral, overall survival and tumor eradication.

Results and Discussions

R-115 treated mice transplanted with high HER2 expressing cells showed a stronger tumor eradication if compared to mice injected with low HER2 expressing cells, underlying the importance of oHSV-mediated oncolysis on HER2 targeted cells. Remarkably, R-115 was capable not only to prolong survival but also to eradicate tumors even in HER2 low expressing tumors. We also evaluated the effect of augmented R-115 administrations which resulted in an improved frequency of tumor eradication and prolonged survival. Moreover, oHSV therapy allowed to establish a solid immune response in surviving mice which were capable to eradicate secondary transplanted tumors. We characterized the immune system of rescued mice which showed an enhanced release of IFN- γ and granzyme B, augmented CD8, CD4 and CD19 proliferation and capability to restore MHC I expression on tumor cells.

Conclusion

Our deeply established murine model gave us the opportunity to analyze the effects of immunovirotherapy on high grade gliomas implanted in immunocompetent mice. Our results underlined the promising and fundamental role of the oHSV oncolytic activity together with its reawakening of the immune system. The resultant therapy worked like an agnostic vaccination since the immune response was directed against an unknown repertoire of tumor neoantigens and the outcome is the development of a durable immune memory that vaccines the host against the tumor.

EACR22-0191

Alanine-based spacers promote a more efficient antigen processing and presentation in neoantigen polypeptide vaccines

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Introduction

Neoantigens are patient and tumor-specific antigens derived from mutations that are a promising target for the development of personalized cancer vaccines. After presentation by APCs, neoantigens can induce a specific anti-tumoral T cell response, whose magnitude and quality strongly depend on how the neoantigen is presented. CD8⁺ T cell responses,

which require MHC-I presentation, may be improved using DNA or RNA sequences that code for a unique polypeptide that concatenates several neoantigens spaced by linker sequences. The selection of these spacers is of special interest, as they might influence the processing and MHC-I presentation of the neoantigens by APCs.

Material and Methods

We designed a chimeric gene containing the immunogenic OVA peptide (SIINFEKL) along with thirteen B16F10-derived neoantigens and separated from each other by a specific short linker (AAA, AAL, ADL, A or GGGS). In addition, SIINFEKL has been tested at the N-terminus and C-terminus of the AAA-linker construct. One individual construction was prepared for each linker and transfected separately into B16F10 melanoma cell. Six hours post-transfection, cells were stimulated with mouse IFN γ for 48 hours, and subsequently stained using a PE-conjugated anti-mouse H-2K^b/OVA, which specifically recognizes SIINFEKL complexed to the H-2K^b molecule.

Results and Discussions

Results showed that the highest SIINFEKL/H-2K^b signal was obtained with alanine-based linkers, especially the AAA-linker, suggesting that this linker might be the most successful spacer for the processing and MHC-I presentation of SIINFEKL. Conversely, the GGGS-linker produced the lower signal, indicating that this linker was the least efficient promoting SIINFEKL presentation.

In addition, the AAA linker allowed for readily detectable OVA peptide presentation when the peptide was presented at the center, the C-terminus or the N-terminus of the polypeptide construct, although the latter position showed lower signal.

Conclusion

Our results show that spacer sequences determine the efficiency of neoantigen processing and presentation by MHC-I molecules. Linkers based on alanine residues promote a more efficient neoantigen presentation than the widely used GGGS linker. Moreover, the results suggests that the position of neoantigen into the construct may affect its processing and presentation efficiency, which might be improved when the neoantigen is located in or close to the middle or C-terminus of the construct.

EACR22-1001

A chimeric Human/Dog DNA vaccine against the Chondroitin Sulfate Proteoglycan (CSPG)4 as an effective strategy to induce CSPG4-targeted immunity for the treatment of melanoma

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Introduction

Melanoma is the sixth most common cancer in the world. Major progress has been made in the treatment of cutaneous melanoma thanks to the introduction of targeted therapies and immune checkpoint inhibitors. These treatments have induced impressive clinical responses in 20-50% of patients, but a still high proportion does not benefit clinically and shows side effects. The other subtypes, i.e. non-ultraviolet (UV)-induced cutaneous, mucosal and uveal melanomas, are less characterized with few therapeutic options and a very poor prognosis.

These evidences prompted us to develop a vaccination strategy against the tumor antigen chondroitin sulfate proteoglycan (CSPG)4. CSPG4 is an attractive immunotherapeutic target, highly expressed on melanoma cells in a high percentage of patients, independently of tumor stage and subtypes, but with a limited expression in normal tissues. However, CSPG4 is a non-mutated self-antigen, and as such it is poorly immunogenic.

Material and Methods

To overcome host's unresponsiveness to the self-antigen, we have generated a chimeric DNA vaccine encompassing both human (Hu) and dog (Do) portions of CSPG4 (HuDo-CSPG4). We have tested the validity of our strategy in mice and in a prospective veterinary clinical trial enrolling 80 client-owned dogs with surgically resected, CSPG4-positive, stage II-IV oral melanoma, representing a clinically relevant model of human non-UV-induced cutaneous, mucosal and uveal melanomas, with the same clinical behavior and a high propensity to metastasize to lymph nodes and lungs.

Results and Discussions

HuDo-CSPG4 vaccination is immunogenic and endowed with an anti-tumor potential in mice. HuDo-CSPG4 adjuvant vaccination, performed in 52 out of 80 dogs, was found to be well tolerated and effective in significantly prolonging the survival of vaccinated dogs as compared to the 28 controls treated with conventional therapies alone. Vaccinated dogs developed a high affinity anti-Do- and anti-Hu-CSPG4 immune response, related with the survival.

Conclusion

HuDo-CSPG4 adjuvant vaccination was safe, effective in breaking immune-tolerance and had a beneficial effect on the course of the disease in dogs with oral melanoma. Thanks to the power of naturally occurring canine tumors as predictive models for cancer immunotherapy response and to the hybrid human/dog structure of the plasmid, these data represent a solid basis for the translation of HuDo-CSPG4 to the treatment of human patients with CSPG4-positive melanoma.

EACR22-1097

Unlocking Pancreatic Cancer to Nano-Immunotherapy

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Introduction

Among immunotherapeutic approaches, immune checkpoint inhibitors (ICI), have revolutionized the treatment of several cancers. However, clinical trials have shown PDAC as one of the most ICI-resistant cancers. PDAC has proven to be refractory to the most conventional anti-neoplastic therapies, like chemotherapy, but also to the most modern, as targeted- and immuno- therapies.¹ Looking into PDAC, the unique dense stroma, the highly immunosuppressive tumor microenvironment (TME), and the immune evasion mechanisms have limited the infiltration by immune cells and therapeutics. Here we show the synergistic therapeutic effect of a nanotechnology-based system in combination with ICI *ex vivo* and *in vivo*.

Material and Methods

PDAC antigens were selected using immune-bioinformatic analysis. Poly(lactic acid) (PLA) and Poly(lactic-co-glycol) (PLGA)-based nanoparticles (NP) incorporating PDAC antigens and immune regulators were formulated following methods already established². Particle size and morphology were determined by dynamic light scattering and Atomic Force Microscopy (AFM), respectively. The anti-tumor immune-mediated effect was evaluated *ex vivo* and *in vivo* in patient-derived organoids and PDAC-bearing mouse model (KPC), respectively.

Results and Discussions

NP presented similar average hydrodynamic diameters, despite the entrapment of large or short peptides, with a low polydispersity index. AFM showed spherical particles with a slightly rough surface. NP showed entrapment efficiency (EE) of 70-90%. Cy5.5-labeled NP was extensively internalized by DC and triggered their activation *in vivo*. Higher levels of DC-related co-stimulatory molecules such as CD80, CD86, and CD40, were observed when compared with non-carbohydrate carriers. NP successfully induced a potent immune-mediated anti-tumor response. Synergistic anti-tumor effects were observed when NP was combined with ICI.

Conclusion

The developed nanotechnology-based system showed the induction of a strong antigen-specific immune response and unlocked PDAC to standard immunotherapeutic approaches, as immune checkpoint modulators.

EACR22-1203

A nano-sized system for the treatment of gastrointestinal cancers

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Introduction

Despite the recent progress in cancer immunotherapies, most patients do not benefit from the currently available immune checkpoint blockers, and many develop severe immune-related adverse events. Therefore, it is urgent to develop alternative effective immunotherapeutic approaches. An example of non-responsive cancers is gastrointestinal (GI) cancers which account for 26% of the global cancer incidence and 35% of all cancer-related deaths. In many GI cancers, the failure of immunotherapies to yield clinical response in GI cancers is partially attributed to the inefficient antigen presentation by DC, which results in insufficient T cells stimulation despite their expression of tumor-associated antigens (TAA). To overcome these hurdles, we designed novel polymeric-based nanoparticles (NP) to deliver combinations of GI cancers antigens and toll-like receptor ligands to DC, thus successfully modulating the tumor milieu-immune cell interactions to control tumor growth and improve overall survival.

Material and Methods

NP was fully characterized using Dynamic Light Scattering, Laser Doppler Electrophoresis, and Atomic Force Microscopy, to determine the mean average size, surface charge, and morphology, respectively. The biodistribution profile and DC accumulation were assessed *in vivo* and evaluated by flow cytometry. The anti-tumor immune-mediated effect was evaluated *in vivo* in mouse models.

Results and Discussions

NP presented an average diameter below 200 nm, narrow polydispersity index, slightly negative surface charge, spherical morphology, and high loading of the antigens and the immune potentiators. Cy5.5-labeled NPs were extensively internalized by DC and triggered their activation *in vivo*, increasing the expression of DC-related co-stimulatory molecules such as CD80, CD86, and CD40. Furthermore, the immunotherapeutic effect of our nano-based platform revealed that the NP successfully induced a potent immune-mediated anti-tumor response, sensitizing the tumor microenvironment to other therapies, ultimately leading to prolonged survival.

Conclusion

The developed NP induced a strong antigen-specific immune response and overall sensitized GI cancers to the clinically relevant treatments that so far have shown disappointing clinical outcomes.

EACR22-0694

ORAL PRESENTATION

Harnessing the microbiota to sensitize cancer cells to immunotherapy with immune checkpoint inhibitors

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Introduction

Recent data have shown that gut microbiota have a major impact on the clinical response to immune checkpoint inhibitors (ICIs). As ICI therapy acts by unlocking tumor antigen (TA)-specific T cells, these data suggest that the presence of certain bacteria can enhance patients' immune responses. A common cause of resistance to ICI therapy is downregulation of HLA class I expression on cancer cells, prompting our hypothesis that microbiota can influence immune responses by upregulating HLA class I expression on cancer cells.

Material and Methods

To investigate the ability of bacterial-based products to upregulate HLA class I expression, we tested proprietary microbial derivatives (MDs) on multiple murine and human primary and immortalized cancer cell lines and examined if the upregulated HLA class I on the cancer cell surface could increase TA-specific T cell activation. To assess translational relevance, BALB/c mice were orthotopically grafted with murine 4T1 breast cancer cells and injected *i.p.* with anti-PD-1 monoclonal antibody (mAb) combined with MDs or vehicle control. Lastly, NLRC5 involvement was evaluated by *in vitro* treatment of cancer cells with MDs in the presence of NLRC5 inhibitor Leptomycin B.

Results and Discussions

Our results show that *in vitro* treatment with MDs can upregulate HLA class I on the surface of cancer cells, with breast and myeloid tumor cells showing highest sensitivity across the cell types tested. The MD-dependent HLA class I upregulation subsequently boosted TA-specific T cell activation (% CD8⁺CD107a⁺), without increasing background reactivity, and was consistent whether the antigen was endogenous ($p < 0.0001$) or added exogenously ($p < 0.01$ and $p < 0.0001$). When combined with anti-PD-1 mAb *in vivo*, MD treatment significantly abrogated 4T1 tumor growth ($p < 0.0001$) and tumors harvested from MD-treated mice expressed higher levels of MHC class I antigens compared to the vehicle control group ($p < 0.01$). Additionally, splenocytes from MD-treated mice showed increased recognition of

4T1 cells when re-challenged *in vitro* (% CD8⁺41BB⁺ cells, p<0.001). NLRC5 plays a major role in MD-induced HLA class I upregulation as blocking of NLRC5 *in vitro* completely abrogated the MD's effect.

Conclusion

Our results thus far confirm that our proprietary MDs can increase HLA class I expression on cancer cells, which leads to increased recognition by TA-specific T cells both *in vitro* and *in vivo*. This suggests that MDs could be explored in combination with ICIs to enhance clinical anti-cancer immune responses.

EACR22-0032

Decrease in HLA class I antigen expression level in progressing metastases of melanoma patients treated with ipilimumab

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Introduction

Breakthroughs in understanding the mechanisms of antitumor immune reactions and escape mechanisms resulted in the development of more effective immunotherapeutic modalities, such as immune checkpoint inhibitor (ICI) therapy. ICIs can induce durable responses in patients with advanced cancer in a wide range of tumor types, however, the majority of the patients fail to respond to therapy or develop resistance during therapy. The knowledge about the underlying mechanisms of primary and acquired resistance is limited. Although HLA class I molecules are crucial in the recognition of tumor antigens by cytotoxic T lymphocytes, few studies have been reported aiming at the role of their expression in ICI resistance.

Material and Methods

We performed longitudinal analysis of HLA class I expression in pre-treatment and post-treatment tumor samples from melanoma patients receiving ipilimumab therapy, by immunohistochemistry using 3 HLA class I specific antibodies. Twenty-nine metastases of six patients were available for the study, including 18 pre-treatment and 11 post-treatment lesions. The mouse monoclonal antibodies used in the study included HCA2, recognizing HLA-A (excluding -A24), -B7301, and -G heavy chains; HC10, recognizing HLA-A3, -A10, -A28, -A29, -A30, -A31, -A32, -A33, HLA-B (excluding -B5702, -B5804, and -B73), and HLA-C heavy chains; and NAMB-1 recognizing β 2-microglobulin.

Results and Discussions

Compared to metastases excised before ipilimumab therapy, post-treatment lesions showed significantly lower HLA class I expression in melanoma cells. The reduction in HLA class I expression was mainly observed in the case of progressing lesions of nonresponding patients while minimal or no change could be seen in responders. These findings are

consistent with the hypothesis of immunoediting in patients receiving immunotherapy, resulting in HLA class I loss and in tumor progression.

Conclusion

Our results indicate the potential importance of downregulation of HLA class I expression as a mechanism of ICI resistance.

EACR22-0150

Circulating extracellular vesicles as biomarkers of response for immune-checkpoint inhibitors in advanced non small cell lung cancer

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Introduction

Today, lung cancer is one of the major causes of cancer related-deaths worldwide. Use of immune-checkpoint inhibitors (ICI) has improved clinical outcomes of patients (pts) with advanced Non-Small Cell Lung Cancer (NSCLC). Programmed death ligand-1 (PD-L1) expression on tumour cells is the only predictive biomarker used in clinical practice for ICI in NSCLC. Despite the efficacy of ICI mainly correlates to high PD-L1 expression, also low expressor pts can achieve long response to therapy. Extracellular vesicles (EVs) were used as biomarkers for cancer progression and can express PD-L1 on their surface. The aim of this study was to evaluate EVs in advanced NSCLC patients with low PD-L1 expression in order to find biomarkers for ICI.

Material and Methods

EVs were isolated using ultracentrifuge from plasma of 64 advanced NSCLC pts treated with ICI or from conditioned medium of primary lung adenocarcinoma (LT73) and metastatic large cell lung carcinoma (H460) cell lines. Pts were classified in responders (R) if they achieved a complete or partial response by RECIST 1.1, non-responders (NR) otherwise. EVs characterization was performed following MISEV guidelines. The presence of co-inhibitory or co-stimulatory immune-checkpoint molecules on EVs was assessed by flow cytometry. T cells were isolated and stimulated with CD3/CD28 beads in presence of EVs (15ug) and analyzed by flow cytometry.

Results and Discussions

R-EVs show significant higher levels of CD9, CD81, CD63, CTLA-4, 41BB, OX40L and CD86 and low levels of TIGIT compared to NR-EVs. Stratifying baseline surface EVs molecules according to median value, higher values of PD-L1 and VISTA were related to worse progression free survival (HR 1.78 and 2.15 respectively). In 20 patients (10 R, 10 NR), evaluated during ICI therapy, EVs-PD-L1 levels increased in R compared to NR (PD-L1 fold change 1.89). Interestingly, changes in CD9, CD81 and CD63 levels on both EVs were observed after ICI. Strikingly, EVs increased CD4 and CD8 T cell proliferation and activation, evaluated as CFSE dilution and IFNg and Granzyme B production, respectively. We did not find any significant difference in checkpoint receptors on T cell surface. Notably, in the same experimental setting, EVs from LT73 efficiently inhibited T cell proliferation whereas EVs from H460 cells had no effect.

Conclusion

Plasma EVs from NSCLC pts treated with ICI showed different features in terms of surface markers. PD-L1 and VISTA on EVs could represent promising biomarkers for ICI response in NSCLC.

Anti-metabolic and chemotherapy drugs combinations enhanced antitumor effects, also plus immunotherapy, in triple-negative breast cancer.

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Introduction

Triple-negative breast cancer (TNBC) is a heterogeneous group that represents 10%–20% of invasive breast cancers. Classical chemotherapy remains the standard of care for advanced TNBC. Immune checkpoint inhibitors (ICIs) have emerged as a promising therapeutic option and are being investigated with encouraging results. Metformin (MTF), a drug used for type 2 diabetes with well-tolerated side-effects, has been reported to have anticancer effects in some tumours such as TNBC. Xenograft mouse models studies of breast cancer reveal a synergistic effect of MTF when combined with chemotherapy. Also, MTF seems to stimulate the immune system in the context of cancer and may enhance the efficacy of immunotherapy. The objective of this work was to assess the antitumor effects of chemotherapy in combination with MTF in TNBC cell lines, and the impact of adding immunotherapy in a TNBC mouse syngeneic model.

Material and Methods

We analysed the combined effect of different anthracyclines and taxanes with MTF in TNBC cell lines. Dose-effect of each drug or drug combination was calculated using the Chou-Talalay method, and synergistic or antagonistic effects of drug combinations were defined. EMT6 cells were implanted subcutaneously in immunocompetent BALB/c mice and tumour response was analysed after 2 weeks of treatment with different drugs combinations, including MTF, chemotherapy and a mouse-adapted anti-PD-L1 antibody equivalent to Atezolizumab (m-ATZ).

Results and Discussions

Chemotherapy and MTF exhibited growth inhibitory effects on TNBC cell lines, showing a synergistic effect. A combination of DOX (6mg/Kg) together with m-ATZ and MTF reduced the tumor size of treated mice while provoking a 20% weight loss on treated mice when compared to the untreated control group. However, treating with the same combination of drugs, a 10-fold DOX reduction dosage (from mg/Kg 6 to 0.6 mg/Kg), produced an equivalent reduction in tumor size, without associated weight loss.

Conclusion

MTF showed a synergistic effect when combined with chemotherapy inhibiting the growth of TNBC cell lines. The incorporation of MTF to a treatment scheme based on chemotherapy and ATZ would allow a reduction in the dosage of chemotherapy in vivo, thus reducing their associated side effects. These results set the basis to develop future studies evaluating the inclusion of MTF in the chemotherapy plus immunotherapy schemes to reduce chemotherapy side effects in TNBC patients.

Implementation of the Patient-Derived Explant (PDE) model for predicting the efficacy of immune checkpoint inhibitors (ICIs)

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Introduction

Melanoma accounts for the highest number of deaths from skin cancer. Although primary tumours are treated by surgery, some patients develop metastasis and resistance to treatment. Ninety five percent of oncology agents fail to reach market authorisation, partly due to a lack of relevant pre-clinical models predictive of patient outcome. Accurate pre-clinical models with the potential to reduce drug attrition rates and better predict patient outcomes are urgently sought, particularly for ICIs. We have established a metastatic melanoma (MM) PDE pre-clinical model, which we have shown represents the “live” culture of MM tumour fragments and retains tumour architecture intact.

Material and Methods

Tumours obtained from surgery were sectioned into explants (1-3mm³) and cultured for 16-24 h. The next day, PDEs were transferred into fresh media containing ICIs. On day 3, PDEs were fixed and formalin fixed paraffin embedded (FFPE) blocks generated. Proliferation marker (Ki67) and cell death (cPARP) in the tumour and stroma areas were assessed by tyramide signal amplification (TSA) based multi-immunofluorescence (mIF) coupled with digital pathology quantitation to assess cell viability in response to ICIs. Biomarkers of interest (CD4, CD8, FOXP3) were also used for mIF to monitor the density and location of key immune cells in response to the ICIs.

Results and Discussions

mIF analyses showed differential levels of CD4⁺FOXP3⁺ T regulatory cells (Tregs) and CD8⁺ Cytotoxic T Lymphocytes (CTLs) infiltration into stroma and tumour areas of the MM-PDEs. Approximately 50% of MM tumours are “hot” tumours i.e. high levels of infiltrating CTLs. We have also observed differential apoptotic responses to ICIs across the MM-PDEs, with ~30% of tumours responding to anti-PD1 inhibition (pembrolizumab/nivolumab) or anti-CTLA4 inhibition (ipilimumab). We are currently evaluating whether there is a correlation between CTL infiltration and apoptosis induction in response to the ICIs and if this is linked to clinical outcomes. These data will be presented at the conference.

Conclusion

PDEs contextually preserve the tumour microenvironment. We have been able to identify tumours that have differential levels of T cell infiltration and are differentially sensitive/resistant to anti-PD1/anti-CTLA4 therapies in *ex vivo* culture. Further analysis of these data with clinical comparisons, will allow us to understand the extent to which the MM-PDEs are predictive of patient outcomes in the preclinical testing of ICIs.

EACR22-0768

Checkpoint Molecule Profiling in Tumor Cell Lines and Immune Cell Lines and Application for Immuno-oncology Drug Screening

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Introduction

The development of cancer immunotherapies has emerged as an exciting new method in treating cancer. Inhibition of immune checkpoint is one of the most promising approaches to unleash the potential of the anti-tumor immune response. Immune checkpoint blockades are monoclonal antibodies that bind to checkpoint molecules on the surface of immune and tumor cells. Tumor cells utilize immune checkpoints to suppress immune response and increase survival. There are several established and novel checkpoint molecules being studied to create cancer immune therapeutics. PD-1 (Programmable Death-1) is one of the most studied of these checkpoint molecules and is the basis for several FDA approved therapeutics on the market. Although immune checkpoint blockades have exhibited anti-tumor effects in multiple cancer types, there are still challenges to overcome such as resistance and low response rate. There is a need for comprehensive data on the expression levels of checkpoint molecules based on cancer type.

Material and Methods

In this study, we profiled a cross-section of those cell lines with several established and novel checkpoint molecules. FACS analysis was used to evaluate expression level of the checkpoint markers including, but not limited to, PD-1, CTLA4, LAG-3, TIGIT, and VISTA. HLA typing was also performed on those cell lines. With this profiling data, we further tested the responses of the cell lines to several on-the-market immunotherapy biologics, such as Pembrolizumab (anti-PD1), Nivolumab (anti-PD1), and Ipilimumab (anti-CTLA4). In our physiological relevant checkpoint assay system, an activated human CD4⁺ T-cell line and primary CD8⁺ T-cells were co-cultured with target cell lines with the addition of a serial dilution of checkpoint blockade treatment.

Results and Discussions

We were able to demonstrate T-cell proliferation and increased cytokine expression based on immunotherapy antibody treatment.

Conclusion

In summary, this study presents a comprehensive checkpoint molecule profiling data set of T cells lines and tumor cell lines derived from various cancer types that can be used to develop custom assays, as required. The combination of established cell lines and primary T cells offers a unique and effective approach in checkpoint assay development, which provides a valuable tool for studying checkpoint molecule interactions and screening biologics as cancer immunotherapy treatments.

EACR22-0772

Mitoxantrone in combination with concomitant blockade of TGF β and PD-1 remodels the tumor immune landscape by enhancing neuroblastoma antitumor immunity

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Introduction

Poor infiltration of functioning T cells renders tumors unresponsive to checkpoint-blocking immunotherapies. Here, we employed a combinatorial *in situ* immunomodulation strategy

based on administration of selected immunogenic drugs and immunotherapy to sensitize two poorly T-cell-infiltrated neuroblastoma (NB) mouse models to the host anti-tumor immune response.

Material and Methods

Multiple *in vitro*, *ex vivo* and *in vivo* approaches were used to select the best drugs able of inducing anti-tumor immune responses in 975A2 and 9464D NB tumors grown subcutaneously or in the adrenal gland of syngeneic mice. The tumor immune microenvironment was comprehensively analyzed by flow cytometry, immunohistochemistry, multiplex immunofluorescence and chemokine arrays. Murine-derived organotypic tumor spheroids (MDOTS) were generated from explanted tumors and used to study immune cell recall towards drug-treated MDOTS using microfluidic devices.

Results and Discussions

We found that mitoxantrone treatment curbed NB growth and promoted tumor regression when combined with TGF β and PD-1 blockade. This combined immunotherapy strategy resulted in the enrichment of a variety of both lymphoid and myeloid immune cells and the concomitant production of inflammatory chemokines involved in remodeling the tumor's immune landscape.

Conclusion

Mitoxantrone in combination with TGF β and PD-1 blockade could be used to reshape the functional repertoire of intratumoral immune cells, restoring the entire immune cell cycle and overcoming the immunosuppressive microenvironment of aggressive NB.

EACR22-0826

Targeting YKL-40 using chitooligosaccharides combined with checkpoint inhibitors and radiation in a syngeneic mouse model of lung cancer

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Introduction

YKL-40 (also named chitinase-3 like-1 protein (CHI3L1)) is a conserved, secreted chitinase-like protein. Elevated plasma YKL-40 is associated with poor prognosis in many solid tumors. YKL-40 is suggested to have several pro-tumorigenic functions. YKL-40 lacks enzymatic activity, but it can bind carbohydrates such as chitin, an abundant glycopolymer found in fungi, insects, and crustaceans. Chitosan and chitooligosaccharides (COS) are derived from hydrolysis and deacetylation of chitin. The use of these compounds represents a potential strategy for blocking YKL-40 *in vivo*.

Material and Methods

YKL-40 expression was investigated in whole tumors and in specific cell populations using public single-cell RNA-sequencing data from healthy and lung-, colorectal-, and ovarian cancer tissue. Animal experiments were conducted following ARRIVE guidelines and with approval from the Danish Animal Experiments Inspectorate. Syngeneic mouse cancer models were screened for expression of YKL-40. The Lewis lung carcinoma model was treated with monotherapies and combinations of COS, a checkpoint inhibitor combination (α -PD-L1 and α -CTLA-4), and radiation (8 Gy x 3). Plasma YKL-40 was determined using ELISA.

Results and Discussions

YKL-40 expression was higher in biopsies from cancer tissue compared to adjacent healthy tissue from the same patients, and the expression primarily increased in the fibroblast and myeloid cell populations. The Lewis lung carcinoma (LL2) model expressed high levels of YKL-40 *in vivo*. There was a trend towards lower plasma YKL-40 after therapy with COS, but monotherapy with COS did not affect tumor growth. Interestingly, COS combined with checkpoint inhibitors led to delayed tumor growth and prolonged overall survival compared to checkpoint inhibitors alone. Radiation also enhanced the efficacy of checkpoint inhibitors, but the addition of COS did not further improve this treatment.

Conclusion

COS improved the effect of checkpoint inhibitors in the LL2 model, and this combination appeared almost as potent as the radiation together with checkpoint inhibitors. COS may thus represent a possibility for a relatively non-toxic treatment of tumors, where the combination with radiation is not feasible. The observed effect of COS could be due to the blocking of YKL-40-receptor binding inhibiting cancer-promoting signaling pathways. Alternative biological functions of COS could also be involved.

EACR22-0962

Molecular and spatial profiling of immune exhaustion and immune checkpoint protein B7-H3 in renal cancer.

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Introduction

Immune checkpoint inhibitors in combination with targeted therapy is improving the response rates of advanced or metastatic renal cancer patients. However, many treated patients either do not respond or develop resistance to therapy, making novel immune checkpoint-based immunotherapies of potential clinical benefit for specific groups of patients.

Material and Methods

B7-H3 mRNA and protein expression has been evaluated in human renal cancer cell lines by quantitative PCR and immunoblot, and *in vitro* functional assays were performed upon B7-H3 knockdown. B7-H3 protein immunostaining was performed by immunohistochemistry on tissue microarray samples from two distinct renal cancer cohorts, and clinical correlations have been explored. Molecular analysis of selected whole tissue sections was performed using Nanostring Immune Exhaustion panel and NanoString's GeoMx Digital Spatial Profiler.

Results and Discussions

B7-H3 is highly expressed in renal cancer cells, and knocking down its expression resulted in lower cell viability and more sensitivity to currently approved targeted therapy (including VEGFR and mTOR inhibitors). In a first cohort of 129 renal cancer specimens, including 96 clear cell renal cell carcinomas (CCRCC), 17 chromophobe renal cell carcinomas (ChRCC), and 16 papillary renal cell carcinomas (PRCC), we explored the correlations between B7-H3 and pathological parameters. B7-H3 was highly expressed in clear cell renal cell carcinoma tumor cells, which correlated with poor outcome and overall survival. In a second cohort of 52 metastatic CCRCC patients, approximately half of primary tumors expressed B7-H3 at the primary site, whereas 1/3 of the metastatic tumors were positive for B7-H3 expression. B7-H3 expression in primary tumors correlated with tumor necrosis, sarcomatoid transformation, disease-free survival, and synchronous metastasis, while B7-H3 expression in metastasis was correlated to metastases to the lymph nodes. Molecular and spatial analysis revealed correlation of B7-H3 expression with increased T cell exhaustion, as well as with specific gene expression profiles and signaling pathways implicated in immune exhaustion and myeloid immune evasion.

Conclusion

Together, our findings suggest a pro-oncogenic and immune evasive role for B7-H3 in renal cancer, and highlight B7-H3 as an actionable novel immune checkpoint protein in combination to targeted therapy in advanced and metastatic renal cancer.

EACR22-1140

Investigation of multiple immune-checkpoints in the context of the disease for personalized therapy selection

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Introduction

The tumors immune evasive pathway programmed cell death-1 (PD-1)/programmed cell death ligand-1 (PD-L1) is the pharmaceutical target of immune checkpoint inhibitor (ICI) compounds. Cancer patient stratification is based on PD-L1 quantification in nowadays clinical practice. The evaluation of a single biomarker does not take into account the complexity of the tumor microenvironment (TME). The use of more sophisticated approach to resolve the different clinical cell phenotypes, their activity and interactions helps to better understand the correlation between tumor features, immune modulatory pathways, and clinical outcome in order to improve the predictivity to single immuno-therapy or their combination.

Material and Methods

Spatial biomarkers characterization on tumor tissues samples from resected NSCLC (adenocarcinoma and squamous cell carcinoma) tumors treated with adjuvant pembrolizumab therapy. The GeoMx® DSP RNA assay (Nanostring®) was used to analyze the spatial transcriptome signature of cells composing the TME and changes in the immune-related mRNAs expression were evaluated. Automatic segmentation was used to define the selected regions of interest (ROIs) using morphological markers for tumor or stroma. All the statistical analysis were performed through the GeoMx® DSP analysis suite.

Results and Discussions

The drug targets, PD1 (PDCD1) and PD-L1 (CD274), together with genes related with cytotoxic T lymphocytes (GZMB, CD8a) and activated T cells (CD44, CD27, TNFRS9) were highly expressed in the sample of the patient that responded to ICI therapy. The analysis also highlighted the increase of the inhibitory ligands CD86 and B7H3 (CD276). significantly overexpressed on the surface of the CD8a+ T cells in the sample of the patient that progressed on therapy. The increase of TIGIT, CTLA4 and TIM-3 in the microenvironment of this sample highlighted the importance of the spatial resistance of the mono-ICI therapy and offered the opportunity for combination therapies to better treat those patients.

Conclusion

Multiplexed spatial high-throughput analysis allowed to investigate at the molecular level several actors involved in immune modulation pathways responsible of drug resistance. These findings highlighted the relevance of investigating multiple biomarkers to obtain a comprehensive strategy for personalized immune therapy selection.

EACR22-1220

Detection of the Proximity of PD1 and PD-L1 in Human FFPE Tissues using a Newly developed Chromogenic *in situ* Proximity Ligation Assay

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Introduction

Blocking the interaction between the immune checkpoint proteins PD-L1/PD-1 in PD-L1-positive tumors by anti-PD-L1 antibodies is a major and challenging therapeutic avenue of cancer immunotherapy.

In this study the interaction between PD-L1 and PD-1 is visualized *in situ*. Molecular assays compatible with FFPE-tissues to accomplish this would significantly aid in the selection of patient responders to anti-PD-L1 or anti-PD-1 therapy and the deselection of non-responders.

To that end, we developed sensitive *in situ* Proximity Ligation assays enabling visualization of the proximity between PD-L1 and PD-1 as shown in this study in normal human tonsil and Hodgkin's lymphoma. **Material and Methods**

For the *in situ* Proximity Ligation assay reactions, tissue sections were processed for staining with anti- PD-L1/PD-1 Navenibodies. The assay development was based on the Naveni™ Proximity Ligation technology, where proximity detection was done by proximity ligation and rolling circle amplification with the NaveniBright kit. Visualization of the *in situ* Proximity Ligation Assay reactions was done by chromogenic detection of the staining products by bright field microscopy.

Results and Discussions

Distinct *in situ* Proximity Ligation assay signals were detected in both tissues, indicating PD-1 and PD-L1 proximity. These stainings were most frequent in the dark zone of the germinal centers and less so in the mantle zone. This study, shows *in situ* PD-1/PD-L1 proximity in tissues which has a very strong diagnostic potential in Immuno-oncology.

Conclusion

Proximity analysis of PD-L1/PD-1 could significantly increase the understanding of this central negative immune checkpoint.

The PD-L1/PD-1 *in situ* Proximity Ligation assays developed at Navinci Diagnostics have the potential to exceed the precision of anti-PD-L1 to assess the eligibility for immune checkpoint blockade therapy by anti-PD-L1 or anti-PD-1.

Characteristic of PD-L1 expression in high grade serous ovarian carcinoma

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Introduction

Ovarian cancer research is focused primarily on high-grade serous ovarian cancer (HGSOC), the most common ovarian cancer histology type. Patients with this highly aggressive form of ovarian cancer often harbor resistance to therapy and have an overall poor prognosis. HGSOC suppresses the host's immune responses by activating checkpoint mechanisms as programmed death protein 1 (PD-1) and its ligand, programmed death-ligand 1 (PD-L1). Such an interaction makes tumor cells resistant to effector lymphocytes. Lymphocyte infiltration is a significant factor that affects PD-L1 expression.

Material and Methods

This study included 91 patients with HGSOC. Immunohistochemical analysis was performed using the tissue microarray (TMA) method and correlated with a carcinoma segment and localization of lymphocytic infiltrate. The following score was used to describe the expression of PD-L1: negative (0), without positive cells or with a single positive cell (<1%); low (1+), with less than 10% positive cells; moderate (2+), with 10–50% positive cells; and strong (3+), with more than 50% positive cells. The lymphocytic infiltrate was analyzed at whole slides before TMA constructions in the central and peripheral tumor parts and its localization was correlated with PD-L1 expression on tumor cells.

Results and Discussions

A high level of PD-L1 expression was more frequently present in the invasive than in the central tumor parts ($p < 0.001$). There was no significant correlation between peritumoral lymphocytic infiltrate and PD-L1 expression regardless of tumor segment. Intratumoral lymphocytic infiltrate was more frequently present (84.3%) in the central tumor parts, with a high level of PD-L1 expression ($p = 0.003$). There is a lot of contradiction about the PD-L1 expression on HGSOC tumor cells concerning the type of lymphocytic infiltrate. Significant heterogeneity in HGSOC could be a factor for this various results. Therefore, a comprehensive analysis of immunological characteristics and the tumor microenvironment in HGSOC could be predictive factors for immune therapy response.

Conclusion

The most prominent PD-L1 expression was observed in the invasive tumor parts of HGSOC. Only the central parts of the HGSOC exhibited significant PD-L1 expression in association with considerable intratumoral lymphocytic infiltrate. Our study results support the hypothesis that the PD-L1 inhibitors could be an effective therapeutic option in aggressive ovarian carcinomas as HGSOC, especially with prominent intratumoral lymphocytic infiltrate.

Proposing a combinatorial treatment modality for Epithelial Ovarian Cancer by untangling the relations between p53 mutations, PD-L1 functions and Amphiregulin

abundance

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Introduction

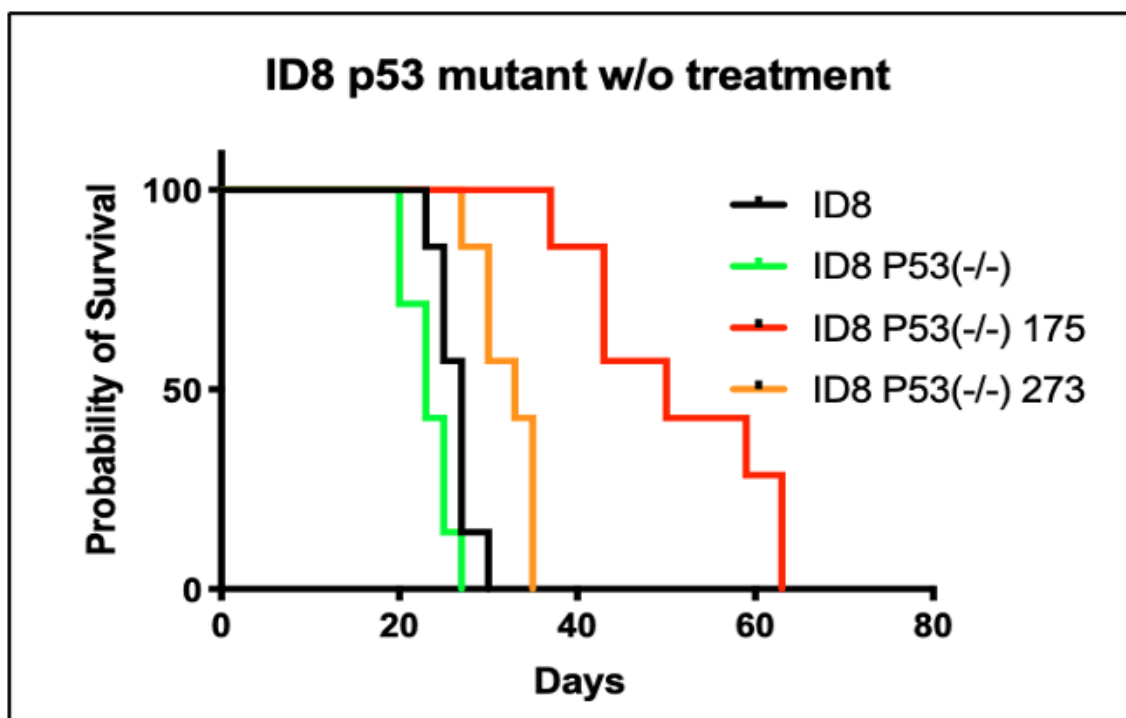
Reports indicate that elevated PD-L1 level induced by IFN- γ from cytotoxic T cells, acts as a prognostic marker by promoting an immunosuppressive environment for epithelial ovarian cancer (EOC). The captivating success of Immune Checkpoint Blockers (ICBs), identification of p53 mutational status dependent Amphiregulin (AREG) expression as a safe target and the established link between PD-L1 and EOC, have initiated studies to evaluate ICBs for EOC. Marginal benefit of ICBs on EOC shifted focus to evaluating combinatorial therapies. We aim to unravel the synergistic effect of a combination therapy considering proper patient stratification and biomarker validation for EOC.

Material and Methods

5 $\times$ 10⁶ cells of ID8, ID8 *p53*^{-/-}, ID8 *p53*^{-/-} R175H and ID8 *p53*^{-/-} R273H were injected into C57BL/6 female mice, followed by 6 weeks of injections with anti-AREG (AR37) and anti-PD-L1 (Avelumab) 400 μ g/ mouse, twice a week. Mice were monitored for abdominal circumference gain and median survival till they reached the end criteria as per IACUC.

Results and Discussions

A previous study from our lab showed abundance of VEGF and AREG in the abdominal fluid of EOC patients. This prompted us to study inhibitory effects of AREG in a syngeneic mouse model. Addressing the association of aberrant p53 with EOC, p53-depleted sublines including two “hotspot” missense mutations R172H (R175H in human) and R270H (R273H in human) were tested. The highly aggressive *p53*^{-/-} line showed loss of ability to secrete AREG, indicating the possibility that EOCs expressing AREG acquire addiction to AREG (M. Lindzen *et al.* Oncogene. 2021). Engraftment of these mutant p53 ID8 derivatives in mice indicated borderline softer aggressive phenotype compared to *p53*^{-/-} cells.



Survival curves for the ID8 p53 mutant ovarian cancer model implanted in C57BL/6 mice.

The median survival days (MSD) of corresponding xenografted animals were significantly prolonged upon individual treatment with Avelumab and AR37 especially in ID8 $p53^{-/-}$ R273H.

Cell type	MSD	MSD (Avelumab)	MSD (AR37)
ID8 $p53^{-/-}$ R273H	33	44	57

Recent studies on mutant p53 showed capacity to alter tumour milieu, support immune dysfunction, modulate toll-like receptor (TLR) signalling and disrupt innate immunity in favour of immune privilege and survival, which is in line with our findings.

Conclusion

Elucidating the varied response of EOC to monotherapies, we aim at evaluating an anti-AREG antibody in conjunction with PD-L1 therapy for synergistic effect. Our aim is to develop a safe and efficacious combinatorial therapy addressing the complexity of AREG and PD-L1 expression in EOC onset and progression.

EACR22-0571

TARGETING LYMPHOCYTE-ACTIVATION GENE 3 AS A NOVEL IMMUNE CHECKPOINT: A NEW PARADIGM FOR UVEAL MELANOMA PATIENT OUTCOME

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Introduction

Immune checkpoint blocking therapy has shown promising results in preclinical and clinical trials for a variety of malignant cancers. Immune checkpoint inhibitors (ICIs) are shown to have poorer response rates in uveal melanoma (UM) trials than in cutaneous melanoma trials. LAG-3 (Lymphocyte-Activation Gene 3) and its ligands may play an important role in regulating the tumor immune microenvironment of T cells and other immune cells that can be used to build monotherapies or add-on agents to current therapy. Therefore, this study was aimed to examine the expression of LAG-3 and CTLA-4 along with T-cell markers (CD4, CD8, CD3 and FOXP3) in UM.

Material and Methods

Prospective analysis of 54 uveal melanoma tumor specimens were taken for this study. Immunohistochemical analysis of ICI tumor targets (LAG-3 and CTLA-4), and T-cell markers (CD3, CD4, CD8, and FOXP3 proteins) were performed on formalin-fixed paraffin embedded specimens, and expression of ICI tumor targets were validated by western blotting. Transcriptional analysis was investigated by qRT-PCR on all the cases for LAG-3 and CTLA-4 gene. Statistical analysis was performed to correlate tumor targets with clinicopathological parameters and patient outcome.

Results and Discussions

Tumor-infiltrating lymphocytes (TILs) were found in 46% of UM tissues. Histopathological high-risk factors were found in 62% of UM cases. Expression of LAG3 and CTLA-4 was found in both tumor and lymphocytic environment of UM tissue. We found higher expression of LAG-3 with higher CD8+ TILs cells in association with epithelioid cell type, high mitotic figures, and large tumor diameter. Disease-free Survival rate were lower in patients with TILs with positive immunoreactivity and mRNA expression of LAG3 ($p < 0.0001$).

Conclusion

This study is the first analysis, to our knowledge, to molecularly and clinically characterize the landscape of LAG3 along with T-cell marker expression in uveal melanoma. Our findings suggest that LAG-3, a new immune checkpoint target, may interact with CTLA-4, and act as a potential therapeutic target in uveal melanoma patients. This could provide more data to improve combination cancer immunotherapy by targeting LAG3, PD1/PDL1, and CTLA4 at the same time.

EACR22-0887

Zebrafish patient-tumor derived xenograft models used for evaluation of check-point inhibitors and autologous T cell activation on primary tumor tissue

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Introduction

Immuno-oncology (IO) is a rapidly growing field and has reported success in the treatment of many cancer patients. The development of these therapies requires advanced and adapted humanized in vivo models, that are both time-consuming to generate and have limited translatability. There is a high need for new in vivo screening systems that are able to recapitulate the biological heterogeneity in the tumor as well as providing efficacy data with high translational value.

Material and Methods

In this study we evaluated efficacy of checkpoint inhibitors, as well as autologous T cells in a zebrafish patient-tumor derived xenograft (ZTX) model. For evaluation of efficacy of checkpoint inhibitors, primary melanoma cells from a metastatic tumor site, and paired tumor-infiltrating lymphocytes (TILs) were used. TILs and tumor cells were dyed and injected into zebrafish larvae either alone or in combination with an anti-PD1 antibody. Photos were taken the day of the injection and 3 days later to compare tumor growth and dissemination of metastatic cells. For evaluation of autologous T cells, 2 muscle-invasive bladder cancer samples with paired personalized tumor trained lymphocytes (pTTLs) and peripheral blood mononuclear cells (PBMCs) were used.

Results and Discussions

Intravenous (i.v.) injection of TILs after subcutaneous implantation of primary melanoma cells significantly reduced primary tumor growth 3 days after tumor implantation, and this effect was further increased in presence of an anti-PD1 antibody compared to isotype control. It was also observed that TILs administered i.v. can infiltrate the primary tumor site and this effect is significantly increased in presence of anti-PD1 treatment.

Compared to negative control, i.v. administration of pTTLs significantly reduced primary tumor size in both clinical samples. Meanwhile, i.v. injection of PBMCs did not affect primary tumor size of the clinical samples.

Conclusion

We here show that this new ZTX model is highly suitable for evaluating efficacy screening of T-cell cytotoxicity activating/boosting drugs within immuno-oncology, providing additional information on T-cell recruitment and cell interaction in the primary tumor and how this process is affected by immune checkpoint inhibitors. Furthermore, this new ZTX IO model provides a powerful tool for IO pre-clinical and clinical drug development, as well as a tool for companion diagnostic. We further conclude that the pTTLs used are highly efficient and show promising results for further clinical development.

EACR22-1051

Resistance to anti-PD-1/anti-PD-L1: GB1211 reverses galectin-3 induced blockade of pembrolizumab and atezolizumab binding to PD-1/PD-L1

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Introduction

Galectin-3 (Gal-3) is a β -galactoside binding lectin highly expressed in cancer¹ with high expression associated with poor response to the PD1 inhibitor pembrolizumab². It has also

been shown in *in vivo* mouse NSCLC models that Gal-3 inhibition enhances the performance of PDL1 checkpoint inhibitors^{3,4}. The mechanism by which Gal-3 impacts the effect of PD1/PDL1 checkpoint inhibitors is not fully understood. We investigated the hypothesis that aPD1/aPDL1 is induced by direct binding & blockade of Gal-3 to the PD1/PDL1 complex itself.

Material and Methods

SPR was used to investigate the effect of recombinant hGal-3 in combination with a Gal-3 inhibitor (GB1211) on the binding of hPDL1 to hPD1 in the absence & presence of either pembrolizumab or atezolizumab. SPR analysis was performed with a BIAcore™ (Cytiva, USA). hPD1 was immobilized onto a CM5 Series S sensorchip via amine coupling & proteins, antibodies or small molecules flowed over in various combinations. Observations made at the protein level were further confirmed in cell binding assays using flow cytometry measuring pembrolizumab & atezolizumab binding to cell lines over expressing high levels of either PD1 (Jurkat cells) or PDL1 (RAJI-hPDL1 cells), respectively.

Results and Discussions

Gal-3 potentiated the binding of PDL1 to PD1 with a 3-fold increase in its K_D observed & the efficiency of pembrolizumab & atezolizumab binding to & blocking the PD1/PDL1 axis was reduced. A 6-fold reduction in K_D of pembrolizumab for the PD1/P-L1 complex was observed in the presence of Gal-3. In addition, atezolizumab PDL1 blockade was reversed in the presence of Gal-3 & dramatically improved by GB1211. Similarly, in cell systems expressing high levels of PD1 or PDL1, Gal-3 reduced the binding of pembrolizumab & atezolizumab to their respective targets on cell surfaces that was also reversed by GB1211. Gal-3 was shown to vastly reduce the binding of the checkpoint inhibitors pembrolizumab & atezolizumab, by potentiating the interaction between PD1 & PDL1. GB1211 restored the binding of the aPD1/aPDL1 therapeutics & may thus reduce tumor resistance to these agents.

Conclusion

These findings agree with *in vivo* & clinical data showing an association between Gal-3 expression & lack of efficacy of PD1/PDL1 targeted checkpoint inhibitors.

¹Li *et al* *Biomedicines* 2021

²Capalbo *et al* *Int J Mol Sci* 2019

³Vuong *et al* *Cancer Res* 2019

⁴Zhang *et al* *FEBS Open Bio* 2020

EACR22-1201

Development of a Chromogenic *in situ* Proximity Ligation Assay for Detection of PD1/SHP2 Proximity in Human FFPE Tissues

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Introduction

Curbing PD-L1/PD-1 mediated immunosuppression through targeting the interaction between PD-1 and the intracellular tyrosine phosphatase SHP2 (PTPN11) remains an appealing clinical strategy to reinvigorate lymphocyte-mediated tumoricidal activity.

The cis-interaction between PD-L1 and SHP2 in T-cells and B-cells depends on tyrosine phosphorylation and has not been demonstrated in tissues. To that end, *in situ* proximity ligation assays were developed at Navinci Diagnostics to visualize PD-1/SHP2 interactions in normal human tonsil and Hodgkin's lymphoma.

Material and Methods

For in situ Proximity Ligation assay, development, human tissues were stained with pairs of monoclonal anti PD-1 and anti SHP2 antibodies (mouse/rabbit) followed by proximity ligation and rolling circle amplification using NaveniBright (anti Mouse/anti Rabbit). Visualization of the PD-1/SHP2 in situ Proximity Ligation Assay reactions was accomplished by chromogenic detection that was assessed by bright field microscopy.

Results and Discussions

This is the first study that demonstrates PD-1/SHP2 proximity in tissues by in situ Proximity Ligation Assay. The PD-1/SHP2 interactions occurred in lymphocytes of the germinal centers and less frequent in the mantle zone of the tissues.

The PD-1/SHP2 in situ Proximity Ligation assay has a vast diagnostic potential for to prediction of clinical responsiveness to anti-PD-1 therapy. A cell-based PD-1/SHP2 in situ Proximity Ligation assay could prove valuable in discovering and developing molecular antagonists disrupting the PD-1/SHP2 interaction.

Conclusion

The PD-1/SHP2 in situ Proximity Ligation assay developed at Navinci Diagnostics for the first time demonstrates the interaction between PD-1 and SHP2 in human tissues. This could have important diagnostic impact in cancer immunotherapy.

EACR22-0895

TOP ABSTRACT

Tumor-directed, myeloid cell-based cytokine gene delivery empowers CAR T cells reshaping the immunosuppressive glioblastoma microenvironment to thwart tumor growth

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Introduction

The immunosuppressive tumor microenvironment (TME) of glioblastoma (GBM) represents a major hurdle for effective chimeric antigen receptor (CAR) T-cell therapies. While specific cytokines can counteract immune suppression, their systemic administration entails risk of toxicity and counter-regulatory responses. We showed that a TIE2-expressing myeloid population (TEM) can deliver interferon (IFN)- α in the TME through a hematopoietic stem cell (HSC)-based gene therapy (GT) to reprogram it toward immunoactivation. Here we investigate TEM-based cytokine delivery as rescue strategy for CAR T cells in the non-permissive GBM TME to safely synergize in thwarting tumor growth.

Material and Methods

We identified the clinically relevant B7H3 antigen on a new murine GBM model (mGB2), closely mimicking the human disease. We generated B7H3-redirected and control mouse CAR T cells at high efficiency by optimizing a lentiviral-based protocol and injected them via systemic or intratumor route in syngeneic mice, previously transplanted with engineered HSCs for TEM-mediated IFN- α or mock delivery, and orthotopically challenged with mGB2. Tumor growth was assessed by magnetic resonance imaging; quantitative, immunophenotypic and transcriptomic analyses were performed on peripheral blood and tumor infiltrate; efficacy and tolerability of the treatments were monitored in survival studies.

To prove portability of TEM GT, we delivered interleukin (IL)-12, whose use is limited by systemic toxicities, in the same setting.

Results and Discussions

While lower peripheral persistence of CAR_{B7H3} T cells was observed compared to control CAR T cells, the former were enriched at tumor site, suggesting a cognate antigen-driven homing/local expansion. IFN- α reprogrammed the TME leading to reduced exhaustion and increased activation of both circulating and tumor-infiltrating CAR_{B7H3} T cells. While IFN- α GT limited tumor growth *per se*, CAR_{B7H3} T cells alone had no effects. Nonetheless, marked inhibition of tumor growth and prolonged survival were achieved in the combination group, in absence of any overt toxicity. This therapeutic effect was potentiated by intratumor injection of CAR T cells. Same safety and efficacy were observed by IL-12 delivery through TEMs in combination with CAR_{B7H3} T cells.

Conclusion

Cytokine-driven TME reprogramming overcame some key limitations of CAR T cells targeting a clinically relevant antigen in a GBM immunocompetent model, rescuing T cell function and synergizing in tumor inhibition while preserving full tolerability.

EACR22-0780

Newly Established CAR-T Target Antigen Luciferase Reporter Cell Lines Facilitate CAR-T Development

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Introduction

Adoptive T cell therapy is an immunotherapy in which T cells are genetically modified to fight tumors. Chimeric antigen receptor (CAR)-T cells have displayed remarkable efficacy in treating malignant cancers, particularly liquid tumors. CAR-T cells have been harnessed, using the patients' immune system to recognize specific tumor-associated antigens and redirect the engineered T cells to more specifically target tumor cells, with efforts to develop new CAR structures to increase the scope of targeted cancers and anti-tumor efficacy. Evaluating the biofunction of CAR-T cells in vitro often involves a series of labor-intensive co-culture experiments and immunoassays. Reproducibility also remains a challenge during the validation of new CAR-T cells due to donor-to-donor variation and other factors.

Material and Methods

In this study, we present a panel of luciferase reporter tumor cell lines that can be utilized to examine the function of CAR-T cells. The panel of selected human tumor cell lines naturally express high levels of CAR-T target antigens on cell surface, such as CD19, CD20 and Her2. After introduction of a Lenti-LUC2 luciferase reporter into the parental cell lines, single cell cloning was performed to isolate stable clones with high luciferase expression. To verify the performance of the target luciferase reporter cell lines, we used commercially available CAR-T cells expressing CD19, CD20, and HER2 and empty vector-transduced T cells as controls in co-culture experiments. The cytotoxicity of the CAR-T cells against target tumor cells was measured using both a luciferase assay and the xCELLigence potency assay.

Results and Discussions

Our results demonstrate that the luciferase reporter system is a relatively simple, robust, and highly sensitive means to measure biological processes. The luciferase reporter cell lines provide an advantage in measuring target cell killing without having to use radioactive ⁵¹Cr

release assay or pre-labeling the cells in CAR-T functional evaluation. In addition, these reporter cell lines were characterized and authenticated using cell morphology, growth kinetics, and STR analysis. The expression stability of both the target antigen and luciferase was verified by comparing the low-passage and the high-passage reporter cells.

Conclusion

In summary, the well-characterized CAR-T target antigen luciferase reporter cell lines from ATCC provide excellent tools for studying CAR-T biofunction and validating new CAR-T agents for cancer immunotherapy.

EACR22-1052

Enhancing the anti-tumor activity of engineered T-cells by manipulating TCR structure and through the use of costimulatory chimeric switch receptors (CSR)

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Introduction

Tumors can employ different mechanisms to evade immune surveillance and function. Overexpression of co-inhibitory ligands that bind to checkpoint molecules on the surface of T-cells can greatly impair the function of latter. Additionally, the absence of positive co-stimulation at the tumor site can further dampen T-cell response.

Material and Methods

As T-cell genetic engineering has become clinically-relevant in the recent years, we devised herein a strategy aimed at enhancing T-cell anti-tumor function by diverting T-cell coinhibitory signals into positive ones using a chimeric costimulatory switch receptor (CSR) composed of exodomains derived from checkpoint receptors (PD1, TIGIT, S9) fused to the signaling domain of CD28 or 4-1BB. In parallel, we also develop protein engineering strategies to improve exogenous TCR expression.

Results and Discussions

We designed optimized CSR with different structural components and co-transduced them along with tumor-specific (enhanced) TCR or CAR into human T-cells. CSR-equipped T-cells exhibited enhanced cytokine secretion and upregulation of activation markers upon co-culture with tumor cells. CSRs enhancing capability was also demonstrated in an original *in vitro* model of T-cell of hypofunction induction upon repetitive antigen exposure. Finally, we tested the function of these molecules in the context of a xenograft model of established human melanoma tumors and showed that CSR-engineered human T-cells demonstrated superior anti-tumor function.

Conclusion

Overall, we propose that CSRs and targeted TCR mutations can substantially enhance T-cell function and thus contribute to the improvement of engineered T cell-based immunotherapy for cancer patients.

EACR22-1272

A new EGFRvIII-T cell bispecific antibody for glioblastoma treatment

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Introduction

GBM is the most common brain cancer of the adult and a clear medical unmet need. Immunotherapy has shown good results in several solid tumors. However, GBM is a non-inflamed tumor with low level of T cell infiltration that barely responds to immunotherapy. Novel agents to re-direct cytotoxic T cells to the tumor may hold promise to improve GBM therapies.

Material and Methods

T cell bispecific antibodies (TCBs) are engineered molecules that bind both the T cell receptor and tumor-specific antigens. EGFRvIII is an EGFR mutation that generates a deletion of exons 2-7 resulting in a constitutively active receptor that promotes cell proliferation, angiogenesis and invasion. EGFRvIII is expressed on the surface of tumor cells and is not expressed in normal tissues making EGFRvIII an ideal neoantigen target for TCBs. We designed and developed a novel 2+1 EGFRvIII-TCB with optimal pharmacological characteristics and potent anti-tumor activity.

Results and Discussions

EGFRvIII-TCB showed specificity for EGFRvIII and promoted tumor cell killing as well as T cell activation and cytokine secretion only in patient-derived models expressing EGFRvIII. Moreover, EGFRvIII-TCB promoted T cell recruitment into intracranial tumors. EGFRvIII-TCB induced tumor regression in GBM animal models, including humanized orthotopic GBM patient-derived xenograft (PDX) models.

Conclusion

Our results suggest that EGFRvIII-TCB may be active in EGFRvIII expressing GBMs, and support further evaluation of this molecule in a first-in human trial (NCT05187624).

EACR22-1131

CD70-directed, IL-15-armored chimeric antigen receptor natural killer cells as promising off-the-shelf cellular immunotherapy to eradicate both tumor cells and cancer-associated fibroblasts

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Introduction

Numerous types of hematological and solid tumors hijack the expression of the immune checkpoint molecule CD70 to facilitate tumor progression and immune evasion. We were the first to unveil that aberrant CD70 expression on cancer-associated fibroblasts (CAFs) in metastatic colorectal cancer provides them with tumor-promoting properties. The limited expression on healthy tissues and growing evidence on its tumor-enhancing effects, make CD70 an attractive target for cancer immunotherapy. Chimeric antigen receptor (CAR) T cells have revolutionized treatment of hematological malignancies but face problems in

terms of safety and production costs. Utilizing natural killer cells (NK) cells as effector cells for CAR-immunotherapy could overcome these limitations. Here, we have generated off-the-shelf CAR NK cells capable of eradicating both CD70⁺ tumor cells and CD70⁺ CAFs.

Material and Methods

The human NK-92 cell line was used as inexhaustible, clinically approved, and cost-effective source of NK cells. Messenger RNA electroporation was used as safe transfection method that limits off-target effects by inducing transient CAR expression. CAR expression and cytotoxic capacity against an array of CD70⁺ cell lines were determined using flow cytometry.

Results and Discussions

Developing CD70-directed CAR NK cells using the NK-92 cell line and mRNA electroporation showed consistent transfection efficiency with high CAR expression (65% positive cells) and competent antigen-specific killing (60% after 4 hours) of the CD70⁺ Raji lymphoma cell line in a co-culture setting. Blocking the CAR's antigen-recognition domain with a neutralizing antibody further validated CAR-mediated killing. Interestingly, arming the CAR NK-92 cells with the highly potent immunostimulatory cytokine IL-15 substantially increased both CAR expression (up to 80% positive cells and 3.4 fold change in mean fluorescent intensity) and cytotoxic capacity (up to 85% after 4 hours). Moreover, IL-15 co-stimulation was required to eliminate harder-to-kill CD70⁺ pancreatic and colorectal cancer solid tumor and CAF cell lines.

Conclusion

We demonstrated that IL-15 armored, CD70-directed CAR NK-92 cells hold great potential in targeting both CD70⁺ tumor cells and CD70⁺ CAFs. Currently we are evaluating their efficacy in vivo in immunodeficient mice. Eventually, these data might pave the way towards clinical application of our allogeneic off-the-shelf CAR NK cell therapy.

EACR22-0909

Hi-Plex RNA Spatial Phenotyping of Human Tissues at Single-Cell Resolution

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Introduction

Spatial Biology has the potential to revolutionize our understanding of disease mechanisms and how to develop new therapies. To date, there has been a trade-off between plex-level, resolution, and sample integrity when mapping the contents of a specimen while retaining tissue context. With next-generation sequencing tools, tissue samples are typically dissociated to enable either bulk or single-cell assessment of gene signatures. This information, however, is void of spatial context and is dependent on equivalent dissociation propensities of different cell types for an unbiased view. While there are technologies that enable spatial profiling, but they typically lack single-cell resolution or support low-plex analysis. Further, different markers benefit from measuring the protein product or the RNA product depending on their expression level, localization, and whether quality antibody products exist.

Material and Methods

The Phenocycler Fusion platform was used to collect data in support of this abstract

Results and Discussions

Here, we present the new Hi-Plex RNA methodology for analysis of dozens of RNA targets on human tissue samples with single-cell resolution using CODEX. This technology uses a targeted, amplification-based chemistry to detect markers through cycles of hybridization, imaging, and dehybridization. In this manner, spatial hi-plex RNA data is generated while retaining tissue integrity. Cell types are identified through the specific panels and associated cellular neighborhoods can be detected based on these annotations.

Conclusion

This capability complements the existing single-cell, whole-slide high-plex spatial protein methodology that is currently enabled by the CODEX instrument. The combination of this new Hi-plex RNA spatial capability with the existing high-plex protein spatial capability makes the CODEX instrument a first-of-its-kind instrument to enable multiomic analysis of samples at a single-cell resolution.

EACR22-0848

Polymorphisms in Transcription Factor Binding Sites and Enhancers as Pancreatic Ductal Adenocarcinoma Risk Factors

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) remains among the most lethal cancers worldwide, with poor early detection rates and poor survival rates. Identification of novel risk factors for PDAC could be instrumental in risk stratification and the implementation of prevention strategies. Genome-wide association studies (GWAS) have been a powerful tool for detecting single nucleotide polymorphism (SNPs) associated with complex traits, but the strict significance threshold commonly used in GWAS makes it likely that many true risk loci will be missed. Functional annotation of GWAS polymorphisms is a proven strategy to identify additional loci. We aimed to investigate SNPs in regulatory regions (transcription factor binding sites (TFBS) and enhancers) that might change expression profiles of multiple genes they act upon, and thereby increase or decrease PDAC risk.

Material and Methods

We analyzed a total of 12,636 PDAC cases and 43,443 controls from the PanScan and PanC4 GWAS (discovery population), the PANDoRA consortium (replication population) and the Asian GWAS summary statistics (meta-analysis population). Seven SNPs showing a significant association in the discovery population were genotyped in the replication population and then meta-analyzed using all populations.

Results and Discussions

We identified three associations (rs2472632, rs17358295, rs2232079) that approached genome-wide statistical significance in the overall meta-analysis. The rs2472632(A) SNP (OR=1.10, 95%CI [1.06,1.13], $P=5.5 \times 10^{-8}$) is located in an enhancer region near the *Leucine Rich Repeat Containing G Protein-Coupled Receptor 4 (LGR4)* gene, that functions as an activator of the Wnt signaling pathway. The rs17358295(G) allele was associated with an increased risk of developing PDAC (OR=1.16, 95%CI [1.10,1.22], $P=6.1 \times 10^{-7}$). This SNP is situated in an enhancer region near the *ETS Homologous Factor (EHF)* gene, whose expression is known to be increased in pancreatic cancer. Additionally, the rs2232079(T) SNP (OR=0.88, 95%CI [0.83,0.93], $P=6.4 \times 10^{-6}$) is located in a TFBS, which is predicted to be associated with lower expression of the *FERM Domain*

Containing *Kindlin 1 (FERMT1)* gene, whose higher expression is known to be associated with growth, proliferation and metastasis in pancreatic cancers.

Conclusion

Our results provide new insights into genetic risk for PDAC through the analysis of SNPs in regulatory regions and demonstrate the usefulness of functional prioritization to identify polymorphisms associated with PDAC risk.

EACR22-0203

Pre-diagnostic selenium status, selenoprotein gene variants and association with breast cancer risk in a European cohort study

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Introduction

The micronutrient selenium (Se) may help prevent breast cancer (BC) development, due to the role of several selenoproteins in combatting the oxidative stress-induced damage implicated in breast carcinogenesis. Owing to conflicting reports from limited observational studies, we investigated whether prediagnostic Se status and/or variants in the selenoprotein genes are associated with BC risk in the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort.

Material and Methods

Se status was robustly ascertained by measures of the major plasma Se proteins selenoprotein P (SELENOP) and glutathione peroxidase 3 (GPX3), and Se in matched BC case-control pairs (2,204 for SELENOP; 1,785 for GPX3 and Se) nested within the European Prospective Investigation into Cancer and Nutrition (EPIC). Single nucleotide polymorphisms (SNPs, n=452) in 55 selenoprotein and Se metabolic pathway genes and an additional 18 variants previously associated with Se concentrations were extracted from existing genotyping data within EPIC for 1,564 case-control pairs. Multivariable-adjusted logistic regression models were used to calculate the odds ratios (ORs) and 95% confidence intervals (CIs) of the association between Se status markers, SNP variants and BC risk.

Results and Discussions

Overall, there were no consistently statistically significant associations of any of the three assessed Se status biomarkers with BC risk. However, higher GPX3 activity was associated with a lower risk of premenopausal BC (4th versus the lowest quartile, OR = 0.54, 95% CI: 0.30-0.98, $P_{trend}=0.013$). Seventeen SNPs in fourteen Se pathway genes and one GWAS Se status-associated SNP (rs234709) were associated with BC risk, with only rs1004243 in the *SELENOM* selenoprotein gene and two SNPs in the related antioxidant *TXN2* gene (rs4821494 and rs5750261) associated with respective lower and higher risks at P -values ≤ 0.01 . However, none of these genetic variant findings retained significance after multiple testing correction. Fourteen SNPs in twelve Se pathway genes (at a significance threshold of $P \leq 0.01$) were found to modify the association between Se status and BC risk.

Conclusion

Higher Se status does not appear to be associated with BC risk, although GPX3 activity may be associated with premenopausal BC, and SNPs in the Se pathway alone or in combination with suboptimal Se status may influence BC risk (both lower and higher risk associations observed).

EACR22-0416

Lack of association of Growth Regulation by Oestrogen in Breast Cancer 1 (GREB-1) rs10929757 polymorphism with bone mineral density and volumetric bone mineral density in breast cancer survivors.

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Introduction

The GREB1 gene is a gene often studied to assess estrogen-stimulated tumor progression, but its function outside the cancer setting is unclear. In the present preliminary report we study the association GREB1 rs10929757 with bone density in healthy postmenopausal women, and in those who have survived breast cancer.

Material and Methods

A cross-sectional study on 49 Spanish women survivors of breast cancer and a control group consisted of 63 healthy women. BMD was obtained by DXA. Volumetric BMD (vBMD) was determined by pQCT. Participants were screened for rs10929757 variant of the GREB1. The Hardy-Weinberg equilibrium (HWE) and comparisons of genotype frequencies were estimated. Associations of rs10929757 with BMD and vBMD in the subgroups were investigated by ANCOVA adjusted for age and BMI. Adjusted odds ratio (OR) with 95% confidence intervals (CI) for low BMD presence (T-score <-1) were calculated.

Results and Discussions

Control and case group conformed to HWE ($P > 0.05$). Genotype distribution was AA 11 (17.5%) AC, 33 (52.4%) and CC 19 (30.2%) in the control group while AA 12 (24.5%) AC, 30 (61.2%) and CC 7 (14.3%) in the breast cancer group. The genotype frequencies of rs10929757 in the subgroups were not significantly different ($P=0.133$). No statistically significant differences were observed in BMD at the lumbar spine as a function of the presence of a history of breast cancer ($P=0.102$), nor as a result of the rs10929757 genotype ($P=0.108$) or the interaction of both factors ($P=0.700$). Nor were statistically significant associations observed at the level of BMD at the hip either for genotype ($P=0.278$), breast cancer ($P=0.320$) or both factors ($P=0.241$). After analyzing the pQCT results, there were no significant associations with total vBMD as a function of genotype ($P=0.926$), breast cancer ($P=0.101$) or both factors ($P=0.615$). Similar results were observed for trabecular vBMD (genotype $P=0.869$, breast cancer $P=0.302$ and both factors $P=0.393$) and cortical vBMD (genotype $P=0.933$, breast cancer $P=0.144$ and both $P=0.693$). The risk of having a low BMD was unrelated to the rs10929757 genotype ($P>0.05$).

Conclusion

Whereas our preliminary findings suggest a lack of involvement of the GREB1 rs10929757 in the regulation of bone density on those women, the small sample size prevents us from definitively discarding a possible association due to the risk of type II error. We recommend that future studies should be developed to increase the sample size and evaluate the possible influence on the temporal trends of BMD.

Colorectal cancer risk associated variation at 11q23.1 is correlated with tuft cell abundance and transcriptional network expression in silico

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Introduction

Colorectal cancer (CRC) is characterised by heritable risk that is not well understood. Genetic variation at 11q23.1 is associated with heritable colorectal cancer (CRC) risk, demonstrating eQTL effects on 3 cis- (*C11orf53*, *COLCA1*, *COLCA2*) and 23 trans-eQTL targets in bulk colonic tissue. We sought to determine the relationship between 11q23.1 eQTL target expression and test for potential cell-specificity.

Material and Methods

scRNAseq from 32,270 healthy colonic epithelial cells was aggregated and subject to weighted gene co-expression network analysis (WGCNA). Following unsupervised clustering, marker and gene set enrichment analysis was performed to identify the cell-specific expression of eQTL targets, replicated in a second dataset of 11,651 cells. Samples were then clustered by the relative expression of 11q23.1 eQTLs. Intra-cluster WGCNA was performed to identify cluster-specific transcriptional networks that were correlated with cis-eQTL expression and sample grouping. The preservation of intra-cluster gene modules was subsequently analysed across clusters. Finally, linear modelling and differential abundance testing were performed to agnostically assess gene association with the abundance of clusters.

Results and Discussions

Across all clusters, one gene module included 17 trans-eQTL targets and was correlated with *C11orf53* expression only. Within clusters, the expression of 19 trans-eQTL targets was greatest and most variable in cluster 11. This cluster transcriptionally resembled colonic tuft cells - a rare, epithelial cell-type. 14 trans-eQTL targets were found to demarcate this cluster, 11 of which were corroborated in a second dataset – the first evidence for tuft cell marker status of several eQTL targets. Intra-cluster 11 WGCNA and module preservation analysis then identified twelve 11q23.1 trans-eQTL targets to comprise a *C11orf53*-correlated network that is cluster 11 specific. Abundance analysis showed 11q23.1 trans-eQTL target expression is predictive of specific and dramatic changes in the abundance of cluster 11. This is the first evidence of CRC-risk associated eQTL expression being associated with cell-specific transcriptional dynamics and abundance changes.

Conclusion

Our findings suggest 11q23.1 trans-eQTL targets comprise a *C11orf53*-related network that is likely tuft cell-specific. These results also potentiate CRC-risk associated reduced expression of these genes correlates with reduced tuft cell abundance *in silico*.

Acceptability of the self-sampling HPV test in Japan: A secondary analysis of the ACCESS trial

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Introduction

In Japan, HPV vaccination has been suspended, and cervical cancer screening rate is notably low. As a result, morbidity and mortality due to cervical cancer has recently increased. The self-sampling HPV test has been shown to improve screening uptake and has already been implemented as an option for screening in several countries; however, it is not yet recommended in Japan. We initiated the Accelerating Cervical Cancer Elimination by Self-Sampling test (ACCESS) trial to evaluate the effectiveness of this test. This study was conducted as a part of the ACCESS trial with the aim to assess the acceptability of the test.

Material and Methods

A questionnaire survey was conducted. Participants in this trial comprised women residing in Ichihara City, Japan, who had not undergone cervical cancer screening for at least 3 years. A questionnaire and a consent form was sent to participants who ordered the self-sampling HPV test along with the self-sampling kit (Evalyn® Brush, Rovers Medical Devices B.V., The Netherlands). After sampling, participants were required to return the sample along with the completed consent form and questionnaire. For statistical analysis, McNemar's test, chi-square test, and logistic regression analysis were performed as appropriate.

Results and Discussions

Of the 7,340 participants in the self-sampling arm, 1,196 (16.3 %) underwent the self-sampling HPV test and 1,192 (99.7 %) returned the questionnaire. The mean (standard deviation) age of the participants was 44.1 (8.2) years. Acceptability of the test was found to be favorable. Most of the participants agreed with the test's positive features i.e., ease of use, convenience, and clear instructions, and disapproved the negative features i.e., painful, uncomfortable, and embarrassing. In contrast, only 21.2 % of participants were confident in their sample collection. The willingness to undergo future screening with self-collected samples was significantly higher than that with doctor-collected samples (89.3 % vs. 44.5 %; $p < 0.001$).

Conclusion

The self-sampling HPV test was confirmed to be highly acceptable amongst the participants; however, concern regarding the accuracy in self-sampling was noted. The screening with self-collected samples was preferable over doctor-collected samples. The self-sampling HPV test may therefore increase screening uptake.

Prevention and Early Detection

EACR22-0540

A new non-invasive concept for detecting endometrial cancer in women over 50 years old

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Introduction

In USA, during the past 20 years, rising by 8% since 2008 with 90% of cases occurring in women older than 50 years. In the clinical practice, when a woman has postmenopausal bleeding (PMB) and her endometrial thickness is $\geq 5\text{mm}$, she should then undergo clinical examination and hysteroscopy. Pain and repeated hysteroscopy often cause fear in women. Cytological high-risk DNA methylation test has a promising application prospect in EC diagnosis.

Material and Methods

88 women over 50 years old who underwent endometrial biopsy in the hysteroscopic room were prospectively selected. If the biopsy result is positive, follow-up treatment shall be carried out. The biopsy or surgical pathology were as the final pathology. Cervical exfoliated cells were collected and stored in ThinPrep solution for methylation test, and office endometrial biopsy was performed in the study. The data of clinical syndrome and tests including bleeding, endometrial thickness (ET) $\geq 5\text{mm}$, CA125 test, BMI measure were collected. The analysis of methylation level of CDO1 and CELF4 were determined by using CisEndo methylation real-time system (CISPOLY Co., China). Positive results were defined in $\Delta\text{Ct-CDO1} < 8.4$ or $\Delta\text{Ct-CELF4} < 8.8$.

Results and Discussions

54 of non-EC group (mean 59 years) and 34 of EC group (mean 58 years) were in the study. The positive rate of all tests in EC/non-EC were menopause [34(100%)/ 42(77.8%)], bleeding [30(88.2%)/ 23(42.6%)], ET $\geq 5\text{mm}$ [26(76.5%)/ 24(44.4%)], CA125 [13(38.2%)/ 11(20.4%)], BMI ≥ 25 [18(52.9%)/ 22(40.7%)], methylated genes [29(85.3%)/ 8(14.8%)], respectively. The BPM was the highest rate in EC group but still with higher rate in non-EC group (42.6%). The combined CDO1 and CELF4 tests were the 2nd highest positive rate in the EC group and with lowest positive rate in the non-EC group. 4 of women with the postmenopausal non-bleeding syndrome were 100% positive results in the methylated gene tests. The data show that methylated gene tests are the non-invasive detection with higher positive rate of EC and lowest rate in non-EC groups. Three EC women with PMB and ET $\leq 5\text{mm}$ were missed following the practice, but 3 of them were positive in the methylation results.

Conclusion

The methylation test of cytological samples may be a new non-invasive method for women over 50 years old and this new discovery need more data for future validation.

EACR22-1090

An in silico approach to prostate cancer biomarkers

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Introduction

Prostate cancer (PCa) is the second most frequently diagnosed neoplasia globally and the sixth cause of cancer mortality. Androgen deprivation therapy has been the gold standard of care for advanced PCa. Initially, this treatment benefits, but eventually, the tumor recurs as castration-resistant prostate cancer, which has restricted treatment options and poor survival benefits. In previous studies, our group reported the role of *Brachyury* (*TBXT*), a T-box transcription factor, which plays a critical role during embryonic development. Thus, a better understanding of these driving resistance mechanisms and their master regulators is crucial to developing more effective therapeutic strategies and potentially the discovery of prognostic and therapeutic biomarkers for prostate cancer. Therefore, we aim to unveil the significantly deregulated genes in prostate cancer using an *in silico* approach, with the prospect of designing a biomarker panel for early diagnostic/prognostic and potentially therapeutic prediction in PCa patients using liquid biopsies.

Material and Methods

Cancer data available *in user-friendly* portals and online databases were used to study gene expression association in prostate cancer. Most of the tools were built based on The Cancer Genome Atlas (TCGA) dataset.

Results and Discussions

A set of 22 genes were identified as potentially diagnostic/prognostic biomarkers since its expression was found significantly modified in prostate cancer across all databases. Moreover, *Brachyury* was found significantly overexpressed in carcinomas and metastasis in different CBioPortal datasets (Varambally, Grasso, Taylor, Yu, and Latulippe). Regarding STRING analysis, it was observed that *Brachyury* directly interacts with *FOXA1/2*, *NKX2-5*, and *WNT3A* genes, but not with *PDE10A* and *NKX2-6*. Additionally, it was observed that the probability of *Brachyury* and AR proteins interacting is 59,8 %, which supports the previous findings reported by the group. Structural analysis of both proteins also corroborates this observation.

Conclusion

The identification of the 22 genes with modified expression in prostate cancer together with the identification of potential binding proteins of *Brachyury* contributes to a step forward in the establishment of new prostate cancer biomarkers.

EACR22-1163

HER2 Overexpression Induces Breast Tumourigenesis By Altering The Energy Metabolism And Causing Oxidative Stress

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Introduction

Breast cancer is the most common cancer type in the world. HER2 overexpression/amplification is associated with 20% of invasive breast cancer cases, whereas almost 80% of the pre-malignant stage breast cancers known as ductal carcinoma in situ (DCIS) overexpress HER2. Although HER2-targeted therapies can significantly improve the prognosis for patients with HER2-positive invasive breast cancer, high levels of therapy resistance and tumour relapse are still observed in clinics. Biological mechanisms of HER2 function in breast tumour cells have been extensively studied, however our knowledge on HER2-driven tumorigenesis is still far from complete, in particular for its role in pre-malignant stages of tumorigenesis.

Material and Methods

In this project, we investigate the *HER2* overexpression driven cellular events at the earliest stages of breast tumorigenesis using *in vitro*, and *in vivo* models. Proteomics and metabolomics analyses of mammary ducts isolated from the mouse model of HER2+ breast cancer (MMTV-Neu) at a stage prior to the development of histologically obvious tumours allowed us to identify pre-malignant stage alterations in the mammary epithelial cells *in vivo*. We have also generated a HER2-overexpressing mouse mammary epithelial cell line as an *in vitro* model to study the effects of HER2 overexpression on metabolism, and its consequent effects. The results obtained from the *in vitro* model have then been verified by immunostaining of sections obtained from the mammary glands of MMTV-Neu mice.

Results and Discussions

Our results have shown that *HER2/Neu* overexpression upregulates glucose and lipid metabolism in mammary epithelial ducts before any apparent tumour formation *in vivo*, whereas ectopic HER2 overexpression in mammary epithelial cells *in vitro* elevates reactive oxygen species (ROS) levels. Consequently, the increase in metabolic activities and ROS levels result in the induction of oxidative stress and DNA damage markers both *in vitro* and *in vivo*.

Conclusion

Our findings indicate that HER2 overexpression in normal mammary glands may lead to a genotoxic tissue microenvironment, which consequently results in the accumulation of further mutations to support tumour evolution during the earliest stages of breast tumorigenesis. A further understanding of the role of HER2-induced tumorigenesis at this Stage-0 breast cancer will allow us to develop more efficient therapeutic and novel preventive strategies against breast cancer.

EACR22-0287

Identification of microbiome markers for colorectal cancer detection

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Introduction

Colorectal cancer (CRC) is the third leading cause of morbidity of all malignant tumours worldwide. It is the most frequently diagnosed cancer in Spain, followed by prostate, breast and lung cancer. There is evidence that the development of colorectal cancer is driven by the interaction between genetic and environmental factors. In particular, the gut microbiome has been described as a critical environmental factor contributing to CRC carcinogenesis and progression, potentially through proinflammatory response, production of microbial

metabolites and interference with energy balance in cancer cells. In addition, the diversity and function of the host bacterial community may be altered in response to changes in diet or physiology. Therefore, the microbiome is now considered a key player in the reflection of the tumour environment, allowing it to be both a marker and a promising non-invasive target for the development of potential treatments that are more specific and less harmful to the host.

Material and Methods

The aim of this study is to develop robust microbiome markers to enable better diagnosis and therapeutic treatment of CRC. Specifically, we want to test the influence of adenoma-associated microorganisms from the microbiota, identified in previous studies, on colorectal tumourigenesis in in vivo assays. To do this, we use *Apc^{min/+}* mice, in which tumour development in the colon and rectum is enhanced, and we use *Fusobacterium nucleatum* subsp. *nucleatum* as a positive control.

Results and Discussions

The study is in progress. No results or conclusions have yet been obtained

Conclusion

The study is in progress. No results or conclusions have yet been obtained

EACR22-0339

Vicia villosa lectin-labelled nanoparticles for detection and targeting of breast and prostate cancer specific Tn-antigen in in vivo and ex vivo models

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Introduction

Glycosylation is a post-translational modification that is altered in cancer. The alterations in the cancer cell glycome can affect tumor growth and spreading. The altered glycomotifs can also function as diagnostic targets. A common glycome modification in breast and prostate cancer is the truncation of O-GalNAc glycans, which leads to the expression of the cancer-specific Tn-antigens. The Tn-antigen can be detected with *vicia villosa* lectin (VVL) and Tn-antigen-specific antibodies. In this project, we investigate the possibility of using stable VVL-labelled fluorescent nanoparticles for the detection of breast and prostate cancer cells.

Material and Methods

In this study, we used red dye-doped particles labelled with VVL. To check the specificity and sensitivity of these nanoparticles towards the Tn-antigen, we used MDA MB 231 and MCF-7 breast cancer cells and PC-3 prostate cancer cells gene-engineered to overexpress the Tn-antigen. Corresponding wild type (WT) cell lines were used as controls. The Tn-antigen overexpressing cell lines were produced by the SimpleCell strategy (provided by Prof. H Clausen, Center of Glycomics; Denmark) (Steentoft et al., *The EMBO journal*, 32(10), 1478–1488, 2013). The specificity of the VVL-labelled nanoparticles towards the Tn-antigen overexpressing cells was studied using flow cytometry and confocal microscopy. Next, we investigated the possibility to use the particles for labelling mouse xenograft tumor tissue sections. These sections were obtained from an experiment, in which immunodeficient

mice were inoculated with gene-engineered Tn-antigen overexpressing and wild type control cells.

Results and Discussions

Flow cytometry showed an increased staining intensity when Tn-antigen overexpressing cells were incubated with VVL-labelled nanoparticles, compared to control cells. Confocal microscopy images of fixed cells showed mainly membrane-associated labelling of engineered cells with the nanoparticles and no or low staining of control cells. The staining of xenograft cryosections indicated that these nanoparticles are also suitable for microscopic visualization of Tn-antigen overexpressing cancer cells on tissue sections.

Conclusion

These studies showed that the stable VVL-labelled nanoparticle complexes can be used to detect cancer cells in *in vitro* and *ex vivo* samples. Due to the high expression levels of Tn-antigen in breast and prostate cancer, these particles are promising tools for detection of cancer in patient samples.

EACR22-0343

Gender and Ethnic Differences in Multiple Myeloma Incidence Trends in Israel

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Introduction

Despite global research on trends of Multiple Myeloma (MM) incidence, which increases worldwide, trends of MM in Israel have not been evaluated yet. This study aims to analyze trends between 1985 – 2018 in Israel, stratified by gender and ethnicity, and to point out some possible risk factors which may explain the differences in trends.

Material and Methods

Data on new MM cases in Israel were retrieved from the Israel National Cancer Registry (INCR). Overall and age-specific accumulated incidence rate ratios (IRRs) between males and females, and between Arabs and Jews for the period 1985 – 2018 were calculated. Joinpoint regression analysis was used to evaluate the time trend of age- standardized incidence. Poisson regressions were conducted to evaluate the interaction between calendar time and gender, and between calendar time and ethnicity with respect to MM rates in Israel.

Results and Discussions

Cumulative incidence rates in Israel between 1985 – 2018 were higher among males compared to females, and among Jews compared to Arabs. Annual age-adjusted MM incidence rates in Israel increased during this period in both Jewish/other and Arabs populations (males and females). Linear regressions show that the rates of increase in age-standardized incidence were higher among Arab males compared to Jewish/other males, and among Arab females compared to Jewish/other females. Poisson regressions demonstrate interactions between calendar time and gender, and between calendar time and ethnicity with respect to MM rates, such that the gap in MM incidence rates between males and females, and between Jews and Arabs are reduced over time. This reduction in gaps in incidence rates between Jews and Arabs were also observed in other

cancer types in Israel. A possible explanation for these gaps may be an epidemiological transition of the Arab population in Israel. Together with the urbanization and progress characterizing the Arab population in Israel during the last decades, the Arab population also acquires various risk factors to chronic, as well as non-chronic diseases. Part of these factors is still unknown.

Conclusion

Trends of MM incidence in Israel is increasing. Gap in trends of MM incidence in Israel between Jews and Arabs is reduced over the years. It is essential to further explore the reasons for the differences in trends of MM incidence in Israel, and the reasons for the gap reduction between Jews and Arabs in Israel.

EACR22-0609

Associations between epidemiological, clinical and lifestyle factors and levels of urinary 7,8-dihydro-8-oxo-2'-deoxyguanosine in colorectal cancer patients: Results from the ColoCare Study

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Introduction

Colorectal cancer (CRC) is the second most frequent cancer in men and the third most frequent cancer in women worldwide. Insufficient physical activity, a Western style diet, smoking, and regular alcohol consumption can favor the development of CRC. Many of these factors contribute to increased oxidative stress which can result in DNA damage. A central biomarker of oxidative stress is 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-oxo-dG), an oxidized form of the guanine base. This study aims to assess associations between levels of urinary 8-oxo-dG in CRC patients and epidemiological, clinical and lifestyle factors.

Material and Methods

This work is nested within the ColoCare Study, an international prospective cohort study among women and men newly diagnosed with a primary invasive CRC of stages I-IV. Pre-surgery urinary levels of 8-oxo-dG of CRC patients (n=198) were measured using an enzyme-linked immunosorbent assay (ELISA, Cayman's DNA/RNA Oxidative Damage EIA competitive assay) and normalized to urinary creatinine. Multivariate models adjusted for potential confounders were applied to investigate associations of epidemiological, clinical, and lifestyle factors such as age, sex, tumor site, tumor stage, body mass index (BMI), alcohol consumption, smoking status, and metabolic equivalent of tasks (METs) h/week with urinary levels of 8-oxo-dG.

Results and Discussions

CRC patients with late tumor stage (III and IV) (n=69) showed significantly higher urinary 8-oxo-dG levels than patients with an early stage (I or II) (n=53); p=0.02. Furthermore, ever active smokers (n=109) showed significantly higher 8-oxo-dG levels compared to never active smokers (n=53); p=0.04. All other variables assessed (age, sex, BMI, alcohol consumption tumor site, and METs h/week) did not reveal statistically significant differences in urinary 8-oxo-dG levels.

Conclusion

Our findings suggest that urinary levels of 8-oxo-dG in CRC patients are associated with advanced tumor stage and smoking.

EACR22-0790

Non-Small Cell Lung Cancer organoids for assessing radiation and targeted drug combination therapies in cancer

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Introduction

Lung cancer remains the leading cause of cancer-related deaths worldwide, the majority of this being Non-Small Cell Lung Cancer (NSCLC). Homogenous cell-line based models are frequently used to study complex mechanisms and treatments combinations. Recent studies of human 3D cancer organoids suggest they may better model tumour responses.

Organoids are cell models directly established and passaged in laminin/collagen-based hydrogels, with defined growth factors. Normal and cancer organoids have been shown to model drug efficacy and toxicity in culture, based in part on their maintenance of complex inter-cellular interactions that are observed in tumours but not 2D cell lines.

Radiotherapy is a standard lung cancer treatment that induces DNA damage. Defects in the DNA Damage Repair (DDR) pathway may influence both cancer development and treatment, as they generate cancer-specific vulnerabilities. In DDR inhibitor-based therapy, tumour cells with specific DDR defects can accumulate DNA lesions that are not recognized or repaired, causing increased tumour cell cytotoxicity and death. This enhances the effects of radiotherapy as a DNA damage agent.

To date, cellular responses to radiotherapy have primarily been studied in plastic-adapted cancer cell line models. In this work, we have evaluated the responses of NSCLC organoids to radiation-drug combinations to determine whether lung cancer organoids might be used to improve the assessment of drug-radiation combinations.

Material and Methods

NSCLC Adenocarcinoma and Squamous Cell Carcinoma organoid-lines were treated with radiation and characterised using a range of imaging and metabolic assays in the presence and absence of DDR inhibitors.

Results and Discussions

Strong differences in the behaviour of individual NSCLC organoid lines to radiation was observed. Characterisation of these responses required the use of a combination of assay formats due to the complexities of organoid 3D structure. A clear radiation dose-dependence was observed in NSCLC organoids. The dose-response was modified by treatment with DDR inhibitors.

Conclusion

The radiation and drug responses of NSCLC organoids suggests that they can be used as models to understand radiosensitive and resistant lung cancer responses in culture. In the near term, these models should be useful for characterising tumour cell niche-dependent inter-cellular response differences. In the longer-term, the response to treatment of patient-derived organoids may help develop personalised medicine pipelines for cancer patients.

EACR22-0960

Epigenetically-induced TP53 in radioresistant prostate cancer model: A new radiosensitizing strategy

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Introduction

DNA damage repair (DDR) signaling pathways are particularly active in stressful circumstances, with the ionizing radiation (IR) therapy leading to the activation of several cellular processes mainly related to cell cycle control and DDR. Currently, radiotherapy (RT) still remains one of the main first-line therapies in prostate cancer (PCa). Although considerable high successful rates, in a significant proportion of those patients the therapy fails. This limitation is mainly due to the increased IR-induced DNA damage repair processes from tumor cells. TP53 display broad molecular functions entailing central roles as DDR facilitator, restoring genomic stability. However, many aspects of TP53 regulation upon IR exposure are still unexplored. Hence, epigenetic regulation of TP53 could be a key to better understand PCa radioresistance.

Material and Methods

In vitro selection of radioresistant cells was conducted to identify relevant epigenetic dynamics. Then, 22Rv1 radioresistant cells (22Rv1-RR) were generated after exposure to 20 fractions of 2.5Gy per day for 28 days. Then, a broad-range transcriptomic array was used to unveil differentially expressed DDR signaling-related genes in RR PCa. γ -H2AX was used as DNA damage sensor, and the % DNA damage was assessed through comet assay. Chromatin immunoprecipitation was performed to study TP53 transcriptional regulation. Moreover, GSK-J4 was used as a selective KDM6A/B inhibitor, with demethylase activity for H3K27me3.

Results and Discussions

Globally, RR PCa cells displayed higher clonogenicity and less DNA damage. In addition, enhanced DDR machinery was accompanied by a significative reduction of histone

repressive-associated markers, particularly H3K27me3. An upregulation of TP53 expression was observed for 22Rv1-RR cell lines in more than 6-fold variation compared with the parental ones (P). Mechanistically, 400x increased KDM6B expression in 22Rv1-RR cells led to a significant decrease of H3K27me3 occupancy at the *TP53* transcription starting site (TSS) promoter (175 bp above TSS). Thus, we believe that GSK-J4 could be a potent PCa radiosensitizer. In fact, GSK-J4 led to a 50% viability reduction at ~13µM and 25µM, for 22Rv1-P and RR, respectively, with 2µM of GSK-J4 already restoring histone methylation levels in 22Rv1-RR cells to 15x higher than the control.

Conclusion

Our data revealed that TP53 epigenetic regulation is mainly initiated through DNA damage triggering by IR in a PCa model.

Radiobiology / Radiation Oncology

EACR22-1024

Targeting serine/glycine metabolism to improve radiotherapy response in Non-Small Cell Lung Cancer (NSCLC)

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Introduction

Lung cancer is the second most common cancer and about 85% of lung cancers are classified as NSCLC. There is a strong need for therapeutic selectivity and a better understanding of the tumor resistance to the treatment. Metabolic vulnerabilities of cancer cells are promising targets in combination with current treatment regimens. Serine/ glycine metabolism has been associated with NSCLC treatment resistance and poor outcome. Certain tumors, e.g. LKB1 mutated, have an increased activation of *de novo* serine/glycine biosynthesis, which branches from the glycolysis and is necessary for the synthesis of proteins, lipids and nucleic acids, as well as regulating the redox homeostasis and methylation reactions. Our main goal is to identify and target metabolic vulnerabilities in NSCLC patients that can cause radiotherapy (RT) recovery and resistance.

Material and Methods

We performed (un)targeted metabolomics on 37 blood plasma samples of stage I/III NSCLC patients receiving stereotactic RT or concurrent chemo-radiation therapy (CCRT). In addition, we landscaped RT responses using ¹³C₆-Glucose metabolic tracing in a panel of NSCLC cell lines. Finally, we combine metabolic targeting with RT.

Results and Discussions

We identified a significant decrease in glycolysis metabolites, i.e. glucose and lactic acid, in the plasma of NSCLC patients as initial response to RT. We also observed a decrease in serine/glycine pathway metabolites, i.e. serine in stage I NSCLC and homocysteine and GSH in stage I/III NSCLC, after the last dose of RT. *In vitro*, we could confirm decreased intracellular levels of the glycolytic metabolite 3-phosphoglycerate and a consistent reduction in serine and glycine in response to RT. Due to this finding, combined with the knowledge that 37% of the NSCLC cases can produce their own serine/glycine from glucose consumption, we challenged to combine sertraline, which inhibits serine/glycine pathway, with RT. Using sertraline in combination with RT, we could achieve synergistic therapeutic responses in NSCLC cell lines in both reducing the cell proliferation and clonogenic survival. Synergistic effects were induced via increased levels of ROS, affecting cell cycle arrest, inducing senescence or programmed cell death.

Conclusion

Our findings emphasize the requirement of NSCLC cells for glycolysis and subsequent serine/glycine pathway metabolites in response to RT, highlighting serine/glycine metabolism as promising target to be exploited in order to improve the therapeutic response to RT.

EACR22-1034

Radio-sensitizing Prostate Cancer cells: A possible role for Zoledronic acid and Pravastatin (ZOPRA)

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Introduction

Prostate cancer (PCa) is the second most commonly diagnosed cancer among men worldwide. Radiotherapy is one of the first-line treatments given to PCa patients. However, it is sometimes coupled with adverse side effects. Therefore, finding a way to radio-sensitize PCa cells and reduce the exposure of healthy tissues to its effect is of high importance.

Material and Methods

Human prostate cancer cell lines DU-145 and PC3 were cultured in RPMI medium, supplemented with 10% fetal bovine serum, and maintained in a humidified incubator with 5% CO₂ at 37°C. The cells were cultured with a medium containing Zoledronic acid (Zo) and Pravastatin (Pra), individually and combined (ZoPra), and subjected to X-ray radiation. MTT assay was employed to test cell viability and drug toxicity. Using the most appropriate drug combination concentration, we will employ different *in vitro* studies including clonogenic assay for cell survival, immunofluorescence analysis of γH2AX, and pATM expressions to study DNA double-strand break repair kinetics in 2D and 3D cell culture models.

Results and Discussions

Our preliminary data, using MTT assay, showed that 1 and 3 μM of Zo and Pra individually are cytostatic and not cytotoxic. It was also deduced that an appropriate combination concentration of 1 μM Zo in combination with 1 μM Pra does not significantly affect PC3 and DU-145 cells, deeming it safe to continue testing their radio-sensitizing potential. Based on data from our laboratory in other cancer models, we expect that ZOPRA will decrease cells proliferation, cell survival, and increase the residual number of γH2AX foci in PCa cell lines.

Conclusion

Amongst all cancer curative innovations, radiation therapy remains one of the primary modes of therapy. The radiosensitization of cancer cells opens the stage to widen the therapeutic window of radiation therapy while maintaining patients' quality of life. Our in vitro study will illuminate the potential use of drugs as radiosensitizing agents.

EACR22-0431

OXYGEN MICROBUBBLES AND METFORMIN RADIOSENSITIZE 4T1 TUMORS BUT INCREASE THE METASTATIC SPREAD

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Introduction

Hypoxia is a major determinant of human tumor sensitivity to radiation therapy. Ultrasound-sensitive microbubbles filled with oxygen (OMB) are promising tissue radiosensitizers. Oxygen from microbubbles can be released locally by an ultrasound impulse, leading to a controlled, local increase in pO₂. Metformin is a drug commonly used in type II diabetes exhibiting also anticancer activity. It has been shown to decrease cellular oxygen consumption, alleviate hypoxia, and inhibit angiogenesis. However, not much is known about the temporal changes in tumor vasculature during metformin therapy. The aim of this study was to investigate the synergistic action of long-term metformin and OMB and the impact of this drug combination on radiotherapy effectiveness and vascular function.

Material and Methods

Anionic pegylated microbubbles composed of DSPC and DSPE-PEG2000 were used. Cavitation of microbubbles was performed with 2W/cm² ultrasound impulse (Vevo SoniGene). In vivo EPR oximetry was conducted with an L-band CW spectrometer (Bruker Elexsys-II E540) in 4T1 tumors growing in a mammary fat pad of Balb/c mice (N = 120). Mice were divided into four groups: control, treated with OMB (iv, twice a week) and metformin (ip, 350 mg/kg, daily) alone and treated with both OMB and metformin. Temporal changes in tumor oxygenation were measured twice a week with OxyChip as an oxygen sensor. Tumor growth and vasculature were monitored twice a week with the use of ultrasonography (Vevo 2100). Radiotherapy (12 Gy, gamma rays) was carried out in the therapeutic window of combined therapy. Vascular morphology and permeability as well as hypoxia and metastasis-related proteins were analyzed in tumor tissue collected at endpoints of experiments and at the point of the highest oxygenation increase.

Results and Discussions

Two weeks of combined therapy resulted in a therapeutic window with higher oxygenation and normalized vasculature. We observed the slowing down of tumor growth after radiotherapy in all treated groups, however, the biggest impact was seen in a group pretreated with combined therapy. Treatment with metformin and oxygen microbubbles effectively increases the oxygenation of tumors and sensitizes them to radiotherapy. However, both metformin and a combination of metformin with OMB increased metastasis of 4T1 tumors.

Conclusion

The combination of oxygen microbubbles and metformin efficiently sensitizes 4T1 tumors to radiotherapy but at the same time increases the metastatic burden.

EACR22-0505

3D rectal cancer spheroid: a new tool for uncovering novel therapeutic targets?

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Introduction

The classification of the 2nd most lethal cancer worldwide, colorectal cancer (CRC), has been questioned in recent years due to the considerable differences between the colon and rectum. In fact, >30% of CRC cases are anatomically located at the rectum and display specific genetic, molecular, and multimodal treatments. Still, most CRC studies are focused exclusively on the colon section, disregarding the rectum. Moreover, most CRC research is performed in 2D, completely ignoring cell-to-cell interaction and in vivo tissue architecture. Altogether, these premises have contributed to the high failure rate in drug discovery. To address this void in cancer research, we developed a biomimetic 3D rectal cancer spheroid (RCS) that can be used as a tool for rectal cancer studies.

Material and Methods

RCS model was developed using SW837 cells in an agarose micro-mould, cultivated in different nutrient composition cell culture media, and generated with different cell-seeding numbers. RCS model was then characterized for their tumour features, namely 3D dynamics and morphology, metabolic and viability properties, as well as proliferative and necrotic areas. Ionizing radiation, mimicking the standard one-week treatment of rectal cancer patients was also used to validate the usefulness of the RCS model involving radiotherapy and resistance mechanisms.

Results and Discussions

RCS model is clearly influenced by the different cell culture media and different cell-seeding numbers. The overall dynamics and morphology of the RCS as well as the metabolic and proliferative activity, change according to the culture conditions. Additionally, our 3D RCS model is characterized by a proliferative region and a well-formed necrotic area, especially in larger spheroids cultivated in a poorer cell culture media. These tumour morphological features were accentuated after ionizing radiation treatment.

Conclusion

This is the first detailed characterization of a 3D RCS model that can not only be used to expand drug screening studies but also metabolic, radioresistance, and therapeutic responses in rectal cancer.

EACR22-0905

Pancreatic cancer cell spheroid control probability assay (SCP) to assess the

outcome of single-dose versus clinical-practice fractionated proton irradiation

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) remains a devastating malignant disease with a 5-year overall survival rate of ~7%. New clinical observations imply that proton therapy might be beneficial for PDAC patients. Our experiments based on single-dose irradiations demonstrate the superiority of proton compared to photon therapy in PDAC spheroid control probability (SCP) assays. The current follow-up study aims at a systematic evaluation of treatment outcome upon fractionated proton irradiation to improve the predictive value of the 3-D test platform.

Material and Methods

Single dose proton irradiation (0-25 Gy) and various fractionation regimens (2.5 Gy/day up to 37.5 Gy; 5 Gy/day and 5 Gy every other day up to 30 Gy) were applied to 370-400 µm Panc-1 and Pa-Tu-8902 PDAC spheroids placed in a proton spread-out Bragg peak using horizontal 150 MeV double-scattered or pencil beam scanning protons. Spheroid integrity and (re-)growth were monitored every 48-72 h up to 60 days to assess treatment response in SCP assays using the “curative” analytical endpoint of SCD₅₀ (spheroid control dose 50%).

Results and Discussions

We here document for the first time the response of PDAC spheroids upon clinically relevant fractionated proton therapy based on a total of >4000 spheroids (>100,000) images. Panc-1 and Pa-Tu-8902 spheroids showed distinct radioresistance, reflected by SCD₅₀ values for the 2.5 Gy daily fractions differing by a factor of two. The spheroid type-dependent difference was more pronounced in the fractionated than single dose applications. SCP values in both models were highest upon single-dose irradiation, intermediate for the 5 Gy and lowest for the 2.5 Gy fractions. Outcome was unaffected by implementing a one-day recovery period in the 5 Gy fractionation regimens.

Conclusion

Hypofractionated 5 Gy irradiation seems favorable to conventional fractionations for PDAC (spheroid) treatment, irrespective of the recovery time that might be preferential for patients. The PDAC SCP assay using fractionated proton irradiation is a sophisticated, valuable tool to be further developed.

NRP1 inhibition by a peptidomimetic increases radiosensitization of medulloblastoma stem-like cells

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Introduction

Medulloblastoma (MB) is the most frequent pediatric malignant brain tumor. Despite the availability of cutting-edge therapies, the risk of morbidity and recurrence remains significant. The neuropilin-1 receptor (NRP1), which plays an important role in the undifferentiated status of MB, has been implicated in the tumor progression of MB. A recent study has shown that NRP1 inhibition stimulates the differentiation of MB stem cells. Targeting NRP1 in medulloblastoma may, therefore, improve treatments such as radiotherapy

Material and Methods

Inhibition of NRP1 by a novel peptidomimetic agent, MR438, was evaluated in combination with radiotherapy (RT) in three MB subgroup models (DAOY, D283-Med, and D341-Med) by in vitro experimentation on stem-like cells and in vivo on heterotopic and orthotopic xenografts by histological analysis and bioluminescence imaging.

Results and Discussions

NRP1 inhibition by MR438 radiosensitized MB stem cells in vitro. The in vivo models show that MR438 improved RT efficacy, measured by inhibition of tumor growth and survival of mice with the heterotopic xenograft DAOY. In addition, the self-renewal capacity of cancer stem cells after tumor dissociation was significantly reduced by NRP1 inhibition concomitant with RT. Similarly, a benefit of the combined therapy was observed in an orthotopic model with a short (one week) treatment period, although this benefit was lost with a longer (two weeks) treatment period.

Conclusion

Our results demonstrate that targeting NRP1 with MR438 is a potential new strategy to radiosensitize MB stem cells, as it can limit the progression of MB by decreasing the number of stem cells, although the schedule needs to be adjusted to optimize the effect.

Targeting energy metabolism to enhance treatment response in rectal cancer

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Introduction

Resistance to neo-adjuvant chemoradiation therapy is a significant clinical problem in rectal cancer. There is an urgent need to identify underlying mechanisms of treatment resistance and novel treatment strategies to improve response. In this study, the role of altered metabolism, and the potential of Metformin, a clinically approved anti-diabetic drug and metabolic modulator, as a novel radiosensitiser was investigated in rectal adenocarcinoma.

Material and Methods

Radiosensitivity and chemosensitivity of SW837 rectal and HCT116 colon adenocarcinoma cells was assessed by clonogenic assay. Energy metabolism of cell lines and *ex vivo* rectal cancer and non-cancer rectal biopsies was assessed by Seahorse. Cell cycle, apoptosis and DNA damage were assessed by flow cytometry. Transcriptomic analysis was performed by Lexogen QuantSeq sequencing and BlueBee, DESeq2 and Ingenuity Pathway Analysis software. MesoScale Discovery 54-plex ELISA was used. Irradiations were performed using the X-Strahl RS225 cabinet irradiator. Experiments in hypoxia were performed using a Whitley H35-hypoxystation.

Results and Discussions

SW837 cells were significantly more resistant to radiation and chemotherapy (5-FU), with altered cell cycle distribution, enhanced DNA damage repair and altered energy metabolism, specifically a decreased reliance on glycolysis, when compared to treatment sensitive HCT116 cells. Transcriptomics demonstrated enhanced oxidative phosphorylation gene expression in radioresistant SW837 cells. Metformin treatment inhibited oxidative phosphorylation in *in vitro* and *ex vivo* models of rectal cancer and induced mitochondrial dysfunction *in vitro*, but didn't alter metabolism of non-cancer rectal tissue. Metformin treatment altered the inflammatory secretome of rectal cancer. Transcriptomic analysis revealed inhibition of oxidative phosphorylation in metformin treated SW837 cells. Importantly, metformin significantly sensitised both HCT116 and SW837 cells to a clinically-relevant dose of X-ray radiation. Metformin significantly affected hallmarks of radioresistance *in vitro*, including cell cycle and apoptosis.

Conclusion

We have identified alterations in metabolism, cell-cycle and DNA damage repair as potential mechanisms underlying radioresistance in rectal cancer. We demonstrate that metformin significantly alters tumour metabolism *in vitro* and *ex vivo*. Importantly, metformin treatment significantly radiosensitises HCT116 and SW837 cells supporting the potential use of metformin as a radiosensitiser in rectal cancer.

EACR22-1229

Ferroptosis induction as a means of combating radiotherapy inefficiency in head and neck squamous cell carcinoma

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Introduction

The sixth most common cancer worldwide is head and neck squamous cell carcinomas (HNSCCs). Conventional treatments, such as radiotherapy (RT), show limited success. Recent research showed that ferroptosis, a type of regulated cell death, can sensitize therapy-resistant carcinomas to RT. Therefore, the study aims to explore ferroptosis' role in RT treatment of HNSCC.

Material and Methods

To understand the low success rate of RT in HNSCC management, protein expression of DNA repair enzymes/damage markers (γ H2AX) and apoptosis activation (CC3) was analyzed in treatment-naïve/treated HNSCC samples. Furthermore, we evaluated the effect of ferroptosis induction on the success of RT. Our study involved irradiating HNSCC cell lines with clinically relevant RT doses, either with or without simultaneous ferroptosis induction by erastin/RSL. Cell death mode, proliferation rate, and accumulation of lipid ROS were analyzed after treatment.

Results and Discussions

Compared to adjacent normal tissue, immunohistochemical analyses of tumors showed elevated levels of DNA repair enzymes (MSH2, MSH6, MLH1, and PMS2) ($p \leq 0.01$). In treated patients, neither CC3 nor γ H2AX expression was significantly different from that in untreated patients. HNSCC cells were generally resistant to irradiation (survival rates $>50\%$ with 10 Gy), as shown in *in vitro* experiments. Together these results suggest that HNSCCs can repair therapy-induced damages. Interestingly, irradiation with simultaneous treatment with erastin/RSL showed a marked synergistic effect which was reflected in a significant growth reduction at doses as low as 2 Gy ($p < 0.01$). The combination treatment led to a reduced proliferation rate, increased cell death, and significantly increased lipid ROS formation.

Conclusion

In summary, our data demonstrate that the proposed combination treatment leads to a significant reduction in tumor cell growth. We believe that this combination represents an important advance in the treatment of HNSCC, especially for patients who undergo RT treatment.

EACR22-1259

Modelling post-radiotherapy recurrence in Group 3 medulloblastoma

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Introduction

Medulloblastoma (MB) is among the most common paediatric malignant brain tumours. Advances in molecular profiling have established the existence of four distinct subgroups of MB including WNT, SHH, Group 3, and Group 4, each with their own distinct molecular and clinical characteristics. Treatment for MB relies on a combination of maximal safe surgical resection, followed by chemo-radiotherapy of the whole cranio-spinal axis. When the disease recurs (in 40% of cases), no therapies have been shown to confer a survival benefit. Group 3 (G3) MB carries the worst prognosis amongst all four subgroups with an urgent need for the development of novel therapeutics.

Material and Methods

We have established clinically relevant mouse models of recurrent Group 3 MB based on the administration of high-dose fractionated and CT-guided radiation to the brain of mice bearing patient-derived xenografts in the cerebellum. Radiation treatment has been shown to increase overall survival and induce the clonal selection of more resistant subpopulations of tumour cells. In order to compare recurrences to treatment naïve primary tumours, single cell RNA-sequencing was used to define a recurrent gene signature predictive of poor prognosis

and radio-resistance. This signature was validated using a targeted CRISPRa library *in vivo* by assessing the enrichment of guides subsequent to radiotherapy.

Results and Discussions

By investigating the mechanism of acquired resistance to radiation in recurrent Group 3 MB, we discovered the overexpression of a distinct set of genes. This gene signature was clinically prognostic in human Group 3 MBs using a large patient cohort. Integrative pathway enrichment analysis of scRNA-seq differential expression data using three PDX cell lines (MB114, MB211, MB411) showed pathways converging on oxidative phosphorylation and nucleoside metabolism. Given that several up-regulated genes constitute druggable hits with existing FDA-approved drugs, these findings will direct the repurposing of existing therapies for pre-clinical testing.

Conclusion

The discovery that recurrent G3 MBs are transcriptionally divergent from primary tumours and harbour a radiation-resistance gene expression signature reveals vulnerabilities that can be leveraged for therapy. Insights from this project present candidate drugs with the potential to be repurposed as an adjuvant to radiotherapy for the treatment of G3 MB.

EACR22-0529

POSTER IN THE SPOTLIGHT

Critical of selecting assay endpoint and critical organ on defining relative biological effects of the born neutron-captured therapy for head and neck cancer

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Introduction

Head and neck (H&N) cancers are the sixth incidence rate in cancer worldwide and are frequently associated with high recurrence with bad life quality after the treatments. The recurrent H&N cancers were hard to be re-treated by conventional protocols, and the boron neutron capture therapy (BNCT) is one of the leading choices. Our recent clinical trial has demonstrated the feasibility and advantage of BNCT for recurrent H&N patients. The destructive effect of BNCT is mainly caused by the heavy charged particles, lithium, and alpha particles, so-called high linear energy transfer (LET) radiation. This study aimed to compare the relative biological effect (RBE) of BNCT on tumors, tumor surrounding tissues, and normal tissues using different biological endpoints.

Material and Methods

The SAS cell line was used for the *in vitro* experiment. The clonogenic assay and γ -H2AX staining were used to quantify the *in vitro* effect on cell survival and DNA damages, respectively, following the therapy. The 4NQO-induced oral cancer model in C57BL/6J mice was the tumor model for the *in vivo* experiment. The H&E staining and DNA damage assay were followed to measure the differential effect on the tumor, surrounding tissues, and normal tissues. To compare the RBE, a clinical linear accelerator and the thermal neutron beam from Tsing Hua Open-pool Reactor (THOR) were used for the irradiation.

Results and Discussions

Our preliminary *in vitro* results show that the RBE value for the SAS cell line depends on the evaluation method by cell survival or DNA damage assay. This is mainly because the RBE values for DNA damage assay varied with different quantitative methods and the time point

for scoring γ -H2AX foci. The in vivo results show that the T/N ratio and RBE not only changed between tumor and normal tissues but also controlled normal and tumor surrounding normal tissues. We not only observed the quantitative difference but also found that the type of DNA damage varied with the kind of radiation. There are more sparsely DNA double-strand breaks (DSBs) following the irradiation by a linear accelerator (low LET radiation) while more clustered DSBs in BNCT (high LET radiation)-treated cells and tissues. The type of DSBs might be the prime factor for the variation of RBE by γ -H2AX foci assay.

Conclusion

The RBE is a critical value for determining the radiation dose or treatment time for BNCT. This study illustrates the variation of RBE on the choice of assay endpoints and tissues. The result cautions the choice of RBE for BNCT.

EACR22-0346

HDAC6 inhibition enhances radiosensitivity in meningiomas by controlling beta-catenin nuclear localisation

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Introduction

Meningioma is the most frequent primary central nervous system tumour (PCNST) which account for one third of all PCNST. Due to the lack of efficient chemotherapy for meningioma, radiotherapy often become a first-line treatment especially when the tumour is not operable. Radiotherapy plays a crucial role in local control but its efficacy is restricted by radioresistance and by normal tissue radiation tolerance. Therefore, developing and evaluating potential radiosensitisers to enhance therapeutic efficacy are urgently needed.

Histone deacetylase (HDACs) expression is generally increased in many cancer types and regulate the expression of numerous proteins involved in tumorigenesis. Targeting HDAC using HDAC inhibitor (HDACi) represent promising radiosensitisers that affect various biological processes, such as cell survival, apoptosis, and DNA repair.

Material and Methods

We investigated whether pretreatment with the hydroxamate-based HDAC6 inhibitor, Cay10603, impacts radiation-induced DNA double-strand break (DSB) induction, cell survival, and cell cycle arrest using immunocytochemistry, clonogenic assay, and flow cytometry in immortalised meningioma cell lines. Low concentration (100 nM) of Cay10603 was treated 24 hr prior to high energy x-ray irradiation (2 Gy) by a medical linear accelerator (LINAC). To investigate the nuclear localisation of beta-catenin, subcellular fractionation and Western Blotting were conducted.

Results and Discussions

We found that surviving rate was synergistically decreased after combination treatment of Cay10603 and radiation. Combination therapy induced DNA damage with activation of histone γ H2AX and increased G2/M arrest compared to drug or radiation alone. To focus on the mechanisms of action of HDAC6 inhibition followed by radiation, we further investigated nuclear localisation of beta-catenin levels. The results showed the both beta-catenin and c-myc expression in the nucleus was suppressed after combination therapy.

Conclusion

In meningioma cells, radiotherapy in combination with HDAC6 inhibitor reduces the nuclear localisation of beta-catenin and synergistically decreases cell survival. Our findings

demonstrate a potential therapeutic strategy of Cay10603 to improve the radiosensitisation for meningioma cells.

EACR22-1159

Assessing the radio protecting effects of ZOPRA on normal human prostate epithelial cells and human fibroblast cells.

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Introduction

Prostate cancer (PCa) is one of the most common types of cancer among men. Radiotherapy is one of the treatments regimens for PCa. However, many patients suffer from unpleasant side effects. Therefore, fine-tuning the effects of radiotherapy on normal surrounding cells is essential.

Material and Methods

We are interested in assessing the radio-protecting properties of these drugs, alone or in combination, on RWPE1 (normal prostate epithelium) and HFF (normal fibroblast) cells. RWPE1 cells were cultured in RPMI medium, supplemented with 5% fetal bovine serum and 5% epidermal growth factor. HFF cells were cultured in DMEM medium supplemented with 10% fetal bovine serum. Both Cell lines were maintained in a humidified incubator with 5% CO₂ at 37°C. The cells were cultured with a medium containing Zoledronic acid (Zo) and Pravastatin (Pra), individually and combined (ZoPra), and subjected to X-ray radiation.

MTT assay was performed to test cell viability and drug toxicity. Cells will be treated with different concentrations of pravastatin, zoledronic acid, and their combination. Anti-γH2AX and anti-pATM immunofluorescence will be performed to study the kinetics of recognition and repair of DNA DSB. Clonogenic assays will be performed to assess cell proliferation and survival in response to radiation, respectively.

Results and Discussions

Recently, a combination of bi-phosphonates (zoledronic acid) and statins (pravastatin), ZOPRA, was shown to radio-protect normal tissues by enhancing DNA double-strand breaks (DSB) repair mechanism.

Our preliminary data, using MTT assay, showed that 1 and 3 μM of Zo and Pra individually are cytostatic and not cytotoxic. It was also deduced that an appropriate combination concentration of 1 μM Zo in combination with 1 μM Pra does not have a significant effect on RWPE1 and HFF cells deeming it safe to continue testing their radio-protecting potential. Based on data from our lab in other cancer models, we expect that ZOPRA will increase cells proliferation, cell survival, and decreased the residual number of γH2AX foci in RWPE1 cell lines.

Conclusion

The fact that ZOPRA was previously shown to have a radio-protective role in the tissues surrounding the tumor, makes it a good candidate to become a therapeutic window-widening drug. In the conclusion of our study, we expect ZOPRA to have a radio-protecting effect on normal prostate and fibroblast cells.

Radiation Nanomedicine for Intraoperative Treatment of Glioblastoma Multiforme*C. Georgiou¹, Z. Cai¹, J.T. Rutka^{2,3,4}, M. Winnik⁵, R.M. Reilly^{1,6,7}*¹*University of Toronto, Department of Pharmaceutical Sciences, Toronto, Canada*²*University of Toronto, Department of Laboratory Medicine and Pathobiology, Toronto, Canada*³*The Hospital for Sick Children, Division of Neurosurgery, Toronto, Canada*⁴*The Hospital for Sick Children,**The Arthur and Sonia Labatt Brain Tumour Research Centre, Toronto, Canada*⁵*University of Toronto, Department of Chemistry, Toronto, Canada*⁶*University Health Network,**Joint Department of Medical Imaging and Princess Margaret Cancer Centre, Toronto, Canada*⁷*University of Toronto, Department of Medical Imaging, Toronto, Canada***Introduction**

Surgical resection and external radiation of glioblastoma multiforme (GBM) combined with temozolomide chemotherapy are not curative and recurrence and death from GBM is common. We hypothesize that intraoperative infusion of ¹⁷⁷Lu-labeled gold nanoparticles (¹⁷⁷Lu-AuNP) into the surgical margins (radiation nanomedicine) may eradicate residual tumour and prevent recurrence of GBM. Our aims were to study the biodistribution, toxicity and efficacy of ¹⁷⁷Lu-AuNP infused peritumorally for treating orthotopic human GBM tumours in mice.

Material and Methods

AuNP (18 nm) were labeled by conjugation to a metal chelating polymer (MCP) with 8 pendant DOTA and 4 lipoic acid groups [PEG-pGlu(DOTA)₈-LA₄] complexed to ¹⁷⁷Lu. Human GBM tumours were established by stereotaxic inoculation of U251/luc cells into the right frontal lobe of NRG mice. ¹⁷⁷Lu-AuNP were subsequently infused peritumorally. SPECT/CT was used to image the distribution of ¹⁷⁷Lu-AuNP up to 21 days post-infusion (p.i.). Biodistribution of ¹⁷⁷Lu-AuNP [percent injected dose/g (%ID/g)] was measured up to 14 d p.i. Toxicity of 1.5 MBq ¹⁷⁷Lu-AuNP or saline infusions was assessed by monitoring body weight, serum biochemistry, and hematology 14 d p.i. Mice were treated with ¹⁷⁷Lu-AuNP (1.0 MBq) and efficacy compared to unlabeled AuNP or saline. Tumour growth was monitored by bioluminescence imaging (BLI) and T2-weighted MRI. Median survival was estimated by Kaplan-Meier analysis.

Results and Discussions

High retention of ¹⁷⁷Lu was measured in the tumour-bearing right hemisphere of the brain 1 h p.i. (359.1 ± 13.5 %ID/g) and 14 d p.i. (225.2 ± 92.3 %ID/g). Low ¹⁷⁷Lu uptake (<4% ID/g) were detected in normal brain and in other organs. SPECT/CT confirmed retention of ¹⁷⁷Lu-AuNP in the brain. No significant differences were found in body weight, serum biochemistry or hematology between treated and control mice. No BLI signal was detected in all mice infused with ¹⁷⁷Lu-AuNP at 21 d p.i. while control mice exhibited increasing BLI signal. MRI showed no tumour in ¹⁷⁷Lu-AuNP treated mice at 28 days p.i. while control mice exhibited GBM tumours. ¹⁷⁷Lu-AuNP treatment prolonged survival of 5/8 (62%) mice up to 150 days p.i. while median survival for mice receiving unlabeled AuNP or saline was 31 and 41 days, respectively (P=0.0001).

Conclusion

Intratumoural injection of ¹⁷⁷Lu -AuNP was safe and effective for treating orthotopic human GBM tumours in NRG mice and may be promising for treatment of GBM in humans.

Complement proteins are associated with radioresistance and can predict response to treatment in rectal cancer

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Introduction

Poor pathological response to neoadjuvant chemoradiation therapy (neo-CRT) is a clinical problem in rectal cancer. There is a need to determine molecular factors influencing response to neo-CRT and identify novel predictive biomarkers. Evidence supports a role for the complement system in tumourigenesis and therapeutic response in cancer.

Material and Methods

Radiosensitivity of colorectal cancer (CRC) cell lines (HCT116, SW837, HRA-19, SW1463) was assessed by clonogenic assay. Cells were irradiated using an Xstrahl RS225 X-ray irradiator at a dose rate of 1.74 Gy/min. Complement gene expression was assessed by qPCR. Complement protein expression and anaphylatoxin production was assessed by ELISA. Transient knockdown of C3 was achieved by reverse transfection using siRNA. Expression of complement regulatory proteins and receptors was determined by flow cytometry. Circulating C3a levels in pre-treatment rectal cancer patient sera (n=39) were assessed by ELISA

Results and Discussions

HRA-19 cells are significantly more radioresistant compared to SW837 and HCT116 cells, whilst HCT116 cells are the most radiosensitive. Complement proteins (C3, C5) and anaphylatoxins (C3a, C5a) were produced by CRC cells, with significantly lower levels produced by HCT116 cells. Complement protein production positively correlated with surviving fraction at a clinically-relevant dose of 1.8Gy X-ray radiation. CRC cells expressed complement regulatory proteins (CD46, CD55, CD59), and complement receptors extracellularly (C5aR) and intracellularly (C3aR, C5aR). Knockdown of C3 in HRA-19 cells was associated with increased radiosensitivity at 2 Gy. C3a levels were elevated in pre-treatment sera from rectal cancer patients with a subsequent poor pathological response to neo-CRT, when compared to those with a good response ($p=0.039$).

Conclusion

Complement is produced by CRC cells, with increased gene and protein expression associated with radioresistance. CRC cells express complement receptors and regulatory proteins suggesting they can respond to complement signals and modulate complement activation, respectively. Transient C3 knockdown resulted in increased radiosensitivity suggesting a functional role for complement in the radioresponse in CRC. In patients, increased C3a levels in pre-treatment sera was associated with a subsequent poor response to neo-CRT, further supporting a role for complement in treatment response and highlighting the potential for complement as a biomarker predicting response to neo-CRT in rectal cancer.

Meitner-Auger Electron Radioimmunotherapy of Metastatic Triple-Negative Breast Cancer in NRG Mice Using Panitumumab-DOTA-¹¹¹In

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Introduction

Triple-negative breast cancer (TNBC) has a high risk of metastatic recurrence. The epidermal growth factor receptor (EGFR) is overexpressed in 50-90% of TNBC and is associated with metastasis. EGFR-targeting antibodies (e.g. panitumumab) radiolabelled with the Meitner-Auger electron (MAE) emitter ¹¹¹In are ideal for irradiation of small volume residual disease and may prevent metastatic progression. Our aims were to demonstrate the safety and efficacy of adjuvant MAE radioimmunotherapy (RIT) with ¹¹¹In-labelled panitumumab in mouse models of recurrent metastatic TNBC.

Material and Methods

Panitumumab was conjugated with DOTA to complex ¹¹¹In. TNBC tumours were generated by orthotopic inoculation of NRG mice with MDA-MB-231/LM2-4 cells. Metastatic recurrence was monitored after local treatment with tumour excision and fractionated radiotherapy (5x6 Gy). MicroSPECT/CT evaluated panitumumab-DOTA-¹¹¹In uptake in metastases. Mice were administered 9.5-24 MBq panitumumab-DOTA-¹¹¹In to determine the no observable adverse effect level (NOAEL) dose. Toxicity was monitored for 14 d by body weight, hematology and blood biochemistry analysis. Controls included irrelevant IgG₂-DOTA-¹¹¹In, murine EGFR reactive Ab30-DOTA-¹¹¹In, panitumumab-DOTA and saline. Tissue uptake was measured from 1 h to 7 d p.i. to estimate absorbed organ doses. The effect of RIT on survival after local treatment was measured by Kaplan-Meier analysis.

Results and Discussions

Metastatic recurrence was observed in the axial lymph node, liver and lungs by 2-3 weeks post-local treatment of the primary tumour. MicroSPECT/CT imaging at 24 h p.i. confirmed uptake of panitumumab-DOTA-¹¹¹In in lymph node, lung and liver metastases. Administration of up to 24 MBq panitumumab-DOTA-¹¹¹In, Ab30-DOTA-¹¹¹In, IgG₂-DOTA-¹¹¹In or panitumumab-DOTA resulted in no significant hematopoietic, hepatic, renal or general toxicity. Organ dosimetry estimates for the NOAEL resulted in 9 Gy absorbed dose to lymph node tumours and 2.5 and 1.5-fold increased dose to metastatic liver and lung, compared to tumour-free mice. The median survival of mice post-local treatment (24 d) was significantly increased (35 d, P<0.01) with adjuvant panitumumab-DOTA-¹¹¹In RIT, while treatment with IgG₂-DOTA-¹¹¹In or panitumumab-DOTA had no significant impact on survival.

Conclusion

MicroSPECT/CT with panitumumab-DOTA-¹¹¹In revealed uptake into metastases in a mouse model of TNBC. MAE RIT at the NOAEL improved survival in mice with metastatic disease and may be a promising new approach for TNBC treatment.

EACR22-1129

Hypoxic tumour cells promote tumour relapse after radiotherapy as revealed by a novel fate-mapping tool

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Introduction

Tumour hypoxia imposes a main obstacle to the efficacy of cancer therapy, especially for radiotherapy and represents an important negative prognostic factor for treatment outcome and tumour progression of most solid tumours. Understanding the fate of individual hypoxic cells prior, during and post-treatment will be of critical importance to understand their role in tumour progression and relapse.

Material and Methods

We use multiphoton microscopy, flow cytometry analysis and immunohistochemistry of H1299 lung adenocarcinoma cells expressing a novel lineage-tracing reporter of hypoxic cells to study the fate of this population after irradiation in tumor spheroids and in xenografts. Lineage tracing relies here on expression of a HIF1a-CreERT2-UnaG reporter, which upon treatment with tamoxifen drives the sustained expression of UnaG in HIF1a-expressing cells.

Results and Discussions

We first confirmed in 2D cultures the ability of the reporter to specifically label cells in hypoxia. (93,7% versus 0,55% UnaG-positive in hypoxia versus normoxia). In 3D cultures of H1299-HIFCre-ER-UnaG cells, UnaG-positive cells are located in the central part of the spheroids, and do not proliferate in untreated spheroids. Irradiation of H1299-HIFCre-ER-UnaG spheroids leads to a significant enrichment of UnaG-positive cells, indicating that the hypoxic cells are the main surviving population that drives spheroid regrowth. Upon IR they retain the integrity of the inner spheroid core while increased cell death is observed in the UnaG-negative cells. The surviving UnaG-positive cells remain arrested for a prolonged period before they start to proliferate and give rise to regrowing spheroids. Lastly, we generated H1299-HIFCre-ER xenograft tumors and found that UnaG-positive-cells coincide with pimonidazole-positive tumour areas and show absence of active cell cycle markers (ki-67, CTD1, Geminin) in untreated tumours. Upon IR, regrowing tumours show significantly higher percentage of UnaG-positive cells compared to untreated controls revealed with cell-by-cell analysis of whole tumour cross-sections.

Conclusion

Collectively, our data demonstrate the feasibility to track the fate of individual tumour cells that were hypoxic at the time of irradiation and provide proof that the hypoxic tumour cells drive tumour relapse after irradiation.

Signalling Pathways

EACR22-1054

ORAL PRESENTATION

Adaptation of colorectal cancer cells to the brain microenvironment: The role of IRS2

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Introduction

Colorectal cancer (CRC) reflects the fourth most frequent etiology of brain metastasis (BM), with rising incidence. Yet, molecular mechanisms supporting the formation of these lesions from CRC are unknown. We aimed to explore drivers enabling tropism and adaptation of CRC cells to the brain environment and decipher mechanisms facilitating the process.

Material and Methods

Genomic alteration of 395 cancer-related genes was analyzed using FoundationOne database, consisting of 35,997 CRC clinical samples including 20,858 local, 8,857 liver metastasis and 297 brain metastasis. Human astrocytes or their conditioned media (A-CM) were used to create an in vitro system mimicking the brain microenvironment. CRC 3D cell model generation was studied. Intracranial CRC BMs mouse model was used. Transcriptomic was studied using RNAseq. Seahorse assessed glycolysis and mitochondrial activities.

Results and Discussions

Analysis of FoundationOne database revealed increased prevalence of IRS2 gene amplification in 13% of BM, compared to 3% of primary or other metastatic sites. Furthermore, IHC of human CRC samples showed increased expression of IRS2 protein in BM. IRS2 is a cytoplasmic adaptor mediating effects of insulin and IGF-1 receptors and is involved in a more aggressive behavior of different cancer types. Mimicking the brain microenvironment showed that IRS2-overexpressed CRC cells survived better and formed larger 3D spheres while IRS2-silencing showed a mirror image. Moreover, intracranial CRC BM mouse model showed that IRS2-overexpression generated larger brain lesions, and the opposite was observed in IRS2-silenced cells. Transcriptomic revealed enrichment of oxidative phosphorylation (OXPHOS) and β -catenin pathways by IRS2. Indeed, IRS2-expression led to increased OXPHOS and glycolysis-independent viability and increased β -catenin transcriptional activity. Interestingly, β -catenin or IRS2 inhibition (using NT219) decreased cell viability, as well as β -catenin and mitochondrial activities, suggesting involvement of IRS2 in modulating OXPHOS through β -catenin. β -catenin is known to confer 5-FU resistance; indeed, 5-FU and NT219 synergistically inhibited formation of BM and extended animal survival.

Conclusion

We show, for the first time, the unique genomic profile of CRC BM and imply the IRS2 role in promoting CRC BM. These effects may be mediated by modulation of β -catenin and OXPHOS pathway. Given the molecular signature described, the approach to patients with BM may be significantly impacted by agents such as NT219.

EACR22-0146

PTCH1 C-terminal domain truncations alter the PTCH1 interactome

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Introduction

We recently identified three somatic mutations in the C-terminal domain (CTD) of the tumour suppressor *PTCH1* in right-side colon cancer, endometrial and stomach cancer, that cause premature truncations after S1203, R1308 and Y1316. *PTCH1*, the Hedgehog (Hh) receptor, is best known for mediating activation of the GLI transcription factors. Its CTD has additional “non-canonical” roles orchestrated through physical interaction with ATG101, ITCH, β -arrestins1/2 and GRB2. Here, we tested whether the CTD mutations affect the *PTCH1* protein-protein interactome and influence non-canonical signalling.

Material and Methods

Plasmids encoding the 3 cancer-associated *PTCH1* CTD truncation mutants were generated by site-directed mutagenesis and expressed in HEK293 cells alone or in combination with known interaction partners. Co-immunoprecipitation was performed to investigate protein-protein interaction. CRISPR/Cas9 was used to generate truncations in endogenous *PTCH1* at S1223 in HEK293 and SW620 cells. Autophagic flux was investigated by bafilomycin A1-sensitive level of LC3BII and p62. Cell proliferation was determined by WST1 assays. GLI-luciferase assays were performed in *Ptc1* KO fibroblasts transfected with *PTCH1* variants.

Results and Discussions

Interaction with the ubiquitin E3 ligase ITCH was disrupted in all *PTCH1* truncation mutants, resulting in increased protein stability. However, none of the mutants showed an impaired capacity to inhibit GLI transcriptional activity. GRB2 and β -arrestins1/2 interaction was greatly reduced in the *PTCH1* Y1316 and R1308 mutants and lost in the *PTCH1* S1203 mutant, consistent with an increased basal ERK phosphorylation in CRISPR/Cas9 cells expressing the *PTCH1* CTD truncation. Interaction of *PTCH1* with ATG101 was disrupted in all mutants. Concomitantly, basal and amino acid starvation-induced autophagic flux was enhanced in HEK293 cells overexpressing each mutant and in HEK 293 and SW620 cells expressing endogenous mutated *PTCH1* compared to their isogenic WT *PTCH1* cells. Proliferation was increased in *PTCH1* mutant cells in the absence of glucose or presence of glycolysis inhibitors 2-deoxyglucose and 3-bromopyruvate or rapamycin, an inducer of autophagy. Apoptosis induced by glycolysis inhibition was also reduced.

Conclusion

The most frequent cancer-associated *PTCH1* CTD truncations dysregulate the *PTCH1* interactome, leading to enhanced autophagy, adaptation to glycolysis inhibitors, and ERK activation, which might contribute to cancer cell fitness.

EACR22-0202

STAT5 is a novel downstream effector in PDGFR β positive Anaplastic Large Cell Lymphoma

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Introduction

Anaplastic large cell lymphoma (ALCL) is an aggressive non-Hodgkin T-cell lymphoma commonly diagnosed in children. A majority of ALCL tumors harbour the translocation t(2;5), resulting in the fusion of *Nucleophosmin (NPM)* and *Anaplastic lymphoma kinase (ALK)* genes. Our lab identified the AP-1 transcription factors cJUN and JUNB, as downstream effectors of the oncogenic NPM-ALK protein and responsible for the transcriptional regulation of *Platelet derived growth factor receptor beta (PDGFRβ)*. Strikingly, therapeutic inhibition of PDGFRβ with imatinib resulted in rapid and sustained remission in late-stage therapy-resistant ALCL ALK⁺ PDGFRβ⁺ patients, however the underlying molecular mechanisms remain elusive.

Material and Methods

To address this mechanism, we generated a transgenic mouse model that mimics human ALK⁺ ALCL expressing or lacking PDGFRβ and manipulated PDGFRβ levels in primary tumor cells using CRISPR/Cas9. Due to the interwoven signalling cascades activated by PDGFRβ and NPM-ALK we developed kinase dead mutants to decipher the downstream targets of each kinase. Having established a kit of *in vivo* and *in vitro* tools we subjected our models to a variety of biological tests including pharmacological kinetic assays, cytokine immuno-arrays and xenograft growth-assays. Finally, we confirmed our findings using human patient biopsies and large cohort data sets.

Results and Discussions

Our data provides evidence that PDGFRβ signals upstream of STAT5 whereas NPM-ALK is responsible for STAT3 activation in ALCL. The PDGFRβ/STAT5 axis offers malignant cells a selective advantage boosting their oncogenic potential. Mechanistically, we identify two modes of action responsible for the observed phenotype. Firstly, PDGFRβ⁺ tumors evade apoptosis via Bcl-x_L fomenting an uncontrolled growth. Secondly, PDGFRβ⁺ tumor cells induce an autocrine-dependent proliferation mediated *via* IL-10. Inhibition of STAT5 activity with AC-4-130 effectively inhibits tumor development and attenuates IL-10 secretion. Our findings hint towards a sequential involvement of STAT signalling in ALCL and suggests imatinib treatment is a successful therapeutic strategy as it targets alternative signalling pathways in end-stage tumors.

Conclusion

We identify a novel axis associated with an aggressive ALCL sub-entity and provide a rationale for the inclusion of PDGFRβ and STAT5 expression as standard diagnostic markers of ALCL. Furthermore, inhibition of PDGFRβ and/or STAT5 represents an effective strategy for the treatment of refractory ALCL patients.

EACR22-0400

Enhanced EDAR signalling alters the immune profile during skin tumourigenesis to encourage development of aggressive squamous tumours

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Introduction

Ectodysplasin A Receptor (EDAR) signalling is directly associated with Wnt and Sonic hedgehog pathways – both of which have been implicated in tumourigenesis. As an NF-κB activator, this pathway is also associated with inflammation – known to be tumour-promoting. Furthermore, EDAR signalling has recently been shown to promote carcinogenesis of colon

and breast, via Wnt regulation. In addition, preliminary work in the Finegan lab has identified increased papilloma conversion to malignant cutaneous squamous cell carcinoma (SCC) as well as altered colon histology in EDAR-overexpressing mice versus wild type – highlighting a role for EDAR signalling in promotion of skin and colorectal cancer.

Due to variant EDAR, signalling activity is enhanced in East Asian compared with Caucasian populations. Moreover, East Asian populations exhibit higher rates of squamous cell versus adeno-carcinomas compared with typically Caucasian populations.

Our work aims to explore *how* EDAR signalling increases susceptibility to skin and colorectal cancers. This will allow us to understand how tumours form in different patient populations, driving personalised treatment decisions and indicating potential issues with use of EDAR activation agents.

Material and Methods

A single topical dose of 25ug DMBA was followed by bi-weekly topical 6ug TPA in EDAR-overexpressing (951) and wild type (FVB) mice. IHC, FACS and qPCR were employed to explore molecular and inflammatory changes during carcinogenesis.

Results and Discussions

Whilst wild type developed earlier papillomas in greater numbers than 951 mice, tumour progression (papilloma-SCC) enhanced with EDAR overexpression. EDAR-mutant papillomas also exhibited more squamous features as well as increased proliferation and macrophage infiltration.

PBMC analysis prior to tumour formation revealed more circulating neutrophils in wild type than 951 mice. This matched with widespread neutrophil staining in wild type skin via IHC. However, whilst fewer neutrophils were seen across EDAR-mutant skin, more neutrophil-containing pustules were observed.

Given that chemokines CXCL10/11 are EDAR targets, such signalling may initially enhance immune responses against cancer cells, before encouraging recruitment and coercion of immune cells for tumour promotion.

Conclusion

Enhanced EDAR signalling alters immune contexture during skin tumourigenesis to create more squamous and aggressive papillomas – primed to form malignant SCC. This has significant implication for human populations with variant, hyper-active EDAR.

EACR22-0458

Decoding Post-Translational Modifications of Notch3 regulating protein activity and stability.

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Introduction

Notch signaling is a conserved pathway whose deregulation has been implicated in the development of several diseases, including cancer. In particular, it has emerged as a promising candidate for innovative target therapies. Notably, a growing body of evidence supports the notion of Post-Translational Modifications (PTMs) as a *modus operandi* controlling Notch activity. Indeed, PTMs play a pivotal role in regulating protein function as they modulate protein activity and stability. Therefore, the exploitation of Notch receptors PTMs is emerging as a novel therapy approach, that allows the prediction of the

interaction between Notch and other proteins. In this field, the peptidyl-prolyl *cis/trans* isomerase Pin1 is able to induce PTMs on its substrate recognizing and binding specific phosphorylated residues. In this scenario, we previously demonstrated that Pin1 positively regulates Notch3 (N3) protein expression in T-cell acute lymphoblastic leukemia (T-ALL) aggressiveness and progression and we wondered whether and how N3-Pin1 cross-talk might occur also in ovarian cancer (OC) context. The main aim of this study is the dissection of key molecular oncogenic mechanisms involved in Pin1/N3 cross-talk which may impinge on tumor progression in OC context.

Material and Methods

Exogenous systems (HEK293T and HEK293T-Pin1KO cells) and endogenous systems (OVCAR3 and SKOV3 OC cells); Transfection; Co-immunoprecipitation assay; Mass-spectrometry (MS) analysis; Insertional mutagenesis.

Results and Discussions

Firstly, we evaluated which aminoacids are responsible for the interaction between Pin1 and N3 by MS and protein conformational analysis. Moreover, we documented that Pin1 sustains the expression of N3 intracellular domain by preventing its proteasomal degradation, promoted by the GSK3 β kinase. Finally, we investigated the potential relevance of the Pin1/N3 cross-talk in allowing the escape from this negative regulation process in OC context, through the evaluation of the N3 protein stability and ubiquitination status.

Conclusion

Our findings showed the presence of two regulators of N3 protein expression, acting one as a positive regulator (Pin1) and the other one as a negative regulator (GSK3 β), suggesting an antagonistic effect that affects the N3 stability.

EACR22-0548

Her2 interaction domain mutations in breast cancer patients are responsible for receptor switch and therapy failure against Her2 targeted medicines

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Introduction

Targeted therapies against HER2 subtype breast cancer (BC) like Trastuzumab and Neratinib, often become futile as patients relapse with aggressive disease. Receptor switching to other HER family members in Lapatinib resistance and HER2 receptor tyrosine kinase domain mutations have been elaborated in promoting resistance. Here we studied the structural and functional relevance of patient specific hotspot HER2 dimerization domain mutations (DDM), which in turn impacts HER2 targeted drug response.

Material and Methods

Analyzing TCGA datasets, HER2 domain II and IV mutants S305C, G309A, S310Y, S310F and P523S were identified. A cumulative 15 μ s MD simulations done for all protein structures. Immuno-blotting and co-IP were performed in HER2 low (MCF7 and ZR75-1) and

high (BT474 and AU565) BC cell lines with +/- Heregulin condition. Mutant HER2 overexpressing ZR75-1 clonal cell lines were selected and used for *in-vitro* and *in-vivo* validation experiments. TRIM-Away method was optimized using HER3 mAb. Altered dimerization and signal activation verified by PLA (Proximity Ligation Assay) and BRET (Bioluminescence resonance energy transfer) biosensor respectively. Response to Her2 targeted medicines, i.e. Trastuzumab, Lapatinib and Neratinib were measured by other standard methods.

Results and Discussions

MD simulation revealed, except S305C, all other DDMs formed unstable HER2 homo-dimer, thereby switching to HER2:HER3 heterodimer. This was further validated by co-IP and PLA assessments in BC cells. TRIM21-mediated HER3 protein knockdown demonstrated specificity of interaction in BC cells. Shift in signalling arm from MAPK activation in cases of WT or S305C mutants to AKT activation in G309A, S310Y and P523S mutants observed in BC cells and TCGA patient cohorts. BRET biosensor determined initial AKT activation predominantly in G309A (3-fold), S310Y (2.5-fold) and P523S (3-fold) expressing BC cells showing interaction switching to HER3; whereas conventional MAPK activation was observed in WT (3-fold) and S305C (3.5-fold) expressing cells. BC cells over-expressing HER2-WT or S305C were found sensitive to Trastuzumab ($P>0.001$) or Neratinib ($P>0.001$), whereas cells with G309A, S310Y or P523S mutation were non-responder to those drugs as revealed by short-term, long-term viability, anchorage-independent growth and Immunoblot assessments.

Conclusion

The structural changes ensued after HER2 interaction domain mutations alters the downstream signalling cascades and impacts the therapy response to HER2 targeted medicines.

EACR22-0832

Crosstalk Between PLCy1 and Estrogen Receptor Modulates the Prognosis and Therapy Resistance of Luminal Breast Cancer

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Introduction

PLCy1 is a direct downstream effector of receptor and non-receptor tyrosine kinases and catalyzes the hydrolysis of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). PLCy1 is involved in the tumorigenesis and progression of different types of cancer, including breast cancer (BC). Previous studies showed that PLCy1 downregulation in BC cell lines resulted in a strong inhibition of metastasis formation in nude mice. Additionally, overexpression of PLCy1 is a risk factor for distant relapse in early luminal A and B BC patients. Our previous work showed that PLCy1 induces cetuximab resistance in metastatic colorectal cancer by activating ERK and AKT pathways. Therefore, this work aims to understand how PLCy1 can impact the prognosis and therapy resistance of luminal BC.

Material and Methods

In silico analysis of the cancer genome atlas (TCGA) BC dataset and an independent cohort of luminal A and B BC patients were performed to correlate PLCy1 expression and BC prognosis. *In vitro* cellular models of parental and PLCy1 knock-down (KD) BC cell lines

were used to investigate the role of PLC γ 1 in cell viability, drug resistance, and downstream signaling pathways.

Results and Discussions

Kaplan-Meier analysis of BC cohort from TCGA revealed that PLC γ 1 expression negatively correlates with patient's overall survival in Luminal A BC. Additionally, *in silico* analysis of PLC γ 1 dependency across different BC cell lines, showed that only luminal A cell lines are negatively affected by PLC γ 1 deletion. These suggest a higher dependence of luminal A BC subtype on PLC γ 1 expression. *In vitro*, PLC γ 1 KD in Luminal A BC cell lines decreased their viability. Additionally, PLC γ 1 KD was able to increase the sensitivity of cells to both tamoxifen and fulvestrant treatment. Western Blot analysis showed that PLC γ 1 KD decreases estrogen receptor (ER) α and ER β expression. Stimulation of cells with β -estradiol showed an increase in PLC γ 1 expression and its activating phosphorylation. Moreover, co-immunoprecipitation and proximity ligation assay showed the binding of PLC γ 1 to ER α in the presence of β -estradiol.

Conclusion

Our results suggest an important role of PLC γ 1 in estrogen-mediated signaling and hormone therapy resistance. These findings can have a significant impact on the prognosis and treatment of luminal BC patients and deserve further investigation.

EACR22-0886

New ways to target the ERK MAPK optimum in malignant melanoma

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Introduction

Oncogenic *BRAF* and *NRAS* mutations activating the ERK mitogen-activated protein kinase (MAPK) pathway are drivers of metastatic melanoma. However, due to the high activating potential of the mutations, the intensity of ERK MAPK signaling must be limited by various negative feedbacks to allow for optimal tumor growth. Therefore, we studied the MAPK optimum in melanoma to identify new essential players controlling the ERK pathway in this aggressive cancer.

Material and Methods

We performed analyses of melanoma cell response to compounds targeting a new essential negative feedback in the ERK pathway using Western blotting and NRAS- and BRAF^{V600E}-mutant human melanoma cell lines stably transfected with ERK activity luciferase reporter constructs.

Results and Discussions

Our results suggest that the ERK MAPK optimum established in malignant melanoma cells bearing oncogenic *BRAF* and *NRAS* mutations is unexpectedly low compared to the total signaling capacity of the ERK pathway in these cells. Furthermore, we show that small molecule compounds can be used to disrupt the control of the MAPK optimum in melanoma.

Conclusion

We identified a new molecular mechanism essential for controlling the MAPK optimum in melanoma. Its pharmacological disruption leads to potently dysregulated ERK signaling.

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EACR22-0215

Disentangling ERBB signaling in breast cancer subtypes - a model-based analysis

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Introduction

Targeted therapies have shown striking successes in the treatment of cancer. However, their specific effects on an individual tumor seem to be variable and difficult to predict. Using an integrative modeling approach that combines mechanistic and regression models we have characterized response mechanisms of breast cancer cells to different growth factor - drug combinations.

Material and Methods

We acquired time-resolved total and phospho-proteomic data of ERBB-receptors as well as of components in downstream MAPK as well as PI3K signaling initially within two luminal breast cancer cell lines, T47D and MCF7. To this end, we tested the response, i.e. quantities and phosphorylation, of proteins in experiments employing an array of four growth factors and five targeted drugs. The data was used to calibrate a mathematical model, capturing signaling at the receptor and downstream pathway levels. The model was then extended to triple negative and HER2-positive breast cancer cell lines, again using experimental proteomic data, and finally used to predict the impact of drug-combinations on the proliferation phenotype of the cell lines.

Results and Discussions

A total of 33,000 datapoints were collected, in experiments testing 80 different conditions and ten time points each. To correctly fit the experimental data, the model requiring adjustments mostly for the respective receptor composition in the respective cell lines. Hot-spot mutations in MAPK and PI3K pathway components that were present in some cell lines needed to be accounted for, to make the model applicable to all three subtypes breast cancer having been tested. The relevance of the oncogenic mutations was identified via L1 regularization. Finally, we predicted and experimentally validated the proliferation response of cells to drug co-treatments.

Conclusion

Based on quantitative proteomic experimental data we have developed a unified mathematical model that can describe ERBB receptor and downstream MAPK- and PI3K-signaling in response to therapeutic drugs targeting this clinically relevant signaling network in cell lines representing three major subtypes of breast cancer. Some cell-line, drug, and growth factor-specific effects have been uncovered.

EACR22-0311

Molecular dissection of GSK3 β -WWP2 crosstalk to downregulate Notch3 activity in

Ovarian Cancer

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Introduction

Ovarian cancer (OC) is the fifth leading cause of female cancer-related deaths. Curative and survival trends have not changed significantly because of lack of a definitive screening tool and clear symptoms to prevent early diagnosis. In this scenario, the identification and characterization of targets for novel therapies are urgently required. It is known that Notch signaling pathway has a crucial role in OC. Notably, over-expression of Notch3 (N3) receptor, among the four Notch receptors, has been detected in a wide panel of OC. Specifically, an increasing number of studies exhibited N3 involvement in the regulation of epithelial-to-mesenchymal transition (EMT), maintenance of cancer stem cells (CSCs) and cancerogenesis. Therefore, N3-specific inhibition may represent a promising therapeutic strategy for OC cancer treatment. The main aims of this work are: 1. to investigate the molecular mechanism responsible for E3-ligase WWP2-dependent negative regulation of N3-intracellular domain (N3_{ICD}) and 2. to analyze the crosstalk between WWP2 and the GSK-3 β kinase, already identified, in our preliminary studies, as a N3-negative regulator in OC context.

Material and Methods

We performed: insertion and deletion mutagenesis of N3, co-transfection experiments and co-immunoprecipitation assay.

Results and Discussions

Firstly, we assessed the interaction between N3_{ICD} and WWP2 proteins by bi-directional co-immunoprecipitation assay. Furthermore, we identified other N3_{ICD} domain, in addition to N3_{ICD} domain containing WWP2-degron site, involved in the WWP2-N3 interaction. Additionally, we evaluated the functional activity of WWP2 upon N3_{ICD}, proving that WWP2 is able to induce the poly-ubiquitination of N3_{ICD}, thus sustaining its involvement in N3_{ICD} degradation. Therefore, we tested if the presence of WWP2 could amplify the negative regulation of GSK-3 β on N3_{ICD}. We documented that GSK-3 β and WWP2 induced higher decrease of N3_{ICD} protein expression with respect to the presence of the GSK-3 β alone. In addition, we also demonstrated that the presence of GSK-3 β increased the interaction between WWP2 and N3_{ICD}. Taken together, these findings strongly supported that the E3-ligase WWP2 is implicated in the GSK-3 β -dependent proteasomal degradation of N3_{ICD}.

Conclusion

Overall, our data will help us to identify and validate new molecular targets involved in negative regulation of N3 oncogenic pathway, finally to develop novel therapeutic strategies.

EACR22-0451

hERG1/b1 integrin interplay: how a non-canonical mechanosensitive ion channel can become a crucial transducer of mechanical signals from the tumor microenvironment.

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Introduction

Ion channels are particularly relevant elements in cancer, mediating interactions between tumor cells and the tumor microenvironment (TME). Cells respond to mechanical cues by exerting forces and regulating their shape. We demonstrated that hERG1, aberrantly expressed in cancer cells where it regulates many aspects of neoplastic cell behaviour, is coexpressed and interacts with β 1-integrins. Based on these data hERG1, although not being a canonical mechanosensitive ion channel, can become a transducer of mechanical signals from the TME upon its link with integrins.

Material and Methods

Cells. Cells were seeded on the micropillar array and allowed to spread for 20h before fixation. After permeabilization with 0.1% Triton-X (Sigma) for 10 min and 1h blocking with 5% BSA (Sigma), actin filaments and nuclei were stained with Alexa-Fluor 561-conjugated Phalloidin and DAPI (Life Technologies). For gene expression profile PANC-1 cells were cultured for 24h on fibronectin-coated elastic substrates, in control and after blocking hERG1 with E4031.

Forces. Hexagonal arrays of PDMS (Sylgard 184, Dow Corning) micropillars were produced using replica-molding from a silicon wafer. The tops of the micropillars were coated with ECM proteins using micro-contact printing. The position of the pillar tops was observed by confocal microscopy and determined using custom software (Matlab, Mathworks). Forces were obtained by multiplying the pillar deflections by the array's characteristic spring constant.

Results and Discussions

The hERG1/integrins interaction in PDAC cells perturbs the force transduction machinery. Using elastic micropillar arrays of varying stiffness, it emerged that the disruption of this interplay increased the average percentage of deflected pillars/per cell and the average maximal forces exerted by cells on a single pillar (significantly only on stiffer micropillar arrays). These findings suggest that the hERG1/ β 1 interaction perturbs the force transduction and rigidity sensing machinery in PDAC cells. Gene expression analysis of PANC-1 cultured on substrates of varying stiffness showed that hERG1 blockade reduced the fold change of 128 unique upregulated genes, with a major effect at 1.5 and 15 kPa. Moreover, direct target genes of YAP, a known mechanosensor, were present within the de-regulated genes.

Conclusion

hERG1/ β 1 interaction alters cells response to mechanical stimuli. This gating-dependent mechanism integrates biochemical and mechanical stimuli from the TME facilitating migratory/metastatic pathways.

EACR22-0457

CXCR4/CXCL12 axis as a potential therapeutic target in mycosis fungoides - an in vitro study

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Introduction

Mycosis fungoides (MF) is the common subtype of cutaneous T cell lymphoma (CTCL). The C-X-C chemokine receptor 4 (CXCR4) is overexpressed in tumor cells of solid and hematologic neoplasms. Its ligand, C-X-C chemokine ligand 12 (CXCL12), is secreted by cells in the tumor microenvironment, mostly by stromal fibroblasts. The engagement of CXCL12 to CXCR4 results in activation of several intracellular signal transduction pathways which fate cell viability. Plerixafor, AMD3100 (AMD), is a CXCR4 antagonist that reversibly blocks the binding of CXCR4 to CXCL12. AMD is already in clinical use as bone marrow stem cell mobilizers and is under clinical trials for cancer therapy. CXCR4 and CXCL12 are known to be overexpressed in MF skin biopsies. We previously have shown that primary MF-fibroblast (MF-Fs) from MF biopsies secrete high CXCL12 which promotes the migration of MyLa (MF cell line), protects them from chemotherapy and both effects were suppressed by AMD.

Material and Methods

Primary MF-Fs were established from skin biopsies of MF patients. The expression of CXCR4 protein was tested by Immunofluorescence staining analyzed by flow cytometry and Apotome microscope. The toxicity of AMD was studied by MTT viability assay, Tripa blue staining and spheroid formation. Apoptosis assay was done by PI and annexin staining analyzed by flow cytometry. The down-stream signaling effect of AMD was analyzed by phosphoproteomics and the phosphorylation of specific proteins was confirmed by western blot.

Results and Discussions

CTCL cell lines express high membrane CXCR4 compared to CD4+ peripheral blood mononuclear cells (PBMCs) from healthy donors. MF biopsies also express high CXCR4 on the membrane of the dermal lymphocytes. AMD is more toxic to MyLa cells co-cultured with MF-Fs than MyLa alone or healthy PBMCs, and promotes their death through apoptosis. The formation of spheroids of MyLa cells with MF-Fs was disrupted by AMD. Deepen into the mechanism of the death induced by AMD in MyLa cells co-culture with MF-Fs shows that: AMD reduces the phosphorylation of the pro-survival protein- AKT leading to apoptosis; eliminates the expression of CXCR4 on the membrane of MyLa cells; decreases the phosphorylation of CXCR4 which might prevent its recycle and upload to the cell membrane.

Conclusion

Our data suggests that CXCR4/CXCL12 axis plays a pivotal role in the survival of MF and disrupting the interaction between the stroma derived CXCL12 and MF derived CXCR4 by AMD might be a new therapeutic option for MF.

EACR22-0459

USP10 is crucial for EGFR signaling in colon cancer cells

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Introduction

Colorectal cancer (CRC) ranks among the third most common human malignancies and is one of the leading causes of cancer-related deaths globally. Epidermal growth factor receptor (EGFR) signaling has been shown to participate in colon cancer development and became an attractive target for therapy with tyrosine kinase inhibitors. However, a high number of CRC patients develop resistance to anti-EGFR therapy. The reasons are largely unknown and therefore, the search for molecular targets that contribute to therapy resistance is of great importance.

Here we show that the ubiquitin-specific protease 10 (USP10), known for its association with

tumor development and progression, is involved in EGFR-mediated cancer cell growth and survival as well as in development of resistance to anti-EGFR treatment.

Material and Methods

CRISPR/Cas9 gene editing was used to generate two independent USP10-deficient human colon cancer HCT116 cell lines. Cells were grown in full DMEM, serum starved and EGF (100 ng/ml) treated when needed. To study the effect of USP10 knockout on cellular behavior and EGF responsiveness, their proliferation, colony formation as well as sensitivity to EGF and the EGFR inhibitor Gefitinib was studied by using Incucyte ZOOM®, MTT tests and immunoblotting.

Results and Discussions

Our results revealed that USP10 knockout are less sensitive to Gefitinib, a potent EGFR inhibitor. Interestingly, USP10 knockout cells displayed an increased proliferation and colony formation when compared to control cells. However, treatment with EGF promoted proliferation and colony formation only in HCT116 control cells but not in the USP10 knockout cells. These findings suggest that USP10 knockout results in the reduction of EGF sensitivity in HCT116 cells.

Indeed, our immunoblotting results verify that EGFR signaling was suppressed in USP10 knockout cells. Although the abundance and phosphorylation of EGFR in response to EGF were not affected in cells lacking USP10, phosphorylation of EGFR adaptor proteins was decreased when compared to control cells. Moreover, EGF-mediated phosphorylation, and activation of downstream signaling pathways of ERK1/2, AKT and S6 kinases were also downregulated in the cells lacking USP10.

Conclusion

Our data show that the deubiquitinase USP10 plays a crucial role in CRC cellular behavior by suppressing EGFR signaling. Although the precise mechanism of this phenomenon is yet to be discovered, the already obtained data reveal a new player that could contribute to EGFR inhibitor resistance in cancer.

EACR22-0533

EWSR1-FLI1-driven dysregulation of heparan sulfate proteoglycan metabolism drives ERK signaling in a novel genetic zebrafish model of Ewing sarcoma

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Introduction

Ewing sarcoma (ES) is a pediatric cancer of bones or soft tissues with extremely poor prognosis for patients with metastatic or relapsed disease. ES is associated with the appearance of one of several chimeric fusion oncogenes, most frequently *EWSR1-FLI1* (*EF1*) driving cell transformation. In other cancers, it has become evident that tumor microenvironment including extracellular matrix (ECM) and secreted soluble factors are important in tumor initiation and progression. It was shown that soluble products (growth factors, cytokines) could be released from the tumor, altering gene expression in surrounding cells. The dependence of a tumor cell on such microenvironment opens new possibilities to target not just cancer cells but also the tumor microenvironment itself. In the absence of a representative *in vivo* model, the role of tumor cell/microenvironmental interactions in Ewing sarcoma initiation and progression is not well-understood.

Material and Methods

To address these concerns, we developed a new *in vivo* genetic model of Ewing sarcoma by integrating the human EWSR1-FLI1 fusion oncogene into the zebrafish genome. Our system allows broad mosaic Cre-inducible expression of human EF1 in zebrafish, which causes rapid onset of Ewing sarcoma at high penetrance.

Results and Discussions

We found that observed tumors express canonical EF1 target genes and stain for known Ewing sarcoma markers including CD99 and PAS and histologically recapitulate key features of human ES. Proteomic analysis revealed that *EF1* expression affects the expression of enzymes involved in heparan sulfate proteoglycan (HSPG) metabolism. Proteoglycans are key molecular effectors of cell surface playing an essential role in signal transduction between cells and regulating signaling from cell surface receptors. We found that dysregulation of proteoglycan metabolism is associated with activation of ERK1/2 signaling pathway in tumor cells resulting in increased tumor cell proliferation and survival. Targeting heparan sulfate proteoglycans with the specific heparan sulfate antagonist Surfen reduces ERK1/2 signaling and decreases cancer cell proliferation and survival *in vitro* and *in vivo*.

Conclusion

Taken together, our novel zebrafish model will open new possibilities to study EWSR1-FLI1-driven cell transformation, as well as mechanisms of tumor development and progression. Our results highlight the emerging role of the heparan sulfate proteoglycans in ES tumor growth. That makes HSPGs attractive an attractive novel target for Ewing sarcoma treatment.

EACR22-0555

The molecular basis for rational targeting of FGFR-driven growth and invasiveness

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Introduction

Receptor tyrosine kinases (RTK) promote growth, survival and dissemination in paediatric tumours including medulloblastoma (MB). We hypothesised that repression of protein-protein interactions downstream of activated RTKs could constitute a novel means to inhibit oncogenic signal transmission. Therefore, we delineated molecular interactions downstream of FGFR and identified and validated small bioactive molecules that repress FGFR-driven growth and invasiveness.

Material and Methods

Using 3D *in vitro*, *in vivo* and cerebellar slice culture models, we identified the FGFR signaling pathway as a driver of growth and invasiveness in MB. Using proteomics and functional assays, we determined key molecular components driving FGFR-induced growth and invasiveness. Finally, by computational screening combined with functional, biophysical, and structural binding analyses, we discovered and validated small molecule compound inhibitors of FGFR-driven growth and invasiveness.

Results and Discussions

We identified the FRS2 protein as an essential up-stream effector of FGFR signaling towards invasiveness in MB. Downstream of FRS2, we found that the Striatin 3 protein integrates antagonistic growth and invasion signals emanating from activated FGFRs. Mechanistically, Striatin 3 interacts with the pro-invasive kinase MAP4K4, couples it to the protein phosphatase 2A, and thereby inactivates growth repressing activities of MAP4K4. In parallel, Striatin 3 enables MAP4K4-mediated phosphorylation of PKC θ and VASP, which combined are necessary to promote tissue invasion. To selectively repress pro-invasive FGFR functions, we identified and functionally validated small molecule ligands of FRS2, that prevent FGFR-induced FRS2 activation and downstream signaling. We demonstrate efficacy of these compounds in inhibiting invasion and growth promoting activities *in vitro* and *in vivo*, confirmed on-target activity and identified potential off-targets of the ligand using a proteome-wide interaction analysis.

Conclusion

Repression of STRN3-MAP4K4 cooperation could reactivate hippo signaling and repress invasiveness. We furthermore describe inhibition of FRS2 by a small molecule ligand as a strategy to repress FGF signaling in FGFR-driven tumours. The development of this ligand, and the *de novo* design of functional analogs thereof bear promise for further pre-clinical evaluation of these structures as anti-growth promoting and anti-metastatic therapeutics applicable to FGFR-driven tumours.

EACR22-0599

Looking for novel interactors of IL-13 receptor $\alpha 2$ (IL13R $\alpha 2$) and tyrosine-protein phosphatase non-receptor type 1 (PTPN1/PTP1B) by using proximity-dependent biotinylation (BioID)

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Introduction

IL13R $\alpha 2$ is a high affinity receptor for IL-13 that is re-expressed in late neoplastic stages. The IL-13/IL13R $\alpha 2$ axis triggers invasion in colorectal cancer (CRC) and other cancers in a process mediated, among others, by the phosphatase PTPN1 (aka PTP1B), involved in the dephosphorylation of the autoinhibitory Src Tyr416 and further Src activation. PTPN1 takes part in IL13R $\alpha 2$ internalization and associates with poor outcome in glioblastoma and CRC patients. Inhibition of IL13R $\alpha 2$ pathway decreases invasion of multiple cancer cell lines (i.e., SKOV-3, PC-3 and KM12SM). To better define the oncogenic properties of IL13R $\alpha 2$ and PTPN1, we aim to identify novel interactors by using proximity-dependent biotinylation coupled to mass spectrometry.

Material and Methods

PTPN1 and IL13R $\alpha 2$ were cloned into pcDNA.5-pDEST-BirA-Flag-Ct by using LR clonase enzyme, obtaining the desired plasmids containing fusion genes PTPN1-BirA and IL13R $\alpha 2$ -BirA. Flip-In™ T-REx™ cells were co-transfected with pOG44 and pcDNA.5 vectors and selected with hygromycin. Then doxycycline and biotin were added to induce the expression of the fusion gene and proximity labelling. Biotinylated proteins were purified by affinity using streptavidin-sepharose, trypsin-digested and analysed in a Q-Exactive mass spectrometer. Raw data were analysed with MaxQuant, followed by Perseus for statistical analysis and SAINT for probabilistic scoring of protein-protein interaction data, which were subjected to gene ontology (GO) analysis.

Results and Discussions

For IL13R α 2, 735 proteins were identified and 94 were scored as significant interactors by SAINT. GO enrichment analysis showed association with cadherin binding and response to reticulum stress, among others. 1009 proteins were identified and 217 were scored as significant PTPN1 interactors. GO enrichment analysis showed important association with mitotic cell cycle regulation, such as CDK1 (G2/M transition) or PCTN (centrosome component). Among the 19 IL13R α 2 and PTPN1 common interactors stand out those related to SNARE complex (YKT6, SNAP29 and VAMP3), which is involved in regulation of cancer invasion, drug resistance and kinase phosphorylation. Another interesting finding was the presence of VRK2, a kinase involved in MAPK pathway.

Conclusion

This study reveals novel individual interactors of IL13R α 2 and PTPN1, but also identifies common interactors of both proteins such as members of the SNARE complex and VRK2, opening new strategies for the inhibition of IL13R α 2 and PTPN1 in cancer metastasis.

EACR22-0805

The emerging role of the dual-specificity tyrosine-regulated kinase 2 (DYRK2) regulating FBXW7 tumor suppressor in cancer development and progression

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Introduction

Dual-specificity tyrosine-phosphorylation-regulated kinase 2 (DYRK2) is considered a key protein in the regulation of several master signalling pathways such as DNA damage response, cell cycle control and proliferation. FBXW7, an E3 ubiquitin ligase, is considered a main tumor suppressor due to its ability to control proteasome-mediated degradation of several oncoproteins such as c-JUN, c-MYC, mTOR and NOTCH1. FBXW7 inactivation in human cancers results from somatic mutation or downregulation of its protein levels. Here we describe a novel regulatory mechanism for this relevant tumor suppressor dependent on DYRK2 with direct consequences in tumor progression and in cancer development.

Material and Methods

Experiments were performed using a panel of representative human cancer cell lines. Silencing of DYRK2/FBXW7 experiments were performed using a pool of siRNA against these proteins and by CRISPR/Cas9 models. Localization of DYRK2/FBXW7 was visualized by immunofluorescence experiments. DYRK2/FBXW7 expression was analysed by immunoblotting. Functional consequences were analysed by pharmacological inhibition of DYRK2, cell viability, clonogenic survival and drug resistant assays.

Results and Discussions

We demonstrate how DYRK2 regulates FBXW7 expression at post-transcriptional level in a kinase activity-dependent manner via phosphorylation after interaction. This regulation is proteasome-mediated and independent of FBXW7 ubiquitin ligase activity. We prove how

these events happen in an oncogenic stress response context, involving the DNA damage response pathway (DDR), but not affecting dimerization capacity. These data were verified modifying DYRK2 expression using siRNAs and the generation of different DYRK2 KO cell lines. We show that DYRK2 profoundly enhances degradation of multiple FBXW7 substrates, implicating this kinase as a general modulator of FBXW7 activity with consequences in tumor progression and proliferation. Finally, the DYRK2-dependent regulation of FBXW7 protein accumulation in response to DNA damage agents contributes to cytotoxic effects. Pharmacological manipulation of DYRK2 levels modulates substrates expression, highlighting this new axis as a suitable target in cancer patient treatment.

Conclusion

We reveal a novel regulation mechanism for the relevant tumor suppressor FBXW7 which might help us to better understand its role in cancer biology, with important consequences on cell-cycle control and DNA damage response pathway in cancer cells.

EACR22-0893

BioID-based proximity labeling identifies ALK interactors in neuroblastoma cells

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Introduction

Alterations of the Anaplastic lymphoma kinase (ALK) such as point mutations, intragenic deletions, genomic rearrangements and gene amplifications have been described in human cancers including neuroblastoma (NB). ALK-associated downstream pathways are highly diverse and it is not clear how ALK oncogenes become wired to different downstream effectors. Analysis of receptor-associated signaling complexes in the context of wild-type and oncogenic ALK activation provides important information for the development of co-therapeutic approaches to target ALK-driven cancers.

Material and Methods

Proximity-dependent biotin identification (BioID) is a powerful method for mapping protein-protein interaction networks. BirA is a biotin protein ligase which converts biotin to reactive biotinoyl-5'-AMP and labels lysine residues of substrates with biotin. BioID utilizes a mutant form BirA* (BirAR118G) which has lower affinity for reactive biotin compared to wild-type BirA and prematurely releases reactive biotin resulting in biotinylation of proximal proteins. Biotinylated proteins are isolated by streptavidin pull-down and identified by mass spectrometry. We have employed BioID to identify and characterize components of ALK signaling in NB cells.

Results and Discussions

To identify the ALK proximitome, we generated two inducible Tet-on NB cell lines expressing ALK.BirA* fusion and compared biotinylated proteins in these cells that were stimulated with ALK ligand in the presence or absence of ALK inhibitor. Known ALK downstream signaling molecules were identified as well as novel candidate proteins. Interaction of SHP2 and PEAK1 with ALK were validated by anti-ALK immunoprecipitations. These interactions were increased in the presence of ALK ligand and abrogated by ALK inhibitor showing that they are ALK activity dependent. Increased SHP2 phosphorylation in response to ALK activation and decreased SHP2 phosphorylation upon ALK inhibition confirmed that SHP2 is a

downstream target of ALK in NB cells. Furthermore, combined inhibition of ALK and SHP2 synergistically abrogated NB cell growth.

Conclusion

BioID-based labeling identified an ALK proximitome in NB cells including known interactors as well as novel potentially important candidates. We have validated PEAK1 and SHP2 as ALK interactors, and further shown that SHP2 activity is important for the growth and survival of ALK-driven NB cells. Importantly, combined ALK and SHP2 inhibition acts synergistically in NB cells making it a potential therapeutic approach for ALK-driven NB.

EACR22-0963

Purified Venom Components Inhibit EGFR Phosphorylation in Triple Negative Breast Cancer

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Introduction

Breast cancer remains the most commonly diagnosed type of cancer in both menopausal age women and adolescent/young adults. 10-20% of diagnosed breast cancers are deemed to be triple negative (TN), lacking expression of hormone receptors and HER2. Triple negative breast cancers (TNBC) present with poor patient prognosis, through their lack of effective treatment options. Studies report that epidermal growth factor receptor (EGFR) is expressed in 15–45% of all breast tumours and its expression is inversely related to hormone receptor expression. Expression of EGFR is indicative of poor prognosis, making it an attractive target for treatment in both TN and receptor expressing cancer subtypes. Development of resistance to current EGFR targeted therapeutics is common, leading to treatment failure and patient relapse, thus novel compound classes are needed. Venom peptides have evolved to be secreted into the lumen of the venom gland and stored ready for rapid delivery; therefore they are exceptionally stable. These proteins naturally act as ligands for a large variety of receptors and ion channels, making them a rich source of potentially novel drug like molecules

Material and Methods

In this study the Venomtech Targeted-Venom Discovery Array^{EGFR} (T-VDATM) containing 320 venom 2D HPLC fractions was screened using Abcam Human EGFR (pY1086) + total EGFR ELISA Kits to identify venom peptides with antagonistic activity against EGFR pY1086 phosphorylation. Optimal cell number, dosing and lysate concentrations were determined empirically. MDA-MB-468 TNBC cells were dosed at 20ug/ml for 2h with fractions, before being stimulated with 1x10⁻⁷M EGF for 5 mins. Further assay protocol was carried out as per manufacturer's instructions. TMB Absorbance signal was measured using a CLARIOstar^{PLUS} plate reader at 450nm (BMG LabTech)

Results and Discussions

Screening of the TVDA^{EGFR} array (N=2) identified 7 hit fractions (2.2% hit rate) from the venoms of three distinct rattlesnake genera from Northern, Central and southern America, and 1 viper species from Northern Africa. These fractions were followed up with dose response, mass spec and drug like properties

Conclusion

Purified protein components from the venoms of pit vipers and vipers show the capacity to antagonistically inhibit the phosphorylation of EGFR at specific tyrosine residues linked to downstream signalling pathways in TNBC cells. Thus, representing a novel chemical class for targeting EGFR

EACR22-1005

Regulation of ALK activity by CD45 phosphatase in anaplastic large cell lymphoma

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Introduction

Anaplastic Large Cell Lymphoma (ALCL) is a subtype of non-Hodgkin lymphoma frequently driven by the chimeric tyrosine kinase NPM-ALK, generated by the t(2;5)(p23;q35) translocation. While ALK+ ALCL belongs to mature T cell lymphomas, loss of T cell identity is observed in the majority of ALCL secondary to a transcriptional and epigenetic repressive program induced by oncogenic NPM-ALK. CD45 is a phosphatase that plays a central role in T cell activation by controlling the JAK/STAT pathway and exists in different isoforms depending on the stage of T-cell maturation, activation, and differentiation. ALK+ ALCL cells mainly express the isoform CD45RO in keeping with their mature/memory T cell phenotype. Because of its regulatory effect on the JAK/Stat pathway that is essential for ALK+ ALCL, we investigated if CD45 expression was affected by oncogenic ALK.

Material and Methods

Human ALK+ ALCL cell lines were analyzed for CD45 expression by flow cytometry, Western Blot, and qRT-PCR. ALK signaling was abrogated by an inducible specific ALK short hairpin RNA (shRNA) or by the ALK degrader TL13-112. CD45-KO ALCL cell lines were generated using CRISPR/Cas9-mediated gene targeting. ChIP-seq analysis was performed on two ALK+ ALCL cells treated with the ALK TKI crizotinib. SD36 degrader was used to induce STAT3 degradation in ALCL cell lines.

Results and Discussions

We demonstrated that ALK+ ALCL cell lines were CD45RO^{high} but CD45RA^{low} and NPM-ALK differentially regulated the expression of these CD45 isoforms. Regulation of CD45 expression was dependent on ALK kinase activity as CD45RO expression was increased when NPM-ALK kinase activity was inhibited by treatment with ALK tyrosine kinase inhibitors (TKIs). Silencing ALK expression through shRNA or degradation of ALK by the PROTAC TL13-112 caused upregulation of CD45RO both at mRNA and protein levels with minimal changes on CD45RA, indicating that oncogenic ALK represses the expression of CD45. CD45 repression was mediated by STAT3 as demonstrated by ChIP-seq data on ALCL cells treated with the ALK-TKI crizotinib and validated on cells treated with a STAT3 degrader. Next, we found that knocking-out CD45 with the CRISPR/Cas9 system resulted in increased resistance to ALK TKI treatment and CD45 was spontaneously down-regulated in ALCL cells that developed resistance in vitro to ALK TKIs.

Conclusion

Overall, these data suggest that CD45 expression is regulated by ALK via Stat3 and acts as a rheostat of ALK oncogenic signaling and resistance to TKI treatment in ALCL.

PDK1, a novel target for KRAS-mutated metastatic colorectal cancer

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Introduction

Colorectal cancer (CRC) is the third most diagnosed type of cancer worldwide and the second kind of cancer causing more mortality. It is estimated that a 40% of CRCs bear a mutation in KRAS, making them resistant to conventional EGFR blockage therapy. Unfortunately, direct pharmacological inhibition of mutated KRAS still remains an unsolved challenge. Thus, the inhibition of a downstream effector of RAS-MAPK pathway might be an interesting pharmacological approach to treat KRAS-mutated cancers. PDK1 is a protein implicated in PI3K-AKT and RAS-MAPK pathways, which has been shown to be critically involved in KRAS-mutated pancreatic cancer. We investigated the role of PDK1 in KRAS-mutated CRC cells and patient-derived organoids (PDO).

Material and Methods

We used a series of CRC cell lines, both mutated and non-mutated in KRAS. We also used our collection of metastatic CRC patient-derived organoids (PDOs), both mutated and non-mutated in KRAS. We treated cells and PDOs with a wide-range of concentrations of PDK1 inhibitors for 3, 5 and 7 days and performed a viability test after the treatment using CellTiter Glo®, based on the measurement of ATP production.

Results and Discussions

We observed that both CRC cell lines and CRC PDOs respond to the inhibition of PDK1 reducing their viability. PDK1 inhibition was effective to hamper the growth of KRAS-mutated CRC cells, both in anchorage-independent and adhesive growth. Treatment with PDK1 inhibitor also reduced the ability to grow of CRC PDOs in 3D cultures, although at higher concentrations compared to 2D cultures. Looking at the mutational profile of CRC cell lines and PDOs that we tested, we found that p53 status affected the response both in CRC cell lines and PDOs.

Conclusion

We propose PDK1 as a novel target for KRAS-mutated metastatic CRC.

The homo/heterotypic interplay between Notch3 and differential levels of Jag1 modulates disease progression in epithelial ovarian cancer (EOC)

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Introduction

Cell-to-cell communication through receptor-ligand interaction forms the basis of Notch signaling. Epithelial ovarian cancer (EOC), well known for its transcoelomic metastasis, displays a dynamic microenvironment due to the presence of ascites facilitating the interplay between both tumor/tumor cells (homotypic) and tumor/cancer-associated fibroblast (CAF) (heterotypic). Still, the underlying functional and molecular cues of these tumor

microenvironmental regulations on EOC progression are not fully understood. Such an uncharted aspect of tumor spread warrants further elucidation for better understanding the disease progression.

Material and Methods

We developed a co-culture-based model of NIH3T3 (differentially overexpressing Jagged1) and SKOV3 cells expressing Notch3-sensor [10XCSL (CBF-Su(H)-Lag2)-driven firefly luciferase] (SNFT) and employed bioluminescence-imaging for monitoring Notch-3 activity. We performed CFSE, MTT, ELISA, Matrigel-invasion, and other assays to study the cellular functions and transcriptional profiling of SNFT post-co-cultures by RT-PCR array.

Results and Discussions

We showed that incremental membranous expression of Jagged1 led to proportional Notch3 activation in SNFT as reflected by the linearly increasing luciferase signal upon co-culturing. With no basal luciferase activity, this system efficiently recorded dose-dependent Notch3 activation by rh-Jag1 peptide, and non-appearance of such induction in co-culture with NIH3T3^{Δjag1} cells indicates its sensitivity and specificity. Such signal modulation was shown for the first time in co-cultures with HGSOc patients' ascites-derived CAFs and Jagged1-expressing EOC cell lines (A2780, OVCAR3, and OAW42). NIH3T3^{J1-A} and OVCAR3 co-cultured SNFT showed maximum proliferation, invasion, and cisplatin resistance among all the heterotypic/homotypic pairs. Among the two most significantly upregulated candidates across co-culture conditions, VEGFA secretion from co-culture induced SNFT reduced cancer stem cell differentiation in platinum-resistant A2780. Upon concurrent treatment of rh-Jag1 peptide-cisplatin, p21(CDKN1A) showed maximum expression and significantly high nuclear-cytoplasmic localization compared to either of the treatments/untreated cells. Additionally, metastatic HGSOc tumors had higher VEGFA than corresponding primary tumors correlating with Notch3 status.

Conclusion

Our study thus brings out a new paradigm of the Notch3/VEGFA axis in EOC progression through the tumoral and non-tumoral cell-mediated differential Notch3 activation.

EACR22-1155

Sensitizing Liver Cancer Cells to c-Met Inhibitors via Targeting Neddylation

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Introduction

c-Met belongs to the family of receptor tyrosine kinases (RTK) and its signaling has profound role in the development and progression of liver cancer. Mechanisms that positively modulate ligand-independent and sustained activation serve to promote worse prognosis and involve post-translational modifications. Neddylation is the covalent attachment of NEDD8 to its targets and deteriorations in neddylation are recently implicated in liver cancer. We aimed to investigate the role of neddylation in c-Met activation and signaling.

Material and Methods

In this study, HGF (10 ng/ml), high glucose (25 mM) and high insulin (100 nM) were used to stimulate c-Met activation. MLN4924 and SU11274 were used as specific inhibitors for NEDD8 conjugation and c-Met activation, respectively. c-Met activation is investigated via

western blotting. Co-immunoprecipitation studies revealed interaction of c-Met and NEDD8. Erk and Akt activations point the initiation of c-Met downstream signaling. Cell proliferation and motility were studied using xCELLigence and scratch assay, respectively. *In silico* analysis revealed lysines 1232 and 1360 as potential neddylation sites on c-Met. Therefore, mutant c-Met plasmids (K1232A-K1360A) were generated, the effect of mutagenesis on c-Met activation were investigated by western blotting and co-immunoprecipitation. MDR2^{-/-} mice (16 mo) were used to represent *in vivo* liver carcinogenesis model.

Results and Discussions

We investigated neddylation pattern of HCC cells in basal conditions and have identified a specific pattern in correlation with basal c-Met activation levels. NEDD8 and c-Met were co-immunoprecipitated under conditions stimulating c-Met activation, SU11274 was shown to suppress c-Met neddylation suggesting that it is directly related to the activation status. c-Met activation and downstream signaling were strikingly blocked when both neddylation and c-Met inhibitors were used together, even in lower doses. Correspondingly, proliferation and migration capacities were also suppressed better. We also investigated lysine mutants and their effect on c-Met activation and identified that neddylation mechanism is an important regulator of c-Met signaling. Analysis of tumor vs. non-tumor tissues in chow and HFHS mice identified high NEDD8 expression and there is a significant change in activation status of c-Met in tumor tissues.

Conclusion

Preventing c-Met neddylation specifically in liver cancer patients could serve as a precisely targeted therapy method in patients with aggressive tumor.

EACR22-0626

SRF fusion genes in myoid soft tissue tumors.

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Introduction

Fusion genes are frequent genetic events in soft tissue cancer. Many of them have been described as strong drivers of tumorigenesis and effective pharmacological targets. We and others have recently identified *SRF* fusion genes as a result of chromosome translocations in different types of soft tissue tumors (such as rhabdomyosarcoma and myofibroma), mostly in children. All of them express skeletal or vascular smooth muscle biomarkers. The exact incidence of these *SRF* fusion-positive neoplasms is unknown since they are not yet routinely detected and have not been systematically studied. *SRF* encodes the serum response factor, one of the best characterized transcription factors. In all cases, the first exons of *SRF*, which encodes the DNA-binding domain, are fused with various partner genes, which encode either transcription factors, transcriptional regulators, the PDGF receptor kinase domain or proteins of unrelated or unknown function.

Material and Methods

In the present study, we characterized four *SRF* fusions: *SRF-RELA*, *SRF-FOXO1*, *SRF-ICA1L* and *SRF-PDGFRβ*. Luciferase assays were carried out on C2C12 cells, with full-length and mutated sequences of the fusions. Stable C2C12 lines expressing the fusions were also generated by retroviral transduction. Muscle differentiation was studied as well as expression of *SRF* target genes.

Results and Discussions

Our data indicated that SRF fusions strongly increased transcriptional activity. Fusions involving a transcription factor as the partner (such as RELA and FOXO1), always retain the transactivating domain in C-terminal. By point mutation or deletion of the domains of interest, we showed the crucial role of both the DNA-binding domain of SRF and the transactivating domain of the partner for transcriptional activity. Interestingly, SRF-ICA1L acted in a similar manner, ICA1L containing a cryptic transcriptional activation domain. By contrast, SRF-PDGFR β activity only relied on the tyrosine kinase domain, which was inhibited by imatinib. In addition, *in vitro* functional assays on stable C2C12 cell lines suggested that SRF fusions induce a shift in gene expression profile, leading to a block in muscle differentiation.

Conclusion

Together, these findings identified the molecular mechanisms whereby these newly reported gene fusions support the development of myoid soft tissue tumors.

EACR22-0230

LOXL2 links BRD4-Mediator nuclear foci to cell cycle transcriptional control in triple-negative breast cancer

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Introduction

Triple-negative breast cancer is the most aggressive breast cancer subtype. On top of the fact that for triple-negative breast cancer there are no effective therapeutic strategies, it also very often develops resistance to chemotherapy and metastasizes to distal organs, condemning patients to death. One strategy to circumvent the insurgence of anticancer drug resistance is the development of efficient combinatorial approaches targeting multiple key cancer proteins. Recently, it has been shown that the inhibition of either BRD4 or LOXL2 dampens breast cancer proliferation, having a more pronounced effect on the triple-negative subtype. Here, we seek to investigate whether the simultaneous inhibition of BRD4 and LOXL2 can be explored as possible triple-negative breast cancer therapy.

Material and Methods

To develop this study, we have been integrating multiple techniques including synergy assays, transcriptomic, epigenomics, and *in vivo* mouse experiments.

Results and Discussions

By combining small molecule inhibitors, we first demonstrate that the inhibition of LOXL2 and BRD4 effectively synergizes to reduce triple-negative breast cancer cell growth *in vitro* and *in vivo*. We further reveal that mechanistically, the synergism is mediated by the direct interaction of the short isoform of BRD4 with LOXL2. Such interaction, which happens in the nucleus, is required to promote the formation of BRD4 and MED1 transcriptional foci and to guarantee the expression of early cell cycle genes. In agreement with these findings, both the pharmacological and transcriptional repressions of LOXL2 lead to G1-S cell cycle arrest in triple-negative breast cancer cells, loss of BRD4 and MED1 nuclear foci, and downregulation of cell cycle-associated gene expression.

Conclusion

Our results indicate that the interaction between LOXL2 and BRD4 short isoform is fundamental for the proliferation of triple-negative breast cancer and that the simultaneous inhibition of both proteins holds potential for the treatment of this unmet medical need

The desmosomal cadherin desmoglein-2 (DSG2) modulates the activity of yes-associated protein (YAP) in liver cancer

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Introduction

Hepatocytes are highly organized in a 3-dimensional network with cadherins being key factors in the formation of junctional structures. It is known that loss of cell-cell contacts and cellular polarity contributes to development of chronic liver disease. In this context, the cell density-sensing Hippo pathway is a regulator of cell autonomous organ growth via negatively regulating the oncogene YAP. Indeed, dysregulation of Hippo/YAP pathway activity contributes to liver tumor initiation. Whether reorganization of junctional complexes contributes to Hippo/YAP-dependent liver cancer development is not well understood.

Material and Methods

To identify junctional factors with potential impact on the Hippo/YAP pathway, expression data from primary human hepatocellular carcinoma (HCC) tissues were analyzed. Candidates that correlated with YAP target gene signatures were further investigated using human HCC tissues (e.g., IHC, IF) as well as in cell culture (RNAi and functional analyses) and *in vivo* model systems. Mass spectrometry-linked proximity-dependent labeling technology (BioID) was used to identify mechanisms that link junctional proteins with Hippo/YAP signaling. For confirmation, co-IP experiments were utilized.

Results and Discussions

In total, the expression of 14 junctional factors statistically associated with YAP target genes in HCC tissues. While some junctional factors were overexpressed (e.g., DSG2), other candidates showed reduced expression levels (e.g., DSG1) in HCC tissues compared to non-malignant livers. Inversely regulated DSG1 and DSG2 expression statistically correlated with reduced patient survival. For DSG2 we showed that reduction led to a nuclear export of YAP in HCC cells and diminished expression of YAP target genes (e.g., CYR61). Functionally, DSG2 inhibition reduced HCC cell viability and therefore phenocopied YAP silencing. In mice, overexpression of DSG2 alone or in combination with other oncogenes such as AKT or c-MYC did not lead to elevated tumor formation. Proteomics data and confirmatory binding studies revealed an interaction of DSG2 with the Hippo pathway constituents Merlin and LATS2, indicating a direct link between DSG2 and the Hippo signaling cascade.

Conclusion

Reorganization of desmosomal structures such as replacement of DSG1 by DSG2 modulates YAP activity via physical binding of Hippo pathway constituents in liver cancer cells. However, DSG2 dysregulation is not sufficient to facilitate oncogenic properties.

oropharyngeal squamous cell carcinoma patients

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Introduction

The incidence of HPV-positive oropharyngeal squamous cell carcinoma (OPSCC) is still rising, making HPV-pos the predominantly OPSCC form. HPV-pos OPSCC patients (pts) treated with standard-of-care have better overall survival (OS) compared to HPV-neg. However, long-term toxicities are relevant and co-morbidities strongly impact the quality of life. To date, treatments are limited and de-escalation protocols did not show positive results. HPV-mediated biology and carcinogenesis are still an open question. It is essential to open up the way to new therapies and to calibrate de-escalation treatment protocols. Viruses are known to disrupt multi-cellular host processes to favor their proliferation, leading the host cell to death. Here, we focused on ferroptosis, iron-mediated necrosis, which could be triggered by viral infection.

Material and Methods

Head and neck squamous cell carcinoma (HNSCC) pts (stage III-IV TNM 7th ed.) treated with curative intent were included in Big Data and Models for Personalized Head and Neck Cancer Decision Support project (BD2Decide, NCT02832102). Gene expression profiles of 286 p16 HPV-positive OPSCC were generated using the Clariom-D platform (Affymetrix) and MIAME-compliant data were deposited (GSE163173). We retrieved 7 HNSCC ferroptosis-related signatures, all trained on HNSCC TCGA dataset. Investigation of the tumor microenvironment, DNA damages, and other biological processes is ongoing.

Results and Discussions

All the ferroptosis signatures were significantly associated with prognosis. The most performing were: Chunyan Li et al. (HR=5.82, 95%CI 2.45-13.8; p=5.8e-06; five genes; PMID: 34539737); Feinan He et al. (HR= 5.33, 95%CI 2.47-11.49; p=1.7e-05; seven genes; PMID: 34130150). Notably, these signatures have no common genes. Ferroptosis is an active but non-apoptotic cell death, causing lipid peroxidation. Metabolic reprogramming is required, including reactive oxygen species (ROS) production, leading to stress accumulation in DNA. Iron is an essential element in several processes, including immune response and genome replication.

Conclusion

We discovered a strong correlation between ferroptosis and outcome of HPV-pos OPSCC. The investigation of ferroptosis-related mechanisms could be beneficial to personalize the treatment for OPSCC pts. Many currently available pharmacological interventions targeting

ferroptosis, ROS accumulation, immune system, and DNA damage, may be considered in this clinical setting in the future.

EACR22-0340

Notch3 deregulates CXCR4 expression in immature thymocytes to sustain Acute Lymphoblastic Leukemia progression.

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Introduction

T-cell acute lymphoblastic leukemia (T-ALL), an aggressive lymphoproliferative disorder results from the malignant transformation of T-cell progenitors and originates in the thymus. Activating mutations of Notch1 or Notch3 receptors are frequently found in humans T-ALL. In the thymus, T-cell precursors develop from CD4-CD8- (Double-Negative, DN-T), to CD4⁺CD8⁺ (Double-Positive, DP) to mature CD4⁺/CD8⁺ T-cells. This developmental program is driven by the cooperation of three receptors: Notch1/Notch3, pre-TCR, and CXCR4. This regulates the survival and proliferation required for the progressive maturation of thymocytes. Overexpression of an active intracellular form of Notch3 receptor in a mouse model (N3-ICtg) develops an aggressive T-ALL form, characterized by impaired T-cell maturation and immature thymocytes in blood circulation. We aim to better understand the role of CXCR4 in deregulated molecular and functional mechanisms driving T-ALL progression in our N3-ICtg model. We will characterize *in vivo* thymocyte dynamics in T-ALL progression and correlate it with miRNAs expression.

Material and Methods

FACS analysis of DN-T in WT and N3-ICtg mice will assess CXCR4 expression in combination with T-cell differentiation markers (CD3, CD5). We evaluate proliferation and apoptosis of CD3⁺ DN-T subset as a function of CXCR4 expression. Based on Bioinformatic studies of the 3'UTR of the CXCR4 gene, we detect the expression of previously identified Notch3-induced miRNAs on CD3⁺ DN CXCR4⁺ and CD3⁺ DN CXCR4⁻ subsets, after sorting.

Results and Discussions

In our Notch3-induced T-ALL model, we investigate the aberrant maturation program of the DN-T subset in the context of tumor progression. The CXCR4 cell-surface expression is reduced in the CD3⁺ DN T-cells of N3-ICtg mice, which in turn appears to be overrepresented in N3-ICtg as compared to WT mice. This suggests that hyperactive Notch3 negatively regulates CXCR4 in CD3⁺ DN cells, interfering with the DN-DP transition and migration. The inverse correlation between CXCR4 expression and Notch3-induced miRNAs may suggest a miRNA-mediated mechanism.

Conclusion

Overall, we suggest that Notch3 by altering microRNA expression might down-regulate the CXCR4 receptor to select an aberrant subpopulation of thymocytes, that sustain leukemia progression. Our present findings may be a more in-depth understanding of the mechanism underlying T-ALL pathogenesis and can propose future perspectives for the development of new therapeutic approaches.

Targeting Nrf2 in non-small cell lung cancer: triptolide as a potential adjuvant chemotherapy

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Introduction

The transcription factor Nrf2 regulates hundreds of cytoprotective genes involved in detoxification, inflammation and the oxidative stress response, such as NAD(P)H dehydrogenase quinone (NQO1). In cancer, the Nrf2 pathway can be 'hijacked', resulting in tumour promotion and chemoresistance. High Nrf2 expression is also linked with poor prognosis and chemoresistance in many patient tumours, including non-small cell lung cancer (NSCLC). Nrf2 inhibitors may therefore be useful in chemotherapy. Here, Nrf2 inhibitors were compared for their effects on cell proliferation, Nrf2 expression, NQO1 activity and apoptosis in 2D and 3D cell culture models of NSCLC.

Material and Methods

A549 NSCLC cells were incubated with Nrf2 inhibitors (brusatol, luteolin, retinoic acid, ML385, triptolide, trigonelline hydrochloride and clobetasol propionate) or gemcitabine for 72h and cell proliferation was observed by MTS assay. Nrf2 protein expression was measured by western blot analysis and NQO1 activity was quantified using an enzymatic assay, following cell stimulation with 10-20µM Nrf2 inhibitors for 4h. A549 tumour spheroids (tumouroids) were cultured in Matrigel for 9 days and were stimulated daily from day 5 with triptolide (1-10µM). Tumouroid area was measured by ImageJ analysis of white light microscopy images. Apoptosis was measured via fluorescence microscopy, following 30-minute incubation with 5µM CellEvent™ Caspase-3/7 Green Detection Reagent.

Results and Discussions

Brusatol and triptolide had the most potent inhibitory effects on cell proliferation in A549 cells, with IC₅₀ values of 60nM and 138nM, respectively (n=3). Brusatol and triptolide also significantly reduced both Nrf2 protein expression (5.75% and 18.4% of vehicle control, respectively (n=3, P<0.01)) and NQO1 activity (49.9% and 58.1%, of vehicle control, respectively (n=3, P<0.001)). Further, triptolide sensitised A549 cells to gemcitabine, reducing cell proliferation by 38.4% compared to gemcitabine alone (n=3, P<0.001). In 3D tumouroids, 1-10µM triptolide significantly reduced tumouroid area (n=3, P<0.001), and induced apoptosis through increasing activated caspase-3/7.

Conclusion

Though several Nrf2 inhibitors have been reported, brusatol and triptolide were the most effective at inhibiting Nrf2, NQO1 activity and cell proliferation in NSCLC cells. Triptolide also sensitised cells to gemcitabine and induced apoptosis in a 3D model of NSCLC. Brusatol has off-target effects; however, triptolide may have potential use as a targeted adjuvant therapy in NSCLC.

Copper imidazopyridine complexes disrupt nuclear factor-kappa B (NF-κB) signaling to promote apoptosis in leukaemic cells

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Introduction

The NF- κ B signal transduction pathway is dysregulated in a variety of cancers including haematological malignancies. In leukaemic cells, NF- κ B is constitutively active as a result of cytokine signaling, chromosomal translocations or mutations leading to myeloid transformation, as well as increased expression and activation of NF- κ B regulators. Over-activity of NF- κ B enhances the transcription of apoptotic inhibitory genes like *BCL2* and *BIRC*. Copper imidazopyridine complexes were previously shown to induce intrinsic apoptosis in leukaemic cell lines. The aim of this study was to investigate the effect of copper imidazopyridine complexes on the transcriptional activity of NF- κ B in leukaemic cell lines undergoing apoptosis.

Material and Methods

An apoptotic qPCR array was used to evaluate the effect of active copper complexes on the mRNA coding for apoptotic associated proteins in two leukaemic cell lines, K562 and HL-60. The effect on the protein profile was evaluated using an apoptotic proteome profiler array.

Results and Discussions

In K562 cells, the mRNA transcript levels of canonical NF- κ B as well as NF- κ B regulated target genes; *BIRC2*, *BIRC3*, *BIRC5*, *BCL2*, *BCL2A1*, *MCL1*, *TNF* and *CFLAR* were decreased. In HL60 cells, a decrease in Akt gene expression and canonical NF- κ B genes; *BIRC1*, *BIRC3*, *BCL2*, *TNF* and *CFLAR* suggested the inhibition of canonical NF- κ B activity via suppression of the Akt pathway. The mRNA transcript levels of non-canonical NF- κ B regulated target genes were decreased, suggesting an inhibitory effect on NF- κ B inducing kinase (NIK), preventing non-canonical NF- κ B activation. The copper complexes decreased the mRNA levels of various key components of tumor necrosis factor signaling like TNF- α , the receptors for TNF- α , TNFRSF1B and TNFRSF21 in both cell lines. Decreased TNF-signaling prevents NF- κ B from entering the nucleus to transcribe pro-survival genes encoding anti-apoptotic factors such as cIAP-1. In this study, the suppression of *BIRC* genes and their gene products serve as evidence of suppressed NF- κ B signaling by the copper complexes.

Conclusion

The copper complexes caused apoptotic cell death in K562 and HL-60 cells, by suppressing the expression of key pro-survival proteins and increasing pro-apoptotic protein expression. This appears to be mediated by inhibiting the transcriptional activity of the NF- κ B pathway, which may be central to the apoptotic effect of the copper complexes.

EACR22-0532

The expression profile of scaffold protein SG2NA in various cancer types and study of its interactome in breast cancer tissue.

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Introduction

SG2NA is a scaffold protein that belongs to the striatin family (other members are Striatin and Zinedin) under the WD40 superfamily. SG2NA generates multiple variants by alternative splicing, RNA editing, and intron retention. SG2NA forms a multiprotein signaling complex called STRIPAK (Striatin interacting phosphatase and kinase). This multiprotein complex is attributed to diverse cellular functions and is associated with developmental abnormalities, cancer, and several other diseases. SG2NA also forms a complex with the cancer-associated protein DJ-1 and the signaling kinase Akt, promoting cancer cell survival. This work aims to study the expression profile of SG2NA isoforms in various cancer cell lines and

cancer tissues. Further, the interactome of the SG2NA was studied in breast cancer tissues to find out their involvement in different cellular pathways.

Material and Methods

Western blotting and qRT-PCR was done to analyze the expression profiling at protein and transcript level. Co-immunoprecipitation followed by LC-MS analysis was performed to find out the interacting partners of SG2NA. Further various bioinformatics tools were used to analyze the LC-MS data.

Results and Discussions

Two isoforms of human SG2NA, i.e., 78 and 87 kDas were found in bioinformatic analysis which was further confirmed. The expression of these isoforms varies between different cancer cell lines of human origin. Also, the protein levels do not corroborate with its transcript levels, suggesting a complex regulation of its expression. A smaller isoform.35 kDa (generated by intron retention) was also observed. In breast tumor tissues, the expression of the 35 and 78 kDa isoforms was higher as compared to the adjacent normal tissues, while the 87 kDa isoform was found in the breast tumor tissues only. With advance stage of the breast cancer, the expression of 78 kDa decreased. In co-immunoprecipitation assays, the profile of the SG2NA interactome in breast tumors vis-à-vis adjacent normal breast tissues showed hundreds of common proteins. Also, some proteins interacted with SG2NA in breast tumor tissues only.

Conclusion

The profiling of SG2NA isoforms differs in different cancer cell lines and tissue. SG2NA is involved in diverse cellular pathways and has roles in cellular reprogramming during tumorigenesis of the breast.

EACR22-0958

SHIP2 promotes akt phosphorylation and esophageal squamous cell carcinoma growth

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Introduction

Esophageal squamous cell carcinoma (eSCC) is among the deadliest cancers worldwide. Current management, consisting of surgery preceded by neo-adjuvant radiochemotherapy, is poorly efficient. Its relapse carries a short-term grim prognosis because of rapid resistance to therapy and the limited number of efficient drugs available. In this context, it is crucial to develop novel targeted therapies against eSCC.

The phosphoinositide (PI) 3-kinase (PI3K) pathway is one of the most frequently mutated signaling pathways in cancer and is being actively pursued today as a therapeutic target. Interestingly, phosphorylation of the AKT protein, one of the mechanisms leading to the activation of the pathway, shows higher expression in eSCC than in corresponding normal tissue, supporting the relevance of the PI3K-AKT path in eSCC development.

The role of PIP3, the product of PI3K is often considered “oncogenic”, but other PIs such as PI(3,4)P2 are also critical signal molecules. SHIP2 (SH2 domain-containing PI-3,4,5-trisphosphate 5-phosphatase 2) is an enzyme able to catalyze the formation of PI(3,4)P2 and plays a crucial role in AKT activation. However, virtually nothing is known about the role of SHIP2 in eSCC.

Material and Methods

In this study, we used publicly available transcriptomics and genomics dataset, a panel of 22 human eSCC cell lines, siRNA and pharmacological inhibitors (K149 and AS194940), xenografts, histology and RNA sequencing upon SHIP2 inhibition or silencing by siRNA.

Results and Discussions

By analyzing data from the Cancer Genome Atlas (TCGA), we found that eSCC is the most frequent cancer type with amplification of *INPPL1* (coding SHIP2) and SHIP2 is the PI 5-phosphatase with the highest expression in human eSCC cells. We used siRNA-mediated SHIP2 knockdown and observed that SHIP2 modulates cell growth in several eSCC lines. We then tested the impact of 2 pharmacological inhibitors (K149 and AS194940) and found that SHIP2 inhibition decreases eSCC cell survival *in vitro* and *in vivo*. SHIP2 inhibition was associated to a decrease in the level of AKT phosphorylation, and AKT inhibition also blocks cell growth, thus suggesting that SHIP2 modulates eSCC cell growth through the regulation of AKT. To determine how SHIP2 regulates cell survival, we profiled eSCC cells treated with inhibitor or siRNA and found that SHIP2 inhibition alters cell metabolism and cell division.

Conclusion

In conclusion, inhibition of the PI3K-AKT pathway may constitute a good axis to treat eSCC.

EACR22-0067

Dynamic combinatorial p53 post-translational modifications regulate cellular outcomes

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Introduction

The tumour suppressor p53 is activated by a range of cellular stresses including DNA damage, oncogenic signals, and nutrient deprivation. Upon activation, p53 transcriptionally upregulates an extensive network of target genes including those in pathways affecting cellular outcomes such as cell cycle arrest, senescence, and apoptosis. What remains unclear is how information on the nature of stresses is precisely conveyed to p53 to elicit responses appropriate for the situation. Changes in p53 temporal expression dynamics as well as specific p53 post-translational modifications (PTMs) are known to differentially regulate target gene inductions. However, with over 100 known PTMs possible on p53, technical limitations have restricted the ability to detect the combinations of modifications on individual p53 molecules.

Material and Methods

We used a novel state-of-the-art mass spectrometry method, I²MS, in order uncover the landscape of combinatorial p53 PTMs on *individual intact* proteins over time from different p53 activation regimes in human cancer cell lines. We further functionally validated the effect of specific phosphorylation or acetylation by generating and expressing (via CRISPR knock-in) p53 containing amino acid substitutions mimicking unphosphorylated or unacetylated forms of p53.

Results and Discussions

We determined phosphorylation states as the major distinction between our p53 activation treatments, which when enhanced, broadly augment the potency of target gene transcription leading to greater extent of cell death. Furthermore, we ascertained dynamic changes in p53 PTMs in response to DNA damage and identified reduction of multiple p53 acetylation with

time. Inhibition of the deacetylase HDAC1, but not SIRT1, prevented the reduction in p53 acetylation, causing overexpression of senescence and apoptotic genes, and corresponding cellular outcomes. Finally, we abrogated lysine (K) acetylation by generating K $\rightarrow$ R (arginine) mutations at single or multiple sites on p53, and found that acetylation sites are functionally non-redundant, with specific combinations exerting unique transcriptional effects on p53 target gene inductions.

Conclusion

Our results highlight the importance of accounting for dynamical combinations of p53 PTMs in order to achieve the desired cellular response and efficacy of p53-mediated cancer therapies.

EACR22-0321

EBV related Nasopharyngeal Carcinoma in non endemic area: a multi-omics study

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Introduction

Nasopharyngeal carcinoma (NPC) is characterized by a higher incidence in Asian endemic area (EA), than in not endemic area (NEA), where it is considered a rare disease. NPC is associated with Epstein-Barr (EBV) infection in both areas but NEA patients (pts) have lower survival rates. Genetic, transcriptomic, epigenomic could explain these differences; however, limited multi-omic data are available for NEA pts. In order to unravel the biology in NEA NPC, we investigated the molecular landscape [mutation, Gene expression (GE) and miRNA] of a cohort of 47 Italian NPC pts that were compared with public data of EA pts.

Material and Methods

FFPE tumor tissue, at initial diagnosis, were collected and histopathologically revised. After DNA and RNA extraction, exome panel sequencing was performed by OCAPlus, while RNASeq and miRNASeq were carried out by QuantSeq 3' mRNA-Seq and by QIAseq miRNA, respectively. Two signatures developed in EA cohorts, and the radiosensitivity indexes (RSI) according to conventional therapy, implicated in prognostic significance were applied on NEA. Survival analysis was defined by Kaplan-Meier. Meta-analysis of 6 public available datasets of EA GE was performed to identify clusters that were then tested on NEA pts.

Results and Discussions

108 mutated genes were observed in NEA pts and compared with EA pts data: only 12 genes were commonly mutated with different frequencies. In addition, several genes, such as TP53, HLA-A, TRAF3, frequently mutated in EA were found wild-type in NEA. The transcriptomic and miRNA landscapes of NEA were investigated and prognostic signatures were tested resulting in significant risk stratification: prognostic signature for tumor-infiltrating immune cells (HR=3.9; 95% CI 1.23-12.34; p=0.013), RSI signature (HR=3.22: 95% CI 1.02-10.16; p=0.035), Mesenchymal subtype-specific miRNAs (HR=3.64; 95% CI 1.15-11.53; p=0.019). Meta-analysis of GE of EA identified 4 clusters but only 3 of them were

expressed on NEA cohort. Correlation between GE clusters and miRNA data, and biological characterization of clusters are ongoing.

Conclusion

A similar trend in the prognostic value of the tested signature was observed between EA and NEA, although a lower significance was recorded in NEA, attributed to limited sample size of our cohort. Further research is warranted to explain the similarities and differences between EA and NEA at biological and epidemiological levels.

EACR22-0341

EZH2 inhibition activates Notch oncosuppressive program in cervical cancer and acute myeloid leukemia cells

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Introduction

Impaired activation of Notch pathway can promote or prevent tumorigenesis depending on the tissue context. Up to date, several strategies to inhibit Notch are under clinical evaluation in cancers in which its signaling promotes tumor progression. However, Notch activators are still in the early pre-clinical investigations in cancers where Notch acts as tumor suppressor. Recent studies unveiled epigenetic players influencing Notch signaling via the regulation of several components of the pathway. In particular, the histone demethylase JMJD3 supports Notch oncogenic program and cell proliferation in T-ALL and colon cancer via sustaining the transcription of the Notch receptors and target genes, suggesting the potential therapeutic anti-cancer efficacy of its targeting in Notch-dependent tumors. Nevertheless, the relevance of the enzymatic antagonist of JMJD3, EZH2, over Notch regulation in cancer contexts where Notch might function as onco-suppressor has not been elucidated yet.

Material and Methods

Cervical cancer (CC) HeLa and SiHa, acute myeloid leukemia (AML) HL-60 and Me-1 cell lines; In vitro pharmacological treatment with GSK-126, DAPT and cisplatin; RNA interference for Notch receptors; Trypan blue exclusion assays; RT-qPCR; Nuclear/cytosol fractionation; Western blotting.

Results and Discussions

We hypothesized that, specularly to Notch activation by JMJD3 in T-ALL and colon cancer, EZH2 could repress Notch signaling in CC and AML in which the pathway is silenced and its activation showed anti-growth effects. Confirming our hypothesis, EZH2 inhibition activated Notch signaling and counteracted the proliferation in SiHa and HeLa CC, and HL60 and Me-1 AML cells. Moreover, we observed that the interference with Notch pathway activation attenuated the biological and molecular anti-cancer outputs induced by the inhibition of EZH2 in squamous CC SiHa and promyelocytic leukemia HL-60 cells, whereas it did not exert effects in adeno CC HeLa cells and acute myelomonocytic Me-1 cells. Furthermore, the treatment with the pharmacological inhibitor of EZH2, GSK126, synergized with cisplatin at lower doses of both drugs, in impairing proliferation and favoring the death in SiHa and HL-60 cells.

Conclusion

Overall, our results indicate that EZH2 plays its oncogenic function in CC and AML, at least partially, via Notch repression and suggest EZH2 inhibition as a potential strategy for

controlling Notch activation and overcoming cisplatin therapy resistance in tumors in which Notch exerts an oncosuppressive function.

EACR22-0665

Transcriptomic profiling in isogenic cell lines reveals key pathways driving ZNF703-amplified breast cancer

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Introduction

Amplification of the oncogenic driver *ZNF703* is found in 14% of breast cancers, enriched for estrogen receptor–positive (ER+) or luminal subtypes, associated with high histological grade and proliferation, and with poor disease-specific survival. *ZNF703* amplification also defines the IntClust 6 subtype as the only gene within the minimal amplicon at the chr8p11/12 locus. *ZNF703* is known to act as a transcriptional repressor in a complex with DCAF7 and PHB2 that recruits HDAC1 to gene promoters. However, genes under regulatory control of *ZNF703* have not been comprehensively defined. Here we sought to profile the transcriptomic changes associated with *ZNF703* expression.

Material and Methods

Isogenic clonal cell lines with CRISPR/Cas9-mediated *ZNF703* knockout (MDA-MB-134-*ZNF703*KO and MCF7-*ZNF703*KO) were generated from MDA-MB-134 and MCF7, ER+ breast cancer cell lines with or without *ZNF703* amplification, respectively, by nucleofection of Cas9 ribonucleoproteins with a gRNA and subsequent single cell cloning. Knockouts were verified by Sanger sequencing and inference of CRISPR edits (ICE) analysis, and western blot. MCF7 cells stably overexpressing *ZNF703* (MCF7-*ZNF703*) and an empty vector control (MCF7-Ctrl) were produced by lentiviral transduction of a *ZNF703* cDNA expression construct or the empty pHIV-ZsGreen backbone, followed by fluorescence activated cell sorting for ZsGreen reporter expression. To identify genes regulated by *ZNF703*, RNA sequencing and differential expression analysis was performed on this panel of isogenic cell lines with perturbations in *ZNF703* expression.

Results and Discussions

We report the generation of isogenic cell lines with verified *ZNF703* knockout from MCF7 and MDA-MB-134. These constitute valuable models for studying *ZNF703*-amplified breast tumours and complement currently available preclinical models. Differentially expressed genes associated with *ZNF703* overexpression overlapped with IntClust6 gene expression profiles identified in METABRIC. Gene set enrichment analysis of differentially expressed genes identified pathways linked with *ZNF703* expression.

Conclusion

Differential expression and pathway analysis of the transcriptome in isogenic cell lines with *ZNF703* overexpression or knockout reveal crucial insights that aid in elucidating the mechanism of tumorigenic activity in breast tumours driven by *ZNF703* amplification.

EACR22-0679

Analysis of MicroRNA expression data using machine learning in non-small cell lung cancer

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Introduction

Non-Small Cell Lung Cancer(NSCLC) is a heterogeneous aggregate of histological types including squamous cell carcinoma(LUSC) and adenocarcinoma(LUAD), which could be further classified in different subtypes depending on the histologic patterns. MicroRNAs(miRNAs) are small non-coding RNAs expressed in various tissues and cell types. They can help understand the carcinogenesis of NSCLC and serve as potential diagnostic biomarkers for differentiating LUAD and LUSC. The present study aims to analyse and compare the expression patterns of miRNAs in LUAD and LUSC samples.

Material and Methods

Fresh frozen tissues from 124 NSCLC and adjacent normal tissues were examined. The expression of 24 NSCLC patients was evaluated by SurePrint human miRNA microarrays (Agilent Technologies). The validation of 14 miRNAs was performed on an enlarged cohort (50 LUAD, 50 LUSC) by qRT-PCR. Supervised machine learning algorithms for binary classification of LUAD and LUSC were performed. Unsupervised machine learning algorithms for subtype classification of LUAD and LUSC were also performed. The preprocessing of RQ values, grid search with cross-validation, model training, evaluation and visualization were performed via scikit-learn.

Results and Discussions

We assessed the expression levels of 2549 human mature miRNAs and found 107 to be significantly differentially expressed ($FC > 2.0$; $p < 0.05$) between the LUAD and adjacent normal tissues, while 240 miRNAs were differentially expressed ($FC > 2.0$; $p < 0.05$) between the LUSC and adjacent normal. Logistic regression and SVM showed 0.80 and 0.83 accuracy on the test data, respectively. K-Means and Hierarchical clustering revealed clusters in LUAD, corresponding to the predominant histologic pattern. In LUSC the clusters correspond to the non-keratinizing and keratinizing LUSC. The expression of miR-650 was associated with acinar, miR-21 with micropapillary, miR-375 with solid, miR-95 with papillary LUAD. The expression of miR-101, miR-195-5p, miR-144 and miR-95 were associated with non-keratinizing LUSC, while miR-195-3p, miR-375, miR-21 were associated with keratinizing LUSC.

Conclusion

Our results were in agreement with previous observations that different panels of miRNAs including miR-375 and miR-21 have better potential to discriminate between LUAD and LUSC. However further analysis in enlarged samples and validation is necessary to ascertain their diagnostic potential in NSCLC.

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In Silico enrichment analysis of top significantly expressed miR-93-5p, miR-210-3p and miR-144-3p after miRNA microarray in patients with advanced laryngeal cancer

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Introduction

Laryngeal Squamous Cell Carcinoma (LSCC) is second most common Head and Neck Cancer (HNC) and is highly aggressive disease, associated with poor prognosis. MicroRNAs (miRNAs) are small non-coding RNAs, with regulatory function, expressed in various tissues and cell types. The aim of current study is to explore and analyze in silico most affected signaling pathways by three of previously investigated top significantly deregulated miRNAs: miR-93-5p, miR-210-3p and miR-144-3p.

Material and Methods

MiRNA expression of 12 advanced LSCC patients (fresh-frozen and paired normal tissue) was analyzed via miRNA microarray technology (published data). The expression of miR-93-5p, miR-210-3p and miR-144-3p was evaluated in a group of 40 fresh frozen LSCC and adjacent normal samples by qRT-PCR analysis. Gene Ontology (GO) and Reactome analysis, as well normalization of data, statistical and target prediction analyses were performed using R version 3.0.2 and GSEA with the Python package, GSEAPy (version0.9.12).

Results and Discussions

We assessed the expression levels of 2549 human mature miRNAs (published data). Confirmed was increased expression levels of miR-93-5p and miR-210-3p (52,38% and 39,47%) while as downregulation of miR-144-3p in 52,38% of cases. Results reveal, that based on target list among GO Biological Processes, regulation of transcription by RNA polymerase II, cellular protein modification process, regulation of transcription, etc. are highly enriched. Most affected GO Cellular Components are intracellular membrane – bounded organelle, nucleus, intracellular non – membrane – bounded organelle and etc. Highly enriched Molecular Functions, according GO analysis are RNA and DNA binding. The Reactom analysis revealed that differentially expressed miRNA target genes were involved in cell cycle, gene expression, transcriptional regulation by TP53, genetic transcription signaling pathways. Common targets of analyzed miRNAs are Cyclin D1 (CCND1), Cyclin-dependent kinase 2 (Cdk2) and CDC25A, which previously are reported to be involved in cancerogenesis.

Conclusion

In silico analysis revealed that these miRNAs were actively involved in major oncogenetic pathways, through targeting specific genes. Analysis could help to demonstrate which cellular pathway is accurate prediction or prognosis target in advanced LSCC. Data validation of obtained results in larger LSCC patients group is a must.

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Cytoplasmic proteotoxicity regulates HRI-dependent phosphorylation of eIF2a via the Hsp70-Bag3 module

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Introduction

The major heat shock protein Hsp70 have been implicated in cancer development. Original hypothesis was that cancer cells experience permanent proteotoxicity stress due to aneuploidy, oxidative stress and other abnormalities, which dictates their stronger requirements for expression of molecular chaperones (non-oncogene addiction) compared to normal cells. This hypothesis was generally refuted, which led to the idea of direct involvement of Hsp70 in signaling pathways that control major stages of cancer development, including initiation, progression and metastasis. Hsp70 forms a complex with a scaffold protein Bag3 that links it to components of multiple signaling pathways. Here, we analyzed effects of the disruption of Hsp70-Bag3 complex on the transcriptome to identify cancer signaling pathways regulated by this complex.

Material and Methods

In this study, JG-98 was used to disrupt the interaction of the Hsp70-Bag3 complex and its effect was measured with the help of stress kinases followed by transcriptome analysis. The approach was supported by GSEA analysis to correlate the pathways that were involved in downstream signaling. The critical pathways identified and were further validated by pull-down experiments for their association.

Results and Discussions

Gene expression profiling followed by the pathway analysis indicated that a set of signaling pathways including UPR was activated by JG-98. Surprisingly, only the eIF2a-associated branch of the UPR was activated under these conditions, while other UPR branches including induction of ER chaperones were not induced, suggesting that the response was not related to the ER proteotoxicity and ER-associated kinase PERK1. Indeed, induction of the UPR genes under these conditions was dependent on activation of a distinct cytoplasmic eIF2a kinase HRI. Hsp70-Bag3 complex directly interacted with HRI and regulated phosphorylation of eIF2a upon cytoplasmic proteotoxicity. Therefore, activation of certain UPR genes can be triggered by cytosolic proteotoxicity via Hsp70-Bag3-HRI-eIF2a axis.

Conclusion

Overall, this work uncovered a novel Hsp70-Bag3-HRI pathway that detect the buildup of abnormal proteins in the cytosol and regulates phosphorylation of eIF2a. In turn, eIF2a integrates proteotoxicity signals both from ER and cytosol.

Ontology-defined subtyping identifies the dominance of a MYC-polycomb repressor complex (PRC) signalling cascade in colorectal cancer

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Introduction

Molecular subtyping of colorectal cancer (CRC) using individual gene-level data, exemplified by the consensus molecular subtypes (CMS), has identified distinct clusters of tumours, each of which associated with molecular features and prognostic value. In this project, we propose that using pathway-level subtyping, rather than gene expression-level, will provide an opportunity for a more holistic assessment of tumour biology, which ultimately may be more informative for therapeutic decision-making.

Material and Methods

Unsupervised *k*-means clustering using single sample ontology scores from bulk CRC transcriptional data (FOCUS; GSE156915) was performed, and subsequently, a classification model for CRC subtyping was generated. A series of *in silico* methods, transcriptional signatures (i.e., Hallmarks, stem, polyp), immunohistochemistry (IHC), and digital whole slide imaging were comprehensively utilised to characterise the defined subtypes. Following classifier development and characterisation, these findings were validated in multiple independent cohorts and further explored in human and mouse preclinical models of CRC.

Results and Discussions

Ontology-led classification captures three distinct ‘**Pathway Derived Subtypes**’ (PDS1-3). PDS1 exhibits epithelial-rich, LGR5-associated, MYC-driven and high Ki67⁺ proliferative features. PDS2, on the other hand, are characterised by mesenchymal-like, highly stroma/immune dense, regenerative-like stem biology, elevated EMT, TGF- β activation and inflammatory-like traits. PDS3 are histologically heterogeneous tumours with low Ki67⁺ proliferation, MYC-repressed, differentiated enteroendocrine-like phenotype, and more importantly, may have dysfunctional polycomb repressive complex 2 (PRC2). An inverse gradient relationship between PDS1 and PDS3 is observed in terms of MYC and PRC2 target gene sets in both patient and mouse data. In the adjuvant colon setting, PDS1 is associated with a good prognosis vs PDS3 with poor prognosis. On the contrary, in response to neoadjuvant radiotherapy in rectal cancer, PDS1 are the least responsive compared to highly responsive PDS3.

Conclusion

Our study proposes a novel ontology-based classification approach for CRC with unique underlying tumour phenotype and prognosis with potential clinical implications.

Tumour Biology

EACR22-0016

A living biobank of patient derived xenograft models of DCIS reveals biomarkers related to invasive progression

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Introduction

Ductal Carcinoma in Situ (DCIS) is a non-invasive non-obligate precursor of invasive breast cancer (IBC). DCIS is usually treated by surgery combined with radiotherapy, which can have a large impact on the life of patients. However, many of these DCIS lesions would never progress into IBC. To reduce the overtreatment of DCIS, but assure proper treatment for high risk DCIS, it is crucial to understand the biology underlying DCIS.

Material and Methods

To study the biology of DCIS we established patient-derived xenograft (PDX) models by intraductally injecting patient DCIS material into the mammary ducts of female immunocompromised mice. We engrafted 130 samples, which have been incubated *in vivo* for a period of 12 months. We obtain a take rate of 89% with 45% of our models showing progression. Histology and molecular subtyping by PAM50 classification are well preserved in the PDX lesions compared to the primary counterpart, ensuring that our models represent the patient disease well. For >80 primary samples we obtained RNAseq as well as >50 matched PDX profiles. In addition WES data is generated from the same primary DCIS samples together with 10 matched PDX WES profiles as well as >50 matched CNV PDX profiles.

Results and Discussions

Together these data revealed multiple biomarkers related to invasive progression, including factors such as high grade, a solid growth pattern, HER2, CCND1 & MYC amplifications and a high Ki67. On top of this we used whole mount imaging of the injected mammary glands extracted from our PDX models, showing two distinct growth patterns correlated with invasion. And as this is all done in the context of the PRECISION consortium this allows us to confirm and validate our findings in larger sequencing and imaging efforts of human samples.

We have also successfully sequentially transplanted >40 PDX models to create models that can be manipulated to validate the role of genes in DCIS progression. As an example we took HER2, where we treated 3 progressive HER2 DCIS PDX models with Herceptin, showing a massive decline in growth. Besides this we infected 2 indolent luminal DCIS PDX models with a HER2 lentivirus showing a transformation to progressive growth.

Conclusion

In conclusion all this data together enables us to create a well-characterized biobank of DCIS models of which we can follow the progression, sequentially transplant >40 models, find mutational and expression profiles related to high risk DCIS and manipulate gene expression to validate the role of genes in DCIS progression.

EACR22-0270

The role of Serum amyloid A in NLRP3 inflammasome signaling in breast and colon cancer

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Introduction

Cancer is a complex disease and to develop more efficient therapeutic regimes, a better understanding on systemic level is required. The role of serum amyloid A (SAA) has gathered significant evidence which highlights its role in the pathogenesis of several

cancers. SAA binds to several pattern recognition receptors, which suggests that inflammasomes play a role in the tumour-promoting effects of SAA. Inflammasomes are cytoplasmic multiprotein complexes characterized by a sensor and adaptor protein and inflammatory caspases. However, the role of inflammasomes in cancer remain controversial. The aim of this study was to investigate the role of SAA in inflammasome signaling in breast and colon cancer.

Material and Methods

Ethical clearance was obtained from the Stellenbosch University Research Ethics committee. In this 3-part study, tissues were subjected to immunoblotting, qPCR, haematoxylin and eosin staining and immunohistochemistry. For the inflammatory model, wild-type and SAA double knockout mice received 2.5% dextran sulfate sodium, administered for a total of 5 days. For colitis-associated cancer, wild-type and SAA double knockout mice received an intraperitoneal injection of 12.5 mg/kg azoxymethane. After one week, dextran sulfate sodium treatment was administered at a concentration of 2.5% for 5 days, followed by a recovery period of 16 days and was administered for a total of 3 cycles. Triple negative breast tumours were established in wild-type and SAA double knockout mice by injecting EO771 cells subcutaneously at the fourth mammary fat pad.

Results and Discussions

This study showed that in a colitis model, SAA ablation exerted pro-inflammatory properties independent of the NLRP3 inflammasome. This was associated with the increased expression of pro-inflammatory cytokines. In contrast, in two cancer models, the ablation of SAA suppressed canonical NLRP3 inflammasome activation and promoted anti-inflammatory properties.

Conclusion

We have shown for the first time, the novel role of SAA in the activation of the inflammasome and the generation of pro-inflammatory cytokines, two mechanisms known to promote tumour development and metastasis. This study emphasizes the notion that the tumour-induced systemic environment acts as a critical regulator of cancer progression and metastasis.

EACR22-0326

Drug screening in Disorders with Abnormal DNA Damage Response/Repair (DADDR) and in vivo validation

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Introduction

Disorders with Abnormal DNA Damage Response/Repair (DADDRs) are inherited conditions caused by constitutional mutations of DNA damage response and repair genes and are characterized by an increased cancer risk. Furthermore, affected individuals also show an elevated risk of secondary neoplasms and excessive toxicity, poor therapy response, as well as increased mortality when treated with standard radiation and chemotherapy regimens.

The main aim of this project is to screen for potential novel chemotherapeutic approaches for these cancer entities, and to employ faithful PDX models for *in vivo* validation.

Material and Methods

In vitro drug screening was performed using a custom library composed of 345 compounds targeting 61 different proteins. For two specific DADDRs, Li-Fraumeni syndrome (LFS) and Constitutional Mismatch Repair Deficiency (CMMRD), two cancerous (glioblastoma and medulloblastoma) and one non-cancerous cell lines were selected to model each of these conditions. Performance of each drug was assessed based on the efficacy (sensitivity score) and genotoxicity (micronucleus assay). For DADDR PDX model establishment tumor material from DADDR patients are currently being injected orthotopically (brain tumors) or subcutaneously (non-brain tumors) into NSG mice. Following engraftment and expansion, the PDX models will be characterized molecularly and compared with original patient material.

Results and Discussions

In vitro screening revealed n=26 drugs that fulfilled the following criteria: a) favorable toxicity in cancerous cell lines compared to non-cancerous cell lines, b) little to no genotoxic effect in non-cancerous cell lines. These characteristics qualify them as potentially suitable candidates for novel therapeutic approaches specifically for DADDR patients. The hits included inhibitors of ATM/ATR, CHK1/CHK2, DHFR, mTOR and PI3K, as well as microtubule-associated compounds.

Conclusion

Combination testing and further validation of these hits using disease-specific *in vitro* and *in vivo* PDX models is ongoing.

EACR22-0352

Investigating Characteristics of the 4T1 Model of Advanced Triple Negative Breast Cancer

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Introduction

Despite improvements in outcome for patients diagnosed with early stage breast cancer, survival remains poor for those with advanced disease. As key mediators of cell communication, Extracellular Vesicles (EVs) have garnered increased attention as both potential therapeutics and biomarkers of disease. Preclinical testing is currently required for all novel therapies so it is critical to employ models that more closely reflect the patient paradigm. The murine 4T1 model of triple negative breast cancer reflects the metastatic pathway of advanced disease. The aim of this study was to establish and characterise the 4T1 model prior to testing a new therapeutic agent, with primary tumour removal included to reflect the patient experience.

Material and Methods

4T1 breast cancer cells (1×10^5) stably expressing luciferase (4T1-luc) were injected orthotopically into the mammary fat pad of immunocompetent mice (n=16) with a further n=6 control mice receiving no cells. Weekly bioluminescent (IVIS) imaging was carried out to

track tumour progression. Five weeks post induction, primary tumours were surgically removed in n=8 mice, while disease was allowed to progress in n=8. At study endpoint, tissues were harvested and paraffin embedded for immunohistochemistry (IHC). Plasma EVs were isolated by Size Exclusion Chromatography (SEC) and characterised by Nanoparticle Tracking Analysis (NTA), Transmission Electron Microscopy (TEM) and western blot.

Results and Discussions

4T1-luc tumours were palpable one-week post induction, with IVIS imaging confirming a primary tumour at the injection site. Primary tumours were successfully resected in n=8 mice, with metastasis to the lungs evident by IVIS after five weeks. Histological staining also confirmed tumour cell infiltration in the lungs. Elevated Ki67 (proliferation), LYVE-1 (lymphangiogenesis) and CD-31(neoangiogenesis) was evident by IHC in tumour and metastatic lung tissue. Isolated circulating EVs were in the size range of 111-180 nm, and had a characteristic lipid bilayer. Expression of EV-associated markers CD63, CD82 and TSG-101, and absence of Calnexin was confirmed. Plasma EV concentration ranged from 1.23×10^{11} – 1.59×10^{12} particles/ml.

Conclusion

While developing treatments for metastatic disease in patients who usually have their primary tumour removed, it is important to use models that more closely reflect this. Establishing reproducible molecular characteristics of this model will support robust analysis of novel therapeutic interventions.

EACR22-0370

Tert mutant promoter mouse model induces cancer progression of BrafV600E-driven thyroid tumors

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Introduction

Hotspot mutations in the proximal promoter of the telomerase reverse transcriptase (*TERT*) gene are the first cross-cancer alterations lying in a gene regulatory region. *TERT* promoter mutations (TPMs) are markers of disease severity: they are enriched in the aggressive poorly differentiated (PDTC) and anaplastic thyroid cancers (ATC), compared to their well-differentiated counterparts, i.e., papillary thyroid cancers (PTC). TPMs reactivate *TERT* transcription, which is otherwise silenced in adult tissues, thus reactivating a *bona fide* oncoprotein with multiple roles in cancer biology.

Material and Methods

To study *TERT* deregulation and its downstream consequences in a biologically accurate model, we generated the first *Tert*-mutant promoter mouse model via CRISPR/Cas9 editing of the equivalent murine *locus* and crossed these animals with thyroid-specific Braf^{V600E}.

mutant mice. In parallel, we employed an alternative model of *Tert* overexpression. *In vivo* genetic and phenotypic characterization of thyroid tumors was performed.

Results and Discussions

Braf^{V600E} animals develop highly penetrant PTCs by week 5, but hardly ever progress. In contrast, Braf^{V600E}+Tert^{MUT} animals showed an increased incidence of PDTCT by 20 weeks (30% vs. 0% in Braf^{V600E}; chi-squared $P=0.03$), mimicking those exhibited by the *Tert* overexpression model (Braf^{V600E}+K5-Tert; 36% PDTCTs). Mouse *Tert* promoter mutation increased *Tert* transcription *in vitro* and *in vivo*, as reported in patients' tumors carrying TPMs. Braf+Tert animals partially responded to MAPK pathway inhibition (dabrafenib plus trametinib), showing that this crucial pathway remains relevant in these specimens. Interestingly, RNA sequencing of *Tert*-reactivated murine thyroid tumors showed unique transcriptomic profiles (compared to Braf^{V600E} alone), suggesting that downstream effects, other than the canonical *Tert*-mediated telomere maintenance, operate in cancers harboring TPMs. These pointed to key cancer functional processes that might inform new routes for therapeutic intervention in telomerase-mediated aggressive thyroid cancers.

Conclusion

These cancer models of telomerase reactivation provide excellent pre-clinical settings to understand the regulatory mechanisms and biological underpinnings of TPM-induced progression of thyroid (and other tumors) to aggressive disease, and to explore novel therapeutic strategies.

EACR22-0422

ESTABLISHMENT OF PATIENT-DERIVED PRECLINICAL MODELS FOR ENDOMETRIAL CANCER.

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Introduction

Endometrial cancer (EC) is the most common gynecological cancer in developed countries. Despite presenting a favorable prognosis, around 15-20% of EC patients will experience

recurrence or die from the disease. Nowadays, adjuvant chemotherapy is estimated to benefit only 10–15% of all EC patients. Under this scenario, the impact of tailored treatments based on a molecular profile and novel strategies remains crucial. For this purpose, patient-derived xenograft (PDX) models have demonstrated their utility as powerful tools in cancer research to study patient treatment response since they preserve both original tumor characteristics and tumor heterogeneity.

Material and Methods

We established a series of 15 patient-derived orthotopic xenografts (PDOXs) mouse models from EC patient tissues. Characterization of the models was done by analyzing tumor histology, expression of cancer related markers by immunohistochemistry (IHC), a mutational panel of 57 cancer-associated genes and gene expression profiles (RNAseq) between paired donor and PDOX.

Results and Discussions

We detected a total of 69 and 85 mutations in primary tumors (PT) and in PDOXs, respectively. Following the TCGA classification, we divided primary tumors according to their assessed mutations and IHC results into *POLE*, *MSI*, *MSS* and *TP53* mutated groups. A total of 48 mutations were coincidental pairwise (69.57% and 56.47%, respectively), suggesting that PDOXs recapitulated most mutations observed in PT. More specifically, same mutations were observed in important genes for EC development such as *PTEN*, *PIK3CA*, *TP53*, and *KRAS*. At the histological level, PDOXs presented a concordance of 66.67% with their PT, some models showed higher tumor grade compared to their PT. More aggressive biology was seen in two PDOXs, which presented undifferentiated tumor areas and serous component, not observed in PT. High concordance was also found by IHC regarding *PTEN* (100%), *TP53* (93.33%), *ESR* (93.33%), *MSH6* (100%), *CTNNB1* (100%), and *PMS2* (100%) expression. At the gene expression level, a high correlation was also observed ($R=0.89-0.95$). GSEA analysis identified that genes with enriched expression in PT were associated with human tumor microenvironment.

Conclusion

PDOXs shared original tumors characteristics, based on histological, IHC, gene expression and mutational analyses, allowing wide-ranging anticancer drug testing.

EACR22-0544

MYC-MIZ1 interaction shapes treatment response in Small Cell Lung Cancer

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Introduction

Lung cancer is the leading cause of cancer related deaths worldwide. Small cell lung cancer (SCLC) is an aggressive, neuroendocrine subtype of lung cancer and accounts for 15% of all lung cancer cases and has a 5-year survival rate below 5%. SCLC is genetically characterized by a biallelic loss of *Rb1* and *TP53*. Furthermore, 20% of all SCLC patients display genomic amplifications of *MYC* paralogs (*MYC*, *MYCN* and *MYCL*). However, no specific drug is currently available to target *Myc* and it remains unclear whether *Myc* activity has an impact on the response to chemotherapy.

Material and Methods

In recent studies we found that *MYC*, but not *MYCN* and *MYCL*, represses *BCL2* transcription via interaction with *MIZ1* and *DNMT3a* leading to increased

sensitivity to cell cycle control inhibition. A dimeric Myc/Max complex activates gene transcription, whereas Myc in complex with Miz1 can represses transcription. To further investigate the complex interplay of Myc and Miz1 in SCLC we developed a novel mouse model combining the RPM (Rb^{fl} Tp53^{fl} Myc^{LSL}) mouse with a delta POZ Miz1 allele (RPMM = Rb^{fl} Tp53^{fl} Myc^{LSL} MIZ^{ΔPOZ fl}). In this model Miz1 is unable to multimerize and consequently unable to bind to the DNA. This in turn means, the interaction of Myc and Miz1 is disrupted and possible gene expression changes may influence the development of SCLC lung tumors.

Results and Discussions

First, we analyzed tumor formation in the RPMM mice and found that all RPMM mice developed tumors after Ad5CGRP-Cre inhalation and showed a prolonged survival compared to the RPM mouse model (RPM 60.6 days vs RPMM 68.1 days). Tumors arising in RPMM mice tended to be smaller when compared to RPM mice and were confirmed to display an SCLC phenotype by H&E and Neural cell adhesion molecule (NCAM; CD56) staining. Furthermore, we observed decreased DNA-damage induction in cell lines derived from RPMM tumors compared to RPM cell lines. In addition to lower steady-state γH2AX signal, preliminary experiments indicate that a subset of RPMM-derived cells may show a higher sensitivity towards chemotherapy when compared to RPM-derived cells expressing wild type Miz1.

Conclusion

Thus, our preliminary data indicate that the Myc/Miz1 interaction may shape the therapeutic response and the development of SCLC tumors.

EACR22-0600

Mouse models for the study of cancer associated cachexia and tryptophan modulation

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Introduction

Cancer cachexia is a multifactorial syndrome identified by involuntary /pathological weight loss with skeletal muscle wasting. It is characterized by systemic inflammation and metabolic alterations that interferes with treatment. Patients suffer a considerable reduction in quality of life and survival. It is estimated that 20-30% of deaths in cancer cachectic patients derive from cachexia rather than from the tumor itself, but there is no effective treatment for cachexia nowadays (Dolly, 2020; Marcena, 2020). Together with the lack of biomarkers and the unknown origin, it is fundamental to increase efforts in cachexia studies. A study previously performed by our group revealed alterations on metabolites related to Trp degradation (Cala, 2018). The aim of this study was to validate mouse models for the study of cancer associated cachexia and to investigate the role of Trp in this syndrome.

Material and Methods

Human plasmas were obtained at 12 de Octubre Hospital (details in Cala, 2018). For animals, we used two syngeneic murine models based on the melanoma B16F10 cells and pancreatic adenocarcinoma KPC cells. Both were injected in the flank of C57BL/6J mice and followed during 21 or 39 days, respectively.

Results and Discussions

B16F10 and KPC injected mice showed a reduction in body weight and a decreased in fat. Also, spleen weight and MCP-1 plasma levels increased in tumor bearing mice compared to control animals. CRP and Myostatin increased in B16F10 but not in KPC injected mice, may be due to differences in tumor growth and different waves of inflammation. Skeletal muscle analysis showed a reduction of cross-sectional area in these mice (especially B16F10) and an increase in the expression of atrophy genes. Altogether, inflammation and muscle affectation, recapitulate cancer associated cachexia syndrome in humans. Based on the results of our previous study, here we confirmed that Trp levels in plasma of cachectic patients were significantly reduced. Interestingly, Trp levels in B16F10 tumor bearing mice were also reduced even at early steps of the tumorigenesis. In KPC injected mice, Trp values were oscillating which may also be due to the longer experimental time.

Conclusion

B16F10 and KPC syngeneic mouse models recapitulate features of cancer associated cachexia in terms of inflammation, skeletal muscle damage, and levels of Trp in plasma. Thus, they can serve as models for the study of pharmacological intervention in the prevention of cancer-associated cachexia.

EACR22-0792

Evaluating the potential of an interventional immunotherapy in a pre-clinical model of lung squamous cell carcinoma

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Introduction

Lung cancer is a leading cause of cancer-related death worldwide. Lung squamous cell carcinoma (LSCC), a lung cancer sub-type, accounts for almost a third of lung cancers in the UK. LSCC is a complex disease with multiple stages of pre-invasive disease leading to invasive LSCC. Immune infiltration has been shown to be influential in pre-invasive lesion progression to invasive disease. This presents the possibility of effective interventional immunotherapies to prevent invasive LSCC development, to reduce patient suffering and mortality. In this work we aim to characterise the immune changes that occur in a chemical carcinogenesis LSCC model and evaluate a tailored interventional immunotherapy.

Material and Methods

The murine N-nitroso-tris-chloroethylurea (NTCU) pre-clinical carcinogenesis model faithfully recapitulates histological characteristics of both pre-invasive and invasive LSCC. Using immunostaining, the immune association and infiltration of NTCU-induced pre-invasive and invasive disease were quantified. The PI3K inhibitor PI-3065, a small molecule inhibitor which impairs regulatory T-cell maintenance and functionality, was applied as an interventional immunotherapy during NTCU-induced carcinogenesis, and its effect on immune infiltration and disease progression was evaluated.

Results and Discussions

The immune composition of the murine lung undergoes dynamic changes during NTCU-induced carcinogenesis. With disease progression, CD4⁺ and CD4⁺/FOXP3⁺ T-cell subsets accumulate in proximity to both pre-invasive and invasive disease lesions, likely promoting an immunosuppressive environment that facilitates disease progression. Interventional treatment with systemically-administered PI-3065 via diet, reduced NTCU-induced tumour incidence. This response was associated with reduced accumulations of CD4⁺ and CD4⁺/FOXP3⁺ T-cells in proximity to pre-invasive disease lesions.

Conclusion

The development of NTCU-induced LSCC in immune-competent mice offers the important opportunity to test the efficacy of immune-targeted interventional therapeutics. This study further demonstrates the importance of the immune microenvironment in determining the fate of preinvasive cancer. Systemic PI3K inhibition shows initial promise as an interventional therapeutic for lung cancer and further cements its relevance to the treatment of solid cancers.

EACR22-1194

DEVELOPMENT OF IMMUNOCOMPETENT AND ORTHOTOPIC PANCREATIC TUMOUR MOUSE MODELS

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most deadly and most aggressive neoplastic diseases in humans. Despite the research carried out in recent years, the mechanisms controlling the progression and poor response to PDAC treatment are still unknown. Therefore, it is necessary to develop reliable, reproducible, and inexpensive orthotopic animal models to better understand the processes of occurrence, development, invasion, and metastasis of this disease. Furthermore, the application of this model can be used to study the mechanism of action of drugs and prompt the discovery of new therapeutic agents.

The aim was to optimize an orthotopic, immunocompetent murine model of pancreatic cancer in mice and study the effectiveness of the applied chemotherapy with gemcitabine.

Material and Methods

In order to implant the tumour into the pancreas, a cellular spheroid model in a hanging drop was used. Spheroids form in this way were embedded in Matrigel® and then inoculated into mouse pancreas during surgical procedures. Tumour progression was imaged by a non-invasive ultrasound (using VeVo 2100). When tumours appeared (20-45 days after inoculation), the animals were scheduled to receive gemcitabine chemotherapy. The therapy lasted 16 days, during which 6 doses of gemcitabine were administered (72 h time interval) at a concentration of 45 mg/kg of the animal's body weight.

Results and Discussions

The new model showed >80% acceptability and presented the response to gemcitabine treatment.

Conclusion

The new mouse model of pancreatic cancer, due to its orthotopic nature, will recreate not only the neoplastic process itself but also the organism responses, including the immune system, which will significantly improve our knowledge of pancreatic cancer, as well as

enable research into new therapies and screening drugs that may prove to be of significant importance in clinical treatment.

EACR22-0021

PDX tumor growth comparisons in the ultra-immunodeficient NOD.CB17-Prkdcscid IL2rgtm1/Bcgen (B-NDG) mouse model

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Introduction

The purpose of the study was to compare the growth of various PDX models in the two ultraimmunodeficient mouse models NSG™ (Jackson Laboratory) and B-NDG (Inotiv).

Material and Methods

Four breast cancer PDX models from the Washington University Human in Mouse (WHIM) collection, ER+ WHIM20 and WHIM81, Her2+ WHIM43, and triple negative WHIM5, and one melanoma PDX models generated by the Wistar institute, WM4071-2, was utilized. For models WHIM20, WHIM5, and WM4071-2 all mice were injected with tumor cells on the same day, whereas for WHIM43 BND-G mice were injected in two separate batches. The WHIM81 studies were carried out at different times for the NSG™ and B-NDG animal cohorts. Studies utilizing WHIM5, WHIM20, WHIM43 and WM4071-2, included a group of athymic nude mice as well. For all models, mice were injected with 1.5x10⁶ cells/mouse. The cells were mixed 1:1 with PBS:Corning Matrigel GFR and the injection volume was 100 µL/mouse. Mice were housed in Innovive individually vented cages and consumed Teklad Global Rodent Diet 2019 throughout the study. Tumors were measured multiple times per week using Bioptron's TumorImager™.

Results and Discussions

For all models except WHIM81, the tumors grew well and with very similar rate in both B-NDG and NSG™ mice. WHIM20 and WHIM43 grew significantly slower in the Athymic nude mice compared to both of the ultraimmunodeficient mouse models, however, the growth of WHIM5 was comparable between all three models. The results from the WHIM81 model suggests faster growth in the B-NDG model compared to the NSG™, however, it is also possible that this is due to the different batches of tumor cells utilized at each implantation event.

Conclusion

Thus, the B-NDG model represents a novel ultraimmunodeficient mouse model that can have great utility for tumor studies, particularly for hard to grow or slower growing tumor models.

EACR22-0263

Single-cell RNA-seq reveals crosstalk between GBM cells and tumor microenvironment in patient-derived orthotopic xenograft models

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Introduction

A major contributing factor to Glioblastoma (GBM) development and progression is its ability to evade the immune system by creating an immune-suppressive environment. GBM-associated myeloid cells, including resident microglia, macrophages, and other peripheral immune cells are generally geared towards tumor-supportive cells. It is however unclear whether such recruited myeloid cells are phenotypically and functionally identical. This study aimed to understand the heterogeneity and functional status of the GBM immune microenvironment, using an unbiased, marker-free approach to systematically characterize cell type identities at the molecular level.

Material and Methods

We applied single-cell RNA-sequencing, multicolor flow cytometry and immunohistochemical analyses to understand the crosstalk between tumor cells and tumor microenvironment (TME) and to determine the heterogeneity of the myeloid phenotypic states in the TME. GBM patient-derived orthotopic xenografts (PDOXs) representing different tumor phenotypes and glioma mouse GL261 models were used.

Results and Discussions

We show that PDOX models recapitulate major TME components found in human GBM. We find that the most prominent transcriptomic adaptations of the brain to a growing tumor are found in tumor-associated microglia/macrophages (TAMs). We revealed inter-patient heterogeneity of myeloid cells in GBM and identified key signatures of different subpopulations within the myeloid compartment, their functional and clinical significance both in mouse models and human GBMs. We show that the majority of myeloid cells in PDOXs and human GBM display a microglia-derived TAMs signature. Notably, GBM-educated microglia show differential expression of genes involved in immunosuppression, phagocytosis and antigen presentation, indicating functional cross talk with the GBM cells. PDOXs models represent a powerful tool for further functional characterization to harness the anti-tumor therapeutic potential of microglia-derived TAMs.

Conclusion

Our data provide insights into the local and molecular adaptation of the heterogeneous TME instructed by GBM tumors. This work further confirmed the clinical relevance of GBM PDOXs for the testing of novel therapeutics including immunotherapeutics designed to target the myeloid compartment.

EACR22-0797

Zebrafish tumor-derived xenograft-models for improved diagnosis and treatment planning in ovarian cancer patients

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Introduction

Precision medicine approaches based on DNA or RNA analyses are often not identifying actionable mutations in the patients, and have not been established for predicting response to commonly used drugs such as Paclitaxel and Carboplatin used as first-line treatment for epithelial ovarian cancer (EOC). A majority of patients with advanced-stage EOC will experience cancer recurrences, which occurs faster for patients that do not respond to first-line therapy. There is a need for methods to predict outcome to first-line therapies, prior to treatment onset, so that the optimal treatment can be identified and offered to every patient.

Zebrafish tumor xenograft (ZTX) models have recently been used to predict treatment outcomes to first-line therapy in multiple myeloma, neuroendocrine, colorectal, lung, and gastric cancer, but EOC and the main drugs used to treat EOC patients have not been studied in ZTX models in the past. With this study, we aim to find a model for prediction of clinical outcomes to Carboplatin and Paclitaxel in EOC.

Material and Methods

25 tumor samples were used from 22 patients with EOC. The tumors were dissociated into a single cell solution, dyed, and then injected into zebrafish larvae. The larvae were kept for 3 days in either Carboplatin, Paclitaxel or without treatment. Photos were taken at the day of the injection and 3 days later to compare tumor growth and dissemination of metastatic cells to the tail. The results were compared to the clinical outcome of the patients.

Results and Discussions

Patients receiving neoadjuvant chemotherapy (NACT) only undergo surgery if responsive to the treatment. All 6 tumors from Carboplatin NACT-treated patients also responded in the ZTX model. Among patients treated with primary surgery, followed by adjuvant Carboplatin, 7 of 11 responded to Carboplatin in the ZTX model, which is similar to the expected clinical outcome. 4 of 6 patients from the group treated with NACT and 7 of 10 in the primary surgery group responded to Paclitaxel.

We have not been able to predict which patients will relapse and progress after treatment in this study. This may be due to patients developing a drug resistance later in the treatment which this model cannot predict.

Conclusion

We have shown that the ZTX model accurately recapitulates response to the first-line treatment with Carboplatin with high sensitivity and specificity and of Paclitaxel with lower sensitivity and specificity. ZTX models could be helpful in the treatment planning of ovarian cancer patients in the future.

EACR22-0831

Establishment and characterization of a new panel of uterine leiomyosarcoma patient-derived xenograft models

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Introduction

Uterine leiomyosarcoma (uLMS) is a malignant mesenchymal tumor that originates in the smooth muscles. Prognosis for patients with unresectable or metastatic uLMS still remains poor and the chemotherapy treatments are mainly palliative. The genetic abnormalities of uLMS are very complex and may contribute to the low sensitivity to chemotherapy. Valid preclinical models are urgently needed to better characterize the molecular basis of this tumor and to identify new therapeutic strategies. For these reasons, we have established a panel of uLMS patient-derived xenografts (PDX).

Material and Methods

Tumor tissues collected from women affected by uLMS were engrafted subcutaneously in nude mice and PDX maintained by serial *in vivo* passages. Pathological analysis was done to compare PDX morphology and phenotype with those of the original tumors. Patients and PDX biopsies were processed for genomic analysis by Next Generation Sequencing. Finally, we characterized the pharmacological profile of the PDX testing the antitumor activity of doxorubicin, dacarbazine, trabectedin and eribulin, all clinically used drugs.

Results and Discussions

PDXs were successfully developed from four patients and named LM01, LM02, LM03, LM04. The obtained PDXs reproduced the main morphological features and maintained the reactivity to the clinical markers of the original tumors. Similarly, somatic variants were comparable in the PDX and in the corresponding human specimen. We observed a high heterogeneity among them, and this aspect represents the complexity of clinical uLMS. In particular, LM01 carried a mutation in SEC31A, LM02 was characterized by variants in TOM1 and SERPINB3, LM03 carried mutations in ARFGEF1, PIK3C2B and TCF7L2, LM04 in TP53, DIS3 and PTPRF. This molecular heterogeneity was paralleled by a different drug sensitivity. Indeed, eribulin caused an impressive tumor regression in LM01 and a long lasting inhibition of LM03 tumor growth, and trabectedin reduced tumor growth in LM03, but was completely inactive against the others uLMS PDXs. All the PDX models were poorly sensitive to doxorubicin and dacarbazine (the current standard of care).

Conclusion

We have obtained a panel of uLMS PDXs that mimics the main pathological and molecular features of the original human tumors. The heterogeneity of drug response highlights the need to expand the uLMS PDX panel to identify possible mechanisms of drug resistance and to identify new therapeutic approaches better than the standard of care.

EACR22-0981

Desmoplastic Small Round Cell Tumor: modelling the disease

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Introduction

Desmoplastic small round cell tumor (DSRCT) is a rare sarcoma whose molecular hallmark is a gene fusion between EWSR1 and WT1. DSRCT remains poorly known and presents a dismal prognosis. The development of research models mimicking the disease is essential to better understand the biology of this tumor and establish new and improved therapeutic strategies.

Material and Methods

HEK293T and hMSC-5H cell lines were transduced with pLV. EWSR1 ex7 - WT1 ex 8 - HA and pLV. EWSR1 ex10 - WT1 ex8 - HA. Candidate clones were isolated and HA positive expression was detected by FACS and immunofluorescence. EWS-WT1 and HA expression were confirmed by western blot and q-RT-PCR, as well as some targets of the fusion. DSRCT cell line JN-DSRCT-1 was transduced with a plasmid encoding the GFP/luciferase-2 genes. Positive cell populations were confirmed by FACS and luciferase assay. Cells were implanted in the retroperitoneal area of male NSG mice. In vivo tracking was evaluated by bioluminescence every 5 days. Tumors and adjacent tissue were resected for histological evaluation.

Results and Discussions

Ectopic EWSR1-WT1 expressing models were successfully generated with 7% of HA positive cells found in the hMSC-5H cell line and over 80% of positive cells in the HEK293T cell line. Protein analysis confirmed that the selected HEK293T clones ectopically express EWS-WT1 and HA. Expression analysis of known target genes is currently ongoing.

A stable DSRCT cell line co-expressing GFP and luciferase was generated.

Functional studies showed that the JN-DRCT-1 GFP-Luc cell line had a slightly lower proliferation rate/cell cycle profile, in comparison to the WT. Nonetheless, given that these differences were not significant, cells were used to perform in vivo studies. Three conditions were implanted: i) cells in matrigel, ii) cells in medium and iii) spheroids. Tumor growth rate was higher in mice implanted with cells in matrigel, where the tumor best resembled DSRCT. Histological evaluation identified tumor cells in all groups.

Conclusion

We have successfully generated in vitro and in vivo models that will help us identify the oncogenic role of the gene fusion and unveil new therapeutic targets.

EACR22-0985

Identification of novel therapeutic strategies against pediatric sarcoma by high-content drug screening in zebrafish xenografts

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Introduction

Ewing sarcoma and osteosarcoma are pediatric bone cancers for which new therapies to improve disease outcome and to reduce side effects of current standard treatment regimens are urgently needed.

High-throughput phenotypic drug screening is a powerful method to identify novel drugs with efficacy against cancer cells. Ideally, such phenotypic drug screens are carried out *in vivo*. Unfortunately, achievable throughput is limited in mouse xenografts, the preclinical standard *in vivo* model for osteosarcoma and Ewing sarcoma. Here, we explored the use of xenografts in zebrafish larvae for high-throughput, high-content drug screening to identify novel therapeutic strategies for Ewing sarcoma and osteosarcoma. **Material and Methods**

Ewing sarcoma and osteosarcoma cell lines as well as patient-derived Ewing sarcoma cells were injected into 2 day post fertilization zebrafish larvae. Drug treatment was performed from day 3 to day 5 post fertilization. Xenografted larvae were imaged on a high-content imager on day 3 prior to drug treatment and on day 5 post drug treatment. Tumor size was quantified at both time points and changes in size were determined to identify effective compounds.

Results and Discussions

We successfully established Ewing sarcoma and osteosarcoma xenografts in zebrafish larvae. We developed an automated workflow for zebrafish xenograft imaging on a high-content imager, tumor detection and tumor size quantification over time. The increase in throughput of our automated setup allowed us to test single compounds, but also drug combinations. We discovered several compounds with activity against sarcoma cells in zebrafish xenografts. Importantly, we identified synergistic compound combinations, which were able to eradicate Ewing sarcoma cells in our assay completely.

Conclusion

In conclusion, we established an automated workflow for drug screening on zebrafish xenografts, which can be easily adapted for various cancer entities. We were able to identify combination therapies highly efficient at eradicating Ewing sarcoma cells in our xenograft model. The identified drug combinations warrant further investigation towards potential clinical application.

EACR22-1185

PDCM Finder: open global cancer research platform for patient-derived cancer models

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Introduction

Patient-derived cancer models (PDCMs) are a powerful oncology research platform for studying tumour biology, mechanisms of drug response and resistance and for testing personalised medicine. Distributed nature of repositories for PDCMs (xenografts, organoids

and cell lines) and the use of different metadata standards for describing model's characteristics make it difficult for researchers to identify suitable PDCM models relevant to specific cancer research questions. PDCM Finder aims to solve this problem by providing harmonized and integrated model attributes to support consistent searching across the originating resources.

Material and Methods

PDCM Finder builds on the success of the PDX Finder resource (<https://www.pdxfinder.org/>, PMID:30535239). Critical PDCM attributes, such as diagnosis, drug names and genes, are harmonized and integrated into a cohesive ontological model based on the PDX Minimal information standard (PDX MI, PMID: 29092942). PDX MI has become established in the community for data exchange, adopted by the PDX providers, consortia and informatic tools integrating PDX data. We are driving the development of and promoting the use of descriptive standards to facilitate data interoperability and promote global sharing of models. We provide expertise and software components to support several worldwide consortia including PDXNet, PDMR and EurOPDX. PDCM Finder is freely available under an Apache 2.0 license (<https://github.com/PDCMfinder>). This work is supported by NCI U24 CA204781 01, U24 CA253539, and R01 CA089713.

Results and Discussions

Users can search for models of interest by either (1) exploring molecular data summaries for models of specific cancer types, or (2) using the intuitive search and faceted filtering options of the web user interface or (3) access resource database via REST API to run their own analysis. The data includes gene expression, gene mutation, copy number alteration, cytogenetics, patient treatment and drug dosing studies. We link external resources like publication platforms and cancer specific annotation tools enabling exploration and prioritization of PDCM variation data (COSMIC, CIViC, OncoMX, OpenCRAVAT).

Conclusion

PDCM Finder is a new portal that aggregates patient-derived models from 27 academic and commercial providers, enables users to search and compare over 6000 models and associated molecular data, and connects users with model providers to facilitate collaboration among researchers.

EACR22-0638

Molecular steps in Lynch-associated endometrial and ovarian tumorigenesis

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Introduction

Lynch syndrome (LS) is a common inherited cancer syndrome with gynecological cancers as one of its main manifestations. Pathogenic germline variants of DNA mismatch repair (MMR) genes *MLH1*, *MSH2*, *MSH6* and *PMS2* characterize the syndrome. Molecular changes in gynecological tumors are well documented but understanding the path from healthy tissue to premalignant lesions needs more investigation. We have previously found that microsatellite instability (MSI) and MMR deficiency may occur even in histologically normal biopsies, and loss of ARID1A protein is common starting from endometrial hyperplasia. In this ongoing investigation, we aim to identify key molecular changes and their timing in LS endometrial and ovarian carcinoma development.

Material and Methods

We conducted PanCancer panel sequencing on 104 follow-up samples from a retrospective dataset from 33 LS patients with endometrial (EnCa) and/or ovarian cancer (OvCa). Patients were recruited from the nation-wide Hereditary Colorectal Cancer Registry of Finland. Endometrial samples were available from different time points up to 15 years before hyperplasia or carcinoma.

Results and Discussions

The average number of somatic nonsynonymous mutations/Mb (tumor mutation burden, TMB) did not significantly differ between ovarian (14.5) and endometrial cancers (20.0) or hyperplasia (12.6) and cancers (17.8), but a significant difference was found between normal (1.9) and hyperplasia (p .01) and normal and cancer samples (p .01). Loss of the wild type allele of the mutated MMR gene was found in one patient's normal endometrial samples (MSI by BAT26 marker) four years before hyperplasia or cancer, and in one patient's hyperplasia (MSI by BAT25 and 26 markers; MLH1 negative by IHC) one year before cancer. Among 721 different significant (p < .01) somatic nonsynonymous mutations with variant allele frequency of at least 10%, mutated *KMT2C* (histone lysine methyltransferase) was found in 39% of cancers and 20 % of hyperplasias, respectively. Mutated *ARHGAP35* (Rho GTPase activating protein) occurred in 35 % of cancers and 25% of hyperplasias, including one hyperplasia seven years before cancer, suggesting a possible driver gene status.

Conclusion

Molecular alterations are detectable in normal endometrium and endometrial hyperplasia years before actual cancer development in LS patients.

EACR22-0034

Epigenetic influence of our (fatty) diet on metastatic-initiating cells: role of intra-tumor Schwann cells

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Introduction

We have recently identified the cells uniquely responsible for the formation of metastasis in different types of human tumors. These cells have intriguing characteristics: i) they form primary lesions as efficiently as their tumor-initiating counterparts, but are exclusive in their ability to generate metastases; ii) they express the fatty acid translocase CD36, and are characterized by a unique lipid metabolic signature; iii) they are exquisitely sensitive to the levels of fat in circulation, and consequently, they relate the predisposition of metastasis directly to the content of dietary fat; iv) they are highly sensitive to CD36 inhibition, which almost completely abolishes their metastatic potential in preclinical models. In my talk, I will present our latest data on the molecular characterization of metastatic-initiating cells. I will place special emphasis showing how specific dietary lipids exert a striking long-term epigenetic effect over metastatic-initiating cells related to activation of intra-tumor Schwann cells, putting forward a novel concept of "metastatic epigenetic memory" elicited by our diet.

Material and Methods

ii) they express the fatty acid translocase CD36, and are characterized by a unique lipid metabolic signature; iii) they are exquisitely sensitive to the levels of fat in circulation, and consequently, they relate the predisposition of metastasis directly to the content of dietary fat; iv) they are highly sensitive to CD36 inhibition, which almost completely abolishes their metastatic potential in preclinical models.

Results and Discussions

In my talk, I will present our latest data on the molecular characterization of metastatic-initiating cells. I will place special emphasis showing how specific dietary lipids exert a striking long-term epigenetic effect over metastatic-initiating cells related to activation of intra-tumor Schwann cells, putting forward a novel concept of "metastatic epigenetic memory" elicited by our diet.

Conclusion

In my talk, I will present our latest data on the molecular characterization of metastatic-initiating cells. I will place special emphasis showing how specific dietary lipids exert a striking long-term epigenetic effect over metastatic-initiating cells related to activation of intra-tumor Schwann cells, putting forward a novel concept of "metastatic epigenetic memory" elicited by our diet.

EACR22-0060

The role of miR-662 in breast-to-bone metastasis

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Introduction

Breast cancer (BC) is the most common cancer in women. Metastatic BC, often in bone (~80%), is responsible for 90% of BC mortality and urgently requires new therapeutic approaches. MicroRNAs (miRNAs) are small non-coding RNA that negatively regulate expression of mRNA targets in cells. MiRNA expression is often dysregulated in BC cells, contributing to tumour progression and metastasis. MiRNAs can be released from cells, being extremely stable in the bloodstream. MiRNAs and their mRNA targets could be therapeutic targets for BC patients, and circulating miRNAs could be used as biomarkers to follow BC progression and predict metastasis risk.

We analysed by TLDA the expression of >700 serum-derived miRNAs from 48 BC patients with a 10-year clinical follow-up. Based on patients' information, we identified a few circulating miRNAs associated with BC bone recurrence. Among them, miR-662 was also associated with BC cell stemness in patient-derived xenograft models. These findings led us to hypothesise that miR-662 could mediate breast-to-bone metastasis progression that we aimed to investigate at the functional level.

Material and Methods

BC cell models: human (MDA-MB-231) and murine (4T1) cells;

Metastasis murine models: young female mice (Balb/c, *nude*), intracardiac injections of BC cells;

BC cell-based assays: proliferation, migration, ALDH activity;

Osteoclastogenesis: murine and human bone marrow-derived osteoclasts, differentiation with/without BC cell conditioned medium;

RNA-sequencing: PolyA-enriched library, Hiseq Rapid.

Results and Discussions

MiR-662 overexpression increased proliferation, migration, and stemness in BC cells *in vitro*. *In vivo*, miR-662 overexpression in highly metastatic 4T1 cells promoted metastasis formation. Interestingly, miR-662 overexpression in bone-tropic MDA-MB-231-NW1 cells inhibited tumour burden, especially in bone, at an early stage of metastasis formation in animals, explained by a transient inhibition of osteoclast-mediated bone resorption, then overtaken at later stages of bone metastasis progression. We also identified by RNA sequencing mRNA targets of miR-662, and observed they were mainly involved in cancer progression, immune suppression, and stemness.

Conclusion

MiR-662 promotes BC metastasis, especially in bone, making this miRNA and its mRNA targets interesting therapeutic targets for BC metastasis. Since circulating levels of miR-662 associate with bone metastasis recurrence, miR-662 could be a good candidate as biomarker to predict bone metastasis recurrence in BC patients.

EACR22-0156

Characterization of invasive cancer cells using catulin based reporter system.

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Introduction

Head and neck squamous cell carcinoma (HNSCC) is an aggressive tumor with a poor prognosis due to late diagnosis and loco-regional metastasis. Partial or more complete epithelial–mesenchymal transition (EMT) plays a role in tumor progression; however, it remains a challenge to observe the EMT *in vivo*, due to its transient nature. Here, we developed a novel catulin promoter based reporter system that allows us to track *in vivo* a small fraction of invasive cancer cells.

Material and Methods

To assess the role of alpha-catulin in tumorigenesis, we have developed a novel Catulin-GFP reporter system that allows us for the first time to isolate and characterize *in vivo* a small population of cancer cells and test *in vitro* their tumorigenic potential. We have performed RNA-SEQ analysis of isolated from tumors (formed after injection of HNSCC reporter cell line) and sorted GFP positive and GFP negative cancer cells to identify a molecular signature of SCC Catulin-GFP invasive cancer cells. We have also sorted SCC15 Catulin-GFP cells from *in vitro* culture and performed a series of *in vitro* tests to verify their tumorigenic potential.

Results and Discussions

Performed series of *in vitro* tests revealed that CatulinGFP positive cells harbor the aggressive phenotype. The analyses of formed tumors revealed that CatulinGFP positive cells were enriched in clusters of cells at the tumor invasion front. A functional genomic study showed that increased alpha-catulin expression correlates with the transcriptional signature of genes involved in cellular movement and invasion. Interestingly, those cells also

activate genes involved in the signaling pathways associated with axonal guidance, and, important for perineural invasion, like the L1CAM protein, highlighting the significance of the interaction between cancer cells and nerves during the invasion in this type of cancer.

Conclusion

In conclusion, the presented data indicate that the catulin reporter system marks a population of invasive HNSCC cells and describe the transcriptional profile of those invasive cancer cells.

EACR22-0206

PHYSICAL AND FUNCTIONAL ASSOCIATION OF POTASSIUM (hERG1) AND SODIUM (NEONATAL NaV1.5) CHANNEL PROTEINS IN BREAST CANCER

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Introduction

Ion channels and transporters have recently emerged as novel mechanisms driving the cancer process, thus offering novel clinical possibilities, especially in the field of diagnosis and therapy strategies. This project focuses on two ion channels members of the voltage gated channels: hERG1 (potassium) and the neonatal form of NaV1.5 (sodium) that have been shown to be expressed in different breast cancer cell lines.

Material and Methods

We first investigated the possible co-expression of the channels in different cell lines (MDA-MB-231 WT, MDA-MB-468, MCF-7, SKBR3) and the possibility of the formation of a macromolecular complex between hERG1/nNaV1.5. In addition to that, integrin receptors and ion channels communicate by diffusible signals as well as by forming macromolecular complexes and hERG1 has been demonstrated to form with the $\beta 1$ integrin subunit. Furthermore, effects of specific potassium and sodium blockers has been tested on cells functional activity. Because of the low endogenous expression of hERG1 in the MDA-MB-231-WT line, a model was built through a stable transfection of hERG1.

Results and Discussions

Results showed that, specifically MDA-MB-231 and 468 cells treated with E4031 and TTX showed a lower invasiveness against control. Through IF experiments cells treated with the two blockers and probed with phalloidin showed a clear alteration of the cytoskeleton, probably related to an effect on stress fibres. Using an orthotopic model of BCa the effects of hERG1 (and nNaV1.5) over expression in vivo using hERG1 transfected MDA MB-231-cells showed that compared to Mock derived masses, in masses derived from MDA-MB231-hERG1 cells, the two channel proteins were expressed at higher levels. This is reflected when considering staining intensity. Also, due to the clinical and biological relevance of TNBCa, we focused our analysis on four molecular subtypes of BCa primary samples (Luminal-A, Luminal-B, HER2-positive, and Basal-like/TNBCa) and a statistically significant positive correlation between hERG1 and nNav1.5 expression emerged.

Conclusion

In conclusion, nNaV1.5 was highly expressed in primary BCa, including TNBCa, samples and this was correlated positively with the expression of hERG1.

Targeting melanocytic markers in uveal melanoma and their clinical significance with patient outcome

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Introduction

Understanding the molecular pathology of pigmentation markers and its association with Uveal Melanoma (UM) risk could be a new approach to identify early metastasis. High melanin content could lead to its high metastatic potential in UM cells. TRP1 (Tyrosine Related Protein 1) and TRP2 (Tyrosine Related Protein 2) are important pigmentation marker that regulates eumelanin synthesis in the Asian population. Literature showed that TRP1 inhibits melanocytic cell death and its high expression has been seen in cutaneous melanoma, but expression of TRP2 has not been explored in melanoma yet. Our aim of the study was to detect the expression of TRP1 and TRP2 in high and low pigmented UM and correlated with patient outcome.

Material and Methods

Fifty formalin fixed paraffin embedded UM tissue samples were included to detect the expression of TRP1 and TRP2 proteins by immunohistochemistry (IHC) and validated by western blot. mRNA expression was also observed by quantitative real time PCR. Cox proportional hazard model and log rank test were used to determine the prognostic outcome of melanocytic markers.

Results and Discussions

High TRP1 and TRP2 expression was found in 61% and 52% cases, respectively which was correlated with high pigmentation and BAP1 loss. Expression of IHC was also validated by western blotting in representative cases. Upregulation of TRP1 was found in 75% cases, whereas TRP2 was found in 69% cases at mRNA level. This was statistically significant with high pigmentation, largest tumor diameter (>15mm) and BAP1 loss. High expression of TRP1 protein showed reduced disease-free survival ($p < 0.001$).

Conclusion

Our study showed that high pigmentation might affect the tumor microenvironment in UM. TRP1 and TRP2 could be a potential melanocytic biomarker with therapeutic value in these patients. However, new approaches are needed to identify which would help in stratifying the patients needed for longer follow up.

Breast cancer brain metastasis formation: Disclosure of hub molecular players

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Introduction

Breast Cancer (BC), is the most frequent neoplasia in women, with 15-25% of cases developing brain metastasis (BM). This disease is a life-threatening condition to which accounts the poor understanding of BC cells' (BCCs) extravasation into the brain precluding the development of preventive strategies. The blood-brain barrier (BBB), which ensures brain homeostasis, is highly impervious to most therapeutics but not to BCCs. Thus, we aimed to unravel the mechanisms involved in BBB endothelial cells-BCCs interaction, underlying BBB alterations and the transendothelial migration of malignant cells, which ultimately lead to brain metastasis formation.

Material and Methods

We used brain microvascular endothelial cells (BMECs) as a BBB *in vitro* model, under conditions mimicking shear stress to enhance *in vivo*-like BBB features. Mixed cultures were achieved by the addition of fluorescently labelled BCCs to differentiate individual cell populations. The temporal profile and major players involved in BBB endothelium-BCCs interplay were evaluated by immunofluorescence and *in-depth* image analysis.

Results and Discussions

Shear stress enhanced BBB *in vivo* like-features, while nurturing BCCs acquisition of a metastasis-like phenotype. BCCs acquired migratory mechanisms uncovered by invadopodia formation and increased expression of β 4-integrin and focal adhesion kinase. Curiously, upon contact, both BBB endothelial cells and BCCs upregulated connexin 43 expression in cell-cell contacts, demonstrating the value of cellular communication for cell interaction. Importantly, barrier integrity became compromised at later timepoints of BCCs-BBB interaction, as disclosed by junctional proteins (β -catenin and zonula occludens-1) disruption. Moreover, cytoskeleton and caveolae-associated proteins (myosin light chain kinase and caveolin-1, respectively) upregulation occurred, suggesting the involvement of both paracellular and transcellular routes along BCCs transmigration.

Conclusion

With this work, underlying players implicated in BC brain metastases formation were uncovered, divulging prospective therapeutic targets for metastases prevention.

EACR22-0706

Characterizing the role of 4E-BP1 in breast cancer metastasis.

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Introduction

Metastasis is the leading cause of death among breast cancer patients. The multi-step process of metastasis (metastatic cascade) is intricate and still poorly understood. However, it is recognized to be a highly inefficient and strenuous process for a cancer cell. mRNA translation, which rapidly and reversibly perturbs the proteome, is thought to be critical in response to cellular stress during metastatic dissemination. Specifically, eukaryotic translation initiation factor 4E (eIF4E) binding proteins (4E-BPs) play a major role in

translational regulation by impeding the assembly of the eIF4F complex that recruits mRNA to the ribosome. In mammals, 4E-BPs are represented by a family of three members, 4E-BP1, 2, and 3. Although literature has supported the role of eIF4E in promoting breast cancer progression and metastasis, the role of the 4E-BPs in this process is not known. Thus, we aimed to investigate the role of the 4E-BPs in breast cancer metastasis.

Material and Methods

To model metastatic breast cancer we have utilized the three following approaches. Firstly, a germline deletion of 4E-BP1/2 was introduced into the Polyomavirus Middle T antigen (PyMT) transgenic murine model of metastatic breast cancer. Secondly, a spontaneous metastasis assay was used to monitor the ability of breast cancer cells to metastasize from the primary site to the lung, following an orthotopic injection of cells and subsequent resection of primary tumors. Lastly, an experimental metastasis model was deployed to examine the metastatic potential of cancer cells specifically within the last steps of the metastatic cascade, following a tail vein injection of cells.

Results and Discussions

We demonstrate that the germline loss of 4E-BP1/2 dramatically reduces lung metastases in the PyMT model, without impacting the growth of the primary tumors. To dissect the mechanism, we reconstituted 4E-BP1 expression in 4E-BP1/2-deficient breast cancer cells. Restoring 4E-BP1 expression in 4E-BP1/2 null cells promoted spontaneous metastasis of breast cancer cells to the lung from the primary tumor site. In turn, 4E-BP1 re-expression had no effect on lung colonization and metastatic outgrowth in a tail vein injection model.

Conclusion

Our findings suggest that 4E-BP1 is specifically necessary for the early steps of the metastatic cascade, including invasion, intravasation and/or survival in the blood stream.

EACR22-0882

Spheroid invasion assay using physiologically relevant collagen concentrations

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Introduction

An aggressive and invasive tumor is often related to a poor prognosis which is why studying the invasion process is essential. It has been well studied *in vitro* but is still not fully understood. Most of the attempts has used collagen, the main component of the extracellular matrix, as it plays a key role in the remodeling of the tumor microenvironment. The collagen closest to the tumor stiffens to help shield from incoming immune cells and can increase several folds in stiffness compared to healthy tissue. The collagen concentration is therefore an important factor when modeling the invasion process and a key factor is to choose a collagen within a physiologically relevant range.

Material and Methods

In this work we used CELLINK's BIO X 3D bioprinter to automatically dispense collagen on-top of preformed spheroids. The cell lines MDA-MB-231, a highly invasive breast cancer and A549, a less invasive lung adenocarcinoma, were preformed for four days in a 96-well ultra-low attachment plate before collagen embedding. Three different collagen concentrations were selected to represent both healthy tissue and tumor stiffness. The telocollagen TeloCol-10 from Advanced BioMatrix was used at concentrations 2, 4 and 6 mg/mL. The area of invasion into the surrounding collagen matrix was measured every day for 7 days. In

addition, the ability of transforming growth factor β (TGF- β) to increase the invasion of A549 was assessed.

Results and Discussions

Rheological measurements of the collagen were performed to be able to select a relevant concentration range. The invasion of the MDA-MB-231 cells into all three collagens increased linearly from day 1 to day 7. Interestingly, at day 7 there was a significant difference in the invasion area between 2 mg/mL and 4 mg/mL as well as 2 mg/mL and 6 mg/mL where we see an increased invasion area in the two stiffer collagens compared to the softest. We also detected an increased invasion of A549 cells into stiffer collagen compared to softer, an effect that was enhanced further with the addition of TGF- β in the medium. These results are in line with previously published *in vivo* data where stiffer collagen is linked to a highly invasive phenotype.

Conclusion

In this *in vitro* assay, we could detect a collagen concentration dependent invasion where the cells invaded more into the stiffer collagens compared to the softer. This work highlights the importance of choosing collagen stiffness within a physiologically relevant range for the target tissue to properly model the invasion process.

EACR22-0890

Circulating tumour cells shed large cytoplasmic fragments to facilitate transit through capillary branches

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Introduction

To mimic the transit of tumour cells (TCs) in the microvasculature, we used capillary-on-chip models, to visualise TCs on real-time. We observed TCs shedding large cytoplasmic fragments (CFs) and postulate that it favours their trafficking in the microvasculature.

Material and Methods

We fabricated constricted branched/linear and non-constricted geometries in polydimethylsiloxane microfluidic devices, simulating human microvessels. Single or clustered breast TCs MDA-MB 231 (formed in pluronic-coated 96 round wells) were **a)** labelled with Tracker dyes, **b)** flowed in microfluidic devices under physiological capillary pressure **20-40 cm H₂O** and **c)** tracked in a fluorescent microscope. TCs shedding frequency, volume loss and lysis were analysed from acquired recordings. We further treated cells with Cytochalasin or Colchicine. Collected cells post-transit were cultured to observe proliferation. Separation of CFs from cells was performed via 10 μ m pore size filters.

Results and Discussions

TCs shedding was a size-dependent phenomenon, where shedding cells were larger than unshed (**mean volumes: 2247 μ m³ vs. 1345 μ m³**, respectively). TCs shedding prevailed in capillary branches than linear or non-constricted geometries (**mean frequencies: 30.42% vs 3.66% vs 9.09%, respectively**), indicating that shedding may be dependent on forces generated from particular geometries. CFs had a wide size range (**1.17-11.32 μ m**) and represented **6.5-57.2%** of cytoplasmic volume. While this volume loss did not lead to major cell lysis (**mean: 3.5%**), it greatly reduced the transit time of shedding cells at levels comparable to their unshed counterparts. These data suggest that shedding functions as a

subtractive process, permitting larger cells to reduce their entrapment time. Interestingly, reducing actin polymerisation has led to an increase of shed CFs and lysis, while Colchicine reduced it below control levels, arguing towards a more involved role of actin. By discriminating between shedding and non-shedding cells, we collected these post-transit, observing similar proliferation over 7 days. Finally, CFs were separated from their TC counterparts with 0% cellular contamination ensuring CFs purity for downstream analyses.

Conclusion

These experiments identify factors that permit TC shedding and suggest that it may be an adaptation of certain TCs to escape faster from capillary beds. In future work we will explore how shedding of CFs may impact the development of metastases.

EACR22-0948

Biomechanical activation of pro-metastatic programs in CTCs by capillary constriction forces.

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Introduction

Circulating tumour cells (CTCs) experience constriction forces during their transit through capillaries that may not only compromise cell survival, but also promote adaptive mechanisms that enhance their metastatic potential. These capillary constriction forces may affect nuclear integrity by inducing nuclear envelope (NE) rupture, resulting in the release of dsDNA into the cytoplasm, the activation of the cGAS/STING signalling axis and its downstream effectors, previously reported to increase metastasis. Microfluidic platforms recapitulating capillary constriction forces were engineered and used to investigate the role of constriction forces in the activation of this pathway.

Material and Methods

Microfluidic capillary-on-chip platforms comprising 5x5µm and 10x10µm-constrictions were fabricated following a standard lithography protocol. MDA-MB-231 cells marked with a NLS-GFP signal were imaged while transiting the constrictions under physiological pressure. Reverse transfected cells with LMNA siRNA and wild type cells were collected upon transiting the constriction and stained for dsDNA, cGAS, STING, p65, p100, RelB and IRF-3, followed by quantitative image analysis.

Results and Discussions

Most NLS-GFP cells transiting 5µm-constrictions (96.74%) experienced NE rupture, whereas no NE rupture was observed in cells going through 10µm-constrictions, suggesting that only smaller capillaries may affect nuclear integrity. Higher levels of cytoplasmic dsDNA, as well as an upregulation of the cGAS/STING axis, were observed in cells that transited 5µm-constrictions when compared to control. Next, we investigated whether nuclear stiffness could influence the activation of this pathway during capillary transit. Cells silenced for LMNA presented higher levels of cytosolic dsDNA. This resulted in an increase of the cGAS/STING axis activation, suggesting that nuclei with lower stiffness are more prompt to experience NE rupture and the activation of downstream effectors previously reported to be involved in metastasis.

Conclusion

Mechanical forces induced by capillaries resulted in an upregulation of the cGAS/STING axis. Lower nuclear levels of lamin A, which have been described to enhance the metastatic potential across different cancers, increased the activation of this axis on cells experiencing capillary constriction forces when compared to wild type. These results suggest that the activation of cGAS/STING pathway during capillary transit may play a role during the metastatic dissemination.

EACR22-0950

Discovery of Candidate Driver Genes for lethal Metastatic Prostate Cancer

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Introduction

Prostate cancer (PCa) is the most common cancer type among men in Europe, but it has a relatively good prognosis, thanks to the efficacy of first line therapies. However, it is still the fifth leading cause of cancer death. This is partly due to the failure of a small subset of patients to respond to first line and subsequent therapies. Importantly, up to 50% of PCa-related mortality is accounted for a 5-10% of patients that exhibits metastatic disease at the time of diagnosis, and respond marginally to available treatment options.

Material and Methods

To identify molecular drivers of this PCa subset (called NDM for short), we created a retrospective cohort of PCa patients with localized PCa vs. NDM (N=91) and performed an RNAseq analysis from their paraffin embedded primary tumours.

Results and Discussions

Our analysis revealed striking differences between the two types of specimens, with nearly 8000 differentially expressed genes between conditions. Integrative analyses allowed to pinpoint stromal alterations as a main feature in this type of disease. In addition, we could address the molecular nature of the epithelial tumour compartment by employing gene signature-based inference, which led to a feasible explanation behind their poor response to current therapies and to the identification of candidate genes that could contribute to the aggressiveness of the disease.

Conclusion

Our experimental strategy could contribute to the identification of molecular processes that translate into new therapeutic opportunities for these patients.

EACR22-0968

RNA sequencing revealed molecular and cellular signatures in ascites from patients with advanced breast and ovarian cancer

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Introduction

The aim of this study was to explore transcriptome data of tumor cell populations in ascites and pleural effusions of breast and ovarian cancer patients in order to identify targets and novel therapeutic strategies for advanced cancer disease.

Material and Methods

Malignant fluids of 40 patients were analysed and cell-sorted by flow cytometry for CD45⁻, CD45⁺ and EPCAM⁺ cell fractions. CD45⁻ cells of 21 breast and ovarian cancer patients were RNA sequenced, including matched primary or metastasis tissues from nine individuals. Different activities of cell type and oncogenic pathway-related gene sets, as well as shared immunotherapy antigens were analysed between matched primary tumour and malignant fluid transcriptome data. Ascites of three patients were deeply investigated on the single cell transcriptome level to characterize cellular heterogeneity, and to search for biomarkers of cancer stem cell populations.

Results and Discussions

The fraction of enriched tumour cells (CD45⁻/EpCAM⁺) was highly variable across different ascites and pleural effusion specimens and patients. In total 35-50% had more than 10% CD45⁻ cells, and about 90% of specimens contained a significant number (>1%) of EpCAM⁺ cells in the CD45⁻ cell fraction reliable for RNAseq. CD45⁻ transcriptome data confirmed strong tumor marker expression such as EpCAM, MUC1 and MYC. Cancer-specific targets were also detected in malignant fluids. In breast cancer specimens, MET activation associated with therapy resistance mechanism was observed in malignant fluids compared to matched primary tumours. In ovarian cancer, IGF2, associated with tumour progression, was stable expressed in matched primary and fluidic specimens. Shared tumour antigens like CLDN6 and PRAME were detected in most of the ovarian cancer specimens presenting a prerequisite to address target-specific immunotherapies at advanced disease stage. Investigation of cell type and pathway-related gene sets indicated differences in cancer-associated fibroblast characteristics between matched primary tumours and malignant fluids. Single cell transcriptome data enabled the detection of different tumour cell clusters including few expressing cancer stem cell-like markers.

Conclusion

Comprehensive transcriptome analyses of malignant fluids of advanced breast and ovarian cancer revealed biomarker and target expression signatures that will be further investigated on the protein level to proof the applicability for specific therapeutic intervention in advanced cancer disease.

EACR22-0980

Can extracellular vesicles transmit vemurafenib resistance in melanoma cells in vitro?

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Introduction

Following a first sensitive period, relapse and resistance to vemurafenib are observed in the vast majority of melanoma cases. Recently, more and more study addresses the crucial role

of extracellular vesicles (EVs) in intercellular cross-talk. Furthermore, EVs are also shown to play tumor-promoting role in melanoma cases. Aim of our investigations were to assess the role of EVs in vemurafenib resistance in syngeneic melanoma cell pairs, which were isolated before and after the vemurafenib treatment from each patient.

Material and Methods

Syngeneic melanoma cell pairs were kept for 72 hours in DMEM, supplemented with 1% EV-depleted FBS, before collecting the supernatant for EV isolation. After ultrafiltration, EVs were isolated with ExoQuick exosome precipitation reagent. EVs were characterized by their size distribution and total protein concentration. The isolated EVs were used to treat both the sensitive and resistant melanoma cells, along with vemurafenib. EVs role in transmitting tumorigenic feature among the cells were investigated by assessing proliferation, migration and apoptosis.

Results and Discussions

Vemurafenib treatment resulted in increased apoptosis, decreased cell viability and migration. Of note, treatment with EVs isolated from both sensitive and resistant cells increased cell motility of sensitive cells. If treated in combination, EVs attenuated the antitumor effect of vemurafenib in a cell line dependent manner.

Conclusion

Thus our results may indicate that extracellular vesicles can prevent vemurafenib cells and they might be a mechanism by which resistance against the BRAF inhibitor vemurafenib emerges.

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EACR22-1046

GRHL2 and TEAD transcription factor involvement in the metastasis of gastro-oesophageal adenocarcinoma.

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Introduction

Metastasis causes the vast majority of cancer deaths. When malignant disease requires complete surgical resection for cure, such as in oesophageal and gastric (gastro-oesophageal) adenocarcinoma, metastasis resigns most patients to palliative treatment. As such, understanding the change in transcriptional programme that allows it to occur is of paramount clinical importance.

Material and Methods

To identify transcription factors (TF) relevant to the metastasis of gastro-oesophageal adenocarcinoma we first compared chromatin accessibility and differentially expressed genes in primary tissue samples with those of cell lines of metastatic origin using ATAC-seq and RNA-seq. scATAC-seq, scRNA-seq and spatial transcriptomics of primary gastro-oesophageal tissue and metastatic tissue of hepatic origin was performed. Targeted TF knockdown was achieved with pooled siRNAs and transcription factor co-activator inhibition was performed with a commercial inhibitor.

Results and Discussions

Differentially accessible chromatin in metastatic cell lines compared with primary gastro-oesophageal tissue was highly enriched for motifs recognised by GRHL2 and TEAD. Comparing single cell clusters of primary cancer cells with metastatic clusters from human tissue also found GRHL2 and TEAD motif enrichment in differentially accessible areas in metastatic clusters. scRNA-seq found upregulation of GRHL2 and TEAD1 genes in distinct cancer cell clusters which we observe in metastatic tissue. Spatial transcriptomics revealed heterogeneous spatial expression for GRHL2 and TEAD factors.

Depletion of GRHL2 reduced the invasive ability of metastatic cell lines of OAC (KYAE-1) and GAC (MKN-28) in vitro. The same effect was seen on YAP-TEAD interaction inhibition with verteporfin.

Differentially expressed genes in OAC cells with GRHL2 knockdown were enriched for epithelial-mesenchymal transition (EMT) pathway and extracellular matrix organisation - two important metastatic processes.

GRHL2 is proposed to maintain a chromatin accessibility profile promoting the epithelial cell state. Reduced GRHL2 activity in primary cells may allow for EMT encouraging metastatic spread. Subsequent re-activation of GRHL2 at metastatic sites may reverse this and promote transition to epithelial cell type.

Conclusion

Transcriptomic and epigenetic evidence in cell lines, corroborated by findings in both human tissue and phenotypic assays, suggest an important role for TEAD and GRHL2 factors in promoting a metastatic cell state in gastro-oesophageal adenocarcinoma.

EACR22-1162

The role of mechanical properties and structure of type I collagen hydrogels on colorectal cancer migration

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Introduction

Mechanical interactions between cells and their microenvironment play an important role in determining cell fate, which is particularly relevant in pathological scenarios, such as metastasis, where cells must invade matrices with different mechanical properties. In vitro, hydrogels made with type I collagen have been commonly used for modelling the microenvironment due to its ubiquity in the human body and, consequently, have served as a platform for studying cell migration. Here, we show that variations in the mechanical properties of the hydrogels and in their structure can modulate cell migration patterns of colorectal cancer cells.

Material and Methods

We modified the mechanical properties of hydrogels by varying their collagen concentration and the gelation temperature (37°C and a two-stage scheme). Hydrogels were characterized by rheology and SEM. Afterwards, we performed two sets of experiments using the mentioned hydrogels and HCT-116 multicellular spheroids. In the first set, the spheroids were placed between two layers of hydrogel. In the second one, they were fully embedded and suspended in a hydrogel layer. Spheroid growth and migration were monitored daily for a total span of 6 days.

Results and Discussions

Regarding mechanical behavior, hydrogels polymerized with the two-stage scheme were stiffer, by approximately one order of magnitude, than those polymerized directly at 37°C. Considering cell migration, the first set showed decreased migration with increasing collagen concentration regardless of the gelation scheme. In addition, cell migrated along the interface between the two hydrogels, which suggests that the collagen structure at the interface generates a barrier to migration, thus stimulating a preferential in-plane cell migration close to such interface. Conversely, isotropic spheroid growth with no cell migration outside the spheroid regardless of the concentration or gelation temperature was observed in the second set. These results imply that the inner structure of the hydrogels may be more uniform than in the surface; thus creating an isotropic environment that is not sufficient for stimulating cell migration.

Conclusion

Based on the above mentioned results, cell migration seems to be modulated by both the mechanical properties of the hydrogels and their structure, suggesting that both parameters may have an essential role in cancer progression.

EACR22-1216

T-cell receptor induction of CCR7 chemokine receptor promotes leukemic T cell dissemination

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Intercellular Communication in Cancer, Porto, Portugal

Introduction

T-cell acute lymphoblastic leukemia/lymphoma (T-ALL/LBL) involves the bone marrow, blood, and lymphoid organs, but can disseminate to other organs, aggravating disease. It was previously reported that absence of T-cell receptor (TCR) expression in T-ALL/LBL mouse models led to reduced dissemination of leukemic cells to peripheral lymphoid organs, most notably lymph nodes. We aimed to understand which molecules could be involved in this TCR-dependent property.

Material and Methods

Gene mRNA expression, flow cytometry immunofluorescence analysis were performed from leukemic cells from the ETV6-JAK2 fusion transgenic mice (EJ-Tg). EJ-Tg mice were bred with Rag2, Ccr7 and Nfkb2 knockout (KO) mice; and with TCR-HY transgenic mice. Leukemic cells were infused intravenously in recipient mice.

Results and Discussions

Infused EJ-Tg mouse leukemic T cells from mice lacking TCR expression (i.e., EJ-Tg;Rag2^{-/-}) colonized much less efficiently the lymph nodes and spleens of recipient mice than EJ-Tg leukemic cells expressing endogenous or transgenic TCR. Interestingly EJ-Tg;Rag2^{-/-}

leukemic cells expressed reduced levels of the Ccr7 chemokine receptor, a T-cell migration mediator involved in both thymic egress and peripheral lymphoid organ homing. By stimulating human T-ALL cell lines with CD3 antibody or the PMA phorbol esters, we confirmed CCR7 expression was induced by TCR signaling. To study the role of CCR7 in vivo, we bred EJ-Tg mice with Ccr7 KO mice. Similarly to EJ-Tg mice lacking TCR (i.e. Tcr α ^{-/-} or Rag2^{-/-}), EJ-Tg;Ccr7^{-/-} mice presented significantly larger thymic lymphomas and reduced splenic and lymph nodal involvement than Ccr7-sufficient littermates. By breeding EJ-Tg mice with TCR-HY transgene and Ccr7 KO, we observed that although the TCR transgene favored disease dissemination from the thymus to spleen and lymph nodes, the absence of CCR7 had a negative impact. To verify whether Ccr7 was involved in homing to lymphoid organs, Ccr7-expressing EJ-Tg leukemic cells were infused in mice KO for the Nfkb2 gene, which express reduced levels of Ccl19 and Ccl21 Ccr7 ligands in the lymph nodes, or control littermates. Infused leukemic cells colonized less efficiently the lymph nodes of Nfkb2-deficient mice, with no differences in the spleen and liver.

Conclusion

We conclude that TCR signaling is associated with expression of proteins associated with leukemic dissemination to specific niches and that CCR7 is a potential mediator of that property.

EACR22-0103

Catulin reporter marks a heterogeneous population of invasive and plastic breast cancer cells that express MCAM and participate in vascular mimicry.

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Introduction

The activation of epithelial–mesenchymal transition (EMT) in cancer cells enhances acquisition of invasive behaviors and expands their generation of cancer stem cells. Increased by EMT plasticity of tumor cells can promote vascular mimicry (VM), a newly defined pattern of tumor microvascularization by which aggressive tumor cells can form vessel-like structures themselves. VM is strongly associated with a poor prognosis, but biological features of this process remains unknown.

Material and Methods

MDA-231 cell line was modified where GFP is driven under catulin promoter reporter. Catulin depleted and control MDA-231 cell lines with turboGFP reporter were developed. Cells were injected into mammary fat pad of Nod.Scid mice. Highly invasive GFP⁺ cells were sorted and RNAseq analysis was performed. *In vitro* spheroid assays and flow cytometry analysis were performed on those cell lines and immunohistochemistry stainings of formed tumors was done.

Results and Discussions

Here we show that catulin is expressed in human BC samples and its expression correlates with the tumor progression. Ablation of catulin in hBC cell lines decreases their invasive potential in the 3D assays. RNAseq analysis of highly invasive cells revealed enrichment in genes important for cellular movement, cell invasion and interestingly for tumor-vasculature interactions. Analysis of tumors unveiled that catulin reporter marks not only invasive cancer cells but also rare population of plastic MCAM positive cancer cells that participate in

vascular mimicry and invasion. Ablation of catulin in the xenograft model revealed deregulation of genes involved in cellular movement and adhesive properties with striking decrease in CD44 expression which may impact stemness potential and plasticity of breast cancer cells. These findings show directly that plastic tumor cells can change the fate into endothelial-like, expressing MCAM and emphasize the importance of catulin in this process.

Conclusion

Expression of alpha-catulin correlates with tumor progression in human BC samples. Ablation of catulin in hBC cell lines decreases their invasive potential in the 3D assays. RNAseq analysis of highly invasive breast cancer cells revealed enrichment in genes important for cellular movement, cell invasion and interestingly for tumor-vasculature interactions. Analysis of tumors unveiled that catulin reporter marks not only invasive cancer cells but also rare population of plastic MCAM positive cancer cells that participate in vascular mimicry and invasion.

EACR22-0209

Activation of innate-adaptive immune machinery by Poly(I:C) exposes a therapeutic vulnerability to prevent relapse in stroma-rich colon cancer

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Introduction

Stroma-rich tumours represent a poor prognostic subtype in stage II/III colon cancer (CC), with high relapse rates and limited response to standard adjuvant chemotherapy.

Material and Methods

To address the lack of therapeutic options for patients with stroma-rich CC, we stratified our human tumour cohorts according to stromal content, enabling identification of the biology underpinning relapse and potential therapeutic vulnerabilities specifically within stroma-rich tumours that could be exploited clinically. Following human tumour-based discovery and independent clinical validation, we utilise a series of *in vitro* and stroma-rich *in vivo* models to test and validate the therapeutic potential of elevating the biology associated with reduced relapse in human tumours.

Results and Discussions

By performing our analyses specifically within the stroma-rich/high-fibroblast (HiFi) subtype of CC, we identify and validate the clinical value of a HiFi-specific prognostic signature (HPS), which stratifies tumours based on STAT1-related signalling (High-HPS v Low-HPS = HR 0.093, CI 0.019 - 0.466). Utilizing *in silico*, *in vitro* and *in vivo* models, we demonstrate that the HPS is associated with antigen processing and presentation within discrete immune lineages in stroma-rich CC, downstream of double-stranded RNA and viral response signalling. Treatment with the TLR3 agonist poly(I:C) elevated the HPS signalling and antigen processing phenotype across *in vitro* and *in vivo* models. In an *in vivo* model of stroma-rich CC, poly(I:C) treatment significantly increased systemic cytotoxic T cell activity ($p < 0.05$) and reduced liver metastases ($p < 0.0002$).

Conclusion

This study reveals new biological insight, uncovering a novel therapeutic option to reduce relapse rates in patients with the worst prognosis CC.

EACR22-0351

Circulating plasma extracellular vesicles are able to support lung cancer pre-metastatic niche formation

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Introduction

Nowadays, lung cancer is still the deadliest cancer worldwide, mostly due to the metastatic spread. In this context, Extracellular Vesicles (EVs) have recently emerged as key players thanks to their ability to deliver biofunctional molecules (as miRNAs and proteins) to recipient cells. Indeed, primary tumor-EVs are involved in the formation of a pro-metastatic environment, called pre-metastatic niche (PMN), in distant organs that favor cancer cells dissemination and growth.

However, only few studies investigated the role of the plasma-EVs from lung cancer patients in the PMN formation.

Thus, we aim to elucidate the role of lung cancer patient's plasma-EVs in the PMN formation and identify new prognostic biomarkers.

Material and Methods

Plasma-EVs were obtained by ultracentrifugation from 20 early stages patients survived at 5 years (ESA-EVs) and 20 died within two years (ESD-EVs). EV's characterization was performed following MISEV guidelines. Functional experiments were carried out in 3D-bioprinting co-cultures (fibroblast, endothelial and epithelial cells) models. Nanostring was used to investigate the EV-miRNA cargo.

Results and Discussions

Patient's plasma-EVs were sized between 100 and 150 nM and possess the EVs markers (CD9, CD81, CD63). Surprisingly, EVs with high levels of CD31 are associated to better prognosis (HR:0.246; p<0.01). Interestingly, in 3D-bioprinted co-cultures, ESD-EVs treatment induced endothelial activation as noted by the increase of VCAM1, CXCR4, CXCL1 that subsequently modulated fibroblast (increase of α -SMA and CXCL12) and epithelial (E-cadherin and EpCAM) phenotypes.

Since we have shown that ESD-EV treatment alone is not sufficient to directly alter fibroblasts phenotype, we treated these cells with the conditioned medium (CM) of ESD-EV-stimulated endothelial cells. Remarkably, we observed that CM administration recapitulated the fibroblast modulations observed in 3D co-cultures.

Moreover, we also treated macrophages noticing after ESD-EVs administration, a phenotypic shift towards M2 polarization and an increase of VEGF and CCL2, factors involved in the recruitment of immune cells.

Importantly, 90 miRNAs were detected in plasma-EVs, 5 of them differentially expressed between ESA- and ESD-EVs. **Conclusion**

Our findings suggested that ESD-EVs are involved in the pMN formation increasing endothelial activation and modulating the phenotype of other stromal cells. Moreover, EV-miRNAs could be used as prognostic biomarkers in early stages lung cancer patients.

EACR22-0429

NGFR as a potential therapeutic target for metastatic immunoresistant melanomas

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Introduction

Although cancer immunotherapies have substantially improved cancer prognosis, melanoma is still a very aggressive disease with few survival expectations, mostly due to the ability of tumours to metastasize and to become refractory to current therapies. Therefore, our main goal is to find new targets to block melanoma metastasis and prevent relapse of resistant tumours. Recently, Nerve Growth Factor Receptor (NGFR) has emerged as a new candidate to be targeted in melanoma. This receptor has been associated with a de-differentiated phenotype responsible for the aggressiveness of tumours and resistance to targeted therapies. Our recent studies showed that NGFR execute its role both intrinsically and extrinsically secreted in small extracellular vesicles.

Material and Methods

We are analysing the use of the NGFR small molecule inhibitor (THX-B) as anti-metastatic therapy in melanoma. Melanoma cells were injected in the flank of immunocompetent syngeneic mice and at day 7, when tumours were established, intraperitoneal THX-B treatment was started, alone or in combination with anti-PD-L1. Primary tumour growth and metastasis were analysed by Luciferase detection using IVIS technology. After sacrificing mice, NGFR expression and phenotype of immune cells was characterized by histology and western blot. We also developed different melanoma models refractory to immunotherapy *in vivo* and re-injected them into new mice to study the THX-B effects in tumour growth and metastasis.

Results and Discussions

Treatment with THX-B showed effective reduction of metastasis. Surprisingly, although anti PD-L1 therapies efficiently counteracted tumour growth, they failed to block melanoma metastasis and several tumours relapse. Interestingly, the combination of THX-B with anti-PD-L1 treatment strongly inhibited metastasis and, importantly, prevent therapy resistance development. Moreover, we have found that NGFR is overexpressed in metastatic melanoma cells and in tumours resistant to immunotherapy. This enhanced NGFR expression correlates with an increase in metastasis development. Therefore, we postulate that NGFR could modify immune cell recognition to generate immunosuppressive environments that favour tumour metastasis.

Conclusion

Overall, our data suggest that combination of immunotherapy with NGFR inhibition could improve melanoma treatment by targeting tumour metastasis and overcoming resistance to immunotherapy.

Molecular characterization and modulation of blood-brain barrier and breast cancer cells interaction: a step closer to brain metastasis prevention

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Introduction

The high mortality of breast cancer (BC) patients has been related with brain metastases (BM). Although the extravasation is crucial for metastases formation, the events arising at the blood-brain barrier (BBB) are unclear. Thus, we aim to dissect and modulate the BC cells (BCCs) and BBB interaction, preventing BCBM.

Material and Methods

We established a human BCBM formation *in vitro* model, composed by human brain microvascular endothelial cells (HBMEC), mimicking the BBB, and BCC line with brain tropism (MDA231 Br4 GFP-fused). Spatiotemporal profile of HBMEC (CellTracker™ Red-labelled)-MDA231 Br4 (GFP+) interaction was observed by live-cell imaging. Adhesion and endothelial-mesenchymal transition (EndMT) markers alterations were assessed by flow cytometry and immunocytochemistry. Transendothelial electrical resistance (TEER) and permeability to sodium fluorescein were used as BBB integrity markers. Cytokines effect on cell viability, migration and adhesion were assessed by a MTT, scratch and adhesion assays, respectively.

Results and Discussions

After 1h of interaction, BCCs acquired an invasive phenotype (decreased circularity and invadopodia formation) and spatially were defined as: “outside” (above to the endothelium); “intercalated” (partially or totally) and “transmigrated” (below). Alterations in adhesion markers expression (CD62E, ICAM1 and CD15) limited the adhesion at 3h of interaction. EndMT occurrence at endothelium was also observed, with an increase of mesenchymal (vimentin and neuronal cadherin) and decrease of endothelial (ZO-1 and β -catenin) markers, with acquisition of elongated morphology, nuclear deformation, and migratory properties. A decreased TEER and increased permeability showed a clear BBB disruption. The BCCs location in the BMECs junctional regions, pointed to the use of the paracellular migration route. Preliminary studies, using inflammatory mediators as potential modulators of the altered events showed that macrophage inflammatory protein-1 β inhibit the BCCs invasion and adhesion, not affecting BMECs viability, pointing to good candidate for extravasation modulation.

Conclusion

These data indicate that during BCCs extravasation, also the endothelium undergoes profound changes with impact on BBB integrity, culminating in successful BCCs transmigration. This work contributes for a better knowledge of BCCs trafficking across BBB, paving the way the development of novel therapeutic strategies, preventing BCBM.

The Effect of Non-Thermal Plasma on Epithelial-Mesenchymal Transition in a 3D Melanoma Model.

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Introduction

Non-thermal plasma (NTP) is an ionized gas consisting of reactive oxygen and nitrogen species and shows very promising anti-cancer capabilities. Our previous preclinical study already showed that NTP, as a local therapy, can induce a systemic immunogenic response in a melanoma cancer model. However, several key aspects about the mechanisms of how NTP interacts with cancer cells remain unknown. One of these aspects is epithelial-mesenchymal transition (EMT). EMT induces a shift from an epithelial towards a mesenchymal phenotype, making the cells more migratory and invasive, leading to a worse prognosis. EMT can be induced by several triggers, of which endoplasmic reticulum (ER) stress is an important one. Since NTP has been shown to induce ER stress, we hypothesized that NTP treatment of cancerous cells may induce this transition. The main aim of this study is to analyze whether we can adjust our plasma parameters to minimize this transition.

Material and Methods

In this study, we used immunofluorescence (IF) techniques to analyze the expression of several EMT markers at different time points following NTP treatment of 3D tumor melanoma spheroids. Furthermore, we performed spheroid migration assays to determine the effect of NTP on the mobility of cancerous cells.

In a second part, we used the Chicken Chorioallantoic Membrane (CAM) model to produce solid tumors for two melanoma cell lines. These tumors were also stained for specific EMT markers using IF. We collected the liver, lungs and lower CAM of the chicken embryo to determine the metastatic properties of tumor cells following NTP treatment.

Results and Discussions

The results of the IF staining showed an increase in the expression of mesenchymal markers and a decrease in the expression of epithelial markers, indicating a shift towards a more mesenchymal phenotype. Additionally, we saw increased cell migration in the spheroid migration assay after NTP treatment.

Conclusion

First experiments showed a shift towards a more mesenchymal phenotype after NTP treatment, dependent on the plasma frequency. These results show that it is possible to tune our NTP treatment parameters to minimize EMT. Overall, these experiments will provide us with new insights into the interactions of NTP with cancer cells to assess its potential as a novel melanoma therapy.

The Role of Atm Deficiency in Prostate Tumorigenesis and Progression

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Introduction

In developed countries, prostate cancer (PCa) is the most common malignancy in men. Genomic studies in PCa have identified germline and somatic deleterious *ATM* alterations as relevant events although little is yet known about their biological and clinical implications.

Material and Methods

To characterize *ATM* deficiency *in vivo*, we first generated transgenic mice (TM), TM allografts and cell-derived xenografts (CDX) that recapitulate the human *PTEN*⁺/*PTEN*⁻ PCa. TM were *TRAMP*^T;*Atm*^{KO} mice and *Pbsn4Cre*^T;*Pten*^{lox};*Atm*^{KO}. TM allografts were established by subcutaneous (sc) implantation of 16 weeks old *TRAMP*^T;*Atm*^{KO} mice prostates in nude mice. To generate CDXs, we first obtained *ATM*⁺/*ATM*⁻ clones using an shRNA against *ATM* in 22Rv1 (*PTEN*⁺) and C4-2 (*PTEN*⁻) cells. Then, we injected sc/orthotopically cells from these clones in NSG® mice.

Results and Discussions

In our TM models *Atm*^{-/-} mice die from thymomas at 16 weeks (w) and do not develop PCa. *Atm*^{+/-} reduces survival (26w vs 28w) and increases tumour burden as soon as initial oncogenesis occurs (20w) in the *TRAMP*^T model, but does not modify the *Pten*^{lox} phenotype. To study *Atm*^{-/-} contribution to the *TRAMP*^T phenotype we generated a TM allograft model. *TRAMP*^T;*Atm*^{-/-} prostate allografts show an accelerated tumour growth rate compared to *TRAMP*^T;*Atm*^{+/-}.

To confirm the potential contribution of *ATM* deficiency to *PTEN*⁺ PCa progression we analysed our human *ATM*⁺/*ATM*⁻ PCa xenograft models. *ATM*⁻ (vs *ATM*⁺) increases tumorigenesis and metastasis of *PTEN*⁺ 22Rv1 xenografts, while the opposite is observed in *PTEN*⁻ C4-2 xenografts.

These differences support that *ATM* deficiency might contribute to cancer progression in *PTEN* competent PCa. Indeed, prior studies suggested a synthetic lethal interaction between *ATM* inhibition and *PTEN* deficiency in the PC3 PCa cell line (McCabe, Walker, and Kennedy, 2016). Interestingly, we also performed *in silico* analysis of cBioPortal datasets and we observed that *ATM* and *PTEN* alterations might be mutually exclusive. Overall, these results support the notion that the contribution of *ATM* deficiency to PCa oncogenesis and progression varies according to *PTEN* status.

Conclusion

The absence of *ATM* function in PCa leads to different phenotypes as evaluated in our *in vivo* models, highlighting the importance of PCa genomic background in this context. Importantly, we unveil a role of *ATM* deficiency in PCa tumorigenesis and suggest its potential prognostic value in *PTEN* competent PCa.

EACR22-0813

Development of a Novel ECAD-EmGFP Reporter Line for Pancreatic Cancer MET

Study and Drug Discovery

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Introduction

World-wide, metastasis continues to be the leading cause of death in cancer patients. Although epithelial-to-mesenchymal transition (EMT) and mesenchymal-to-epithelial transition (MET) have been implicated in the progression of cancer metastasis and drug resistance, their mechanistic pathway and specific role in disease progression is not fully understood. As such, the development of a reporter line that enables the real-time monitoring of the changing status of cells will not only aid in dissecting the EMT/MET pathway in the research field, but could also become a robust platform for new cancer drug development.

Material and Methods

E-cadherin, an adhesion protein expressed in epithelial cells, is upregulated in pancreatic cancer cells during MET and is associated with an increase in tight junctions and apico-basal polarity, as well as a change in morphology. Here, we developed a novel PANC-1 ECAD-EmGFP reporter line using CRISPR/Cas9 genome-editing technology. In this cell line, the emerald green fluorescent protein (EmGFP) reporter was incorporated into the last exon of the endogenous E-cadherin gene, enabling real-time monitoring of MET status in live cells. The ECAD-EmGFP knock-in allele was confirmed at genomic, transcriptional, and translational levels.

Results and Discussions

Functional data revealed that miRNA-200 treatment induced the increased expression of ECAD-EmGFP, and decreased expression of Snail, a transcription factor associated with mesenchymal traits. These changes in marker gene expression, as well as the decrease in invasive capacity upon induction, suggest that cells have undergone MET. This cell line is a valuable tool for dissecting the molecular mechanisms underlying EMT and MET and for evaluating or screening compounds targeting EMT and MET in pancreatic cancer.

Conclusion

We created the pancreatic cancer PANC-1 ECAD-EmGFP reporter cell line using CRISPR/Cas9 genome-editing technology to incorporate a GFP gene into the last exon of the endogenous ECAD gene. PANC-1 ECAD-EmGFP cells undergo MET upon induction, enabling real-time monitoring of the dynamic MET states in live cells. The PANC-1 ECAD-EmGFP cell line is a valuable tool for studying MET and EMT cancer biology and for use in the screening compounds that target these processes.

EACR22-0911

Characterization of components of the premetastatic niche in the bone marrow

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Introduction

Breast cancer cells home to the bone marrow, where they may stay dormant or proliferate and form metastases. Presumably the components of the niche affect the behavior of tumor cells. This niche however consists of poorly defined stromal populations. Our group had shown that a stromal subpopulation is diminished whenever homing increases. In this work we aim to use transgenic mouse models to better characterize the cellular components of the premetastatic niche and define the role of matrix produced by them.

Material and Methods

To evaluate homing of cancer cells, MDA-MB-231/Bluc⁺ were injected intracardially and the number of tumor cells in the bone marrow was evaluated by qPCR after 24 hours. Transgenic mouse models of tissue specific promoters attached to cre-recombinase were used: osterix-cre, vav-cre, leptin-receptor (lepR)-cre and Mx-cre. Using animals with floxed genes, we deleted fibronectin or its main receptor β 1-integrin in the populations that express cre. dTomato-reporter mice allowed quantification of the expression of promoters in bone marrow cells.

Results and Discussions

Although osterix is expressed in only 1% of bone marrow stromal cells (BMSCs) (CD45⁻ Ter119⁻) and hematopoietic cells (HCs) as determined with the reporter mice, deletion of fibronectin using osterix-cre increased homing of cancer cells 4.1-fold ($p < 0.05$). In contrast, deletion of β 1-integrin did not affect cancer cell homing. This suggests that fibronectin originating from a small subpopulation exerts an inhibitory effect on cancer cell homing to the bone marrow. Vav-cre is expressed in many more cells of the bone marrow (80% BMSCs and almost all HCs). Nevertheless, deletion of fibronectin using this promoter had no effect on cancer cell homing. In contrast, loss of β 1-integrin increased homing 3.1-fold ($p < 0.05$). Similarly, deletion of β 1-integrin in lepR-expressing cells enhanced homing 2.5-fold ($p < 0.05$) and its deletion in Mx-expressing cells also increased homing 2.1-fold ($p < 0.01$) (lepR is expressed on 82% BMSCs and 93% HCs, while Mx is expressed in 50% BMSCs and 60% HCs). However, because vav, lepR and Mx are all expressed in HCs, a contribution of hematopoietic cells to the increase in homing cannot be excluded.

Conclusion

In summary, fibronectin from preosteoblastic cells prevents cancer cell homing to the bone marrow. This effect is not mediated by an auto-feed-back loop in the preosteoblasts. It is possible, however, that fibronectin inhibits homing by acting on cells that express vav, lepR and/or Mx.

EACR22-0442

Role of microglia on the chemoresistance and invasiveness of astrocytoma cells

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Introduction

Astrocytoma is a common tumor in the primary central nervous system and has a high death rate worldwide. Although the chemotherapy showed promising results in both in vivo and in vitro experiments, the clinical outcome and prognosis are poor because of the chemoresistance because of the complex brain tumor microenvironment. In our previous study of the astrocytoma animal model, we found a group of microglia cells surrounding the tumor peripheral region, particularly the invasive tumor islands. This study aimed to examine the effect of microglia on the chemoresistance and invasiveness of astrocytoma cells.

Material and Methods

An in vitro co-culture system including the astrocytoma cell line, ALTS1C1, and microglia cell line, BV2, was used to simulate the brain tumor microenvironment. The TK-gene transfected ALTS1C1 cell line was used to explore whether BV2 affects astrocytoma chemoresistance in response to TK-GCV therapy. Flow cytometry and migration assay were performed to assess the effect of BV2 on the response of ALTS1C1-TK cells to the cytotoxicity of pro-drug, GCV, and the migration ability of ALTS1C1, respectively.

Results and Discussions

Results showed that co-culturing with BV2 could render ALTS1C1 to form a colony and reduce the cytotoxic effect of GCV on ALTS1C1-TK cells. On the other hand, the migration and invasion abilities of ALTS1C1 were increased in the presence of BV2. In addition, we also found that BV2 could inhibit cell proliferation, arrest cells at G0/G1 phase, and reduce drug uptake. When Connexin 43 expression was suppressed by siRNA and Connexin 43 inhibitor, the BV2-mediated chemoresistance, but not BV2-associated migration, was abolished.

Conclusion

This study demonstrates that the direct contact of microglia and astrocytoma plays a crucial role in the development of drug resistance, migration, and invasion of astrocytoma and is responsible for the therapeutic failure and tumor recurrence after the therapy.

EACR22-0122

Identification and characterisation of prostate cancer-associated stromal cell subtypes

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Introduction

Stromal fibroblasts and mural cells are well established as key contributors to the development, progression and therapy resistance of prostate cancer (PCa), the second leading cause of male cancer-related death in Western nations. However, it is evident that stromal cells in the tumour microenvironment are functionally heterogeneous with some types of cancer-associated stromal cells (CASCs) mediating tumour-supportive effects, whereas others display tumour-restrictive properties. In view of this functional heterogeneity, a better characterisation of CASC subtypes is needed in order to specifically target onco-promoting stromal entities. This study therefore aims to specifically identify, characterise and isolate those CASC subtypes present in the PCa microenvironment.

Material and Methods

Based on in-house single cell RNA sequencing data from digested PCa biopsy cores, a cell surface marker panel was developed for the detection and future isolation of putative CASC subtypes using flow cytometry-based sorting. Furthermore, we established a quantitative real time PCR gene panel for additional validation and implemented spatial localisation analyses via multiplex immunohistochemistry.

Results and Discussions

We will present data from ongoing analyses using these panels on (i) immortalised fibroblast and smooth muscle cell lines, (ii) heterogeneous *ex vivo* cultures of primary prostate fibroblasts derived from PCa biopsy cores via outgrowth and (iii) cell culture-naïve primary fibroblasts in single cell suspensions of bulk digested PCa biopsy tissue. Preliminary findings thus far suggest a dynamic emergence of CASC subtypes contingent on pathophysiological states.

Conclusion

It is hoped that application of these marker panels in future studies will provide a means to better characterise the prostate tumour microenvironment at the cellular level and evaluate the contribution of distinct CASC subtypes to PCa progression.

EACR22-0166

The interaction of ovarian cancer stem cells with the tumor microenvironment revealed by a platform of patient-derived organotypic models

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Introduction

High-grade serous ovarian cancer (HGSOC) has the highest mortality rate of all gynecologic malignancies, because most patients are diagnosed when the disease has already disseminated into the peritoneal cavity. Moreover, despite an initial response to chemotherapy, most patients undergo tumor relapse within three years from surgery, and the recurrent disease is often chemoresistant.

This unfavorable prognosis is driven by a small subset of tumor cells, named ovarian cancer stem cells (OCSC), which are endowed with tumor-initiating ability and are intrinsically resistant to cytotoxic treatments. They can therefore escape chemotherapy and drive tumor relapse. These characteristics are sustained by signals coming from the surrounding tumor microenvironment (TME), which for metastasizing HGSOC cells is mainly the omentum. Thus, defining the biological mechanisms of this OCSC/TME cross-talk may lead to innovative therapeutic strategies.

Material and Methods

We reconstructed the architecture of human omentum *in vitro* culturing together primary human omental fibroblasts and mesothelial cells. We then integrated in these organotypic cultures primary, patient-derived HGSOC cells, grown both in adhesion and as monoclonal spheres enriched in OCSC. Single-cell suspensions of matched adherent and sphere-derived cells were labeled with the green fluorescent dye CMFDA, cultured for 48h on TME, re-isolated by FACS sorting and subjected to whole-transcriptome RNA sequencing, comparing them to control cells that have not been in contact with TME.

Results and Discussions

The TME induced a strong transcriptional reprogramming in HGSOC cells, with 1154 genes differentially regulated in bulk tumor cells and 2431 genes in OCSC. An Upstream Regulator analysis identified a panel of transcription factors (TFs) that were differentially regulated by the TME in OCSC. The gene networks orchestrated by these TFs might represent OCSC vulnerabilities and, hence, potential targets in relapsing tumors. On this basis, we are testing inhibitors of selected TFs (chosen on the basis of novelty, relevance and druggability) for their ability to eliminate OCSC in the context of the organotypic TME, as a proxy for the eradication of OCSC from the omental niche.

Conclusion

Our patient-derived platform holds the ability to identify genes and pathways modulated by the TME in OCSC, and can be exploited to test whether interfering with such pathways represents an innovative HGSOC-eradicating approach.

EACR22-0178

SAA1 is the tumor derived molecule regulating adipocyte reprogramming in TNBC cells

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Introduction

Cancer development and progression depend on tumor cell intrinsic factors, the tumor microenvironment and host characteristics. Despite the identification of the plasticity of adipocytes, the primary breast stromal cells, both in physiology and cancer, we lack a complete understanding of mechanisms that regulate tumor-adipocyte crosstalk.

Material and Methods

Tumor-adipocyte crosstalk was studied *in vitro* treating ASC52 adipocytes with triple negative breast cancer (TNBC) cell conditioned medium (CM). Adipocyte phenotype was analyzed by qPCR, Oil Red O staining and gene expression profiling. SAA1 in BC-derived CM was identified by MS/MS analysis. A cohort of 1311 TNBCs was *in silico* analyzed according to SAA1 expression. Cancer associated adipocyte (CAA) score was generated comparing the transcriptomic profile of 42 matched tumor-adjacent and -distant adipose tissues. CAA score was also applied to scRNAseq of 5 TNBC specimens.

Results and Discussions

SAA1 was identified as the protein that differentiates the CM proteinogram of MDA-MB-231 from that of MDA-MB-361 cells, which were able and not able to induce adipocyte dedifferentiation, respectively. SAA1 silencing significantly reduced the induction of CAA by TNBC cells. Adipocyte treatment with inhibitors of SAA1 receptors (CD36, P2X7R, TLRs) and their downstream signaling molecules (MyD88, NF-kB, JNK, ERK), during adipocyte dedifferentiation induced by TNBC-CM or recombinant SAA1, indicated that CD36 and P2X7R receptors and NF-kB and JNK are causally involved in SAA1-mediated induction of adipocyte dedifferentiation program. NF-kB was also one of the significant activated upstream regulators of the gene expression profile of CAAs. *In silico* analysis revealed that TNBCs with high SAA1 expression are significantly enriched in CAA score, which identified specific subsets (i.e. impVL and myCAF) of mesenchymal cells among the stromal and immune cell clusters found in the TNBC microenvironment. Moreover, SAA1-high tumors are characterized by high inflammation, stimulated lipolysis, stem-like properties and pro-tumor immune microenvironment, all features that we demonstrated to be regulated by CAAs in TNBC cells *in vitro*, supporting the relevance of tumor-released SAA1 in mediating the tumor-adipocyte crosstalk.

Conclusion

Our findings provide evidence that interaction between tumor cells and adipocytes through SAA1 release is relevant to the aggressiveness of TNBC.

HSP90 as a key effector of Cancer Associated Fibroblasts (CAFs) functions in aggressive tumor microenvironments

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Introduction

Cancer Associated Fibroblasts (CAFs) are a key component of the tumor microenvironment (TME) as they actively remodel the extracellular matrix (ECM) to promote tumor progression, cancer cell invasion and chemotherapy resistance. Despite their prominent role in cancer development, specific targeting of CAFs remains a therapeutic challenge. CAFs can arise from normal resident fibroblasts (NFs) in response to a wide range of corrupting insults emanating from the emerging tumor, including cytokines, growth factors and physical inputs. Still, how cellular stress characteristic of proliferating tumors modulates CAF behavior remains ill-defined. HSP90 is a chaperone protein with two major isoforms (HSP90 α and HSP90 β) that participates in cellular responses to stresses such as hypoxia, nutrient-deprivation and DNA damage, which are all common conditions in cancer. In this context, HSP90 controls the correct folding and stability of several key proteins related to cancer functions. HSP90 is also a major player in fibrotic disorders, but its role in cancer stroma and CAFs is not well understood.

Material and Methods

To identify novel regulatory nodes modulating CAF function, we used an integrated transcriptomic analysis of patient-derived tumor stroma samples with an *in silico* gene-expression based chemical screen. We identified HSP90 as a critical gene expression modulator in the stroma, suggesting possible implications in fibroblast activation in stressful environments. We assessed this question by depleting HSP90 α/β expression in CAFs, and performed extensive molecular and functional characterization *in vitro*. We will also interrogate our *Hsp90aa1*-knockout murine model for further *in vivo* studies.

Results and Discussions

Our data demonstrates that HSP90 α specifically modulates the ability of CAFs to migrate, remodel collagen matrices and generate anisotropic ECM. These processes are associated with the ability of CAFs to promote cancer cell migration and invasion. Similar results were observed when treating NFs with TGF β , a well-known activator of fibroblasts. On the other hand, modulation of HSP90 α/β had no effect on CAF-dependent cancer cell growth *in vitro*. Interestingly, depletion of HSP90 β or treatment with anti-HSP90 drugs did not have any observable effect in these processes.

Conclusion

Our results suggest a particular role of HSP90 α in modulating specific CAF functions associated with cytoskeletal regulation, which may provide an unexpected molecular link between stress responses and mechanotransduction programs.

Superior effects of polymeric micelles in reprogramming tumor microenvironment

and enhancing nano-immunotherapy

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Introduction

Nano-immunotherapy has high potential to improve cancer patient outcomes, as already demonstrated in triple negative breast cancer with the combination of nanoparticle albumin-bound paclitaxel and the immune checkpoint blocker antibody, atezolizumab. This regimen, however, does not lead to complete cure with median survival lasting less than two years. To increase patient survival, research focuses on repurposing common drugs to modulate the tumor microenvironment (TME), aiming to increase delivery of nanoparticles, antibodies and other drug regimens and improve immunostimulation.

Material and Methods

Taking advantage of the TME reprogramming capabilities of the approved antihistamine drug tranilast, we developed polymeric tranilast-loaded nanoparticles and explored their potential to modulate the TME more efficiently than the free drug.

Results and Discussions

The micellar formulation of tranilast administered in a dose 100 times lower than that of the free drug reported superior reprogramming effects in two syngeneic triple negative breast tumor models. These effects were due to the enhanced intratumoral accumulation and cancer-associated fibroblast uptake of the nanoparticles compared to the free drug. Tranilast micelles were also found to promote significantly the delivery and efficacy of epirubicin micelles, overcome immune checkpoint resistance and induce tumor immunogenicity. Overall, the proposed micelles improved the efficacy of nano-immunotherapy in both tumor models leading to complete cure. Next, we employed the non-invasive and clinically applied ultrasound shear wave elastography method to monitor changes in tumor stiffness, which is the first effect of tranilast micelles, and thus to determine an optimal dose-scheduling. Importantly, we found that tumor stiffness before the initiation of nano-immunotherapy was able to predict the efficacy of the treatment providing strong evidence for the potential of shear wave elastography to be used as a biomarker predictive of response.

Conclusion

Our findings indicate that a) encapsulation of TME modulating drugs improves their pharmacokinetic properties, while allows for drastic reduction in the dose of administration, b) modulation of the TME can lead to complete cures of nano-immunotherapy in resistant to this therapy breast cancer tumor models, and c) ultrasound shear wave elastography can be employed for optimizing the use of TME modulating agents and for the prediction of tumor response to nano-immunotherapy allowing for optimized treatment protocols.

EACR22-0224

Syndecan-1 interaction with MT4-MMP promotes HER2+ breast cancer progression and sensitivity to HER2 inhibitors

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Introduction

The expression of EGFR and HER2 confers an aggressive tumor phenotype and worse prognosis in Breast Cancer (BC). Here, we revealed a new tri-molecular association formed by HER2, syndecan-1 (SDC1) and MT4-MMP in HER2+BC. We aim at exploring the functional significance of this tri-molecular complex in HER2+BC malignancy and resistance to anti-HER2 therapy.

Material and Methods

Lentiviral transduction was used to overexpress MT4-MMP in four HER2+BC cell lines: HCC1954, SKBR3, BT474 and HCC1569. *In vitro* assays and xenografts were used to assess the effect of MT4-MMP on tumor growth and response to HER2 inhibitors. Phosphoproteomic analysis was conducted to highlight pathways activated by MT4-MMP in HER2+ cells (HCC1954 and SKBR3).

Results and Discussions

The interaction of MT4-MMP and SDC1 in HER2+ BC cell lines was confirmed by PLA (Proximity Ligation Assay) and co-immunoprecipitation assays. These interactions were decreased in cell expressing the inactive form MT4MMP-(E249A), suggesting a proteolytic release of SDC1 by MT4-MMP. While the biological effect MT4-MMP was not evident on cell proliferation in 2D culture *in vitro*, it promotes HER2+ xenografts growth and their sensitivity to anti-HER2 therapy (like dacomitinib, a pan-HER inhibitor) *in vivo*. The phosphoproteomic analysis of HCC1954 and SKBR3 cells revealed that MT4-MMP induces 10-20-fold changes in the phosphorylation of PIP5K1A (responsible of AKT/mTOR pathway activation), cofilin (implicated in actin remodeling), NF-kappa-B-inhibitor-like-protein-1 and serine/arginine repetitive matrix protein-1 and -2, involved in mRNA metabolism. Finally, immunohistochemistry analysis of primary tumors and relapses of human HER2+BC, revealed an association of MT4-MMP and SDC1 in 94% of HER2+BC samples. The expression of MT4-MMP but not SDC1 was correlated with TNM staging.

Conclusion

Intercellular protein phosphorylation resulting from interaction of MT4-MMP with SDC1/HER2 might provide new molecular insights in HER2+ BC progression and therapy response. Further analysis of SDC1 shedding in serum from cancer patients and in the cell-based assays will determine the functional significance of SDC1 shedding in the progression of HER2+ BC.

EACR22-0236

Colorectal cancer patient-derived scaffolds as tools to study the role of the extracellular matrix on cancer stemness and immunogenicity

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Introduction

Colorectal cancer (CRC) is the second leading cause of cancer-related mortality worldwide. To improve conventional diagnosis and treatment, important elements present within the tumor microenvironment require to be considered. The extracellular matrix (ECM) is a

dynamic macromolecular structure that provides mechanical support to tissues, serves as storage of several bioactive domains and growth factors and provides cues for cell behavior. In CRC tumors, the ECM is extensively remodeled, with increased stiffness, and has been described to sustain the cancer stem cell (CSC) niche. CSC represent a small subpopulation of cells with the ability of self-renewal and clonal tumor initiation, considered key drivers of malignancy. Abnormal ECM changes are known to alter cell differentiation, polarity and promote epithelial-to-mesenchymal transition. Our main objective was to evaluate the impact of tumor ECM on CRC cell stem-like markers.

Material and Methods

Paired normal and tumor tissues, derived from CRC patients surgical resections, were decellularized and repopulated with CRC cells. Repopulated matrices were characterized by hematoxylin/eosin, Masson's trichrome staining and immunofluorescence. Expression of stem cell and markers and immune escape molecules was evaluated by flow cytometry and qRT-PCR, and soluble factors, such as TGF-beta and TNF-alpha determined by ELISA. Proteolytic activity was analyzed by gelatin zymography.

Results and Discussions

Notably, in tumor matrices, there was an enrichment in CD133⁺CD44⁺ cells and an up-regulation of *NANOG*, *SOX2*, *OCT4* and *SNAI1* genes. TGF-beta production was also enhanced significantly when cells were cultured within tumor PDS than within their normal counterparts. MMP-2 and MMP-9 proteolytic activity was also higher than in normal matrices. PD-L1 expression was also increased in CRC cells cultured in tumor PDS than those in paired normal PDS. These significant differences observed in cancer cells were due to specific modulation by the tumor ECM. PDS from several patients were used in this study and, despite CRC heterogeneity and inter-patient variability, our results are solid.

Conclusion

Overall, our work suggests that factors (biochemical and/or biophysical) inherent to the tumor ECM modulate/favor CRC cells' stem-like phenotype and inhibition of anti-tumor immune response. The underlying molecular mechanisms are currently being explored, envisaging the identification of novel therapeutic targets against cancer progression.

EACR22-0260

MYC associates with MIZ1 to convey immune suppression via inhibition of inflammatory signaling in TNBC .

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Introduction

One of the leading causes of cancer associated deaths in women is breast cancer. Treatment of triple-negative breast cancer (TNBC) still proves problematic, even though advances were made by utilizing immunotherapy in other cancer types. Unfortunately, these therapies are only applicable in a part of TNBC cases with high immune infiltration. In a large part of TNBCs, lymphocyte infiltration is low and thus classical immune-therapies with Pd-L1

blocking antibodies are not effective. Mining of breast cancer databases showed that scarce immune infiltration is often associated with MYC overexpression.

Material and Methods

We generated mouse models of TNBC using WAP-cre driven mammary gland specific deletion of Trp53 without (WP) or with MYC overexpression (WP-MYC) as well as an additional deletion of Brca1 (WB1P and WB1P-MYC). To show a possible causality of MYC expression with immune cell expulsion, we also generated a WB1P-MycERT2 mouse, allowing us to dynamically follow immune cell infiltration in relation to MYC expression. We could mimic the morphology of TNBCs in all the models.

Results and Discussions

We observed a stark decrease in infiltrating lymphocytes in the models overexpressing MYC, confirming our hypothesis that MYC is responsible for creating immune deserts. Infiltrating immune cells in established WB1P-MycERT2 tumors were expelled upon induction of MYC expression. RNA-sequencing showed strong downregulation of inflammatory signaling in MYC expressing tumors. Inflammatory signaling was shown to be associated with DNA damage caused by loss of BRCA1 through the cGAS-STING pathway, which we found to be strongly downregulated in MYC expressing tumors. Co-cultures of organoids generated from WB1P and WB1P-MYC tumors with immune cells showed that MYC expression leads to repulsion and reduced proliferation of immune cells. Addition of CCL5 and CXCL10, two cytokines we found to be strongly downregulated upon MYC expression, restores T-cell killing of MYC organoids. ChIP-sequencing experiments confirmed association of MYC together with its co-repressor MIZ1 with interferon regulating genes playing key roles in transducing the signal in several inflammatory signaling cascades. Finally, we show that activation of inflammatory signaling via STING agonist treatments increases survival of tumor bearing mice.

Conclusion

We conclude from these results that MYC expression in TNBCs creates immune deserts by directly repressing STING/IFN signaling and thus blocking lymphocyte attraction.

EACR22-0430

Generating novel prostate cancer 3D models for prognostics, diagnostics and immunotherapy.

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Introduction

Prostate Cancer (PrCa) represents the second most common malignancy in men worldwide. Currently, clinicians and pathologists have difficulty distinguishing aggressive PrCa from

relatively indolent disease; this is partly due to the lack of accurate models representing the disease, necessary to develop more conclusive prognostic and diagnostic platforms as well as effective therapeutic strategies. Three-dimensional (3D) culture methods, such as spheroids and organoids, allow cells to organise into structures that resemble the *in vivo* architecture and allow the incorporation of the tumour microenvironment components. This makes them a great tool for the study of cellular changes associated with PrCa development and its interactions with vascular and immune elements. Specific immune cells, such as unconventional lymphocytes, are currently of great interest because of their potentiality as an immunotherapy approach. We aim to generate 3D co-cultures of prostate cells and $\gamma\delta$ T lymphocytes to investigate their cytotoxic activity along with a potential immunotherapeutic value.

Material and Methods

We have obtained co-cultures consisting of combinations of mice healthy primary tissue-derived organoids or spheroids containing different proportions of five human PrCa cell lines (RWPE-1, WPE1-NA22, WPE1-NB26, PC3, DU145) of various malignancies and expanded human $\gamma\delta$ T immune cells isolated from healthy blood donors. Confocal microscopy, ptychographic quantitative phase imaging and flow cytometry were used to carry out quantitative and qualitative analyses of the effect that $\gamma\delta$ T lymphocytes have on cancerous cells.

Results and Discussions

Our group has developed organoids and spheroids which display the acinar architecture of the prostatic epithelium and recapitulate the histological features of the prostate by organising in a double layer of basal and luminal cells. We are generating the mixed-cells spheroids to reproduce the heterogeneous profile of the cancer lesions in the prostate. Each cell line has shown different proliferation and migration rates, making them a potential PrCa diagnostic and prognostic tool. We are also optimising the conditions for the co-cultures of prostate 3D models with unconventional immune cells, called $\gamma\delta$ T lymphocytes, to investigate their cells targeted cytotoxicity against cancerous cells.

Conclusion

We have developed a model which faithfully recapitulate PrCa cellular biology and that is now amenable for the investigation of new immunotherapy approaches.

EACR22-0446

The differential expression of apolipoprotein E among glial cells in tumor-bearing brain during the progression of murine astrocytoma

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Introduction

Gliomas arise from glial or precursor cells in the central nervous system (CNS). Gliomas represent 81 % of malignant brain and CNS tumors. Brain tumors have proven challenging to treat due to the complex and sensitive architecture of the brain. Lipid is in high demand in the brain, and thus lipid carrying proteins such as apolipoprotein E (APOE) is required to transport lipid among tissues or cells. APOE has been extensively studied in Alzheimer's disease. Despite the critical role of APOE in maintaining brain lipid homeostasis, few studies have taken them into account on brain tumor development. Here, we analyzed the APOE expression in both tumors and the surrounding tissues during the tumor progression.

Material and Methods

Mice were intracranially injected with ALTS1C1 astrocytoma cells. Flow cytometry analysis and immunohistochemistry (IHC) analysis were employed to analyze the immune cell composition and APOE expression.

Results and Discussions

Flow cytometry analysis showed that APOE was upregulated in the tumor-infiltrating microglia, macrophages, and lymphocytes. Astrocytes with stronger GFAP expressed lower levels of APOE in the normal or tumor-bearing brain. With IHC analysis, CD68⁺ macrophages were highly correlated with the APOE signal, while F4/80 did not. Little to no APOE was observed in the GFAP⁺ cells from IHC analysis. It has been reported that APOE can participate in immune regulation in various cancers. Previous research also suggested that APOE participates in regulating the immune response of microglia. Further studies to increase or decrease the expression of APOE may help uncover their roles in the glioma microenvironment.

Conclusion

This study demonstrated that APOE was differentially expressed in different cells and tissue types. Tumor-infiltrating immune cells showed an elevated APOE expression, while GFAP⁺ astrocytes expressed a lower level of APOE. Although it is still not clear about the mechanism and the influence of these changes, this study provides a basis for future studies about the roles of APOE in the brain tumor microenvironment.

EACR22-0464

BCAS1-expressing cells contribute to the proliferative phenotype of oligodendroglioma

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Introduction

Breast carcinoma amplified sequence 1 (BCAS1) has been proposed as a key gen in non-central nervous system tumors as breast, colorectal or prostate cancer, due to its relevance in these neoplastic entities. Furthermore, this molecule has been established as a novel marker that defines an immature oligodendrocyte population in human brain. As a novelty, our group has recently found a BCAS1 positive cell population in a case of oligodendroglioma (OGs), but studies about this protein on glial-derived tumor are scarce.

Material and Methods

In this study, we analyzed the expression of BCAS1 in tumor tissue surgically removed from patients with OGs (n=17), glioblastomas (GBs, n=60) and astrocytomas (ASs, n=8). The distribution, microenvironment and proliferative status of this cell subpopulation was analyzed by immunofluorescence and confocal microscopy. Parallely to each previous analysis, a series of stereological quantifications were performed. In addition, we studied the BCAS1⁺ cells ultrastructure by immunoelectron microscopy.

Results and Discussions

Our results show that glial-derived tumors have a BCAS1⁺ cell population morphologically different and heterogeneously distributed: stellate BCAS1⁺ cells can form tightly packaged nodules or staying individualised, whereas spherical BCAS1⁺ cells, which are isolated. Both cell types have similar organelles, but they differ in its size and their chromatin compaction, being the stellate BCAS1⁺ cells bigger and with a lax chromatin. The nodules present a high proliferative rate and they have been detected only in OGs, becoming a distinctive feature of more aggressive clinical behaviour. Finally, we detected that part of this cell population co-expresses markers of immaturity (EGFR).

Conclusion

Our findings provide the first evidence for the presence of a BCAS1 positive cell population in brain tumors. Their proliferative feature suggests their important role in the tumor malignancy, specifically in OGs.

EACR22-0538

Mimicking tumor cell heterogeneity of colorectal cancer in a patient-derived organoid-fibroblast model

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Introduction

Tumor evolution and clinical response to cancer therapy are both strongly influenced by the intratumoral heterogeneity and the surrounding non-malignant stroma. The aim of this study was to establish and molecularly define a human-derived culture model of patient-derived organoids (PDOs) and their matched stromal fibroblasts isolated from colorectal cancer (CRC) specimen to mimic *in vivo* tumor complexity.

Material and Methods

Differences between normal (NFs) and cancer associated fibroblasts (CAFs) were based on quantitative label-free MS/MS proteomic analyses, secretome cytokine arrays and spheroid motility assay in collagen gels. Immunohistochemistry was used to detect expression of cell specific proteins. RNA expression changes in organoids and fibroblasts were analyzed by RNA sequencing and validated in primary tumors by deconvolution strategy inferred from published single cell RNA sequencing data of CRC tumors.

Results and Discussions

Characterization of patient matched NFs and CAFs revealed 235 significantly differentially expressed proteins, 12 altered secreted cytokines and increased motility of CAFs. Co-culturing of PDOs and matched fibroblasts (NFs or CAFs) allowed for organoid growth over a prolonged period of time without the addition of growth factors. Immunohistological analyses of three patient tumors, and the corresponding PDO mono- or fibroblasts- co-cultures revealed striking resemblance between patient material and co-cultures as evaluated by Ki67, CK20, PAS, Fibronectin and CDX2 staining. Organoids induced expression of genes in both NFs and CAFs, including *CXCL14*, *IL6*, *ICAM1* and *ITGB2* supporting CAF-like phenotypes. We found 1742 differentially expressed genes in organoids grown in different conditions - regular growth medium, without growth factors, or in co-culture with NFs or CAFs. Importantly, while the regular growth medium mainly supported stem-cell features of PDOs, co-culture with fibroblasts showed presence of stem, tuft, enterocyte, and goblet cells. Deconvolution of bulk RNA sequencing data generated from PDOs under the different conditions using published single cell RNA-seq datasets confirmed this finding. These data suggest that organoid-fibroblasts cultures retain greater tumour heterogeneity.

Conclusion

Organoid fibroblast co-cultures preserve tumor cell heterogeneity and reflect patient tumor biology, hence representing a valuable preclinical model. Additional studies will be needed to evaluate and compare patient chemotherapy response to the established co-culture model.

EACR22-0564

Heterogeneity in the experimental set-up of three-dimensional cancer cell cultures determines data interpretation and reproducibility.

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Introduction

Spheroids are three-dimensional (3D) cellular models that show widespread application across academic and industrial research groups. However, methodological transparency and guidelines are missing. Our international consortium presents a crowdsourcing knowledgebase (www.mispheroid.org) that assembles experimental parameters of more than 3000 published spheroid-related experiments. Interrogation of the knowledgebase revealed heterogeneity and lack of transparency in the methodological setup of spheroids.

Material and Methods

The impact of this heterogeneity was assessed through empirical evaluation of various spheroid set-ups, using 11 different cancer cell lines (of which 3 are early passage patient-derived) from 8 different tissue types, 6 different medium types, 2 different spheroid formation methods and different seeding cell numbers. The impact was evaluated by measurements of ATP concentration, dead staining, glucose consumption and lactate production, cytokine/chemokine production, spheroid circularity and size, response to 10 and 20Gy irradiation and RNA sequencing.

Results and Discussions

With the plethora of different metrics we were able to show highly significant variations in the metabolic activity, presence of cell death, metabolome, secretome, circularity, size and transcriptome of spheroids cultured in the various spheroid set-ups. This heterogeneity was observed by multiple laboratories part of the international consortium. Furthermore the culture medium influences the response to irradiation significantly. These results strongly suggest that reporting of these 4 experimental parameters; cell line, culture medium, formation method and size is an absolute prerequisite to interpret and reproduce a spheroid experiment.

Conclusion

Our consortium contributes to the field of 3D spheroid biology by providing 1) a knowledgebase that catalogues spheroid setup, characterization and applications enabling the community to share and access key technical and biological insights in 3D experimentation; 2) minimal information parameters combined with a tool to implement them; 3) a resource containing experimental data demonstrating the impact of experimental variations on spheroid metrics. In conclusion, our consortium aims to advance 3D biology in both an academic and industry environment by removing barriers of inconsistency and unawareness.

EACR22-0662

Nischarin receptor agonist rilmenidine decreases production of pro-tumorigenic cytokines and growth factors in pancreatic ductal adenocarcinoma stellate cells

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal types of cancer, with the five-year survival rate of just 6%. PDAC is characterized by a dense desmoplastic cancer stroma that can constitute up to 80% of the tumor volume. Cancer-associated fibroblasts (CAFs) in the PDAC stroma produce extracellular matrix and soluble factors that can support cancer progression. The major source of CAFs in PDAC are pancreatic stellate cells (PSCs). Nischarin (NISCH) is an Imidazoline-1 receptor protein that has been described as a tumor suppressor in breast and ovarian cancers. Importantly, there are several clinically approved agonists for this receptor. *In silico* analysis of the laser-microdissected epithelial and stromal compartments of the PDAC tumor tissue showed that NISCH was expressed in both the compartments. We have previously examined the effects of NISCH agonists on PDAC cancer cells. The aim of this study was to determine the effects of NISCH agonist rilmenidine on the phenotype of activated PSCs isolated from the PDAC tissue.

Material and Methods

We isolated PSCs from the patient's pancreatic tissue by the outgrowth method and characterized expression of CAF markers (αSMA, FAP, COL1, FN) by immunocytochemistry and expression of NISCH by western blot. To model cancer-stroma interactions we used transwell co-cultures of PSCs with PANC-1 cancer cells in the presence or absence of the NISCH agonist rilmenidine. Expression of growth factors and cytokines in co-cultures was determined by the qRT-PCR array in PSCs and PANC-1 separately, and by ELISA in the common supernatant.

Results and Discussions

PDAC patient-derived PSCs expressed collagen 1 and fibronectin and presented a heterogeneous population with αSMA and FAP positive fractions. Rilmenidine treatment of PSC-PANC-1 co-cultures decreased the production of pro-inflammatory cytokines IL-6, IL-8, and CCL-2, known drivers of the metastatic progression. In addition, qRT-PCR array of PSCs from co-cultures showed that rilmenidine decreased mRNA expression of JAG2, IL-10 and IL-11, markers of poor PDAC prognosis.

Conclusion

Systemic therapy affects both cancer and stromal cells in the tumor tissue. Our results imply that rilmenidine, an FDA-approved drug for the treatment of hypertension, decreases the production of pro-tumorigenic factors in PDAC stellate cells. These results lay a ground for further examination of rilmenidine potential for drug repurposing in PDAC.

EACR22-0670

Light microscopic graded oxygen-sensing using novel genetically encoded dual fluorophore reporters

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Introduction

Tumor hypoxia is investigated in the context of vessel function, metastasis formation and therapy resistance. For hypoxia imaging, various technologies have been developed, however, all ridden by specific shortcomings. These include, among others, the dependence on molecular oxygen (O_2) due to its particular relevance for the maturation of genetically encoded fluorescent protein (FP)-based hypoxia-/ O_2 -reporters. However, discovery of the green FP UnaG, which matures O_2 -independent, enabled our group to describe a family of hypoxia-reporters (Erapanedi et al., 2016).

Developed from these sensors, here we report two novel, genetically encoded UnaG- O_2 reporters: ratiometric dUnORS (destabilized UnaG-CyOFP1 Ratiometric Sensor) and the FRET-sensor dUnOFLS (destabilized UnaG-mOrange2 Fluorescence Lifetime Sensor). In both sensors, UnaG fluorescence provides an O_2 -independent reference signal, combined with an O_2 -dependent orange FP performing as the sensing signal for graded microscopic O_2 imaging.

Material and Methods

Various stably dUnORS or dUnOFLS expressing cell lines were generated and for *in vitro* characterization either incubated at various ambient O_2 concentrations, analyzed during live cell experiments or grown to tumor cell spheroids. Further, human glioblastoma reporter-cell lines were intracranially injected into mice to assess intratumoral oxygenation patterns *in vivo*. Analysis was performed via ratiometric microscopy or FLIM-FRET imaging.

Results and Discussions

With a live cell imaging approach, we could successfully correlate various O_2 concentrations with the provided reporter readout. Further, the sensors were suited to visualize O_2 gradients within tumor spheroids. Both sensors were sensitive in 1-10% ambient O_2 , resembling the physiological O_2 -range within most mammalian tissues, also covering the respective tissue hypoxia. Based on these correlations, we present first data demonstrating the visualization of O_2 gradients within solid intracranial tumors *ex vivo* in mice. Microscopic visualization revealed that the maturation of the O_2 -dependent orange FP was limited within scarcely and abnormally vascularized tumor regions, altering the sensors' responses.

Conclusion

Our genetically encoded UnaG-based O_2 -sensors were after extensive *in vitro* calibration shown to discriminate between tumor areals of sufficient and insufficient O_2 -supply *ex vivo*. Therefore, dUnORS and dUnOFLS promise to be novel, valuable tools to approach long-standing questions concerning the role of hypoxia in e.g. tumor biology.

EACR22-0690

Microbiome analysis of human breast cancer primary tumour tissue based on whole transcriptome analyses

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Introduction

Studies show that breast tissue considered previously as sterile is a place where multiple microorganisms can be present. As elsewhere in the human body, they can affect the environment *in situ* and thus be associated with various potentially clinically relevant effects. The objective of the study was to analyze the microbial composition (microbiome) of primary tumour tissue compared to normal breast tissue based on transcriptome analysis. Moreover, study cohort and available datasets allowed for a limited analysis focused on different breast cancer phenotypes, as well as comparison of our results with those obtained from dataset originating in China.

Material and Methods

Whole transcriptome analysis (rRNA depleted RNA-Seq) of breast tumours and normal tissues (from cancer-free women) of 23 individuals from Slovakia was performed and different bioinformatic tools (Kraken2 and Metaphlan3) were used and compared to reveal differences in the microbial composition of analysed tissues and cohorts. Kraken2 has showed higher reliability for our data. Additionally, RNA-seq from 91 samples obtained from SRA database, originating in China, and submitted by Sichuan University were used for comparison of findings in our original cohort.

Results and Discussions

Breast tumour tissue was different in microbial composition compared to healthy breast tissue. Normal tissue appears to be more abundant in the number of microbes, while many microbes have been found to be overrepresented there. Altogether, the most prevalent groups were *Proteobacteria*, *Firmicutes*, and *Actinobacteria* for both datasets (primary tumour and healthy breast tissue). In original tumour samples from Slovakia, *Bacteroides* genus was more frequent, while in Chinese samples *Cyanobacteria* was more frequent. Changes in microbiome profiles have been recorded not only between tumour and healthy tissues and different populations, but also based on circulating tumour cells status and markers HR, HER2 and p53, proliferation index Ki67, T and N stage of tumour and tumour grading.

Conclusion

The reasons and biological significance of microbial presence and amounts of transcripts are not yet clear and further studies will be needed to understand the impact on breast cancer and to exploit the microbiome for benefit of cancer patients.

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EACR22-0700

Inhibition of hypoxia-induced carbonic anhydrase IX sensitizes triple-negative breast cancer cells and murine models to cisplatin

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Introduction

Eradicating resistance and improving sensitivity to chemotherapy is a critical unmet clinical need in triple negative breast cancer (TNBC). Interestingly, studies of reversible

mechanisms of chemoresistance have shown that chemoresistant tumors can revert to a chemosensitive state. It is well known that hypoxic TME is the crucial driver in the onset of insensitivity to cisplatin through multiple mechanisms. To this aim we evaluated the possibility to sensitize TNBC to chemotherapy by targeting hypoxia-induced carbonic anhydrase IX (CA IX) with a sulfonamide inhibitor SLC-0111 (in Phase Ib/II clinical trials) and to monitor *in vivo* the efficacy of combinatorial therapy using different molecular imaging.

Material and Methods

In order to investigate the role of hypoxia in modulating response to cisplatin, TNBC cell lines (MDA-MB-231, BT-549, and 4T1) were grown under normoxic (21% O₂) or hypoxic (1% O₂) conditions. The ability of CA IX inhibitor, SLC-011, to sensitize TNBC cells to cisplatin was assessed by MTT and clonogenic assays as well as by apoptosis analysis performed with Annexin V. Furthermore, the efficacy of combinatorial therapy was assessed on mammospheres with stemness characteristics and patient-derived organoids (PODs). The main signal pathways involved in drug reversible resistance such as epithelial-mesenchymal-transition (EMT), stemness, and apoptosis markers were analyzed by western blotting. The reversal of acidic TME by SLC-0111 will be analyzed using a Fluorometric pH assay. The ability of SLC-011 to boost the cisplatin effect was analyzed in syngeneic orthotopic 4T1 xenografts using high-frequency ultrasound (HFUS) and PET/CT with 18F-FDG.

Results and Discussions

When TNBC cells grown under hypoxic conditions in 2D and 3D were treated with cisplatin (1mM) and SLC-0111 (100 mM) a greater reduction of proliferation, tumor growth, and induction of apoptosis with respect to single drugs was observed. The combinatorial treatment most hampered the EMT program, stemness features (Nanog and Sox-2), and apoptotic markers (caspase-3 and PARP). Interestingly, the addition of SLC-0111 to cisplatin caused a greater reduction of tumor growth and 18F-FDG uptake in a TNBC murine model as assessed by molecular imaging.

Conclusion

Our overall results highlighted the possibility to reversal cisplatin-resistant caused by hypoxic TME in TNBC by an integrated therapeutic approach

EACR22-0708

Primary Cilium BBS5 gene expression occurs in human malignant mesothelioma cells as opposed to human benign mesothelial cells.

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Introduction

Malignant pleura mesothelioma (MPM), a rare and aggressive tumor of the pleura is characterised by significant heterogeneity in terms of histology and molecular profiling. The Primary Cilium (PC) is an important but understudied cell organelle that is instrumental in receiving signalling cues from the extracellular space and the BBSome an important part of it's molecular machinery. Gene expression data relative to the ciliary complex in the pleura mesothelium are lacking. In this study we sought to assess the expression patterns of the BBS5 gene in human mesothelial cells (MeT5A; benign, M14K; epithelioid MPM, MSTO; biphasic MPM and ZL34; sarcomatoid MPM).

Material and Methods

Primers specific for the human BBS5 transcript were designed with the NCBI Primer-BLAST tool (template NM_152384.3). For mRNA expression, quantitative real time PCR was performed using b-actin as the reference gene. Total RNA was extracted using the TRIzol™ Reagent (Invitrogen™) and quality was assessed with a NanoDrop spectrophotometer (Thermo Fisher Scientific). 200 ng of RNA were reverse transcribed into cDNA in a 20 µL reaction volume using the the SuperScript™ III first-strand synthesis system (Invitrogen™). The cDNA was diluted 1:5 with nuclease-free water (Sigma-Aldrich®). Amplifications of the diluted cDNA were performed using the PowerUp™ SYBR™ Green Master Mix (Applied Biosystems™) and 0.4 µM primers on the ABI 7300 Real-Time PCR System. The thermocycler conditions were as follows: 50°C for 2 min, 95°C for 2 min followed by 40 cycles of 95°C for 15 sec, 55°C for 30 sec and 72°C for 1 min.

Results and Discussions

The gene expression of BBS5 relative to b-actin ($1/\Delta Ct$) in M14K, MSTO and ZL34 MPM cells was 0.11, 0.12 and 0.08, respectively. We did not detect BBS5 expression in MeT5A cells. These results indicate that the primary cilium-related BBS5 gene may have a role in the pathogenesis of MPM. The available evidence suggests that the primary ciliary complex is associated with a variety of malignancies including renal, pancreatic and breast cancer.

Conclusion

Our findings highlight the potential importance of the primary cilium molecular component BBS5 in pleural mesothelioma pathophysiology and should be further explored.

EACR22-0838

Improving response to treatment in head and neck cancer by targeting both tumour cells and microenvironment

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Introduction

Head and neck squamous cell carcinomas (HNSCCs) are aggressive tumours, associated with poor treatment responsiveness and poor prognosis. The adaptive immune response plays a significant role in the tumorigenesis. In fact, immune cells are major components of the tumour microenvironment (TME), particularly, the tumour-associated macrophages (TAMs) which are involved in many tumorigenic steps. The macrophages are basically categorised into pro-inflammatory M1 versus pro-tumoral M2 (TAMs). A lot of strategies aimed to induce a switch between macrophage phenotypes to create an anti-tumoral TME. To improve the response to treatment, we highlight strategies targeting both TME and cancer cells. Iron oxide nanoparticles (IONPs) are increasingly used in biomedical field as they may notably influence the redox balance. We evaluated effect of a concomitant IONPs exposition with X-Ray radiations on HNC cells and macrophages.

Material and Methods

First, we evaluated macrophages infiltration in HNSCCs. Immunostaining of CD68, CD80 (M1) and CD163 (M2) were performed on 60 patient tumour samples. Additionally, we validated a monocyte-to-macrophage polarization protocol to generate M0, M1 and M2 macrophage subtypes. Each phenotype was characterized by RT-qPCR and immunofluorescence (IF) using a panel of markers. In parallel, we designed IONPs vectorised with a peptide 22 targeting EGFR in order to favour their internalisation in FaDu and 93-VU cancer cell lines. Macrophages and cancer cells were exposed for 24h with 50µg/ml IONPs and internalisation was evaluated by a Prussian blue quantification and IF. Then, cells were irradiated at 2, 4 and 8 Gy, cells viability and ROS production were assessed.

Results and Discussions

Clinical results showed 37% M1 and 63% M2 infiltration in 60 tumour samples ($p < 0.001$). In vitro studies pointed out that M1 and M2 exhibited distinct phenotypes but also differences in metabolism (KGA, $p = 0.005$) and reactive oxygen species (ROS) production (NOX2, $p < 0.001$). M1 and M2 were able to uptake 7 pg iron/cell, while FaDu and 93-VU cells internalized only the vectorized IONPs ($p < 0.001$, $p < 0.005$, respectively). After X-Ray exposure, cancer cell viability decreased and ROS production increased.

Conclusion

In the oncology field, targeting specifically TAMs in addition of cancer cells will be a promising therapeutic approach based on the continuum plasticity between macrophage phenotypes. With this study we highlight that vectorized IONPs may affect TAM polarization and cancer cell radiosensitization.

EACR22-0842

Snail1 expression in the melanoma microenvironment drives immunosuppression and regulates tumour growth

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Introduction

Melanoma is a very malignant form of skin cancer due to its high metastatic abilities and resistance to therapies. MAPK pathway is hyperactivated in 90% of melanomas, with mutations in BRAF responsible for approximately 50% of the cases. In addition, melanoma cells reside in a highly heterogeneous tumour microenvironment (TME) that has been identified as a crucial regulator of its biology. Cancer-associated fibroblasts (CAFs), a very heterogeneous and plastic population, constitute one of the main cells within the TME and possess many tumour-modulating effects including regulation of tumour development, metastasis, and therapeutic resistance. Snail1 is an epithelial-to-mesenchymal transition transcription factor expressed during development and reactivated in pathological situations including fibrosis and cancer.

Material and Methods

To investigate the impact of stromal Snail1 expression in melanoma we have established BRAF-driven murine melanomas in conditional mouse models that allow Snail1 depletion in the TME.

Results and Discussions

Our results show that Snail1 is highly expressed in cells from the melanoma microenvironment, predominantly in CAFs. Importantly, Snail1 silencing in the TME greatly

decreases tumour size and metastasis burden in the lungs. Transcriptomic analysis of melanoma-associated CAFs and analyses of the immune infiltrate from the tumours in our models indicate that Snail1 expression in the melanoma microenvironment drives immunosuppression and promotes tumour and metastases growth.

Conclusion

Therefore, the investigation of a combination therapy that includes targeting Snail1 expression in the melanoma microenvironment may provide a promising new strategy to improve patient's prognosis.

EACR22-0926

Depicting the cellular architecture of the tumour microenvironment by integrating hyperplex immunofluorescence and automated image analysis

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Introduction

The tumour microenvironment (TME) is emerging as an important factor that shapes the dynamic of the tumour growth, its heterogeneity, and response to therapies (Binnewies et al., Nat Med 2018). Thus, efforts are undertaken to understand better the biology of cells within the TME (Rao et al., Nature 2021) and to provide spatial mapping of TME components and their interactions (Lewis SM, et al. Nat Methods 2021). One of the new technologies opening new avenues of research for spatial biology is multiplex immunofluorescence. This antibody-based approach enables the study of dozens of biomarkers within a single tissue section while preserving the spatial context (Hickey et al., 2021). In this study, we focus on the phenotyping of cells across different tumour types with an immuno-oncology panel encompassing 20 biomarkers. We interrogated their TME with the use of the COMET™ instrument: an automated microfluidic-based staining and in situ imaging device that allows the study of up to 40 antigens simultaneously (Migliozzi D et al., Microsyst Nanoeng 2019).

Material and Methods

Here we present a complete workflow “tissue in – data out” that allows the investigation of TME. Formalin-fixed paraffin-embedded samples from primary tumour biopsies assembled in tissue microarray of 9x9 mm were stained using COMET™. The resulting hyper-plex images were analysed through advanced tissue and cell segmentation using HALO® and HALO AI™ image analysis platform (Indica Labs, Inc.).

Results and Discussions

By developing a deep learning classifier using HALO AI™ software, the tumour and stroma areas as well as the vessels were automatically segmented. This classifier was then embedded within the Highplex FL module to quantify the various tumour and immune cell subpopulations of the TME such as proliferative tumour cells, lymphocytes, and macrophages within the different classes. Finally, by utilizing the Spatial Analysis module the spatial distribution and inter-relationship of the TME components such T cells and macrophages with vessels and tumour areas were assessed.

Conclusion

We present here a robust workflow to interrogate TME composition that is easily adaptable to any tissue type and any set of markers. Synergy of automated staining on Lunaphore's COMET™ instrument and Indica Labs' HALO® image analysis software eases the adoption

of multiplex immunofluorescence technology and data extraction to researchers at every stage of their scientific project.

EACR22-0974

Characterization of the visceral adipose tissue in the colon cancer microenvironment: a multi-omic approach

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Introduction

Even though colon cancer (CC) is the most preventable cancer, it is currently one of the deadliest. Worryingly, CC incidence is increasing in people <50 years. The hypothesis is that the drastic shift in lifestyle (nutritional habits, physical activity) may play an important role. Obesity, a CC risk factor, whereby adipose tissue promotes the secretion of adipokines, cytokines and reactive species, may lead to tumorigenesis. In this context, visceral adipose tissue (VAT) in particular is considered the culprit of causing major health disturbances, including cancer. Further, adipocytes can be part of the tumor microenvironment (TME), especially in tumors associated with AT. In colon, the interaction of tumor cells with adipocytes can be either indirect via production of hormones and inflammatory mediators that can reach the TME (as in T2 stage) or caused by cell-to-cell contact once the tumor mass extends VAT (as in T3 and T4). Our aim was to characterize the CC-associated AT at different tumoral stages using a multi-omic approach, focusing on lipid metabolism.

Material and Methods

Anthropometric data, CT-scans, plasma, peritumoral VAT (pVAT) and mesenteric VAT (mVAT) were collected from patients undergoing surgery for CC (HUSE). Three groups were established according to the pathologist classification: T2, T3, and T4. Cytokine and adipokine levels were analyzed in plasma and adipose tissue (Luminex). The lipidomic profile of pVAT and mVAT isolated adipocytes was established using UHPLC/MS. Transcriptomic analysis was carried out in whole adipose tissue (Affymetrix). Differentially expressed candidate genes were validated by qPCR.

Results and Discussions

Patients <50 years and those with a higher body mass index (>25 kg/m²) were at a more aggressive tumor stage (T3/T4). Plasma TNF α , IL-6, insulin, IL-8, resistin and leptin levels showed no significant differences between groups, while FABP4 levels decreased as tumor aggressiveness increased. Transcriptomic data revealed significant differences between mVAT and pVAT in genes involved in cholesterol and phospholipid metabolism, immune

system, and inflammation. Additionally, a differential lipid fingerprint was observed when comparing mVAT vs. pVAT and between the different tumoral stages.

Conclusion

The different transcriptomic and lipidomic profile of VAT depending on its association to tumor (pVAT) or non-tumor (mVAT) cells reinforces the concept of a crosstalk between adipocytes and cancer cells, and thus highlights the importance of the study of the TME.

EACR22-1002

DISSECTION OF TUMOUR MICROENVIRONMENT AND MELANOMA INTERACTIONS DURING THE INVASION PROCESS

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Introduction

Despite recent progress in melanoma management, patients frequently experience relapse and die of metastases. The development of new approaches to better understand the metastatic process and test novel combination therapies is therefore urgently needed. Recent knowledge on the interaction between cancer cells and the microenvironment suggests a prominent role of tumor surrounding cells in driving its progression and metastases formation.

Material and Methods

To unveil how melanoma cells interact with the microenvironment and respond to therapy, we established an innovative and highly reproducible three-dimensional (3D) melanoma model, making use of our platform of PDXs and primary patients' cultures. 3D organotypic cultures (OCs) were generated by hanging drop techniques from cancer cells alone or in combination with the two main melanoma TME components, fibroblast and endothelial cells.

Results and Discussions

The 3D cellular organization of the OC recapitulates patients' tissue structure, better than previous models used in the melanoma field. The proliferation rate of melanoma cells remains constant in the OCs over time and it is not affected by co-culture with fibroblasts or endothelial. Conversely, we demonstrated that the addition of TME components fosters melanoma invasion of collagen matrix in vitro and increases its metastatic potential upon transplantation in immunodeficient mice. To better characterize melanoma cells behavior, we examined the transcriptional status and the phenotype of our 3D models, as well as their secretome profile. Pathways related to invasion, EMT and ECM remodelling are upregulated during the development and growth of the organotypic cultures. Notably, L1 cell adhesion molecule (L1CAM) expression is highly increased in melanoma cells in hetero-culture and its depletion strikingly abolishes the collagen matrix invasion potential of melanoma cells.

Conclusion

3D OCs shed new light on the mechanisms behind the relationship among malignant melanoma cells and the surrounding ecosystem during tumor progression, exploiting the potential of a new in vitro system. We proved that fibroblasts and endothelial cells reprogram melanoma cells toward a more aggressive phenotype. Our model might represent a powerful tool also to identify new targetable vulnerabilities and biomarkers during melanoma dissemination, allowing to test novel combination treatments in a complex and relevant system.

Selective depletion of reactive hepatic stellate cells impairs liver metastasisA. Benedicto¹, B. Arteta¹, S.L. Friedman², M.D. Boyano¹, E. Olaso¹¹University of the Basque Country, Cell Biology and Histology Department, Leioa, Spain²Icahn School of Medicine at Mount Sinai, Division of Liver Diseases, New York, United States**Introduction**

Hepatic stellate cells (HSCs) are non-parenchymal liver resident cells with a wide array of functions. In normal liver, they remain in a quiescent state in the space of Disse, in close contact with hepatocytes and liver sinusoidal endothelial cells. However, upon injury they activate and acquire a myofibroblast-like phenotype, with contractile ability. Activated HSCs play a major role during tissue repair, extracellular matrix regulation, immune responses and inflammation. Even though HSCs are major actors in liver fibrosis, their involvement in the generation and regulation of the hepatic tumor microenvironment needs further attention

Material and Methods

We used a previously described transgenic (TG) mice expressing the herpes simplex virus thymidine kinase gene (HSV-Tk) driven by the mouse GFAP promoter, based on the differential expression of GFAP in the liver cells, mostly constrained to HSCs. Therefore, the limited expression of HSV-Tk on proliferating GFAP-expressing HSCs makes them susceptible to apoptotic cell death upon Ganciclovir (GCV) administration. *In vitro*, we analyzed the responses of freshly isolated HSCs from wild type (WT) or TG mice to tumor cell supernatants and GCV treatment. *In vivo*, either B16 melanoma or MC38 colon carcinoma were intrasplenically injected and allowed to generate liver metastasis in (WT) and TG mice.

Results and Discussions

Primary isolated mouse HSCs from both WT and TG mice proliferated 50 % more than upon activation with either B16 or MCA38 cell supernatants, compared to untreated ones. Thus, tumor activation made transgenic HSCs susceptible to apoptotic cell death through GCV treatment *in vitro*, reducing cell viability in 80 %. *In vivo*, the treatment of MC38 or B16 tumor bearing mice with GCV substantially reduced the foci number and total area occupied by liver metastasis by ~80 %. Moreover, livers from TG mice showed reduced key tumor microenvironment parameters, such as intratumoral collagen accumulation, neoangiogenesis and recruitment of myeloid-derived suppressor cells, accompanied by lower serum levels of IL-1, IL6, TNF- α and VEGF.

Conclusion

Our results point out HSCs as the required spark for the progression of liver metastasis, making them a good candidate for new targeted therapies to treat liver metastasis of different primary origins.

The oncoprotein myoferlin as potential new participant in myCAF activity in pancreatic cancerR. Peiffer¹, S. Anania¹, Y. Boumahd¹, F. Agirman¹, N. Maloujahmoum¹, A. Masamune², E. Letellier³, A. Bellaïche¹, O. Peulen¹¹University of Liège, Metastasis Research Laboratory, Liège, Belgium²Tohoku University Graduate School of Medicine, Division of Gastroenterology, Sendai,

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Introduction

Early metastasis formation and a highly desmoplastic tumor microenvironment (TME) are hallmarks of pancreatic ductal carcinoma (PDAC) that contribute to a 5-year survival rate of only 9%. Another major PDAC hallmark is cancer-associated fibroblast (CAF) heterogeneity, as several CAF subpopulations reside within the pancreatic TME such as myofibroblastic CAFs (myCAFs), inflammatory CAFs (ICAFs) and antigen-presenting CAFs (apCAFs). Pancreatic stellate cells (PSCs) have been described as key players in the pancreatic TME, as they support cancer cell proliferation via metabolic fueling, and contribute to CAF heterogeneity. Orchestrated by TGF- β 1, quiescent PSCs are activated and differentiate into myCAFs, the most prominent producers of extracellular matrix in PDAC. Thus, forging not only a physical barrier to drug delivery but also preventing immune cell infiltration. Here we investigate for the first time the role of myoferlin, a PDAC transmembrane oncoprotein, in PSC activation and their differentiation into myCAFs.

Material and Methods

Stromal myoferlin quantification with survival correlation and Masson's trichrome staining were performed on PDAC patient biopsies (n=47). As a bottom-up approach we designed bulk RNA sequencing on 4 immortalized human PSC cell lines while comparing myoferlin depleted PSCs to control groups. Western blotting and RT-qPCR on 2D cultured PSCs were used to analyze the impact of myoferlin depletion on TGF- β 1 signal transduction and myCAF gene signature. The importance of myoferlin in myCAF mitochondrial metabolism was assessed via extracellular flux analysis.

Results and Discussions

High stromal abundance of myoferlin was detected in PDAC patient biopsies and trended towards a negative correlation with patient survival rates. Following myoferlin knockdown in PSCs, RNA sequencing in combination with gene ontology analysis revealed a down regulation of mitochondrial activity as well as several cellular protein processing and trafficking routes. We revealed in myCAF promoting culture conditions of PSCs, that mitochondrial respiration decreases after myoferlin knockdown, validating RNA sequencing data. Additionally, we were able to show that this metabolic change is accompanied with a decreased TGF- β 1 signal transduction in myCAFs.

Conclusion

This study not only suggests a metabolic switch in myCAFs after myoferlin knockdown but also a decreased response to TGF- β 1, underlining a supportive role of myoferlin for myCAF activity.

EACR22-1152

Modification of BBSome gene expression components in malignant pleural mesothelioma cells under the influence of the Primary Cilium modulating agent, ammonium sulphate.

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Introduction

Emerging evidence has demonstrated that several Primary Cilium (PC)-associated genes are deregulated in cancer. The role of the PC in the pathophysiology of pleural malignant

mesothelioma is not well understood. Compounds modulating the function of the PC could provide more information regarding at least one important part of the PC which is the BBSome. The aim of this study was to examine the effect of ammonium sulphate (AS) on the gene expression of PC associated genes in the epithelioid malignant pleural mesothelioma (MPM) cell line, M14K.

Material and Methods

M14K MPM cells were cultured as monolayers. 30mM AS was used for PC shedding (incubation for 24hrs prior to experiments). Primers specific for the human BBS2, 5, 7, 18 transcripts were designed with the NCBI Primer-BLAST tool. For mRNA expression, quantitative real time PCR was performed using b-actin as the reference gene (2 biological replicates, 3 technical replicates each). Total RNA was isolated by TRIzol™ Reagent (Invitrogen™). 200 ng of RNA were used to synthesize cDNA in a 20 µL reaction volume with the SuperScript™ III first-strand synthesis system (Invitrogen™). The cDNA was diluted 1:5 with nuclease-free water (Sigma-Aldrich). Diluted cDNA (8.4 µl) and primers (0.4 µM) were then added in a 20 µl PCR mix (PowerUp™ SYBR™ Green Master Mix, Applied Biosystems™), and amplified in the ABI 7300 Real-Time PCR System. The thermal cycling conditions for all genes were: 50°C for 2 min, 95°C for 2 min followed by 40 cycles of 95°C for 15 sec, 55°C for 30 sec and 72°C for 1 min. Relative gene expression was calculated with the $2^{-\Delta\Delta CT}$ method.

Results and Discussions

Our analysis showed that the fold difference in the gene expression of BBS2, BBS5, BBS7 and BBS18 in M14K cells treated with AS relative to untreated M14K cells was 0.43, 0.3, 0.52 and 0.76, respectively. These results indicate that treatment of epithelioid MPM cells with AS induces the downregulation of the corresponding PC-associated genes.

Conclusion

We report here that AS treatment of epithelioid MPM cells leads to significant reduction in BBS2, BBS5, BBS7 and BBS18 genes. Our results highlight that BBSome components in MPM cells are declining when PC shedding agents are used and this may be of value for further investigation in MPM.

EACR22-1178

Target proteomics discovery in muscle invasive bladder cancer cell lines

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Introduction

Bladder cancer (BC) is the eighth most common cancer in men in the UK. Around 25% of non-muscle invasive BC (NMIBC) progress to muscle invasive BC (MIBC). Abnormal expression of protein function is one of the most important factors in disease pathologies. Understanding the cellular mechanisms, microenvironment, disease progression, and the effect of hypoxia in gene or protein expression should improve prognostication and treatment options. Therefore, we aimed to use mass spectrometry (MS) to: 1) generate a spectral library of all of the detectable peptides in MIBC cancer cell lines; 2)

identify differentially expressed proteins in cells cultured for 24h and 48h in hypoxia (1% and 0.1% O₂) versus normoxia (21% O₂).

Material and Methods

Three MIBC cell lines (J82, HT1376, T24) were seeded and incubated for 24h. The cells were then cultured for 24h or 48h in 21% O₂, 1% and 0.1% oxygen levels, collected and lysed. SWATH-MS samples were prepared and used for library preparation and gel fractionation. Proteins were identified using MS. Proteins were verified using www.uniprot.org/.

Results and Discussions

75 MIBC samples were processed and combined for the spectral library generation. Over 6000 proteins were generated for the spectral library with 3553 proteins found in ≥30% of the samples in the SWATH analysis. SWATH-MS quantified 4150 proteins across all samples at a peptide FDR of 1%. Protein expression was affected by both the level and duration of hypoxia and cell line. We identified the number of proteins differently upregulated (fc>2) under 24h of 1% vs control compared with 48h of 1% vs control for each cell line which is 242 (HT1376), 472 (J82) and 82 (T24) proteins with 73 common proteins upregulated in all three cell lines, hypoxia level and hypoxia duration relative to normoxia. The list of changing proteins filtered to specifically identify plasma membrane proteins which could be subsequent candidates for tumour imaging or targeted therapies.

Conclusion

There is a wide variety of proteins the expression of which change in response to reducing oxygen levels in bladder cancer cells. Further analysis might identify potential new biomarkers and targets for further study.

EACR22-1188

Matrix-remodelling subtype of cancer-associated fibroblasts promotes tumour growth and metastasis

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Introduction

Cancer-associated fibroblasts (CAFs) are one of the most abundant cell types within the tumour microenvironment. Besides their matrix-remodelling capabilities and their role in promoting primary tumour growth and metastasis, CAFs have been proposed to have immunosuppressive functions. However, high *in vitro* plasticity, high heterogeneity and a lack of specific markers hamper the functional analyses of CAFs.

Material and Methods

In this study we combined *in vivo* tumour growth and metastasis assays with gene expression and immune cell profiling and *in vitro* 3D co-culture assays and mechanistic studies to investigate the role of the stromal receptor Endo180 (Mrc2) in breast cancer progression.

Results and Discussions

Here, we show that loss of Endo180, mainly expressed on a subpopulation of matrix-remodelling CAFs, profoundly reduces tumour growth and metastasis in a range of breast cancer *in vivo* models.

Neither secreted factors nor deposited extracellular matrices of wildtype or Endo180 knockdown fibroblasts have a differential impact on in vitro tumour cell growth. Investigation of CAF intrinsic characteristics revealed that Endo180 depletion is associated with a contractility defect and increased stress-induced cell death. Consistent with these findings fewer Endo180-deficient fibroblast can be recovered from 3D co-culture spheroids and importantly, reduced levels of α SMA-positive cells are found in primary tumours in Endo180-/- mice. These tumours have also reduced levels of collagen but no change in endothelial cell and macrophage infiltration. However, co-implantation of D2A1 tumour cells with Endo180-wildtype expressing CAFs reduces the number of tumour-associated CD8+ T cells; effects that are lost when co-implanted with Endo180-deficient CAFs.

Finally, we addressed how tumours might adapt to a defective microenvironment. We selected the D2A1 tumour subline by serial in vivo passage in Endo180-/- mice for its ability to overcome the impaired microenvironment and to metastasize to the lung. RNASeq analysis of the generated tumour subline suggests an enhanced ability of the tumour cells to secrete, remodel and respond to changes in the extracellular matrix even if CAFs are impaired.

Conclusion

We demonstrate that upregulated Endo180 expression by a specific, potentially targetable CAF subset is required to generate a permissive tumour microenvironment and provide further evidence for the critical role of the tumour stroma in driving tumour evolution.

EACR22-1200

Dissecting the interplay KRAS/Galectin-3/Tumor-associated macrophages in colorectal carcinogenesis

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Introduction

KRAS mutations ($KRAS^{mut}$) are present in 30%-50% of colorectal cancer (CRC) cases, contributing to CRC cell survival, increased tumor aggressiveness and resistance to anti-EGFR therapies. Galectin-3 (Gal-3) is a multifunctional protein also important in CRC and correlated with cancer cell invasion, metastasis and angiogenesis. Interestingly, Gal-3 interacts and affects KRAS expression levels. Furthermore, Gal-3 secreted by cancer cells is also capable of influencing tumor-associated macrophages (TAMs), which are important players at the tumor microenvironment (TME). On its turn, our group has demonstrated that TAMs stimulate CRC cell motility and invasion.

To the best of our knowledge, the role of KRAS on Gal-3 expression and function in CRC is not fully understood. Since KRAS seems to interfere with Gal-3, which is known to regulate TAMs, here, we aim to explore the interplay $KRAS^{mut}$ /Gal-3/TAMs and uncover their impact on CRC.

Material and Methods

For this purpose, KRAS^{mut}/Gal-3 complex and its regulation was analyzed in CRC cells through co-localization, co-immunoprecipitation and western blot. Additionally, KRAS and Gal-3 impact on proliferation, invasion and migration of CRC cells was also evaluated. Further, we established co-cultures of CRC cells with human macrophages isolated from healthy blood donor buffy coats. The impact of co-cultures on macrophage polarization and on CRC cell immunogenicity were evaluated, analyzing by flow cytometry the expression of specific pro-inflammatory/anti-inflammatory and cancer cell immunogenicity surface markers.

Results and Discussions

Co-immunoprecipitation and co-localization studies demonstrated that KRAS/Gal-3 physically interact and form a complex in CRC. Moreover, KRAS/Gal-3 exhibits a feedback loop regulation, being Gal-3 able to interfere with KRAS expression, but also KRAS affects Gal-3 expression levels. Additionally, we also found that the disruption of the KRAS/Gal-3 complex significantly decreases CRC cell migration and invasion. Co-cultures of CRC cells with human macrophages resulted in alterations on inflammatory profile, rising anergic macrophages and evasive CRC cells.

Conclusion

In summary, our results appoint KRAS and galectin-3 as relevant players in CRC. Additionally, we expect that understanding the KRAS^{mut}/Gal-3/TAMs interaction and its impact in CRC phenotypic alterations will bring valuable insights to unveil the role of TME in resistant CRC harboring KRAS mutations and to the development of novel therapeutic approaches.

EACR22-0055

Bidirectional paracrine communication between head and neck cancer cells and stromal fibroblasts

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Introduction

Increasing evidence has demonstrated that the tumor microenvironment (TME), and especially, cancer-associated fibroblasts (CAFs), are closely related to the tumorigenesis process and actively participate in several hallmarks of cancer. Secreted factors are known to be important mediators in the communication between cancer cells and the surrounding TME. This study was aimed to investigate the paracrine crosstalk between head and neck squamous carcinoma cells (HNSCC) and stromal fibroblasts.

Material and Methods

Different cell populations of HNSCC cells (FaDu, UT-SCC-38 and LSC1), normal keratinocytes (Kc), primary cancer-associated fibroblasts (CAFs), and normal fibroblasts (NFs) were grown, and conditioned media (CM) produced. Reciprocal functional effects of secreted factors were studied on cell growth (by MTS), invasion (3D spheroid invasion in collagen) and stemness (tumorosphere formation). In addition, expression of MMPs and stemness markers was analyzed by RT-qPCR.

Results and Discussions

Firstly, we analyzed the functional effects of CM from fibroblasts on HNSCC cells. The HNSCC cell lines (FaDu and UT-SCC-38) were able to form tumorospheres more efficiently when grown in the presence of CAF-CM than in NF-CM and were unable to form tumorospheres in non-supplemented medium. Consistently, CAF-CM robustly increased anchorage-independent growth and the expression of several cancer stem cell markers (ALDH1, NANOG, SOX2, and OCT4) in both HNSCC cell lines, compared to NF-CM.

Reciprocally, we investigated the effects of CM from HNSCC cells (FaDu or LSC1) or Kc (as normal control) on stromal fibroblasts. HNSCC-CM specifically and significantly increased fibroblast invasion (both NFs and CAFs), whereas Kc-CM had no stimulatory effect. Moreover, mRNA levels of several MMPs were found to concomitantly increase in both CAFs and NFs upon treatment with HNSCC-CM. However, HNSCC-CM and Kc-CM exhibited a similar stimulatory effect on fibroblast viability and growth.

Conclusion

This study unveils important insights on the reciprocal paracrine communication in the head and neck TME, which could cooperatively drive tumor progression. Specifically, our results evidence that CAF-secreted factors enhance stemness in HNSCC cells. In turn, HNSCC-secreted factors strongly promote fibroblast invasiveness, at least in part, by increasing MMP expression and matrix degradation.

EACR22-0107

A model for the origin and context of cancer associated fibroblasts

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Introduction

Tumors are evolving organs where cancer cells communicate with several cellular components of the tumor microenvironment (TME). Cancer associated fibroblasts (CAFs) are the most abundant constituents of the TME in many cancers and have been linked to most hallmarks of cancer.

We have employed single cell RNA-sequencing (scRNAseq) on a murine model of breast cancer and identified four distinct subsets of CAFs based on their transcriptional signatures and spatial distribution throughout time (Bartoschek M. et al., 2018). Notably, some of these CAFs are linked to a worse prognosis.

However, the origin of the CAF subtypes and how their phenotype is shaped during tumor progression remain unclear.

Material and Methods

We used the Seurat package V3.2 (Stuart T., Butler A. et al., 2019) to re-analyze our scRNA-seq data and identify differentially expressed genes (DEGs). The transcriptional signatures obtained were mapped onto a human breast cancer spatial transcriptomic atlas (10X Genomics® Visium v1 chemistry). The Iris (Abbas A.R. et al., 2005) and Immunome (Ortutay C. et al., 2006) databases were used to identify immune-related genes among CAFs DEGs.

Results and Discussions

Re-analysis of our scRNA-seq data at a higher resolution led to the identification of eight CAF sub-groups. More specifically, the CAF populations associated with ECM deposition (mCAFs) and vascular development (vCAFs) have been refined to comprise three subclusters each termed mCAFs_1-3 and vCAFs_1-3, respectively.

Moreover, we have generated 20-gene signatures for each CAF subset and mapped them back onto a human breast carcinoma spatial transcriptomic dataset. Preliminary analyses show different localizations for the distinct CAFs, suggesting that the cellular context in which CAFs reside determines their functional programs.

By overlapping the lists of DEGs to the Iris and Immunome databases, we observed that the mCAFs are the most enriched for immune-related genes, especially the mCAF_2 subset. Similarly, within the vCAFs, the vCAF_3 subset is the most immune-related.

Conclusion

Our preliminary data suggest that CAFs have different origins, and the context in which they reside determines their phenotype during tumor evolution. Spatial and functional characterization of the different CAF subpopulations will allow to establish if distinct CAFs have a different origin and immune impact thus potentially hampering or facilitating tumor progression and offering new therapeutic strategies.

EACR22-0123

Elucidating the molecular pathways regulated by Nox4 in a subtype of cancer-associated fibroblasts in prostate cancer

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Introduction

Prostate cancer progression is associated with a reactive stromal environment, characterized by the loss of well-differentiated stroma and the presence of activated fibroblast subtypes, termed cancer-associated fibroblasts (CAFs). Single cell analyses have shown that there are distinct CAF subtypes across tumor types, but their identification and functional characterization is still lacking in prostate cancer.

We identified a population of CAFs in prostate cancer that is characterized by the expression of the redox enzyme NADPH oxidase 4 (Nox4). Nox4-expressing CAFs primarily localize in close proximity to tumor foci and are associated with higher gleason grade, reduced progression-free survival and express a transcriptional program related to high contractility and ECM production. In order to evaluate the potential of targeting Nox4-expressing CAFs in prostate cancer, we currently aim to characterize the driver molecular pathways regulated by Nox4.

Material and Methods

Primary prostate CAFs and patient-matched normal prostate fibroblasts were treated with GKT831, a small molecule Nox4/Nox1 inhibitor, and analyzed using an integrative bioinformatics approach. Identified pathways were validated by functional assays and Western Blot. For identification of direct oxidative targets of Nox4, dimedone-labeling of oxidized protein residues was performed.

Results and Discussions

Integrative bioinformatics suggest that Nox4 influences adhesion and migration pathways in CAFs via the transcription factor TEAD1 and its coactivator YAP. These results were

experimentally verified wherein Nox4 inhibition reduced migration and adhesion of CAFs to collagen I and reduced expression of total YAP and its phosphorylation at tyrosine 357, which promotes translocation of YAP into the nucleus. Analysis of upstream pathways revealed that Nox4 activates a FAK-BCAR1-Src signaling cascade that has previously been described to regulate YAP activation. Ongoing experiments suggest that during adhesion, Nox4 regulates this cascade via oxidation of a redox-sensitive protein tyrosine phosphatase.

Conclusion

Our data suggests that Nox4-expressing CAFs in prostate cancer are characterized by a contractile, myofibroblastic phenotype. Specifically, we found that Nox4 regulates a FAK-Src-YAP signaling axis which has been shown to drive adhesion and mechano-transduction pathways and the development of a tumor-promoting microenvironment.

EACR22-0145

ASPA as a new regulator of Cancer-Associated Fibroblasts phenotype and its implication in cancer

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Introduction

The crosstalk between cancer cells and the tumor microenvironment (TME) plays a critical role in the acquisition of molecular and cellular features underpinning tumor progression. Cancer Associated Fibroblasts (CAFs) are the major population of the TME and their contribution to most of the hallmarks of cancer is gradually emerging. CAFs present a pathologically activated phenotype that promotes extracellular matrix (ECM) remodeling and pro-tumorigenic signaling to cancer cells. CAFs may also present metabolic adaptations such as enhanced anaerobic glycolysis that generate metabolites to fuel cancer cell growth. However, how metabolic reprogramming assists in the activation of fibroblasts, and influence signaling and transcriptomic adaptations controlling their pro-tumor behavior is not well defined.

Material and Methods

To identify new stromal modulators of cancer progression, we analyzed gene expression datasets of cancerous and normal stroma from human breast, ovary, colon, prostate, and lung tissues. Consistent up- or down-regulated genes were shortlisted based on their association with disease-free survival, and further investigated using genetic manipulation in murine and human models, molecular characterization, and functional *in vitro* and *in vivo* experiments.

Results and Discussions

These analyses revealed that the metabolic enzyme Aspartoacylase (ASPA) is consistently downregulated in CAFs of different tumor types, and it is correlated with a poorer prognosis in human breast and prostate cancer. Modulation of ASPA expression in normal fibroblasts or CAFs affect their levels of CAF marker expression, extracellular matrix remodeling capacity and the crosstalk to cancer cells. Our preliminary results show that ASPA expression is downregulated by the transcription factor TAZ downstream of TGF β signaling. They also suggest that ASPA downregulation may negatively affect the intracellular pool of acetate, potentially affecting acetylation processes such as histone acetylation and epigenetic reprogramming.

Conclusion

Understanding the role and mechanisms of action of ASPA in CAFs may illuminate the intricate metabolic crosstalk between tumor and stroma, and how metabolic adaptations affect signaling and epigenetic reprogramming to enable the emergence of a fully pro-tumor CAF phenotype. We expect these advances to inform strategies to restrain tumor-promoting behaviors in CAFs and compromise tumor progression.

EACR22-0149

Deciphering the role of CTHRC1 on prostate cancer aggressiveness

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Introduction

Prostate cancer (PCa) is the most common cancer in men worldwide. Although most patients are curable, 15% of them still relapse and die due to the disease. To find a better therapy for these patients, first we need to understand which mechanisms drive the development of metastatic features in the cancer cells. Recent research has demonstrated that cancer cell-derived secretome modulates tumor microenvironment, especially the extracellular matrix (ECM). Although it has been shown that in different tumor types this event has a strong impact on aggressiveness (cell proliferation, dissemination, and invasion), no data is still available in the context of PCa.

We have previously described that transcriptional alternation of metabolism has tumor and metastasis suppressor activity in PCa both *in vitro* and *in vivo*, and this was associated with cell intrinsic alterations of signaling pathways downstream of cell-ECM interaction, leading to cytoskeleton organization and cell adhesion changes. Based on this data and the aforementioned opened questions, we hypothesize that the alteration of secreted factors is a driving force in the acquisition of aggressive properties by PCa cells and it may impact the biology and progression of the disease.

Material and Methods

1. Computational analysis of clinically relevant PCa patient's cohorts.
2. *In vivo* analysis of a PCa GEMM that is faithful to the human disease.
3. *in vitro* experimental assays to exploit the potential of secretome-related genes in PCa progression.

Results and Discussions

By integrating computational screenings of clinically relevant patients' cohorts and functional assays, we have exploited the potential of secretome-related genes in PCa. Our approach has led us to identify and select, from among the more than 600 genes tested, Collagen Triple Helix Repeat Containing 1 (CTHRC1) as the top gene with the strongest prognosis potential in PCa.

CTHRC1, a secreted ECM-related glycoprotein involved in vascular remodeling, is increased in PCa patients and associated with bad prognosis. We observed that silencing of CTHRC1 induces a decrease in proliferation, migration and invasion of PCa cells. Proteomics analysis reveals that CTHRC1 deletion alters the expression of several protein functionally related to cell communication and ECM pathways.

Conclusion

CTHRC1 expression is increased in PCa patients and has prognostic potential. Its deletion and/or silencing in PCa cells has tumor suppressive activity possibly through the alteration of ECM-related events.

EACR22-0157

Annotating stromal populations in the metastatic pancreatic cancer microenvironment

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is frequently metastatic, with lung and liver among the preferred metastatic sites. Two main transcriptional subtypes, classical and basal, have been defined, the latter being associated with a more aggressive disease and enriched in metastatic lesions. In addition, PDAC presents with an intense desmoplastic reaction and the tumour stroma has been proposed as a therapeutic target in PDAC. However, the success has been limited, possibly due to a limited understanding of the interplay between the different cell populations in the tumour microenvironment (TME) and how they might differ between molecular subtypes and between primary and metastatic lesions. In this study, we analyse the impact of PDAC molecular subtypes on the TME in the pancreas and at metastatic sites.

Material and Methods

We use Cytometry by Time of Flight (CyTOF) to characterize the mesenchymal stroma and immune cell populations in healthy and tumour-bearing mouse pancreas, liver, and lung using syngeneic injection models of classical and basal PDAC cancer cell lines.

Results and Discussions

Here we describe that the phenotypes of mesenchymal stromal cells, and fibroblasts in particular, are diverse and specific for the different organs studied. In tumours, we observe important differences in the cell composition of the TME induced by the same cancer cells in different organs, with a different profile of cancer associated fibroblasts (CAFs) in pancreas, liver, and lung. Notably, we still detect subtype-associated features that are maintained across the three organs. For example, we consistently observe differences in the relative abundance of myofibroblastic and inflammatory CAFs between the different molecular PDAC subtypes, as well as differences in infiltration by granulocytes, monocytes and macrophages and CD8 T cell phenotypes. Moreover, we identified specific subsets of CAFs that are associated with the abundance and phenotypes of immune cell subpopulations, suggesting a possible immunomodulatory role for these CAF subsets.

Conclusion

The cell composition of the TME is highly dependent on local factors. However, we found subtype-associated features that are conserved across tissues. These phenotypes might point towards environmental dependencies that differ between subtypes and which could be exploited to design subtype-specific stromal targeting therapies.

EACR22-0183

C-type lectin domain family 3 member A (CLEC3A) extracellular matrix protein sustains breast cancer aggressiveness

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Introduction

The idea that extracellular matrix (ECM) plays a crucial role in tumor progression has become increasingly evident. We identified an ECM gene expression pattern (ECM3) in approximately 40% of breast carcinomas (BCs) that significantly associates with a high risk of relapse in patients with grade III BCs (E3G3). In this study, we investigated the biomechanical characteristics of the ECM in human BC samples according to their ECM pattern and grade to identify molecules responsible of E3G3 aggressiveness.

Material and Methods

Soluble extracts, obtained from decellularized BC matrices (dECMs), were analyzed by a shotgun, label-free proteomic approach. Immunohistochemistry (IHC) was used to study the expression of CLEC3A and Gomori trichrome stain to evaluate collagen density in FFPE sections of human BC samples. Stiffness was assessed by measuring the elastic moduli of frozen specimens. BC cells transiently transfected with CLEC3A and grown on ECM were analyzed in *in vitro* assays.

Results and Discussions

In accordance with gene expression profile of E3G3 BCs, dECMs proteome of these tumors was enriched in pathways related to the interaction between tumor cells and the ECM and about 50% of E3G3 unique proteins were cytoskeleton-related, supporting peculiar tumor-ECM teamwork in these tumors. E3G3 BCs did not have higher amount, but significantly higher collagen density and a fibrillary cytoskeleton network. Moreover, they showed the highest stiffness, which significantly positively correlated both with the amount of dense collagen and of tubulin. Among the E3G3 unique proteins, we selected CLEC3A, a secreted C-type lectin domain family 3 member, as putative matrisome molecule implicated in tumor-ECM crosstalk. IHC analysis in two BC cohorts validated higher CLEC3A, which localized at the tumor nodule edge, in E3G3 BCs and its significant association with worse prognosis. CLEC3A transfection in MDA-MB-231 and MDA-MB-361 cells increased intracellular mediator of adhesion (pFAK, RhoA and Rac1), modified actin cytoskeleton organization together with an increase in YAP activation, supporting CLEC3A activity as mechanosensor. Moreover, CLEC3A expression was significantly associated with higher migration and invasion in MDA-MB-361 cells. Accordingly, YAP/TAZ signature level, mirroring YAP activity, was higher in CLEC3A positive BCs and positively correlated with tumor stiffness.

Conclusion

A distinct tumor/ECM interaction mediated by CLEC3A occurs in E3G3 BCs, increasing tumor stiffness and aggressiveness.

EACR22-0243

Deciphering the Crosstalk Between Cancer-Associated Fibroblasts and Endothelial Cells and its Role in Tumor Progression

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Introduction

The tumor microenvironment (TME), composed of resident and infiltrating stromal cells, is critically hijacked by the emerging tumor to support its progression, dissemination and therapeutic resistance. Both tumor-endothelial cells (TECs) and the cancer-associated fibroblasts (CAFs) are key components of the TME impinging in multiple tumoral processes. By forming vessels, TECs support the high demand of the proliferating cells. But vascularization in tumours is often aberrant which hinders blood perfusion and the deliverance of anti-cancer agents. CAFs are an abundant stromal population that profoundly shapes the properties of the TME and alter the behaviour of both cancer and stromal cells. However, whether there exists a specific communication between TECs and CAFs, and whether this reciprocal interplay affects their pro-tumor behaviour is not well-defined.

Material and Methods

To address these questions, we used functional in vitro assays for TECs and CAFs coupled with cellular and molecular characterization, as well as in vivo models such as chick embryo angiogenesis/tumorigenesis assays. In addition, integrative analyses of patient-derived material, as well as murine and human models were used to identify potential factors mediating the reciprocal crosstalk.

Results and Discussions

Our data shows that interaction with CAFs, but not normal fibroblasts (NFs), induces an inflammatory state in endothelial cells that profoundly affects their ability to form networks. This phenotype is reminiscent of the abnormal vasculature that characterizes most solid tumors and that negatively affects anti-cancer agent perfusion. This response was in part mediated by the upregulation of secreted factors (SFs) and activation markers (V/ICAM1). Interestingly, the same set of SFs was upregulated in TECs in human tumors and was significantly associated with poor prognosis. In addition, there was a correlation between the expression of these genes with desmoplastic responses in tumors, suggesting a potential crosstalk with CAF. Our data shows that SFs produced by inflamed ECs promote migration, invasion and ECM remodeling in NFs.

Conclusion

Altogether, our data suggest the generation of a feed-forward loop that modulates the emergence and maintenance of pathological behaviors in TECs and CAFs and underlies stromal co-evolution as critical factor in the generation of aggressive TMEs. We propose this may be particularly relevant for strategies aiming to normalize tumor vasculature and increase drug delivery within tumors.

EACR22-0355

UGDH expressed by ovarian cancer tumour initiating cells remodels the tumour microenvironment to establish new metastatic niches

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Introduction

Ovarian cancer (OC) is a broad classification for tumours of the ovary and fallopian tubes, of which several histological classifications and molecular subtypes are defined. Among the distinct subtypes of high-grade OC, the mesenchymal molecular subtype has particularly poor five-year survival rates, a complex tumour microenvironment (TME) and high recurrence rate. OCs form spheroids in malignant ascites to promote survival of stem-like tumour-initiating cells (TICs) that drive recurrence. The TME facilitates disease recurrence by augmenting mesothelial clearance and invasion by TICs and by creating a desmoplastic stroma that presents a significant challenge to treatment. We hypothesise that TICs, supported by this complex TME, are a target for therapeutic eradication. We identified the enzyme UDP-glucose-6 dehydrogenase (UGDH) through high-throughput screening as highly influential to TIC survival and show that targeting this enzyme specifically weakens OCs of the highly aggressive mesenchymal molecular subtype.

Material and Methods

OC spheroids viability was examined in a whole genome siRNA screen to identify genes critical for TICs. We concurrently found UGDH as a target in RNAseq of TICs. Inducible shRNA against UGDH was used to validate screen findings. UGDH expression was examined by immunohistochemistry in histological and molecular subtypes of OC. Cell lines were characterised by clustering analysis to represent the OC molecular subtypes. Using OV90 as representative of the mesenchymal subtype, we examined the effect of UGDH knockdown on spheroids alone, and on stromal support cells in co-culture with spheroids.

Results and Discussions

Knockdown of UGDH in spheroids reduced viability, sphere-formation, hyaluronan production and molecular markers of TICs. High UGDH expression was observed in high-grade serous OC histotype and variable expression was observed in clear cell, mucinous and endometrioid histotypes. A distinctive survival prognostic was revealed specifically in the mesenchymal molecular subtype of OC, where high UGDH was associated with poor prognosis. Importantly, knocking down UGDH in the spheroids influenced the stromal cells' expression of extracellular matrix signaling and remodeling proteins.

Conclusion

These data show that UGDH expression in OC influences heterotypic cells in the TME and reveals a distinct role for OC-expressed UGDH in the mesenchymal molecular subtype of OC. UGDH is a strong prospective therapeutic target in TICs, for the prevention or treatment of recurrent OC.

EACR22-0361

Ultra-high plex immunofluorescence imaging for deep spatial analysis of human cancer tissues at single cell resolution

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Introduction

Spatial omics technologies like Co-Detection by indEXing (CODEX) have transformed cancer research from discovery to translation. CODEX enables the simultaneous detection of more than 100 protein biomarkers at single cell resolution within their native tissue environment and thus combines the advantages of multi-parameter analyte readout with high optical resolution. The Phenocycler Fusion, a novel imaging platform, seamlessly integrates with and accelerates the CODEX workflow to achieve ultra-high plex spatial mapping within a matter of hours. The data presented here demonstrate the utility of the Phenocycler Fusion platform for unbiased, whole tissue analysis of cellular metabolism, signaling, proliferation and stress responses at unprecedented speed, scale and resolution.

Material and Methods

The CODEX technology centers around a DNA-based barcoding strategy, in which oligonucleotide-labeled antibodies are imaged in iterative cycles of hybridization and removal of complementary oligonucleotide fluorescent reporters. The key advantages of this approach - (1) unbiased whole tissue imaging, (2) high single-cell and subcellular resolution, (3) compatibility with a wide range of FFPE tissues, (4) customized panel design and plexing capability and (5) sophisticated analysis of cell phenotypes and cellular neighborhoods – make this a comprehensive end-to-end solution for deep spatial phenotyping.

Results and Discussions

A novel 100+ antibody panel comprising markers for cell lineage, tissue structure, immune activation, apoptosis, DNA damage and cellular metabolism markers was developed and validated in human FFPE tissues. This comprehensive panel provides the means to study critical processes in healthy and diseased tissues. Single-cell spatial phenotyping of the tissue proteome via deep bioinformatics analyses provides quantitative and spatial information to further our understanding of the role of different cellular neighborhoods in regulating immune and metabolic functions.

Conclusion

Our data demonstrate the power of an integrated and unbiased single-cell based spatial profiling approach of cell lineage identification along with analyses of tissue metabolomics and immune signaling. These types of data provide critical information for both basic discovery research into tumor biology, as well as translational research aimed at the identification of therapeutic targets and drug mechanisms.

EACR22-0415

Deciphering tumour-stroma crosstalk signalling driven by Pten loss in high grade serous ovarian cancer

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Introduction

HGSOC, the most common histotype of ovarian cancer with 5 year survival rate of 30%, is a copy number-driven cancer characterized by ubiquitous Trp53 mutations. However, loss of

Pten occurs in 6% of cases, and alterations in genes downstream of Pten signalling have been found in 70% of HGSOC patients. Moreover, loss of function mutations of Pten accelerates tumour growth in murine and in vitro models of HGSOC, supporting the relevance of Pten loss in HGSOC.

Despite an initial response to standard treatments in most patients, relapse occurs frequently, highlighting the need of new therapeutic strategies. Notably, high proportion of cancer associated fibroblast (CAF) rich stroma correlates with chemoresistance and with poor overall survival in HGSOC, suggesting that CAFs may offer new therapeutic opportunities. There is some evidence that oncogenic signalling affects the tumour microenvironment, including CAFs, but not much is known in the context of HGSOC. A deeper understanding of the crosstalk signalling between cancer cells and fibroblasts could represent a unique opportunity to identify new therapeutic targets.

Material and Methods

We developed a murine cancer cell-fibroblast heterotypic co-culture system that models HGSOC, using ID8 cell lines carrying Trp53 alone or Trp53 and Pten deletion and fibroblasts. To decipher the Pten deletion-dependent crosstalk signalling established between fibroblasts and cancer cells, we have used the cell type-specific labelling using amino acid precursors (CTAP) methodology and MS-proteomics.

Results and Discussions

Proteomic characterization of ID8 cell lines in monoculture showed that both Trp53 or Trp53 and Pten deletion induced sizable changes of the ID8 proteome, including levels of matrix associated proteins. When in co-culture with fibroblasts, however, Pten loss in the cancer cells was a major driver of proteomic changes in the fibroblasts. Interestingly, these changes included the expression of markers typical of CAF subtypes that have been shown to have distinct functions in HGSOC.

Conclusion

So far, our work suggests a key role of Pten deletion in HGSOC cells in modulating CAF phenotype and potentially their pro-tumorigenic function. In the future computational modelling will be used to pinpoint relevant pathways/molecules through which Pten deletion can control CAF phenotype and function in HGSOC.

EACR22-0501

Targeting connexin43 in triple negative breast cancer progression, tumour microenvironment and antitumour immunity

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Introduction

Triple negative breast cancer (TNBC) is the most aggressive subtype of mammary carcinoma and also the most challenging, as it currently lacks any kind of targeted therapy. Subsequently, the study of novel and valuable therapeutic targets is of paramount importance. Connexins are transmembrane proteins involved in gap junction (GJ)-mediated intercellular communication (GJIC) as well as in GJ-independent functions.

Material and Methods

Samples comprise core needle biopsies of invasive lesions from patients of all BC subtypes, immortalized TNBC cell lines, patient-derived breast cancer-associated fibroblasts (CAFs) and patient-derived and immortalized natural killer (NK) cells. Methodology involves immuno-fluorescence/-histochemistry, flow cytometry, western blot, qPCR, parachute, proliferation and migration assays under 2D/3D conditions.

Results and Discussions

Immunohistochemical analysis in a 40-patient cohort found TNBC expressed the lowest Cx43 levels among all BC subtypes. Cx43 restitution in primary and metastatic TNBC cell lines partially restored GJIC and reverted EMT, enhanced SASP (senescence-associated secretory phenotype) *IL-1* and *IL-6* synthesis, reduced 2D and 3D-spheroid migration and colony formation, diminished 2D and 3D-spheroid proliferation and resensitized the cells to Anoikis. Physiologically appropriate 3D heterospheroid models, made up of TNBC cells and patient-derived *ACTA2*+*FAP*+*ITGB1*+ CAFs, further validated these results, as Cx43 restitution in TNBC cells led to a significant halt of heterospheroid proliferation and migration. These CAFs showed a steep reduction of Cx43 and GJIC when compared to their normal counterparts (NAFs). When CAFs/NAFs were cocultured with TNBC cells, heterocellular GJIC was only present if tumour cells were positive for Cx43, and happened unidirectionally from the fibroblasts towards Cx43-restored TNBC cells. Cocultures of patient-derived, immortalized and EGFR-chimeric antigen receptor (CAR)-modified NK cells with the primary TNBC cells showed that Cx43 restitution in these tumour cells led to a significantly higher antitumour cytotoxic effect of NKs.

Conclusion

This work validates Cx43 as a double therapeutic target in TNBC, by (i) showcasing a tumour-suppressive role in both primary and metastatic scenarios and (ii) resensitizing primary tumour cells to NKs cytotoxicity. Notably, the aforementioned results have been confirmed in physiologically relevant homo- and hetero-spheroid models comprising breast CAFs.

EACR22-0545

Examining the influence of adipose tissue metabolism and secretome in oesophageal adenocarcinoma

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Introduction

Oesophageal Adenocarcinoma (OAC) is the most strongly associated cancer with obesity. Approximately 75% of OAC patients are obese which results in chronic systemic low-grade inflammation, which is believed to drive carcinogenesis as well as influencing radiation treatment response. Adipose tissue is a regulatory organ with many downstream effects which are still not understood. This study aims to elucidate what influence adipose tissue metabolism and secretome has in treatment resistance

Material and Methods

Following patient consent, *ex-vivo* Visceral Adipose Tissue (VAT) explants were exposed to increasing doses of radiation with/without Palmitate and Oleate. Agilent Seahorse Xfe24 was used to measure oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in VAT explants. Dendritic cells (DCs) and Macrophage's (Mφs) were treated with adipose conditioned media (ACM) to assess mitochondrial metabolic parameters. Levels of DCs maturation and Mφ polarisation following ACM exposure and analyzed by flow cytometry. ACM was analyzed via MSD 54plex ELISA to assess secreted pro-inflammatory factors and whether these correlated with immune cell function.

Results and Discussions

VAT explant energy metabolism showed significant increase for OCR and ECAR with obesity and increasing radiation exposure however OCR was significantly decreased following Palmitate treatment and increased following Oleate treatment ($p<0.05$). Altered secretion of proinflammatory mediators were observed from the obese adipose secretome compared with non-obese patients, with increasing radiation doses and with fatty acid treatment ($p<0.05$). Key inflammatory mediators also showed significantly increased secretion correlating with increasing OCR rather than with ECAR in VAT explants as well as correlating with a series of clinical factors ($p<0.05$). DCs showed altered Proton Leak, Basal and ATP linked respiration following exposure to ACM ($p<0.05$). M1 polarised Mφ showed decreased basal, and ATP-linked respiration following exposure to ACM ($p<0.05$).

Conclusion

We have demonstrated that obesity and increasing radiation doses can significantly alter the metabolic influences of adipose tissue with fatty acid treatment further influencing these changes. Alterations in the secretome of adipose tissue could potentiate the tumour microenvironment and deleteriously affect immune cell function therefore further interrogation is required to fully elucidate the influence adipose tissue may have in treatment response.

EACR22-0591

The role of tumor microenvironment in regulation of Serpine1

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Introduction

Serpine1/PAI-1 (Plasminogen activator inhibitor) is a serine protease inhibitor of plasminogen degradation and hence fibrinolysis, but also plays a role in cancer progression. Clinically, high tumoral PAI-1 expression correlates with poor survival and fast progression in RCC (Renal Cell Carcinoma) patients. Regulation of Serpine1 expression may be mediated by chemokines, cytokines, growth factors and miRNAs. Hypoxia (low oxygen tension) is characteristic feature of tumor microenvironment (TME), but in RCC often occurs dysregulation of HIF-1 α signaling, due to frequent VHL mutation, causing a pseudo hypoxic state. Since the regulation of PAI-1 expression is insufficiently understood, we aimed to elucidate how the TME regulates PAI-1 level in kidney cancer.

Material and Methods

Murine kidney cancer Renca cells were exposed *in vitro* to several stress factors related to the TME: hypoxia (culture in 1% pO₂), pseudohypoxia (VHL inactivation by siRNA), chemotherapy (Gemcitabine treatment), inflammation (LPS addition). The expression of PAI-

1 was examined at the level of transcript (qRT-PCR), protein (Western Blot) and secreted protein (ELISA). Global miRNA analysis in cells cultured in normoxia and hypoxia was performed by NGS and verified by qRT-PCR. Selected results were confirmed in human RCC cell lines: Caki-1, RCC4/vect, RCC4/vhl.

Results and Discussions

In response to hypoxia strong upregulation of Serpine1 gene expression, protein level and secretion were observed. LPS stimulation also increased PAI-1 secretion. However VHL silencing, causing deregulation of the HIF-1 α signaling, did not induce PAI-1, which was also confirmed in the human VHL mutant model (RCC4). No changes in PAI-1 expression was observed after stress related with Gemcitabine treatment. In Renca cells, hypoxia caused changes in the expression of several miRNAs, including the hypoxic miR-210-3p, but also the miR-224-5p, targeting PAI-1 as shown by bioinformatics analysis. The link between miR-224-5p expression and PAI-1 secretion was also indicated in human renal cancer model Caki-1 (decrease in miR224-5p with a simultaneous decrease in PAI-1 secretion).

Conclusion

Tumor microenvironment influences interaction between PAI-1 and miR-224-5p, suggesting a significant role of such cross talk in RCC progression.

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EACR22-0593

Laying the groundwork for the use of MALDI-IMS for an in-depth characterisation of the TME: lipids as spearhead.

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Introduction

Overall survival of patients with advanced stages of colon cancer (CC) has not increased despite all the progress made in the field. The tumour microenvironment (TME) plays a crucial role in tumourigenesis, tumour expansion, and metastasis. Thus, a better understanding of the intricate network orchestrating TME heterogeneity may hold the key for identifying immunotherapy biomarkers, and the development of new tools for patient stratification. To address this complexity, we aim to characterise the membrane lipid fingerprint of tumour-infiltrating immune cells, which intimately influence tumour fate and its response to therapy.

Material and Methods

First, 12 CC patients and 5 healthy controls were enrolled at the *Gastroenterology Services*. Cells were isolated from peripheral blood by *FACS*. Sorted cells ($\sim 10^5$ cells) were applied on poly-L-lysine-coated glass slides and analysed by *Matrix-Assisted Laser Desorption Ionization mass spectrometry* (MALDI-MS). In parallel, 5 CC patients who underwent colon surgical resection were enrolled at the *General Surgery Unit*. Tumour-infiltrating Lymphocytes (CD4⁺, CD8⁺ T Cells, and B Cells) and tumour-associated macrophages (M1-

M2-like) were isolated using *FACS* after tumour enzymatic disaggregation and plated following the same method.

Results and Discussions

The setting-up of this methodology allowed us to establish the lipidome using at least 1 to 2 orders of magnitude fewer cells than usual. Main membrane lipid species were characterised in circulating monocytes, NK cells, B and T lymphocytes, neutrophils, and NKT-like cells, besides the above-mentioned infiltrating immune cell subsets. Such analysis revealed a distinctive pattern of lipid species distribution for every cell type, confirming the lipid fingerprint's specificity and accuracy for our proposal to describe the immune compartment shaping the TME. Furthermore, the comparison between healthy controls and CC patients revealed a significant impact of the clinical condition on arachidonic acid-containing phosphatidylethanolamine (PE) and PE-plasmalogen species.

Conclusion

Each immune cell type shows a unique lipid fingerprint that is altered in CC patients. The sensitivity of the methodology developed provides a unique chance to analyze the lipidome of immune cell minor subsets. Once characterised, these profiles can be recognised and localised within the tumour tissue slide using imaging techniques (MALDI-IMS), enabling an in-depth approach for the TME characterisation.

EACR22-0663

Long-term culture of tissue slices as a platform to identify tumor-initiating cells: Human uterine leiomyoma as a model

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Introduction

Uterine leiomyomas or fibroids are monoclonal tumors that originate from a single myometrial smooth muscle stem cell (SC) that undergoes a mutational event, becoming a fibroid progenitor cell. Previous studies suggested that fibroid CD34+/CD49b+ stem cells differentiate to CD34+/CD49b- intermediary cells, which terminally differentiate to CD34-/CD49b- cells. In addition, the CD24 and CD73 surface markers are highly expressed in fibroid smooth muscle cells.

Albeit fibroids are ovarian steroids dependent tumors, SCs lack estrogen and progesterone receptors. However, adjacent differentiated cells respond to sex hormones and send paracrine signals to stem cells residing in particular niches. Organotypic culture allows the cells to communicate while maintaining the original niches. We have set up an organotypic culture of fibroid slices explant for 7 days. After that, apoptosis increase led to a slice devoid of cells. Unexpectedly, after 20 days of culture, new cells repopulated both leiomyoma and myometrium tissues. This finding prompted us to determine the mechanism behind myometrial and leiomyoma cells regeneration.

Material and Methods

Fresh tissue sections were cultured for a month. We collected slices before culture (T0) and after 20, 23, 26, and 29 days of culture. We carried out hematoxylin-eosin staining and immunofluorescence analysis of desmin, vimentin, alpha-smooth muscle actin, the stemcell and undifferentiated cell markers CD49b, CD34, CD24, CD73, KIT, HMGA, sex ovarian receptors, and Ki67 proliferation marker. Genes from the above markers were analyzed by qPCR.

Results and Discussions

Repopulated fibroid and myometrium slices showed smooth muscle cells and fibroblasts. Significant upregulation of CD49b, CD24, CD73, and HMGA2, at the mRNA and cellular level were detected in fibroids. Increased KIT mRNA and cells were detected in the myometrium. CD49b SCs were found in clusters or dispersed in tumor and normal slices, respectively. In both tissues, ovarian receptors expression gradually increased over time as cells differentiated. Few proliferating cells were seen in the myometrium, whereas most cells were dividing in fibroids.

Conclusion

Stem cells are able to proliferate and fully differentiate after long-term culture. The presence of the SC niches allows exploring the nature of paracrine interactions and the mechanisms controlling temporal regulation during normal homeostasis and tumorigenesis. This platform may constitute a preclinical model to investigate drug resistance mechanisms in human cancer.

EACR22-0677

Differentiation therapy for leukemia affects stromal cells

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Introduction

Interaction between acute myeloid leukemia (AML) cells and bone marrow stromal (BMS) cells is known to contribute to development of resistant AML clones. Our recent study demonstrated that two drugs that inhibit pyrimidine synthesis, 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) and brequinar, induced differentiation of AML cells by activating checkpoint kinase-1 (Chk1). In addition, Chk1 was necessary for AML differentiation in response to low doses of cytarabine. The murine BMS cell line MS-5 is known to attenuate cytarabine-mediated cytotoxicity, but the role of stromal cells on AML differentiation is unknown. The aim of this study was to investigate the effects of drugs that activate Chk1 in a co-culture system.

Material and Methods

MS-5 cells (ACC 441, DSMZ) were seeded 24 hours prior to human AML cell lines U937 (85011440, ECACC) and THP-1 (a kind gift from Paolo Gallipoli). The cells were incubated with AICAR (0.2 mM), brequinar (0.5 µM) or cytarabine (10, 100, 1000 nM) for 72 hours. In some experiments, nucleosides were added 15 minutes after the addition of agents. Viable cells were counted using hemocytometer and trypan blue exclusion. Cells were stained with May-Grünwald-Giemsa and morphology was analyzed by AxioVert 200 microscope and AxioCam MRc 5 camera. Cell cycle analysis of propidium iodide-labeled cells and the expression of differentiation markers were analyzed by flow cytometry.

Results and Discussions

In AML cell lines, the presence of MS-5 reduced cytarabine-mediated toxicity and S-phase arrest caused by pyrimidine synthesis inhibitors, but inhibited the expression of

differentiation markers only in U937 cells treated with low-dose cytarabine. However, AICAr and high-dose cytarabine induced significant changes in morphology of stromal cells. AICAr inhibited the growth and induced fibrocyte-like appearance in MS-5 cells. The addition of nucleosides completely prevented cell cycle arrest and differentiation of AML cells induced by AICAr and brequinar, but had no effects on AICAr-induced changes in stroma. Preliminary data suggest that AICAr activates AMPK and inhibits mTOR in MS-5 cells.

Conclusion

The presence of stromal cells decreases differentiation of leukemia cells only in response to low-dose cytarabine. AICAr inhibits proliferation and induces fibrocyte-like changes in stromal cells that are independent of pyrimidine synthesis inhibition. These results suggest that mechanisms responsible for phenotypic changes in response to AICAr are different in AML and stromal cells.

EACR22-0685

ROLE FOR THE LYSYL OXIDASE LIKE 2 (LOXL2) ENZYME IN STROMAL MATRIX REMODELING AND INVASIVE PROPERTIES OF DEDIFFERENTIATED MELANOMA CELLS

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Introduction

Cutaneous melanoma is a highly malignant and invasive skin cancer. Despite therapies targeting the BRAFV600E oncogenic pathway, resistances occur. Upon microenvironment and therapeutic pressures, melanoma cells can switch from a melanocytic differentiated state to dedifferentiated states associated with increased expression of tyrosine kinase receptors (RTKs) and mesenchymal markers. Such adaptive plasticity was described as a driver of resistance to targeted therapies (TT). This study relies on original observations that dedifferentiated cells acquire extracellular matrix (ECM) remodeling activities and that BRAF inhibitors (BRAFi) induce tumor matrix stiffening. Analysis of the matrisome of BRAFi-resistant cells revealed an accumulation of the collagen-crosslinking enzyme LOXL2. LOXL2 is a member of the Lysyl-Oxidase family that drives tumor stiffening and the epithelial-to-mesenchymal (EMT) process in breast cancer, but its role in melanoma is still unknown. We hypothesized that LOXL2 production by melanoma cells and its presence within the stroma could influence phenotypic plasticity towards a drug-tolerant dedifferentiated state.

Material and Methods

The experiments are carried out on a panel of melanoma cells chosen according to their melanocytic MITF^{high}/AXL^{low} or dedifferentiated MITF^{low}/AXL^{high} phenotype. The impact of LOXL2 is evaluated by loss of function approaches.

Results and Discussions

LOXL2 is preferentially expressed by dedifferentiated MITF^{low}/AXL^{high} cells and induced by BRAFi/MEKi combination and hypoxia, cues known to remodel and aggravate the tumor niche. On the contrary, LOXL2 induction by TT is reversed by PDGFR and AKT inhibitors and by silencing the plasticity transcription factor ZEB1. Using si/shRNA approaches, we revealed that LOXL2 plays a role in focal adhesion formation and cell morphology and promotes melanoma cell migration. LOXL2-dependent migration required the expression of

the EMT factor SLUG. Interestingly, we also show that targeting LOXL2 in melanoma-associated-fibroblasts impaired their ability to contract a collagen matrix and to assemble an organized ECM, suggesting the implication of LOXL2 in the dialogue between melanoma cells and the stromal matrix.

Conclusion

Together, these findings provide an original link between LOXL2, ECM remodeling and melanoma cell biology. This study improves our understanding of the biochemical and biomechanical cues from the tumor microenvironment that affect melanoma cell plasticity and adaptation to anti-melanoma therapies.

EACR22-0733

Conditioned media from renal cancer cells reprogram transcriptome of MSC cells: possible role of microRNAs.

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Introduction

Renal cell carcinoma (RCC) is the most common subtype of kidney cancer and it is highly aggressive and chemoresistant. Mesenchymal stromal cells (MSC) are multipotent cells with the ability to differentiate into different cell lineages. It was shown that MSC are able to promote the progression of RCC. We have recently found that conditioned media (CM) from RCC cells contain multiple microRNAs and induce migration of MSC. Here, we analyzed transcriptome of MSC treated with RCC CM in a search for mechanisms by which RCC cells attract MSC.

Material and Methods

MSC cells were treated with CM collected from the control cell line (RPTEC) and two RCC cell lines (Caki-1 and KIJ265T) for 48h, then RNA was isolated using Universal RNA/miRNA Purification Kit (Eurx). Analysis of MSC transcriptome was performed using microarrays with Human Gene 2.1 ST (Thermo Fisher) and validated using qPCR and specific primers. Bioinformatic analysis was performed using miRNET and pantherdb.

Results and Discussions

Treatment of MSC cells with CM from both RCC cell lines led to upregulation of 16 while downregulation of 36 genes expression in MSC cells. Gene ontology analysis revealed that these genes are involved among others in the regulation of angiogenesis, CCKR signaling, plasminogen activating cascade, and VEGF signaling pathways. Previously, we have shown suppressed expression of miR-10b-3p and miR-125a-5p while increased expression of miR-17-5p, miR-21-5p, miR-193a-5p, miR-365a-3p, miR-365b-3p and miR-455 in exosomes isolated from RCC CM compared with CM from control cells. To find the potential genes whose expression is regulated in MSC by microRNAs secreted in exosomes by RCC cells, we searched for targets in the MSC microarray results. Bioinformatic analysis predicted ATP1B1 and SLIT2 as miR-125a-3p targets, KCNMB1, KLF10, LMCD1, TGFB1I1, ATP1B1, SLIT2, and SLC22A3 as miR-17-5p targets as well as KLF9, PTX3, CD44, RAB27B, MCAM,

TSPAN2, ATP1B1, and SLIT2 as miR-21-5p targets. Further validation studies are needed to confirm the interactions between these miRNAs and genes.

Conclusion

These results suggest that RCC cells could modulate MSC cells transcriptome possibly through the release of microRNAs.

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EACR22-0783

Heterogeneous tumour-stroma interactions in breast cancer patient-derived tumour xenografts

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Introduction

Patient-derived tumour xenografts (PDXs) retain the molecular properties and clonal complexity of the original tumour but invariably lose its interacting human tumour microenvironment (TME). We and others reported that the human TME is replaced in PDXs by cells of mouse origin and observed different TME composition in models established from different patients. This suggests a tumour-driven education of the mouse TME, but the determinants of this process are not well understood. In this work, we expand previous observations and use in-depth phenotyping to better understand tumour-stroma interactions in breast cancer PDXs.

Material and Methods

We extensively characterize the TME of selected xenografts using single cell RNA-sequencing (scRNA-seq), three-dimensional imaging with serial two-photon tomography (STPT) and imaging mass-cytometry (IMC). We then widen our observations using transcriptome and histological analysis on more than 60 independent models from the large PDX biobank we have established.

Results and Discussions

High-resolution scRNA-seq reveals unexpected complexity in the PDX TME: we describe 23 distinct mouse cell phenotypes, including multiple innate immune populations, that are present at varying frequencies in different models. Using STPT and IMC, we demonstrate that the structural distribution of these cells is largely model-specific, indicating a concerted and reproducible organisation of the murine TME by the human tumour compartment. In agreement with this, we predict multiple exclusive interactions between cancer and stroma and show that regional variations in tumour phenotypes result in heterogeneous TME features within each sample. We confirm these observations using bulk RNA sequencing and histological analysis on a larger sample set and further show that PDXs share key stromal features with the original patient tumours. This suggests that the processes governing TME organisation are conserved during xenotransplantation.

Conclusion

Our work unveils the complexity of the PDX TME, which represents an additional layer of heterogeneity between models and is largely controlled by the tumour cell-autonomous compartment. These findings reveal that the mouse TME is a key element of PDX biology

and have strong implications for the interpretation of xenograft studies and for the characterisation of tumour-host interactions *in vivo*.

EACR22-0837

How carboplatin alters the tumour microenvironment in breast cancer.

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Introduction

Platinum chemotherapy drugs, such as carboplatin and cisplatin, are used to treat various cancers. While many studies have investigated their anticancer properties, the impact of them on fibroblasts in the tumour microenvironment is not yet fully understood. There is growing evidence that chemotherapeutics can modulate fibroblasts to become more pro-tumourigenic. Fibroblasts are known to deposit and remodel the extracellular matrix (ECM) and can in turn influence tumour cell behaviour. Here, we examine the impact of carboplatin on normal (NF) and cancer-associated fibroblasts (CAF), their ability to deposit ECM after treatment, and how this influences tumour cell proliferation and migration.

Material and Methods

In this study we combined *in silico* analysis of existing gene expression datasets of untreated and platinum drug-treated fibroblasts with *in vitro* ECM deposition assays. The ECM structure was analysed by quantitative image analysis. Tumour cell behaviours such as proliferation, colony formation and migration were assessed by seeding breast tumour cells as 2D and 3D cultures on top of fibroblast-derived ECM.

Results and Discussions

Gene expression analysis after platinum treatment revealed distinct changes in matrisome-related gene expression. Within the significantly up- and downregulated genes the top three are CXCL5, COL9A3, COL10A1 and PARM1, NELL2, SPARCL1 respectively. To further investigate the impact of platinum drugs on structural components of the ECM, we conducted ECM deposition assays and assessed matrix orientation. Carboplatin treatment of NFs and CAFs stimulates the deposition of a more anisotropic ECM with larger pores. Tumour cells seeded on top of the carboplatin-treated fibroblast-derived ECM are viable and proliferate. Interestingly, tumour cell colonies are larger on the treatment-derived ECM compared to ECM from untreated fibroblasts. While proliferation is stimulated, migration of tumour cells on top of ECM from carboplatin-treated fibroblasts is reduced.

Conclusion

Carboplatin treatment of fibroblasts results in greater matrix anisotropy. This ECM re-organisation influences tumour cell behaviour, such as promoting proliferation while impairing migration, which can impact metastasis formation post treatment. Future work will investigate *in vitro* and *in vivo*, how matrisome genes and pathways aberrated upon platinum treatment impact tumour progression and metastasis, providing us with better strategies to target stromal directed cancer progression and therapy resistance.

EACR22-0841

Exposure of malignant pleural mesothelioma cells to malignant pleural fluid in 3D, induces differential expression of the primary cilium acetylated tubulin

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Introduction

Malignant Pleural Mesothelioma (MPM) is an aggressive, chemo-resistant malignancy of the pleural cavity. In MPM, Epithelial-to-mesenchymal transition (EMT) is an important mechanism involved in the invasive behavior of the tumor. The aim of this study was to assess whether the exposure of mesothelial and MPM cells to malignant pleural effusion fluid has a biological effect on their contractile EMT behavior.

Material and Methods

MeT-5A benign mesothelial cells and MSTO, biphasic MPM cells were used in a gel contraction assay with 10% FBS or 10% FBS with mesothelioma diagnosed pleural fluid (PF). Gels were cast in 24 well plates and imaged on a flatbed scanner on day 1, 2 or 3, and contraction was calculated from area of the gels. Gels were subject to western blot procedure. The expression of acetylated tubulin (ac-tuba1a) a component of the Primary Cilium (PC), a cell sensory organelle, was assessed.

Results and Discussions

In MeT-5A cells, contraction until day 2 was significantly increased in PF condition (0.63 ± 0.01 , $p < 0.001$) compared to control (0.57 ± 0.009), $n=13$. In MSTO cell, contraction, in PF media (0.65 ± 0.007 , $p < 0.001$) was significantly greater till day 3 compared to control (0.43 ± 0.02), $n=14$. Under PF condition ac-tuba1a expression was significantly lower in both cell types. Gel contraction assay is indicative of EMT phenomenon of cells, on a 3D setting. Our findings suggest an effect of malignant pleural fluid on inducing a mesenchymal behavior of the cells.

Conclusion

Our results demonstrate that exposure to malignant PF causes greater contraction. Primary cilium protein ac-tuba1a expression is reduced in cells that undergo EMT.

EACR22-0843

Preclinical study of tumor hypoxia by multimodal MRI and PET imaging in brain metastases: interest of ⁶⁴Cu-ATSM?

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Introduction

Lung cancer patients frequently (40%) develop brain metastases (BM). Despite aggressive treatment including neurosurgery and external radiotherapy (RT), overall survival remains poor. The hypoxic intratumoral microenvironment generates expression of many factors, in particular through Hypoxia-inducible Factors (HIFs), known to participate in tumor growth and resistance to RT making hypoxia a factor of poor prognosis. Clinical studies on biopsies and preclinical studies using multimodal MRI and PET ¹⁸F-FMISO imaging both proved the existence of a hypoxic microenvironment in lung cancer-derived BM. Recently, ⁶⁴Cu-ATSM has been suggested as a promising radiotracer for moderate hypoxia imaging and potentially to follow cell redox state. The aim of this study is to assess the interest of ⁶⁴Cu-ATSM in the

characterization of tumoral microenvironment in preclinical models of lung cancer-derived BM using PET and MRI imaging, completed by *ex vivo* and *in vitro* approaches characterizing HIFs expression.

Material and Methods

PET ^{64}Cu -ATSM, ^{18}F -FDG and multiparametric MRI were carried out in intracerebral injection model in nude rats of H2030-BrM3 cells (MSKCC, Dr Massagué, USA), on a hybrid MRI/PET machine between 21 and 24 days post-implantation. After imaging exams, brains were collected and immunostaining of pimonidazole, HIF-1 α , HIF-2 α , and copper transporter were performed. HIF-1 α and HIF-2 α expressions and that of their target genes were studied by RT-qPCR and immunocytochemistry on H2030-BrM3 cells under normoxia and hypoxia conditions (1% O₂).

Results and Discussions

The results showed ^{64}Cu -ATSM uptake in BM with more pronounced uptake for cortical BM than striatal BM at 4h and 24h post-injection. Interestingly, the ^{18}F -FDG images did not reveal any major change in glucose consumption at these analysis times. Immunohistology analyses revealed a staining for pimonidazole, HIF-1 α , HIF-2 α , and copper transporter in BM. *In vitro* studies confirmed an increase of expression of HIFs and their target genes (TUBB3, VEGF, GLUT-1, CCDN1, CA-IX) by hypoxia in H2030-BrM3 cells

Conclusion

These results confirm our previous studies showing the existence of a hypoxic microenvironment of BM from lung cancer with an inter-metastasis heterogeneity and add a new angle on redox metabolism with ^{64}Cu -ATSM uptake. This study underlines that ^{64}Cu -ATSM imaging could be complementary to other hypoxia imaging modalities (^{18}F -FMISO, MRI oxygen saturation) to target and guide RT depending on the presence or not of tumoral hypoxia

EACR22-0919

Studies on fibroblastic contribution to tumor growth

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Introduction

The characteristics of cancer-associated fibroblasts remain elusive. We had shown that stromal bone marrow cells possess the ability to inhibit tumor growth. In addition, differentiating osteoblasts modulate the immune response against cancer by producing fibronectin. We therefore aimed to determine how fibroblasts might inhibit tumor growth.

Material and Methods

Mice received subcutaneous injections of 10⁵ stromal cells isolated from murine bone marrow mixed with 10⁶ B16 melanoma cells. 14 days later, lesions containing stromal/tumor cells were 67% smaller compared to lesions containing only B16 cells (p<0.001). We then deleted fibronectin (FN), an extracellular matrix protein, or β 1 integrin, a major matrix receptor using one of the following promoters attached to cre: collagen α 1(I) (col, expressed in differentiating osteoblasts), osterix (osx, expressed in preosteoblasts), vav (expressed in vascular progenitors), Mx (active in various stromal cells).

Results and Discussions

Injection of stromal cells, in which FN was conditionally deleted using col-cre or vav-cre, led to suppressed growth similar to wildtype stromal cells. In contrast, deletion of FN in stromal cells using Mx or osx failed to reduce tumor growth (p<0.001). Since Mx deletes in most

stromal cells and osx in a subpopulation, we concluded that FN originating from osx-expressing cells is required for suppression of tumor growth. Using the same promoters to delete $\beta 1$ integrin showed that the Mx, but not osx, population increased growth back to that in the absence of stromal cells ($p < 0.05$). Thus, FN originating from osx cells acts on other cells that neither express osx, col, or vav but are included in the Mx-expressing population leading to suppressed tumor growth.

T-cells were suggested to be involved in stromal-mediated growth modulation. We therefore repeated all experiments in mice that lack mature T-cells (*foxn1nu*). The same findings were detected suggesting that mature T-cells are not required for growth suppression. Instead an increase in a subpopulation of Ly6G-expressing myeloid cells was seen when growth was suppressed ($p < 0.05$), but not when growth was no longer affected as is the case with osx-FN deleted cells or Mx- $\beta 1$ -deleted cells.

Conclusion

In summary, FN produced by preosteoblasts suppresses tumor growth, suggesting that FN production by cancer-associated fibroblasts is relevant in cancer development. This is not mediated by T-cells but might involve a myeloid subpopulation that infiltrates the tumor in the presence of FN.

EACR22-1009

PGC-1 α and PGC-1 β as players in ovarian cancer: tumor-host crosstalk

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Introduction

Energetic metabolism has emerged as key factor on cancer cell behavior and could play a role in cancer-immune cell interaction in tumor microenvironment, promoting tumor progression.

Recently, we found that PGC-1 α and PGC-1 β , two master regulators of mitochondrial activity, promote oxidative metabolism (OXPHOS) in ovarian cancer and confer susceptibility to OXPHOS inhibition.

Here, we aimed to explore how PGC-1 α and PGC-1 β regulate ovarian cancer cell features and their crosstalk with macrophages, the most abundant immune cells in ovarian tumor microenvironment.

Material and Methods

High (OVCAR-3) and low (IGROV-1) PGC-1 α and PGC-1 β expressing cell lines were used. PGC-1 α or PGC-1 β expression was silenced in OVCAR-3 cells using CRISPR-Cas9 technology, and direct co-culture with human monocytes were performed. The cancer cell

immunogenicity (CD47 and HLA-ABC) and the inflammatory profile of differentiated macrophages (CD86, CD163) were assessed by flow cytometry. Cancer cell migration was evaluated by wound healing assay.

Results and Discussions

Highly expressing PGC-1 α and PGC-1 β OVCAR-3 cells showed higher expression of CD47, “don’t eat me” signal molecule, and lower expression of HLA-ABC, involved in antigen presentation, compared to low expressing PGC-1 α and PGC-1 β IGROV-1 cells.

PGC-1 α and PGC-1 β silencing increased the HLA-ABC expression, suggesting that silenced cells are more immunogenic compared to parental cells. This finding suggests that PGC-1 α and PGC-1 β modulate cancer cell immunogenicity and might influence cancer cell-macrophage crosstalk.

In co-culture with parental and PGC-1 α silenced cells, macrophages shifted to a more anti-inflammatory profile, reducing the expression of CD86 and increasing the expression of CD163. On the other hand, in presence of PGC-1 β silenced cells, the modulation of CD86 and CD163 by macrophages was prevented, suggesting that PGC-1 β silencing affects the cancer cell ability to modulate macrophages towards an anti-inflammatory phenotype. Upon the stimulation with naïve macrophages, PGC-1 β silencing reduced the ability of cancer cells to migrate compared to parental cells, while PGC-1 α silencing did not affect migration.

Conclusion

Our results suggest that albeit PGC-1 α and PGC-1 β affect cancer immunogenicity, only PGC-1 β plays a role in the modulation of cancer cell-macrophage interaction.

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EACR22-1031

Factor inhibiting HIF (FIH) promotes lung cancer progression and limits anti-tumor T cell responses

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Introduction

Factor inhibiting HIF (FIH) is an asparagine hydroxylase that acts on hypoxia inducible factor (HIF) to modulate the cellular adaptation to hypoxia. Hydroxylation of HIF by FIH blocks the association of HIF with the transcriptional co-activators CBP/p300, inhibiting HIF activity. FIH is constitutively expressed in a wide range of tissues, including lung cancer. The role of FIH in tumour progression and immunity remains unexplored.

Material and Methods

CRISPR/Cas9 editing technology and short hairpin RNA (shRNAs) were used for FIH deletion in mouse and human lung cancer cell lines (LLC1, NCIH1581, A549 and NCIH460). RNA-seq analysis of FIH-KO Lewis Lung cancer cell line (LLC1) was performed to elucidate molecular pathways dysregulated in the absence of FIH. C57BL/6 mice were injected subcutaneously with LLC WT or FIH-KO cells. Tumor volume was monitored, and immune infiltrates were characterised by multi-colour flow cytometry.

Results and Discussions

Deletion of FIH in mouse and human lung cancer cell lines results in a cellular phenotype characterised by an increased glycolytic metabolism and upregulation of HIF target genes (Vegfa, Bnip3). FIH-deficient lung cancer cells showed a reduced in vitro cell proliferation and increased apoptosis under hypoxia. RNA-seq analysis revealed altered molecular pathways, including the Hippo tumour suppressor pathway. FIH-deficient LLC1 tumors show a reduced in vivo growth rate. Characterisation of the tumor infiltrate in FIH-KO tumors revealed changes in immune infiltrates characterised by an increase in T and NK cells. Moreover, CD4+ and CD8+ T cell infiltrating FIH-deficient tumours express higher levels of CD137, OX40, PD-1 and LAG-3, suggesting an enhanced effector phenotype.

Conclusion

Our findings demonstrate that FIH controls lung cancer cell proliferation and apoptosis under hypoxia. This is particularly relevant in the tumor microenvironment where FIH influences immune responses. These data unravel FIH as a new therapeutic target for treating lung cancer.

EACR22-1045

Galectin-9 regulates PD-L1 expression on human macrophages: implications for cancer immunotherapy

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Introduction

Solid tumors co-opt surrounding immune and stromal components to establish an immunosuppressive tumor microenvironment (TME) that limits effective immunotherapeutic responses. Cancer cells drive tumor immune evasion by promoting suppressive features of key TME components, including tumor-associated macrophages (TAMs). Galectin 9 (Gal-9) is a tandem-repeat C-type lectin overexpressed in multiple human cancers, which is associated with poor patient outcomes. The best-characterized role of Gal-9 in cancer is the suppression of adaptive immune responses through interaction with the co-inhibitory receptor TIM-3, which leads to T cell inhibition and apoptotic death. Recently, it has been shown that Gal-9 enhances the protumor properties of TAMs, but the underlying molecular mechanisms and implications for immunotherapy remain unexplored.

Material and Methods

We analyzed Gal-9 expression data across multiple cancer types from the TCGA and GTEx databases. To study the functional consequences of Gal-9 overexpression in the TME, we produced recombinant full-length human Gal-9 (Gal-9 FL) and truncated versions of the Gal-9 C-terminal (Gal-9 C-ter) or N-terminal (Gal-9 N-ter) domains. Human primary macrophages were derived from peripheral blood CD14+ monocytes and treated with different Gal-9 domains or classic macrophage polarizing conditions (IFN γ or IL-4). Macrophage phenotype—including PD-L1 and TIM-3 expression—was characterized by flow cytometry.

Results and Discussions

Analysis of cancer datasets confirmed Gal-9 overexpression across multiple cancer types and association with reduced patient overall survival. Recombinant Gal-9 FL or its truncated domains are sufficient to reshape macrophage polarization and induce a distinct phenotype,

characterized by reduced surface expression of TIM-3. Importantly, Gal-9 FL induced robust surface expression of PD-L1 on macrophages, a phenotype that depends on Gal-9 C-ter domain. Moreover, expression data analyses showed a positive correlation between the levels of Gal-9 and PD-L1 in cancer patients.

Conclusion

Our data revealed an unexpected link between the Gal-9/TIM-3 and PD-L1/PD-1 axes in the TME, suggesting Gal-9 as a potential predictive biomarker and therapeutic target in cancer immunotherapy.

EACR22-1061

Single-cell Glycomics - parallel assessment of cell transcriptome and surface epitopes using single-cell multimodal expression profiling by sequencing in ovarian cancer

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Introduction

Each eukaryotic cell is equipped with a defined glycocalyx on its cell surface. Cancer-associated changes in the glycocalyx composition are reported and even clinically exploited as tumor markers (e.g. CA125 or CA19-9). However, the non-templated assembly and vast structural diversity make the glycome a difficult molecular framework to interrogate and current technologies do not allow an integrative analysis in complex samples on the single-cell level. To overcome this bottleneck, we developed a powerful single-cell glycomics platform by targeting cell surface glycosylation in combination with transcriptomics.

Material and Methods

Our technology builds upon the Cellular Indexing of Transcriptomes and Epitopes by Sequencing technology that relies on DNA-barcoded antibodies and lectins for simultaneous profiling of transcriptomes and surface glycan epitopes in single cells. Immortalized and patient-derived cells are labeled before droplet encapsulation. Raw reads are assigned to their respective cells, mapped to the human reference genome using Cell Ranger in parallel with antibody-derived tag data analysis, and observed gene expression and antibody abundance counts are further normalized and undergo downstream DE analysis with Seurat. Flow cytometry and immunofluorescence are performed as technology-independent quality controls.

Results and Discussions

We successfully conjugated unique DNA-oligonucleotide barcodes to glycan-binding proteins (anti-glycan antibodies and lectins) that can be read out by state-of-the-art droplet-based single-cell platforms in combination with high-throughput next-generation sequencing. First experiments in gene-edited ovarian cancer cell lines and patient-derived cells determined optimized lectin and antibody concentrations. These experiments revealed cell type-specific lectin binding (e.g. AAL, SNA, or Jacalin).

Moreover, differential expression analysis on individual genes and pathways revealed a correlation of cell surface markers and underlying transcriptional changes.

Conclusion

We are currently exploring different computational pipelines and performing scGlycomics on cancer and immune cells in an extended patient cohort. The designed experimental setup offers not just a unique opportunity to close the current gap of knowledge investigating the transcriptome in parallel with the surfaceome but also to open a novel chapter for future personalized medicine applications.

EACR22-1073

Cancer-associated Fibroblast Heterogeneity is Investigated in a Spatially Defined Co-culture Model and Sustains Drug Resistance in Non-Small Cell Lung Cancer

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Introduction

Lung cancer is the most lethal cancer worldwide with a 5-year survival rate of merely 17%. This is due to the development of therapy resistance, which leads to failure of treatment in virtually all lung cancer patients. Cancer-associated fibroblasts (CAFs) are cells found in the tumor stroma that are emerging as mediators of therapy resistance. However, CAFs are a heterogeneous cell population and while subtypes of CAFs with distinct transcriptomic profiles and spatial distributions across tumor tissue areas have been identified, the impact of drug treatment on CAF biology in lung cancer has yet to be explored. Additionally, mechanistic studies of CAF subtype contribution to drug resistance are hindered by a lack of models that can sustain CAF subpopulation diversity *in vitro* in a reproducible manner.

Material and Methods

We present a novel protocol for establishing a 3D *in vitro* model of Non-Small Cell Lung Cancer (NSCLC) tissues, wherein tumor cells are surrounded by a stromal zone populated with CAFs. This spatially defined setup is inspired by NSCLC tissue histology and allows for the establishment of CAF subtype-specifying signalling axes between CAFs and tumor cells. In this model, patient-derived CAFs NSCLC organoids, which retain a tight genotype-phenotype similarity to their parent tumors, are utilized as building blocks.

Results and Discussions

We have identified suitable culture parameters (media and ECM) for generating NSCLC organoid-CAF co-cultures, thereby unlocking the possibility of assessing the interactions of these cells at baseline and after drug treatment *in vitro*. Preliminary data shows that in these conditions CAFs maintain plasticity and that key markers of CAF heterogeneity are differentially expressed depending on spatial proximity to tumor cells, indicating that instructing CAF phenotype spatially is attainable in this model. ScRNA-Seq of baseline co-cultures compared to *in vivo* tissues further confirms the presence of relevant CAF and tumor subpopulations. Further, the cytotoxic effects of chemotherapeutic drugs are significantly altered in co-cultures.

Conclusion

We propose that drug treatment alters CAF heterogeneity to sustain drug-resistance in NSCLC tumor cells. To investigate this, we have established a novel co-culture platform that recapitulates key aspects of the stroma-tumor cell spatial relationship and consequent phenotypic heterogeneity. Using this model, we will characterize post-treatment CAF phenotypes in NSCLC and the mechanisms behind their pro-resistance functions.

Targeting pancreatic cancer stroma increased natural killer cell-mediated cytotoxicity in tumor microenvironment-mimicking spheroids

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Introduction

Pancreatic cancer (PaCa) is one of the solid tumors with the poorest prognosis. PaCa tumors characteristically have a rich tumor microenvironment which is mainly responsible for the aggressive cancer characteristics. Pancreatic stellate cells (PSCs) are the most abundant cells in the pancreatic cancer microenvironment and the principal source of collagen in the tumor stroma. Periostin, a matricellular protein, is specifically produced by PSCs and promotes the aggressiveness of PaCa cells. Periostin is known to facilitate in collagen assembly in the tumor microenvironment. Although the studies indicated that tumors which have denser extracellular collagen were insensitive to cytotoxic T-cell infiltration, there is no study showing the effects of periostin on NK cell infiltration and activity. Therefore, in this study, we aimed to decrease extracellular collagen assembly by targeting periostin and thus increase the cytotoxic activity of NK cells.

Material and Methods

Periostin expression was knocked down in PSCs (we named PSC-P) using CRISPR-Cas9 system. BxPC-3 pancreatic cancer cells co-cultured with PSC or PSC-P cells in a 3D environment to form tumor microenvironment-mimicking spheroid structures. The extracellular collagen in spheroids was evaluated via Masson's trichrome staining. The invasion ability of the spheroids was evaluated by matrigel drop evasion assay. Then, to analyze the cytotoxic activity of NK cells, NK-92 cells were added to the spheroids and incubated for 24 h. The cytotoxic activity of NK-92 cells was analyzed by flow cytometry and confocal microscopy via CD107a staining. In addition, to evaluate cell death in BxPC-3 cancer cells, Annexin-V and PI positivity were evaluated by flow cytometry.

Results and Discussions

We showed that periostin knockdown in PSC-P caused a decrease in extracellular collagen levels. We also observed that the size of spheroids that were formed by PSC-P+BxPC-3 formed smaller than the spheroids formed by PSC+BxPC-3. The invasion ability of the spheroids was also found to be decreased in the periostin knocked down-group. In addition, periostin knockdown caused an increase in the infiltration of NK-92 cells into spheroids and their cytotoxic activity and thereby induced pancreatic cancer cell death.

Conclusion

In conclusion, these results suggested that periostin might be a therapeutic target that may increase the cytotoxic activity of NK and thereby induce cell death in tumors.

Reduced Enteric Neuron Density Influences Tumour Phenotype in Colorectal Cancer

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Introduction

Even though the gastrointestinal tract is highly innervated and contains a nervous system of its own (the enteric nervous system (ENS)), knowledge on the role of neurons in the context of colorectal cancer remains sparse. The ENS regulates diverse gut functions and gastrointestinal homeostasis by (paracrine) communicating with different other cell types in the gut. In line, we recently elucidated that enteric neurons can enhance tumorigenesis via the release of extracellular matrix proteins. This follow-up study aims to investigate if reduced enteric neuronal density affects CRC development, progression and phenotype.

Material and Methods

Hand2^{fl/+}Wnt1Cre2 (hypo-innervated) and Hand2^{fl/+} (wildtype) mice were subjected to a colitis-associated CRC induction protocol using azoxymethane and dextran sulphate sodium. To track tumour initiation, growth and burden, we acquired computed tomography scans during cancer induction and histologically assessed tumour specimens after sacrifice. Isolated tumour tissues from hypo-innervated and wildtype mice were subjected to RNA sequencing, followed by a gene ontology enrichment analysis (GORilla). In addition, gene-set enrichment analysis on differentially expressed genes was performed for *hallmark of cancer* genes using Fisher's exact test. Finally, the microbiome was assessed using 16S rRNA sequencing on stool samples.

Results and Discussions

Despite the confirmed reduction in enteric neurons, Hand2^{fl/+}Wnt1Cre2 mice presented with a similar tumour burden (i.e. tumour initiation, growth and progression) as the Hand2^{fl/+} mice. RNA sequencing of tumours showed differentially expressed genes belonging to the cancer hallmarks '*avoiding immune destruction*' and '*deregulating cellular energetics*' when comparing both genotypes. Moreover, an enrichment of the gene ontology (defence) response to bacterium was found. Interestingly, analysis of the microbiome revealed that a reduced density of enteric neurons does not affect the microbiome, irrespective of the CRC-induced dysbiosis.

Conclusion

A reduced number of enteric neurons prior to tumorigenesis does not directly impact murine tumour growth or microbiome, but affects tumour phenotype. Further research is needed to elucidate these phenotypic differences and corresponding mechanisms.

Serotonergic Receptor Ligands interfere with Mammary Adipocytes function compromising their ability to sustain Breast Cancer phenotype

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Introduction

Adipose cells in the tumor microenvironment provide a relevant contribution toward breast cancer (BC) progression. Serotonin (or 5-Hydroxytryptamine, 5-HT) signals in mammary gland becomes dysregulated in cancer and contribute to proliferation, metastasis, and angiogenesis. Noteworthy, 5-HT leads to fat accumulation in white adipose tissue. Thus, the discovery of novel compounds targeting serotonin signaling may pave the way to modulate plasticity of peri-tumoral adipose cells and, in turn, offer new prognostic tools and therapeutic opportunities for treating BC. The effect of 5-HT on both cancer and adipose cells are mainly mediated by 5-HT_{2A} receptor. We have previously synthesized serotonergic receptor ligands (SER) with high affinity and selectivity towards 5-HT_{2A} receptors. Here, we investigated Mammary Adipose Cells-mediated impact of SER137, SER167, SER195, SER196, SER142 onto BC phenotype.

Material and Methods

Mammary Adipose-derived Mesenchymal Stem Cells (MAd-MSCs) - isolated from adipose tissue biopsies of healthy women (N=5) - were differentiated into mature adipocytes (MAd) in presence and absence of SER (15 µM). Cells were collected at different time points upon the induction of the adipogenic process. MAd were co-cultured with ER⁺ BC cells in 2D and 3D. Mammospheres' number was obtained after 10 days; mammospheres' diameter was calculated with a dedicated software coupled to optical microscope. Gene expression were checked by RT-PCR; mRNA levels were measured by qPCR. Data were statistically analyzed using GraphPad Prism 7.

Results and Discussions

MAd expressed 5-HT_{2A} receptor at higher levels compared to undifferentiated cells. MAd differentiated in presence of SER showed a delayed increase of adipogenic markers expression (*PPARG*, *ME1*, *FABP4*, *LPL*, *ADIPOQ*) compared to untreated cells. Notably, SER determine a significant down-regulation of lipid droplets (*PLIN1*, *PLIN2*) and insulin signaling (*IRS2*, *GLUT4*) markers in MAd. Interestingly, the average size and number of mammospheres were significantly reduced when BC cells were co-cultured with MAd differentiated in presence of SER; thus, SER reduced the ability of MAd to prompt a stem-like phenotype in ER⁺ BC cells.

Conclusion

SER interfere with adipocytes function compromising their ability to sustain BC phenotype. SER may represent new molecules potentially usable in combination with endocrine therapy to improve its effectiveness on ER⁺BC patients.

EACR22-1209

Lipid accumulation in cancer associated macrophages: insights into functional consequences and impact of diet

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Introduction

Tumor associated macrophages (TAMs) are abundant in tumors and correlate with disease progression in most cancers. Cancer cells confer to infiltrating macrophages pro-tumoral features, that span from support to tumor growth and invasion, to immunosuppression and resistance to therapies. The mechanistic basis of such interaction are still partially uncovered and therapeutic strategies to target TAMs remain poorly defined.

Material and Methods

Here, high dimensional single-cell RNA-seq was used to profile the transcriptional landscape of TAMs in human tumors. Immunofluorescence and Flow cytometry analysis were applied to investigate the functional features of tumor infiltrating macrophages in mouse models of cancer and patients samples. In vitro assays were conducted to dissect the mechanisms underlying the cancer-macrophage axis. Transgenic and transplantable mouse models were implemented to test immunotherapies that target tumor macrophages in vivo.

Results and Discussions

Our approach lead to the identification of a subset of macrophages characterized by a dysregulated lipid metabolism. Lipid accumulation in TAMs correlates with immunosuppression and tumor invasion. Importantly, administration of high fat diet augments the amount of lipid droplets in tumoral macrophages and impacts on the transcriptional profile of TAMs. Notably, the abundance of lipid-loaded macrophages correlates with tumor growth and resistance to immunotherapy. Importantly, targeting lipid intake in vivo restores immunesurveillance and hinders tumor progression.

Conclusion

Together, our findings identify a heterotypic signalling involving lipid-loaded TAMs and cancer cells that drives tumor progression and cancer invasion. Our results point at lipid-loaded TAMs as a novel therapeutic target for cancer treatment.

EACR22-1221

A transcriptomic atlas of human PDAC and normal pancreas reveals interaction networks and deregulated signalling at the cell-type and patient levels

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) tumors are complex highly desmoplastic masses formed by a mixture of different cell-type populations. Interactions between these different cell-type populations (tumor compartments) can influence tumor biology and progression in both pro- and anti-tumorigenic manners. Identification of the specific signals and interactions between compartments in actual human tumors and at the patient level is key to better understand PDAC and inform new target therapy options.

Material and Methods

Here, we have FACS-processed 32 human primary resected PDAC tumors and derived the whole transcriptome of the pure major cell-type populations (cancer associated fibroblasts, immune, endothelial and epithelial cancer cells) in each sample. To understand the molecular changes happening at the cell-type level we have isolated and profiled the same populations from 9 tumor-free distal-adjacent normal pancreases.

Results and Discussions

The generated data provide a comprehensive expression landscape of 125 samples with a deep sequencing coverage that outperforms previous single cell or laser microdissection studies. Using these data, we have identified the transcriptional changes occurring in PDAC at the cell-type level and the interactions between different compartments. We highlight changes occurring in a Pan-cancer manner *versus* those appearing in specific subsets of patients. As an example, our data revealed a distinct tumor-stroma communication network in PDAC patients with an activated interferon signaling status. The data suggest that a stratification according to interferon status would outperform the classical/basal subtype classification of patients when considering ICB therapies. Additionally, we have derived cell-type population specific signatures that allow the robust deconvolution of public and self-generated bulk tumor data in a total of 5 populations (cancer cells, immune, fibroblast, endothelial, and normal contamination). We provide access to all expression data, interaction networks, and deconvolution method through a novel interactive platform in which investigation and analyses can be carried out in a user-friendly and custom-based manner.

Conclusion

Our work provides a comprehensive landscape to study the deregulated signaling and communication networks employed by different cell-type populations in human PDAC. Understanding which cell-type compartment activates which genes and pathways in human PDACs is a precious information to unveil potential personalized targeted therapies.

EACR22-1269

Reprogramming of the immune infiltrate by targeted inducible delivery of stimulatory cytokines inhibits growth and promotes survival in a mouse model of glioblastoma multiforme

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Introduction

We previously demonstrated that is possible to exploit a population of tumor-associated macrophages (TEM, Tie2 expressing macrophages) to deliver interferon-alpha (IFN- α) to tumors through a cell and gene therapy approach. Transplant of hematopoietic stem cells (HSPCs) transduced with a lentiviral vector (LV) expressing IFN- α under the control of the Tie2 promoter induces TEM-specific release of the cytokine in the tumor microenvironment (TME), reprogramming it towards a pro-inflammatory state. Glioblastoma multiforme (GBM) is the most common and aggressive primary brain tumor. Due to the immunosuppressive and pro-angiogenic features of its TME, GBM represents the ideal candidate for our gene therapy approach.

Material and Methods

Mice were transplanted with HSPCs transduced with Tie2-Ifna or Tie2-IL12. To test our strategy in the context of GBM we took advantage of a syngeneic orthotopic mouse model of GBM, closely recapitulating several features of the human pathology. To further improve our platform we developed an inducible strategy based on destabilizing domains (DD) to control the timing and amount of IFN- α or IL-12.

Results and Discussions

Upon GBM challenge, mice transplanted with HSPCs transduced with Tie2-Ifna or Tie2-Ifna-DD showed remarkable tumor inhibition and improved survival, up to tumor clearance in a fraction of them.

Remarkably, the TEM-mediated delivery of IFN- α outperformed the systemic treatment with the recombinant protein both in terms of efficacy and specificity.

Single-cell RNA sequencing of the tumor immune infiltrate revealed a dramatic reprogramming of the GBM TME towards a pro-inflammatory state accompanied by the suppression of a macrophage gene expression program predicting poor prognosis in human GBM.

Notably, the spatial and temporal control of cytokine release was crucial in the case of IL-12, to overcome its otherwise lethal hematopoietic toxicity while allowing to fully exploit its antitumor activity.

Conclusion

These results indicate that the tumor-specific release of IFN- α and IL-12 can reprogram the GBM TME and stimulate an anti-cancer immune response.

Moreover, the possibility to turn ON and OFF cytokine allows the better control of the cytokine levels and time of expression, thus preventing their potential systemic toxicity.

Overall, our findings demonstrate a novel therapeutic approach for GBM and contributed to the recent launching of a first-in-human clinical trial of the Tie2-IFN- α strategy in this lethal tumor

EACR22-0168

PCSK9 plays a key role during liver sinusoidal endothelial cells activation in the angiogenic process of hepatic metastasis produced by colorectal carcinoma

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Introduction

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is an enzyme member of proprotein convertases family involved in LDL cholesterol metabolism due to its role in the degradation of LDL cholesterol receptor. PCSK9 has been described mainly in liver, brain and lung. In the liver, it has been deeply studied in hepatocytes but there is no any references about its presence in liver sinusoidal endothelial cells. We have identified for the first time expression of PCSK9 in liver sinusoidal endothelial cells and studied also the cellular localization and role during liver metastasis produced by colorectal carcinoma.

Material and Methods

Human immortalized liver sinusoidal endothelial cells were used to study the presence and localization of PCSK9 in physiological condition and after receiving human metastatic colorectal cancer cell lines SW620, SW480 and HT-29 secretomes. The protein expression study was performed by RT-PCR, western-blot and immunofluorescence observed by confocal microscopy. Motility study of liver sinusoidal endothelial cells was done by the wound healing assay and proliferation by XTT previous siRNA silencing of the protein. PCSK9 was cloned with His tag and produced in cells to isolate and study the interactome by mass spectrometry. PCSK9 was silenced by siRNA in activated by tumor secretome and non-activated liver sinusoidal endothelial cells and the transcriptome was studied by RNA sequencing. Human biopsies samples from patients with liver metastasis produced by colorectal carcinoma were used to confirm PCSK9 expression pattern.

Results and Discussions

For the first time we described PCSK9 expression in liver sinusoidal endothelial cells and how this proprotein convertase is upregulated when the cells are activated by tumor secretome. The upregulated isoform of PCSK9 is the immature one and it is located in the nucleus, this fact has been also demonstrated in human biopsies. Silencing experiments show direct correlation between proliferation capacity and PCSK9 upregulation in the nucleus. Sequencing and interactome analysis show that EpCAM is one of the substrates of PCSK9. Silencing of PCSK9 provokes downregulation of EpCAM and blocks proliferation cascade by cyclin inhibition.

Conclusion

We have described a new role for PCSK9 beyond that described as carrier of LDL receptor in hepatocytes, which includes a new location in the nucleus by the immature isoform. This discovery open new options to study the possibility to use PCSK9 inhibition in liver metastasis treatment.

Recapitulation of the alternative vasculature in neuroblastoma using bioengineered models

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Introduction

Neuroblastoma (NB) is a highly vascularized pediatric tumor originating from undifferentiated neural crest cells in the first years of life. NB displays an alternative vasculature constituted by cancer cells that can transdifferentiate into tumor-derived endothelial cells (TECs), a phenotype associated with drug resistance and tumor relapse. We currently lack treatments against TECs, mainly because of the challenge of developing predictive models that can mimic this phenomenon *in vitro*. This study aims to recapitulate the alternative vasculature of NB using bioengineering tools.

Material and Methods

NB-on-a-chip: idenTx 3 Chips were connected through Tygon tubes to a 50 mL culture medium reservoir, which was also acting as a bubble trap system. The culture medium was pumped using a high precision IPC-N 8 peristaltic pump. NB cells and HUVEC cells were mixed with a hydrogel and introduced in the main chamber of the chip.

Polydimethylsiloxane (PDMS)-based platform: PDMS Sylgard 184 was mixed in a weight ratio of 1:75 and 1:70 to mimic the artery stiffness range. PDMS Sylgard 527 was mixed with proportions of 1:1 and 1:2 to obtain the stiffness for the venous tissue.

Results and Discussions

We deconstructed the NB microenvironment to induce endothelial transdifferentiation and developed two technological platforms using bioengineering techniques.

The first approach consisted in mimicking the NB physiological stiffness by fabricating a collagen I/fibrin-based hydrogel of around 1 kPa designed to support the survival of NB cells. NB cells cultured under multiple combinations of microenvironment factors, but static, did not result in transdifferentiation. However, the fabrication of an NB-on-a-chip by including shear stress into a microfluidic chip device induced conversion into TECs.

The second strategy consisted of mimicking the reported stiffness for physiological angiogenesis and inducing angiogenesis *in vitro*. We developed a biocompatible PDMS-based platform of different stiffnesses. We demonstrated that the most aggressive NB cell phenotypes were mainly transdifferentiated in stiff substrates in the arteries range, while the less aggressive cell lines lacked transdifferentiation capacity.

Conclusion

These model systems that recapitulate tumor heterogeneity will help defer a specific treatment against the TECs subpopulation to avoid resistance and tumor relapse in pediatric patients. We found that NB cells with aggressive phenotypes can evolve into TECs by mimicking the artery stiffness.

PTPRZ1 negatively regulates endothelial cell activation in vitro and lung adenocarcinoma growth and angiogenesis in vivo

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Introduction

Protein tyrosine phosphatase receptor zeta 1 (PTPRZ1) is a transmembrane tyrosine phosphatase that has been shown to have a positive or negative regulatory effect in different types of cancer. Although it has been shown that PTPRZ1 is decreased in lung adenocarcinoma (LUAD), its role in LUAD development or growth is unknown. We have previously shown that PTPRZ1 is expressed in human umbilical vein endothelial cells and regulates their growth factor-stimulated migration but the role of PTPRZ1 in angiogenesis *in vivo* has not been addressed up to date.

Material and Methods

We isolated lung microvascular endothelial cells (LMVEC) from *Ptprz1*^{+/+} and *Ptprz1*^{-/-} mice and studied their proliferation rate, migration, tube formation on Matrigel and signalling *in vitro*. In the same mice, we also employed the urethane model of lung carcinogenesis to study LUAD growth and angiogenesis *in vivo*.

Results and Discussions

Ptprz1^{-/-} LMVEC have increased proliferation rate, migration, and tube formation on Matrigel, Akt activation and elongated mitochondria compared to *Ptprz1*^{+/+} LMVEC. These data are in line with the increased angiogenesis in the lungs of *Ptprz1*^{-/-} mice, and the enhanced chemically induced LUAD development and angiogenesis in *Ptprz1*^{-/-} compared to *Ptprz1*^{+/+} mice, accompanied by decreased overall mice survival. RNA sequencing analysis of *Ptprz1*^{-/-} and *Ptprz1*^{+/+} LMVEC identified differentially expressed genes linked to regulation of angiogenesis, among which decreased expression of integrin beta 3 (itgb3) in *Ptprz1*^{-/-} LMVEC, verified at the protein level. Up- or down-regulating itgb3 levels in *Ptprz1*^{-/-} and *Ptprz1*^{+/+} LMVEC highlighted the significance of the decreased itgb3 expression for the enhanced angiogenic phenotype of *Ptprz1*^{-/-} LMVEC. Crizotinib, a small molecule tyrosine kinase c-Met inhibitor, abolished the enhanced angiogenic phenotype of *Ptprz1*^{-/-} LMVEC *in vitro*, and the enhanced LUAD growth and angiogenesis in *Ptprz1*^{-/-} mice *in vivo*.

Conclusion

PTPRZ1 seems to have a significant regulatory role in endothelial cell activation and LUAD growth and angiogenesis *in vivo* through regulation of the expression of itgb3 and activation of c-Met.

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EACR22-1104

Oncostatin M signalling is required for tumour-promoting inflammation in the liver and for hepatocellular carcinoma progression

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Introduction

Hepatocellular carcinoma (HCC) is one of the deadliest cancers partly due to the lack of knowledge about its pre-tumorigenic stages and onset. Among others, inflammation is a key process in liver carcinogenesis: chronic inflammation triggers cirrhosis and, later on, HCC by promoting tissue remodelling and fibrosis, mediated by hepatic stellate cells (HSCs) and Kupffer cells (KCs).

Oncostatin M (OSM) is a cytokine from the Interleukin-6 family that, through its receptor OSMR, promotes tumorigenesis, tumour microenvironment (TME) remodelling and metastasis in different cancer types. OSM/OSMR pathway also plays relevant roles in liver development, regeneration and fibrosis, although the mechanistic insights have not been clearly defined. Thus, we hypothesised that OSM signalling may contribute to HCC onset and development by affecting not only tumour cells, but also inflammation in the TME.

Material and Methods

We studied HCC formation in mice lacking OSMR (OSMR-KO) compared to the wildtype (OSMR-WT) in two *in vivo* models with different inflammatory status: low-inflammation Diethylnitrosamine (DEN)-induced model and high-inflammation DEN combined with CCl₄ (DEN+CCl₄) model. Tumours were monitored by Magnetic Resonance Imaging (MRI) and analysed by visual inspection and histology. Liver functionality was determined by serum cholesterol levels and Alanine Aminotransferase (ALT) activity. Mice liver subpopulations (hepatocytes, cholangiocytes, HSCs and KCs) were isolated for *in vitro* experiments.

Results and Discussions

We found that the lack of OSMR did not affect inflammation-independent tumorigenesis, while it decreased tumour number and size in a pro-inflammatory model. In DEN+CCl₄ model, OSMR-KO decreased liver inflammation and prevented HCC-associated liver damage. Interestingly, we found that *OSM* and *OSMR* were mainly expressed by HSCs and KCs liver subpopulations. Tumours also present an upregulation of *OSMR* compared to their adjacent tissue which may implicate an additional role of OSM/OSMR in cancer cells. Finally, we also checked *OSMR* expression in samples from Donostia University Hospital and from public databases. We discovered that *OSMR* is overexpressed in cirrhotic/pre-HCC samples, and that high expression of *OSMR* is associated with decreased survival in HCC.

Conclusion

Our research supports that OSMR is a key regulator of HCC-promoting inflammation, mainly expressed by HSCs and KCs, and, consequently, the potential role of OSM/OSMR pathway as a new therapeutic target in HCC should be further studied.

EACR22-0779

Loss of STAT1 promotes KRAS-driven lung adenocarcinoma

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Introduction

Lung cancer is the leading cause of death worldwide with a low 5-year survival rate. One third of lung adenocarcinomas (LUADs), the most abundant form of lung cancer, harbors activating mutations in the KRAS oncogene. Since human KRAS-mutant LUAD is correlated to an inflammatory phenotype, we hypothesized that the JAK-STAT pathway plays a crucial role in disease progression. Indeed, preliminary data demonstrated that human KRAS-mutant LUADs are associated with the activation of the JAK-STAT signaling pathway,

including STAT1 expression upregulation. Therefore, we aimed to elucidate the tumorigenic functions of STAT1 in KRAS-mutant LUAD.

Material and Methods

To analyze the functions of STAT1 in KRAS-driven LUAD, we used C57BL/6 mice which were genetically engineered in order to develop KRAS-driven autochthonous LUAD deficient for P53 and either proficient or deficient for STAT1 (KP and KPS mice, respectively). Additionally, we deleted STAT1 in an inflammatory model where KRAS driven, p53 deficient tumors express ovalbumin (OVA) which acts as a strong neoantigen and increases immune infiltration (KPO and KPOS mice). Overall survival was assessed by Kaplan Meier analysis. Tumor burden and infiltration was analyzed in lungs via H&E and IHC staining 6, and 10 weeks after tumor induction. Bronchoalveolar lavage was performed and the supernatant used for an inflammatory cytokine antibody array.

Results and Discussions

Survival decreased significantly after STAT1 deletion in mouse autochthonous LUAD only in the OVA expressing model. This was concomitant with increased tumor burden and grade in KPOS mice 6 and 10 weeks after tumor induction suggesting that STAT1 controls tumor progression. Interestingly, KPO and KPOS mice derived tumors had similar T cell infiltration levels, which suggests that deletion of STAT1 does not result in T cell tumor exclusion, but rather in T cell exhaustion. Cytokine analysis showed an upregulation of CCL9, sTNF-RI and II, as well as VCAM-1 in STAT1 deficient tumors, which may contribute to a remodeling of the tumor microenvironment (TME) towards a more protumorigenic status in STAT1 deficient tumors.

Conclusion

This data suggests a tumor suppressive role for STAT1 in KRAS-driven autochthonous LUAD due to the induction of an antitumorigenic TME. Further studies are conducted to characterize the impact of STAT1 on the TME, focusing in changes on the immune infiltrate to clarify the mechanism standing behind these intriguing findings.

EACR22-0791

Endogenous retroviral and LINE1 reverse transcriptases derived RNA/DNA hybrids induce inflammation in Muscle Invasive Bladder Cancer

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Introduction

Endogenous retroviral (ERV) derived genes make up to 4.6 % of the human genome. Epigenetically silenced ERVs become activated in a variety of tumors. Expressed ERVs strongly associate with tumor inflammation, induced by ERV derived dsRNA and RNA/DNA intermediates, the latter synthesized by the reverse transcriptase (RT) activity of ERV-K pol or LINE1-ORF2, another human retrotransposon. We focused on the molecular basis of ERVs and tumor inflammation, especially of RNA/DNA intermediates in muscle invasive bladder cancer (MIBC), which accounts for ~20 % of newly diagnosed bladder cancer cases.

Material and Methods

MIBC cell lines, RT112 (luminal) and SCaBER (basal) were transfected with synthetic 60 nt long non-homopolymeric dsRNA and RNA/DNA structures. Gene expression of 60 different genes involved in immune response and inflammation pathways was determined via qPCR. LINE1-ORF2 was transfected into the MIBC cell line RT112 for 20 hrs. RNA/DNA hybrids were visualized by immunofluorescence using the S9.6 antibody. A tissue microarray of 124 MIBC tissues was evaluated by immunohistochemistry (IHC) with antibodies against LINE1-ORF2 and ERV-K pol. Correlation of ERV-K pol expression was confirmed via qPCR and IHC.

Results and Discussions

Transfected MIBC cells with LINE1-ORF2 showed significant higher levels of RNA/DNA hybrids using the S9.6 antibody, which supports that the RT enzyme is functional synthesizing RNA/DNA hybrids. Synthetic dsRNA and RNA/DNA induced immune responses in transfected cells, especially interferons (IFN α or IFN β 1), cytokines (CXCL8, TNF α) and interferon stimulated genes (IFI6, IFI27). Striking differences of gene expressions were found between the luminal and basal MIBC cell lines, but also after transfection with either dsRNA or RNA/DNA. A high correlation coefficient of 0.74 could be shown between gene and protein expression of ERV-K pol in human MIBC samples.

Conclusion

These findings, along with a better survival of MIBC patients with high ERV expression and a high inflammation signature, highlight the importance of ERVs in bladder cancer. The future focus will be on the molecular and cellular functions of RNA/DNA hybrids derived from ERV-K pol and LINE1-ORF2. In addition to LINE1-ORF2, we are currently transfecting ERV-K pol in MIBC cell lines. Additional RT activity and inhibitory assays will provide more information about the importance of ERV-K pol and LINE1-ORF2 in MIBC tumorigenesis and inflammation.

EACR22-0897

LRG1 as a novel modulator of the tumour immune microenvironment

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Introduction

Leucine-rich alpha-2-glycoprotein 1 (LRG1) is a pleiotropic innate immune system signalling molecule associated with a broad range of human conditions including cancer. Of particular interest is the role that LRG1 plays as an angiopathic factor where it disrupts the normal crosstalk between endothelial cells and pericytes, thus contributing to the formation of leaky, poorly perfused, and dysfunctional tumour vessels. We have previously observed that restoring vessel function with a LRG1 function-blocking antibody (15C4) limits growth of solid tumours and improves the efficacy of current standards of care and immunotherapies, including checkpoint inhibitors. The aim of this study is to investigate the effects of LRG1 blockade on the immune tumour microenvironment (TME).

Material and Methods

Following subcutaneous grafting of syngeneic melanoma (B16) and lung cancer (LLC) cells into wild-type mice, treatments with 15C4, anti-PD1, or a combination of both were administered. Tumours were measured at defined intervals and, at end point, analysed by immunohistochemistry or FACS analysis on tumour-infiltrating leukocytes.

Results and Discussions

In both tumour models, dual therapy was associated with a significantly higher density of CD3⁺ lymphocytes and activated cytotoxic CD8⁺ cells, as characterised by increased expression of PH3 and Granzyme B, compared to monotherapy. Notably, a significant reduction in the ratio between regulatory and helper CD4⁺ cells was also observed after combination therapy. Other additive effects that 15C4 exerts in a tumour-specific fashion includes: i) the reduction of exhausted (PD1^{high}) lymphocytes; ii) an increase in cytotoxic NK cells and iii) the modulation of the myeloid compartment. Thus, while in the B16 model the co-presence of 15C4 enhances a higher recruitment and/or formation of anti-tumoral M1 macrophages and antigen-presenting activated dendritic cells, in the LLC model LRG1 blockade acts towards the suppression of pro-oncogenic neutrophil function.

Conclusion

These data show that LRG1 blockade orchestrates a shift in the TME from being immune silent to immune active. Whether this outcome is achieved as a consequence of improved vessel function and oxygenation or whether LRG1 also directly disturbs immune cell function remains to be elucidated. Nonetheless, the evidence here reported suggests that targeting LRG1 may represent a novel co-player in current immunotherapies whose clinical success strongly rely on the presence of a highly immune-supportive *milieu*.

EACR22-0650

ORAL PRESENTATION

Long non-coding RNAs as outcome predictors in pediatric Acute Lymphoblastic Leukemia

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Introduction

Acute Lymphoblastic Leukemia (ALL) remains an important cause of death from disease in children, despite the progress made in risk assessment and treatment outcome over the past decades. Therefore, the identification of new outcome predictors and therapeutic targets is still a need. In this context, long non-coding RNAs (lncRNAs), which represent an important fraction of the functional genome that has been poorly studied to date, could be novel candidates with great potential. This work aimed to identify new biomarkers of ALL relapse,

analyzing expression differences in lncRNAs through a high throughput RNA sequencing approach.

Material and Methods

Total RNA was extracted from tumor samples at diagnosis of 52 pediatric ALL patients treated following the same protocol (SEHOP-PETHEMA 2013) in three Spanish hospitals. RNA libraries (stranded and without rRNA) were generated with TruSeq Stranded Total RNA Library Prep Kit and sequenced on NovaSeq 6000 System (Illumina), with a depth of approximately 150 million paired-reads. Alignment to hg19 human genome was performed with HISAT2, and featureCounts was used to quantify all lncRNAs annotated in LNCipedia v5.2. Differential expression analysis according to relapse status 5 years after diagnosis was performed using DESeq2, and Kaplan-Meier and Cox Proportional Hazard Model methods were used to perform 5-year event-free survival (EFS) analyses.

Results and Discussions

A total of 25,783 expressed lncRNAs were identified in our patient population from 56,846 lncRNAs annotated in LNCipedia. Among them, 18 lncRNAs were differentially expressed ($p_{adj} < 0.01$; $\log_2FC > 2$ / < -2) comparing relapsed ($n=7$) vs. non-relapsed patients ($n=45$). Patients were divided into three groups for survival analyses: “High expression”, “Low expression”, and “No expression”. From the 18 significant lncRNAs, 14 showed significant differences ($p < 0.05$) in Kaplan-Meier survival analyses. Notably, higher expression of all these lncRNAs was associated to worst survival, *lnc-CEP120-6* and *lnc-AAED1-1* being the most statistically significant. These results point to an important role of lncRNA expression in treatment response, and warrant further downstream studies to understand their role in the pathogenesis of the disease and as potential new therapeutic targets.

Conclusion

Our high throughput approach identified 14 lncRNAs associated with relapse and EFS, suggesting that lncRNA expression pattern could serve as prognostic predictor in pediatric ALL.

EACR22-0608

Splicing machinery dysregulation in hepatocellular carcinoma: a central role of EIF4A3-FGFR4-FGF19 axis in hepatocarcinogenesis.

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Introduction

Hepatocellular carcinoma (HCC) is characterized by the presence of aberrant oncogenic splicing variants involved in tumour development/progression. A dysregulation of the machinery responsible for the splicing process (spliceosome) could be responsible for these

alterations. Here, we explored the putative dysregulation and role of key spliceosome components (SCs) in HCC.

Material and Methods

Expression (mRNA/protein) of key (n=72) SCs and clinical implications were assessed in HCC patients from two retrospective (n=154 and n=172) and six in silico cohorts. Gene Set Enrichment Analysis (GSEA) and mutational landscape were analysed in TCGA patients. The functional and molecular consequences of EIF4A3 manipulation (silencing, overexpression, and pharmacological blockade) were assessed in liver-derived cell lines (HepG2, Hep3B, SNU-387) and in vivo in Hep3B-induced xenograft tumours. RNAseq from EIF4A3-silenced HepG2 cells was analysed. Rescue experiments were performed by overexpressing FGFR4 in cell lines.

Results and Discussions

A consistent, significant dysregulation in the expression of several SCs (mRNA/protein) was found in HCC vs. control-tissues in all cohorts. Among them, EIF4A3 was robustly overexpressed in all HCC cohorts and associated with clinical and molecular parameters of aggressiveness. EIF4A3-silencing (and/or its pharmacological blockade) reduced proliferation, migration and tumorsphere/colony formation in vitro, and reduced tumoral growth in vivo, while EIF4A3 overexpression had the opposite functional effect in vitro. RNAseq showed that EIF4A3-silencing altered the expression and splicing pattern of key genes associated to HCC (e.g., FGFR4). In fact, in vitro assays demonstrated that EIF4A3 controls FGFR4 splicing. FGFR4 overexpression partially rescued the EIF4A3 silencing-mediated decrease in proliferation and tumorspheres/colony formation. Thus, EIF4A3 is essential to maintain the pro-oncogenic signalling triggered by FGF19/FGFR4.

Conclusion

The splicing machinery is profoundly dysregulated in HCC, wherein it may represent a promising source of novel diagnostic, prognostic or therapeutic targets. Indeed, EIF4A3 is consistently elevated in HCC patients and associated with tumor aggressiveness and mortality, which may be exerted through the modulation of FGFR4/FGF19 axis.

EACR22-0856

Involvement of the RNA-binding protein HuR in the resistance acquisition to EGFR-tyrosine kinase inhibitor gefitinib in non-small cell lung cancer

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Introduction

Advanced non-small cell lung cancer (NSCLC) patients with sensitive mutations in epidermal growth factor receptor (EGFR) are treated with tyrosine kinase inhibitors (TKIs), including gefitinib. However, acquired resistance invariably develops, associated with immune suppressive phenotype involving expression of pro-inflammatory cytokines such as IL-6 and IL-8 and of immunomodulatory factors including CD47 and programmed death ligand 1 (PD-L1). The RNA-binding protein HuR is an emergent regulator of cancer through the enhancement of mRNA stability/translation of key effector genes by binding to their 3'-

untranslated region, including cytokines and PD-L1. Here, we investigated the TKI-mediated modulation of HuR in TKI-sensitive and resistant NSCLC cell lines and, reciprocally, HuR participation in TKI-treatment response.

Material and Methods

EGFR-TKI-resistant cell lines (HCC827GR and PC9GR) were generated by treating EGFR-TKI-sensitive HCC827 and PC9 cells with gefitinib; H1975 harboring EGFR-secondary mutation T790M were also used. To study the involvement of HuR in gefitinib treatment, PC9 HuR-KO cell line was generated by CRISPR/Cas9 technology. Protein expression was studied by immunoblotting, flow cytometry and ELISA assay. HuR association with 3'-UTR of CD47 and PD-L1 mRNAs was validated by biotin pull-down assay.

Results and Discussions

Gefitinib treatment reduced HuR expression in TKI-sensitive but not in resistant cells, in which HUR protein is more localized in the cytoplasm. Development of TKI resistance was associated with significant increase of IL-6 and IL-8 secretion and enhanced expression of CD47 and PD-L1 on cell surface compared to parental cells. Loss of HuR reduced the release of both cytokines in PC9 HuR-KO cells and decreased superficial expression of CD47 without affecting the total level of this protein. Additionally, loss of HuR impaired *in vitro* TKI-resistance acquisition and migratory ability of PC9 cells and enhanced the apoptotic rate in gefitinib-treated cells, accompanied by down-regulation of the anti-apoptotic protein Bcl-2.

Conclusion

Overall, our findings indicate that HuR, whose expression pattern is influenced by the acquisition of TKI-resistance, contributes to the adoption of pro-tumor hallmarks in NSCLC, through the modulation of migratory capacity, the promotion of an immunosuppressive tumor micro-environment, the limitation of TKI-induced apoptosis and subsequent acquisition of resistance to target therapy.

EACR22-0878

Role of the epitranscriptome and its implication in immune infiltration in cancer

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Introduction

Prostate cancer (PCa) is the second most diagnosed cancer in men worldwide and the first in terms of prevalence. Although most patients initially respond to conventional therapies, advanced disease develops resistance to therapy and metastasis. Immune checkpoint blockade (ICB) has emerged in recent years as an effective tumour therapy, but the immunosuppressive tumour microenvironment (TME) of this cancer type makes this therapy ineffective. Epigenetic inhibitors have been shown to be effective in the reprogramming of immune cells in tumoricidal endotype. After analyzing cancer expression databases (TCGA), a significant number of RNA epigenetic regulators (RER) were found to be altered. To explore the interactions between the RERs and the inflammatory status of the tumour in PCa, deconvolution methods were applied, founding a striking inverse correlation between RERs expression and immune cell infiltration with more tumoricidal endotype. These observations lead us to hypothesize that the combined actions of BCI agents together with

targeted agents that inhibit aberrant RER may elicit significative immunotherapy responses in cancer.

Material and Methods

By using genomic screenings, *in silico* analysis, deconvolution algorithms (CIBERSORT), CRISPR/Cas9 technology, secretome analysis, cell and mouse models and patient samples, we aim to explore the role of RERs in the activation and polarization towards inflammatory or immunosuppressive activity of immune cells associated with cancer, and their influence in the development of PCa.

Results and Discussions

We have found an inverse correlation between the RNA m⁷G methyltransferase METTL1 expression and pro-inflammatory macrophage and T cell infiltration in human PCa biopsies. We have observed the same results *in vivo*, in *Mettl1*-deleted tumour mice, and *in vitro* PCa METTL1-KO cells polarize macrophages into a pro-inflammatory endotype, indicating that METTL1 deletion in the tumour reprograms the tumour microenvironment. Mechanistically, tRNA methylation, through stress pathway and IFN γ response pathway activation, regulates the crosstalk between cells and the immune microenvironment.

Conclusion

Thus, our study concludes that METTL1 over-expression in PCa induces a immunosuppressive TME, and that its inhibition in PCa may be a promising therapeutic tool to improve immune therapies, as is the case of ICB.

EACR22-0906

Role of RNA methyltransferases in cancer progression

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Introduction

Non-mutational events, particularly RNA post-transcriptional modifications, are emerging as key players in tumour development and progression in several cancer types. Prostate cancer is the most prevalent cancer and the third cause of death by cancer in European men. Although most patients respond to therapy, a high percentage of them progress to metastatic castration resistant prostate cancer, highlighting the need to find the molecular pathways that regulate survival and metastasis. Emerging evidence show that self-renewal, survival and migration are regulated by epitranscriptomic marks, which may be a potential therapeutic target to specifically eliminate cancer cells.

Material and Methods

By using genomic screenings, epitranscriptomic RNA-seq tools, scRNA-seq, deconvolution algorithms, CRISPR/Cas9 technology, proteomics, cell and mouse models and patient samples we aim to decipher the epitranscriptome in prostate cancer in order to implement novel therapeutic strategies.

Results and Discussions

We find that overexpression of novel RNA methyltransferases correlate with poor prostate cancer prognosis. Loss and gain-of-function assays result in reduced cell proliferation, invasion, tumour formation and metastasis *in vivo*. Mechanistically, our data show that reduced RNA methylation results in reduced global protein synthesis and aberrant activation of catabolic, interferon response and stress pathways, resulting in decreased tumour growth and increased infiltration of pro-inflammatory immune cells.

Conclusion

In summary, we find that RNA modifications regulate cell proliferation, metabolic pathways and the tumour microenvironment crosstalk by adapting the translational machinery. Targeting RNA methylation is emerging as an effective therapeutic tool to eliminate cancer cells. Whether targeting RNA methylation alone, or in combination with agents targeting catabolic pathways, cytotoxic agents or immunotherapy can be used as effective therapeutic tool needs further validation.

EACR22-0922

Long non-coding RNAs CARMN and MIR100HG are downregulated in ovarian cancer

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Introduction

Ovarian cancer (OC) is one of the most lethal gynecological cancers in the world because of its late detection when the disease is already spread and, in combination with the arising of treatment resistance and relapse, OC is associated with poor patient prognosis. Long non-coding RNAs (lncRNAs) are transcripts longer than 200 nucleotides that regulate gene expression at several levels, playing a role in the pathogenesis of various diseases, including OC, correlating with important features for the clinical setting.

Material and Methods

RNA-seq data of unmatched ovarian serous cystadenocarcinoma and normal ovary tissues was obtained from The Cancer Genome Atlas and Genotype-Tissue Expression, respectively, both included in the database GEPIA. lncRNAs were manually identified in the top significantly dysregulated gene list and selected according to their fold change. The endogenous expression levels of the selected transcripts were measured by RT-qPCR in A2780, PEO1 and SKOV3 cancer and IOSE80T normal ovarian cell lines.

Results and Discussions

Our analyses from the RNA-seq data show 2 non-coding transcripts, namely *CARMN* and *MIR100HG*, that are previously unrelated to OC according to the literature. Both of them are significantly downregulated in ovarian carcinoma tissues compared to normal ovary, as well as in the three studied OC cell lines in comparison to the normal one.

Conclusion

Our results point that the two lncRNAs may play a tumor suppressive role within ovarian cancer cells. This fact opens the possibility of using them as novel biomarkers for OC, with diagnostic and, presumably, therapeutic value.

EACR22-1076

Splicing Machinery is Dysregulated in Craniopharyngiomas: A novel source of diagnostic, prognostic and therapeutic biomarkers

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Introduction

Craniopharyngiomas (CP) are relatively benign epithelial tumors that typically arise in the sellar/suprasellar region and are classified in adamantinomatous (ACP) and papillary (PCP). Diagnosis is usually performed when tumor development is already advanced, and serious comorbidities are present. The first-line therapy is usually surgery, but frequently the resection is not complete, causing high rates of recurrence. Therefore, the identification of alternative diagnostic/prognostic/therapeutic tools to improve CP management is necessary. Recently, growing evidence indicates that defects in the splicing process are frequent in cancer, leading to the appearance of altered spliceosome components (SCs), splicing factors (SFs) and/or aberrant splicing variants (SVs), which are associated to the development/progression/aggressiveness of various cancer types.

Material and Methods

Expression profile of key splicing machinery components [major/minor spliceosome (n=13/4) and 28 relevant SFs] in ACP (n=36) and PCP (n=4) vs. control samples [normal pituitary glands (NP, n=11)] was determined. Different approaches [i.e., bioinformatics, RNAseq analyses (18 ACP vs. 3 NP) and functional experiments (i.e., overexpression consequences of key SCs in primary CP cell cultures)], were performed to identify and explore the putative oncogenic role of key SCs.

Results and Discussions

Expression of a substantial number of SCs and SFs were drastically altered in ACP vs. NP, and also when primary vs. recurrent ACP were compared. RAVR1, RBM22, FBP11 and PRPF8 were identified as the most discriminating diagnostic/prognostic factors of ACP, being corroborated in additional human ACP and PCP cohorts. Expression levels of some of these components were associated with key clinical parameters suggesting a potential oncogenic role in CP. Dysregulation of RAVR1 and PRPF8 was corroborated in RNAseq of 18 ACP vs. 3 NP. In vitro overexpression of PRPF8 and RAVR1, and treatment with pladienolide B (splicing machinery inhibitor), in primary ACP-derived cells revealed a critical role of these factors and of the splicing machinery in CP. Finally, key SVs that are associated to CP development/progression (e.g., GNAS) were identified as potential oncogenic linkers with the observed splicing machinery dysregulation.

Conclusion

The spliceosome is drastically altered in CP wherein RAVR1 and PRPF8 could represent attractive novel diagnostic/prognostic and therapeutic targets for this pathology.

EACR22-0299

Investigating the role of microRNA in the resistance of oesophageal adenocarcinoma to therapy

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Introduction

Poor pathological response to neoadjuvant chemoradiation therapy (neoCRT) is a significant clinical problem in oesophageal adenocarcinoma (OAC). Using a novel proprietary algorithm (miR Scientific LLC), we identified *miR-Predict*, a 32-microRNA tumoral signature (confidential), altered between OAC patients having a good and poor response to neoCRT. However, the functional role of these individual microRNAs (miRNAs) in the response to treatment of OAC is largely unknown.

Material and Methods

TargetScanHuman 8.0, miRTarBase, and KEGG pathway analysis were used to identify miRNA target genes and pathways. Firebrowse and miR-TV were used to assess The Cancer Genome Atlas (TCGA) miRNA expression and survival data in oesophageal cancer (OSCC) and OAC. Radiosensitivity of OAC cell lines was assessed by clonogenic assay. RNA was isolated from a panel of OAC cell lines (OE33 P, OE33 R, OE33, OE19, Flo-1^{Par} and Flo-1^{LM}) following irradiation at a clinically relevant dose of 2 Gy. miRNA expression was assessed by qPCR.

Results and Discussions

Using a publicly-available data set, nine *miR-Predict* miRNAs demonstrated altered expression in OSCC tumour vs. normal tissue ($n=182$ vs. $n=14$) and/or altered survival in OAC ($n=78$). This demonstrated that *miR-Predict* miRNAs may play a role as cancer biomarkers and/or prognostic biomarkers in OAC. A number of these miRNAs were demonstrated to regulate pathways associated with treatment resistance. Basal miR-C expression was significantly lower in inherently radioresistant OAC cells (OE19), compared to inherently radiosensitive OAC cells (OE33) but was significantly increased in OE19 cells following irradiation with 2 Gy. Flo-1^{Par} cells, derived from a distal OAC, were significantly more radiosensitive than Flo-1^{LM} cells, derived from Flo-1^{Par} liver metastasis. Basal miR-D expression was significantly lower in Flo-1^{LM} compared to Flo-1^{Par} cells but was significantly increased in both Flo-1^{Par} and Flo-1^{LM} cells following irradiation with 2 Gy. These data suggest a role for miR-C and miR-D in treatment response in OAC cell lines.

Conclusion

Decreased miR-C and miR-D expression were associated with radioresistance in OAC cell lines, supporting previous data in patient tumour biopsies, wherein these miRNAs were decreased in OAC patients having a poor response to neoCRT. We are currently investigating the functional role of these miRNA in modulating the tumour response to radiation *in vitro* to determine their potential as novel therapeutic targets to boost response to treatment in OAC.

EACR22-0693

Progesterone modulates DSCAM-AS1/miR-130a/ER axis to suppress the invasion and migration of breast cancer cells

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Introduction

In a randomized clinical trial on operable breast cancer patients, a single dose of Progesterone--the dominant hormone during the luteal phase-- administered before surgery (preoperative)-- showed an increased disease-free survival period in patients. Of several hypothesis-generating results from this trial, we earlier showed that differential regulation of *SGK1* by Progesterone underlines the survival benefit to breast cancer patients by interfering with the migration and invasion of breast cancer cells. Here, we set to study the regulatory role of non-coding RNAs to explore the potential utility in monitoring the response of Progesterone in human cancer.

Material and Methods

Transcriptome sequencing of breast cancer cells was performed to analyze differential expression of lncRNAs in response to progesterone treatment. The candidate genes were validated using qPCR analysis, siRNA-mediated knockdown, and luciferase assay.

Results and Discussions

We identify significant down-regulation of *DSCAM-AS1*, an oncogenic lncRNA in hormonal receptor-positive T47D and BT474 breast cancer cells in response to progesterone treatment, that could effectively be blocked by siRNA mediated knockdown of progesterone receptor (PR) or treatment of cells with PR antagonist, mifepristone. Our data further suggest that knockdown of *DSCAM-AS1* mimics the progesterone treatment by impeding the cellular migration and invasion property of T47D and BT474 cells. On the contrary, *DSCAM-AS1* overexpression could rescue the effect of mifepristone, suggesting the essentiality of PR and *DSCAM-AS1* to mediate the effect of Progesterone in breast cancer cells. Interestingly, ChIP-Seq data analysis of T47D cells treated with Progesterone reveals a dynamic binding of estrogen receptor (ER) to the *DSCAM-AS1* regulatory regions suggesting *DSCAM-AS1* regulates the ER level in breast cancer cells. Using luciferase assays, we show *DSCAM-AS1* sponges *miR-130a* to regulate the 3'-UTR of the ER. Consistent with the finding, overexpression of *miR-130a* mimics progesterone treatment by downregulating ER levels in breast cancer cells.

Conclusion

We show an intricate interplay of *DSCAM-AS1* mediates progesterone treatment of breast cancer by regulating the expression of ER through sponging *miR-130a* that binds to the 3' UTR of the ER. Our study reveals a novel molecular axis, *DSCAM-AS1/miR-130a/ER*, mediates the Progesterone effect in breast cancer cells to impede invasion and migration of breast cancer cells in response to Progesterone.

EACR22-0823

Tissue miRNAs as a tool to predict and improves benefit from trastuzumab

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Introduction

Trastuzumab has revolutionized the clinical management of HER2 positive breast cancers. Unfortunately, 50% of patients develop resistance. Researchers have already designed alternative anti-HER2 agents, such as Pertuzumab and Lapatinib. Still, predicting which patients will benefit from the therapy would prevent overtreatment and avoid unnecessary risks of side effects. Analyzing tissue samples from the phase III NeoALTTO trial, we recently (*Pizzamiglio S, Cosentino G et al; Cancer Med 2022*) identified a predictive signature (hsa-miR-31-3p, OR 0.70, 95%CI: 0.53-0.92 and hsa-miR-382-3p, OR 1.39, 95%CI: 1.01-1.91; AUC value of 0.73, 95%CI: 0.60-0.87) associated to response to neoadjuvant Trastuzumab. Here, we explored the functional role of miR-31-3p and miR-382-3p.

Material and Methods

MiRNA modulation was performed in HER2 positive breast cancer cell lines SKBr3 and HCC1954 by transient transfection of miR-382-3p or miR-31-3p mimics (ThermoFisher), or a miR-31-3p inhibitor (LNA-31-3p, Exiqon), or negative controls, at a final concentration of 50nM. Trastuzumab was given at a final concentration of 10 ug/mL. MiRNA expression was evaluated by qRT-PCR (Taqman Assay, ThermoFisher), protein modulations assessed by Western blot, cell growth analyzed by 2D and 3D assay on matrigel.

Results and Discussions

Overexpression of miR-31-3p, which negatively associated to pCR in HER2 positive patients, in HER2-addicted SKBr3 cells was able to counteract the reduction of phosphorylated HER2 levels induced by trastuzumab treatment and increased the proliferation of both treated and non-treated SKBr3 cells in a 3D setting.

On the contrary, HER2 non-addicted HCC1954 cells were transfected with LNA-31-3p alone or in combination with miR-382-3p, which positively correlates with pCR. This combination significantly increased response to trastuzumab *in vitro* and reduced total HER2 levels, as assessed by western blot analysis.

Finally, preliminary data obtained *in vivo* in HCC1954 xenograft models suggest that miR-31-3p inhibition by LNA peritumoral delivery reduces tumor growth.

Conclusion

Predictive miR-31-3p and miR-382-3p play a functional role in the responsiveness to anti-HER2 trastuzumab.

EACR22-0891

N6-adenosine methyltransferase complex regulator ZC3H13 deletion as malignant prognosis factor in prostate cancer

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Introduction

Prostate cancer (PCa) is the most common malignancy in men and the cause of highest mortality in developed countries. Despite improvement of diagnostic tools and therapies part of the patients still evolve to a more aggressive form, where treatment options are very limited revealing the need to develop new therapies. Compelling evidence indicates that epitranscriptome, and specially 6-methyladenosine (m⁶A), plays a fundamental role in

oncogenic or tumor suppressor process. M⁶A deposition is catalyzed by a methyltransferase complex formed by *METTL3/METTL14* as the catalytic unit and whose non-catalytic components are still emerging.

Material and Methods

By *in silico* screening using TCGA genomic and expression data, CRISPR-ko and CRISPRa technology, pre-clinical models for indolent and metastatic PCa, and patients samples we aim to decipher the role of novel m⁶A regulators in PCa to implement novel therapeutic strategies.

Results and Discussions

We identified that the epitranscriptomic regulator *ZC3H13* is deleted and down-regulated in PCa and mCRPC patients, which is associated with the recurrence, progression and poor prognosis. Gain- and loss-of-function analyses show that *ZC3H13* regulates the proliferative, migratory and invasive capacities of PCa cells. Mechanistically, we find that *ZC3H13* stabilizes the core methylation complex *in vitro* and in patient samples, resulting in decreased m⁶A deposition changes upon *ZC3H13* deletion.

Conclusion

In summary, we identify *ZC3H13* as a prognostic factor in PCa. Our data further indicates that targeting m⁶A deposition levels in PCa inhibiting m⁶A mRNA demethylases alone or in combination with other therapeutic agents is a promising strategy to affect tumour growth and metastatic invasion

EACR22-0901

SRSF6 (Serine/arginine-Rich Splicing Factor 6) as an exploitable target for prostate cancer treatment

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Introduction

Prostate cancer (PCa) treatment is mainly based on the blockade of the androgen receptor (AR) signalling pathway, known as androgen-deprivation therapy. Unfortunately, some patients acquire resistance to this pharmacological approach, developing castration-resistant prostate cancer (CRPC), the most aggressive phenotype of this pathology, which remains lethal nowadays. Therefore, new molecular targets with potential to identify more effective therapeutic strategies are extremely needed. In this sense, alteration of RNA-splicing process has raised as a new hallmark of cancer. Herein, we studied the presence and pathophysiological role of SRSF6, a splicing factor that has been reported to have an oncogenic potential in other tumor-types (e.g., lung and colon).

Material and Methods

SRSF6 levels were interrogated by qPCR and immunohistochemistry in two well-characterized cohorts: 1) fresh PCa (n=42) and control (n=9) samples, and 2) formalin-fixed, paraffin-embedded PCa and non-tumour adjacent tissue samples (n=84). Additionally, multiple functional and mechanistic assays were performed in different models [PCa cells (LNCaP, 22Rv1, DU145, and PC-3) and non-tumour prostate cells (PNT2)] in response to SRSF6 silencing (using specific siRNA). Moreover, the *in vivo* effect of SRSF6 silencing was also tested using a xenograft mouse model. Finally, we analysed the putative relationship between the expression levels of SRSF6 with the AR signalling and the resistance to androgen-deprivation therapy.

Results and Discussions

SRSF6 is upregulated (at mRNA and protein levels) in PCa samples, and its levels directly associated to key clinical and molecular parameters of PCa aggressiveness [e.g., Gleason grade, AR signalling pathway]. Furthermore, SRSF6 silencing significantly decreased critical functional parameters in PCa cells (i.e., proliferation and migration rates, and tumorsphere formation). Mechanistically, SRSF6 silencing altered AR splicing, by specifically reducing the oncogenic splicing variant AR-V7. Consistently, a preclinical *in vivo* approach showed that the treatment with SRSF6 siRNA reduced the size of 22Rv1-derived tumours, impacting key molecular pathways (e.g., AR signalling).

Conclusion

Taken together, our results suggest that SRSF6 impacts AR signalling by altering the splicing of the oncogenic AR-V7 variant and, that SRSF6 targeting could represent a novel and promising therapeutic strategy for the treatment of advanced PCa.

EACR22-1017

Ovarian cancer-related mRNA and lncRNA detection in blood plasma samples and their derived small extracellular vesicles

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Introduction

Ovarian cancer (OC) is one of the most diagnosed gynecological cancers in women. Due to the lack of effective early stage screening, women are more often diagnosed at an advanced stage; therefore, it is associated with poor patient outcomes. Cancer extracellular vesicles (EVs), including small EVs (sEVs), contain biomolecules (protein, nucleic acid, and lipids) at various stages of carcinogenesis. An emerging role of long non-coding RNAs (lncRNAs) in tumor progression has been revealed in the last decade. The difficulty in detecting lncRNAs in liquid biopsies lies in their low expression levels, in addition, many of these RNAs have a very short half-life and are easily degraded, their role not being significant in the processes of malignancy and metastasis. However, those lncRNAs released by cancer cells and that increase their stability, through interaction with proteins, through their inclusion as cargo in sEVs or through both processes, may be more aggressive and more useful as biomarkers.

Material and Methods

Peripheral blood plasma samples of participant women were obtained from Hospital of A Coruña. sEVs were isolated from plasma samples using ultracentrifugation, followed by a

characterization by Western blot, Transmission Electron Microscopy, and Dynamic Light Scattering. RNA was purified from both isolated sEVs and original plasma samples, carried out by Phenol:chloroform extraction. The expression levels of lncRNAs and mRNA were determined by qRT-PCR, in which a reverse transcription with random hexanucleotides was performed.

Results and Discussions

The isolation and characterization of sEVs from blood plasma samples was successful, as they contained Flotilin and CD9 protein, were cup-shaped, and their size ranged from 50 to 150 nm. As described in the literature, the RNA ranges obtained from the sEVs samples are relatively low, with concentrations lower than 20 ng/μl. Despite the low amount of RNA obtained from the exosomes, the analysis by qRT-PCR showed that several mRNAs and lncRNAs related to the diagnosis and treatment of ovarian cancer, were detected both in plasma and in sEVs.

Conclusion

At the extent of the participant cohort our finding confirms the usefulness of our methodology to detect and analyze the expression levels of mRNA and lncRNAs related to ovarian cancer representing a further step towards establishing liquid biopsy in the clinical setting.

Tumour Evolution and Heterogeneity

EACR22-0433

ORAL PRESENTATION

Exploring clonal plasticity in breast tumour xenografts by integration of single-cell transcriptional profiles with inferred genomic clones

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Introduction

Intra-tumour heterogeneity increases as the tumour grows, gains mutations and diversifies into distinct genomic clones. The original transcriptional identity and the set of mutations largely dictate the transcriptional phenotype of the clone. We set out to explore the remaining transcriptional plasticity within a genomic clone and its potential to cover the full phenotypic repertoire of the tissue. To do so, we used single-cell RNA-seq (scRNA-seq) to profile a large and diverse cohort of patient-derived tumour xenografts (PDX). The scRNA-seq profiles were used to infer genomic clones and to create high-resolution transcriptional maps. Integrating the genomic and transcriptional profiles sheds light on the extent of clonal plasticity in different types of breast tumours and reveals shared phenotypic features that affect this plasticity.

Material and Methods

We generated scRNA-seq profiles from 30 breast tumour PDXs, including ER+, HER2+ and TNBC primary and metastatic tumours. *Metacell* was used to partition cells into small cohesive groups that facilitate quantitative analysis. Genomic clones were inferred by

integrating copy number (CN) and single nucleotide variant (SNV) profiles, called by *inferCNV* and *Souporcell*, respectively. Whole exome sequencing (WES) was used to test if SNV identification from WES can improve clonal inference. Single-cell DNA-seq (scDNA-seq) was generated for 4 samples to compare the scRNA-seq inferred genomic clones with the gold-standard scDNA-seq derived clones.

Results and Discussions

Inferring genomic clones solely from scRNA-seq is challenging due to data sparsity and low genomic coverage. Integrating CN and SNV data and corroborating the clones with high quality scDNA-seq enabled us to assess the accuracy and limitations of our method. High resolution transcriptional maps facilitated sub-clonal analysis which revealed clones with variable levels of transcriptional heterogeneity. Furthermore, we utilised the scRNA-seq data to understand tumour specific gene expression, sub-clonal transcriptional features, and to detect global and sub-type specific transcriptional programmes.

Conclusion

High resolution scRNA-seq data enables inference of genomic clones and highlights different levels of transcriptional plasticity within them. Examining this heterogeneity together with additional sub-clonal phenotypes promotes our understanding of the heterogeneity and dynamics of tumour clones.

EACR22-0927

ORAL PRESENTATION

TRACERx: Characterizing the evolution of non-small cell lung cancer metastases

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Introduction

Primary lung cancer is the leading cause of cancer-related mortality, with metastatic disease being responsible for the majority of deaths. To gain insight into the lethal process of metastasis, we report on the longitudinal evolutionary analysis of the TRACERx 421 paired primary-metastasis cohort.

Material and Methods

485 primary tumor and 227 metastatic samples, from 129 metastatic non-small cell lung cancer patients were collected and whole exome sequenced. Mutations and copy number events were integrated to resolve the evolutionary history of each tumor.

Results and Discussions

We observe that metastases diverge relatively late in molecular time, after the majority of mutations in the primary tumor have accumulated, with a substantial minority (33%) diverging prior to the last clonal sweep in the primary tumor. For these cases, divergence probably occurred at tumor volumes below the limit of computed tomography (CT) detection. Sampling bias may result in erroneous inference of metastatic trajectories. 79% of metastases diverging after the last clonal sweep in the primary tumor would be misclassified as diverging prior to the last clonal sweep if only a single region of the primary tumor is considered.

Patterns of dissemination range from monoclonal, involving a single metastatic clone (68% - where metastatic potential is likely acquired once in the life-history of the tumor), to polyclonal and polyphyletic (17%), where metastatic potential may have arisen multiple times during lung cancer development, or at a single time point early in the development of the tumor. We find that thoracic lymph node disease resected at surgery was responsible for less than 20% of subsequent disease dissemination, suggesting that lymph nodes likely represent metastatic potential rather than a gateway to further metastases.

Furthermore, we observe that clones which seed the metastases are generally dominant within the primary, reflecting positive selection and acquisition of subclonal mutations in specific cancer genes. In squamous cell carcinomas, we find that non-metastatic primary tumors show no significant evidence of positive subclonal selection. We also identify somatic copy number alterations that are enriched in metastases.

Conclusion

These data highlight the potential to apply evolutionary measures to predict metastatic risk, the limitations to current screening approaches particularly for early tumor divergence, and the importance of future precision adjuvant therapies to target disseminated micro-metastatic clones.

EACR22-0938

ORAL PRESENTATION

Genomic-transcriptomic evolution in TRACERx Lung Cancer and Metastasis

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Introduction

Genomic intra-tumour heterogeneity (ITH) drives tumour evolution, leading to immune evasion and resistance to therapy. Emerging evidence implicates the transcriptome as a source of important variation that impacts tumour phenotype.

Material and Methods

Here, we perform a genomic-transcriptomic analysis of intra-tumour diversity upon 941 tumour regions taken from 357 TRACERx non-small cell lung cancers (NSCLC) across primary and metastatic disease subjected to high-depth bulk DNA and RNA sequencing, as well as 91 tumour-adjacent normal tissue samples. We developed novel bioinformatic pipelines to identify complex transcriptional features such as allele-specific expression (ASE) and RNA editing.

Results and Discussions

We find that mutations in driver genes such as *STK11* or *KRAS* are associated with specific transcriptional phenotypes ($q=0.013$ and 0.007 for *STK11* and *KRAS* with fourth and fifth principal components of transcriptomic diversity, respectively). Similarly, we find that patterns of expression ITH can be used to infer which genes are more likely to be under positive or negative selection in cancer evolution. An integrative analysis of sources of transcriptome-specific diversity reveals that copy-number (CN) independent ASE, affecting 1% ($\pm 0.5\%$) of genes, is underpinned by allele-specific methylation ($OR=7.58$, $p<2.2\times 10^{-16}$) and provides a window to the NSCLC epigenome. Driver mutations in *SETD2* lead to

increased global levels of CN-independent ASE, a finding we validated using isogenic cell lines. CN-independent ASE is also involved in genomic-transcriptomic parallel evolution converging on disruption to specific cancer genes. Expression of *ADAR* and *APOBEC* enzymes is linked to novel RNA editing signatures, uncovering otherwise undetected *APOBEC* activity within tumours at sampling.

By characterising the transcriptome of primary-metastatic tumour pairs we find enriched expression signatures of proliferation within regions of the primary tumour that seeded metastasis ($q=0.03$). We generalise this finding through a machine-learning algorithm that relies on multiple genomic and transcriptomic features to characterise the phenotype of regions from multi-sampled tumours that seeded a paired metastasis.

Conclusion

This work highlights the importance of the transcriptome during tumour evolution, as well as the power of integrative multi-omic assessments of ITH, and provides novel insight into the role of transcriptomic variation in lung cancer.

EACR22-0558

ORAL PRESENTATION

Dissecting the melanoma ecosystem one cell at the time during immunotherapy

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Introduction

Melanoma is notorious for its high degree of intratumoral heterogeneity (ITH) and plasticity. The advent of single cell genomics allows to study ITH to an unprecedented resolution and identify clinically-relevant and rare cell populations, which are missed in bulk sequencing studies. Leveraging single cell RNA sequencing (scRNAseq), we aim to establish a comprehensive view of the transcriptomic landscape of the melanoma ecosystem and measure its dynamics during immune checkpoint blockade (ICB).

Material and Methods

The scRNA-seq data (10X Genomics) was generated from 46 human metastatic lesions (11 responders, 11 non-responders according to RECIST1.1) from treatment naïve and two weeks after treatment with ICB, stage III, and IV melanoma patients. More than 55K cells were identified and analyzed using standard Seurat pipelines. To overcome the challenge of identification of malignant cells clusters on the integrated data across patients posed by the high degree of inter-patient genetic variability, this analysis was further guided by deep scRNAseq from *Tyr::NRas*^{Q61K/p}; *Ink4a*^{-/-} murine melanoma lesions, which showed high transcriptomic, but little genetic, ITH. Moreover, to spatially map the identified cell types/states we leveraged spatial transcriptomic approach Visium (10X Genomics).

Results and Discussions

Our analyses allowed the identification of novel shared (evolutionarily conserved), and thereby biologically relevant, melanoma states. Intriguingly, one of the melanoma cell states expressed higher levels of genes involved in the antigen processing and presentation machinery and was more abundant in tumors from responding, whereas mesenchymal-like state from non-responding patients. Moreover, our results suggested that ITH is not solely driven by genetic makeup, as shown by the discrepancy between transcriptional and genetic (CNV) cluster identity. Instead, we conclude that ITH is partly driven by specific cell-cell interactions with the TME. This was consistent with a non-random geographical distribution of the melanoma cell states as revealed by spatial transcriptomic analyses.

Conclusion

Taken together, analyses of our newly generated scRNA-seq data from melanoma lesions unraveled previously undescribed melanoma cell states and their molecular identity and further emphasized the critical role of the TME as a driver of ITH and its association with responses to ICB.

EACR22-0076

Evolution of pancreatic adenocarcinoma is associated with differential modulation of cancer-associated fibroblast subtypes

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Introduction

Heterogeneity of cancer-associated fibroblasts (CAFs) in pancreatic ductal adenocarcinoma (PDAC) is well-known. Notably, molecular subtypes have been identified including our patient-derived four subtype signature pCAFassigner and the iCAF, apCAF and myCAF classification. We demonstrated prognostic status of pCAFassigner subtypes and through an unbiased analysis, we found that our pCAFassigner subtypes were synonymous with iCAFs, apCAFs and myCAFs (>90 %). We performed a comprehensive study into the prognostic impact of these subtypes and evaluate over PDAC evolution the changes in CAF subtypes to inform of early detection and biomarkers.

Material and Methods

Whole tumour and normal, non-adjacent patient samples were classed into a dominant CAF subtype or a mixture using the Nearest Template Prediction (NTP) algorithm or average cutoff of subtype-specific genes, respectively. Kaplan-Meier overall survival (OS) and cox regression analyses was performed. For analysis of mouse tumour/normal single-cell data, the R package Seurat was used.

Results and Discussions

A significant difference in OS was observed with apCAF- and myCAF- dominant patients having poorer prognoses than iCAF-dominant samples (n = 618, log-rank p < 0.01). Similarly, to account for intra-tumoural heterogeneity by characterising samples with mixed subtypes, there was a trend of prolonged survival in patients with an increased iCAF population, irrespective of the presence or absence of apCAF/myCAF (log-rank p < 0.01). Strikingly, we also noted a decrease in iCAFs and an increase in myCAFs from grade-2 to grade-3 tumours in a publicly available dataset (Fisher's test, p = 0.011). This suggests that iCAFs are potentially early populations in evolving tumours. In support of this hypothesis, adjacent normal pancreas (n = 96) was enriched in dominant iCAFs, whereas the matched tumour predominantly were myCAFs (Fisher's test, p < 0.01). We further noticed reduced

iCAF gene expression and increased myCAF gene expression in a single-cell dataset from a mouse model from normal pancreas to early PDAC and eventually to late PDAC.

Conclusion

We shed light into the prognostic impact of CAF subtypes and the evolution of iCAFs and myCAFs during PDAC progression. Our data potentially suggests that during normal-to-tumour evolution, suppression of iCAFs is occurring allowing the emergence of a larger myCAF population. Consequently, by preventing the progression of myCAFs, we may better manage this deadly disease and improve the quality of treatment given to patients.

EACR22-0552

PI3K signaling mediated whole genome doubling drives pancreatic cancer progression and defines subtype specific therapeutic vulnerabilities

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Introduction

Whole-genome doubling (WGD) and subsequent chromosomal instability occur in 30% of pancreatic ductal adenocarcinomas (PDACs). WGD⁺ PDACs are bound by poor prognosis and a fundamental lack of mechanistic knowledge regarding the molecular bases of the disease. Here we aimed at uncovering molecular determinants of WGD and exploit them as new therapeutic vulnerabilities.

Material and Methods

To overcome the limitations posed to genetic studies due to the rich stromal content of PDAC, we generated a unique and molecularly characterized mouse primary cell culture and tissue resource. Exploiting this cohort, we first we integrated whole genomes, transcriptomes and phenotypic data to investigate WGD. Second, we screened 246 primary PDAC cell lines with more than 400 drugs in clinical and preclinical investigation to identify WGD specific vulnerabilities.

Results and Discussions

We identified that *Kras*^{G12D}-driven PDACs are characterized by WGD in 24% of cases, in line with reports of human PDAC. To analyze the molecular determinants of WGD and probe the role of KRAS and its downstream signaling pathways, we systematically activated KRAS effectors *in vivo* in the pancreas. Interestingly, activation of PI3K signaling via expression of oncogenic *Pik3ca*^{H1047R} increased the fraction of WGD⁺ PDAC to 70%. To study the processes underlying these events, we initially reconstructed the evolutionary history and timed the genetic alterations through analysis of whole genomes. Then we functionally investigated the consequences of oncogene induction in primary pancreatic ductal cell cultures and mouse embryonic fibroblasts. We show that PI3K-signaling activation selects for chromosome-specific aneuploidies and increases tolerance to WGD.

In *Pik3ca*^{H1047R} WGD⁺ PDACs, we demonstrate that arms of chromosome 4, 12 and 14, containing tumor suppressor barriers and WGD enabler genes, such as *Cdkn2a*, the p53-pathway and *Rb1*, are lost prior to WGD. This allows WGD⁺ PDAC cells to survive and give

rise to chromosomal instability. Subsequently, with a high-throughput drug screen, we identified that WGD⁺ PDAC cells are highly susceptible to proteasome, cell cycle and PI3K signaling inhibition.

Conclusion

Collectively, our results demonstrate a link between PI3K signaling, WGD and loss of specific tumor suppressor barriers in PDAC and open new avenues for targeting WGD⁺ PDAC.

EACR22-0033

Exploring stimulus-driven heterogeneity with in vitro models of pancreatic ductal adenocarcinoma

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a dismal disease with a 5-year survival rate of around 10%, most patients present in the clinic with metastatically advanced disease. Given a conventional understanding of metastasis formation, it is likely that Epithelial-Mesenchymal Transition (EMT) plays a role in advancing PDAC. Transcriptional analysis of PDAC has identified two subtypes: Classical and Basal. These classifications have more epithelial and mesenchymal phenotypes, respectively. More recently, cells of both subtypes have been identified in the same tumour, demonstrating that heterogeneity of EMT status exists within tumours. PDAC is also characterised by extensive stroma which saturates the Tumour microenvironment (TME) with pro-and anti-EMT signals. How cells integrate and respond to these signals is underexplored. Here I begin to decode the response to various stimuli from the complex microenvironment that may induce this epithelial-mesenchymal heterogeneity.

Material and Methods

Publicly available mass cytometry datasets were analysed using Cytofkit2, and scRNAseq were analysed using Seurat Package. CellphoneDB, CellChat and the CellTalk Database were used to identify stromal signals that may regulate EMT. These ligands were screened *in vitro*. Briefly, PDAC cell lines and murine organoid models were treated with ligands and assessed by Western Blotting (WB) and immunofluorescence (IF) respectively. Ligands that were inducers of EMT were then measured using a mass cytometry panel signalling proteins to identify signalling pathways that may control stimuli-induced changes in cell phenotype.

Results and Discussions

Analysis revealed that PDAC tumour cells demonstrate heterogeneity in expression of EMT markers. Computational analysis was used to identify subsets of Fibroblasts and Macrophages as potential inducers of EMT in tumour cells. Further analysis identified a list of potential secreted factors from these cells. These secreted factors were screened for their capacity to induce EMT. WB and IF analysis revealed HGF, among others, to induce EMT in both cancer cell line and murine-organoid models of PDAC. Ligands were also analysed by Mass Cytometry to show differential engagement of signalling pathways.

Conclusion

Computational tools can be effectively used to identify critical TME factors associated with a change in EMT phenotype. Stimuli from the TME demonstrates the capacity to induce a change in cellular phenotype in cancer cells alongside accompanying rewiring of signalling pathways.

EACR22-0499

Transference of biomolecules from platelets to tumor cells promotes tumor heterogeneity and acquisition of aggressive phenotypes

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Introduction

Besides their function in blood homeostasis and immunity, platelets have an essential role in cancer. In fact, an increased platelet count is a hallmark of cancer that is often accompanied by unexplained thrombotic events preceding diagnosis of an occult malignancy. Tumor dissemination relies on the release of circulating tumor cells (CTCs), their ability to escape the immune system and their metastatic potential. Interaction of CTCs with platelets forms aggregates (microemboli) promoting CTC survival although this communication process is still poorly studied. The aim of this project was to understand the impact of this interplay between platelets and tumor cells.

Material and Methods

CTCs were isolated from peripheral blood (10ml) of prostate cancer patients (N=15) using our previously established protocol and standard immunocytochemistry for CD61 was done. Co-culture experiments were done using 10 human cancer cell lines of different tumor types (prostate, lung, colon and breast cancers) and platelets isolated from peripheral blood using a three-centrifugation protocol. Functional assays included: cell tracking, flow-cytometry, cell monitoring assay, wound healing assay, confocal microscopy, electron microscopy and qRT-PCR.

Results and Discussions

Platelets became activated after 1 hour of co-culturing with tumor cells, with a dynamic transference of lipid membrane components to tumor cells including platelets-specific biomarkers (such as PAC-1 or CD61/CD42) as well as from tumor cells to platelets (such as EpCAM). Different exchange mechanisms were observed (direct contact, membrane fusion, platelet internalization and microparticles-dependent interaction) acting at any cellular level (cellular membrane, nuclear membrane, cytoplasm and nucleus). RNA transference was also observed between the two cell types, although at lower levels than lipids transference. Finally, platelets-associated markers (CD61) were detected in CTCs from prostate cancer patients and gene expression data suggested that epithelial-to-mesenchymal transition (EMT) and stemness was induced in cancer cell lines after co-culture with platelets.

Conclusion

Our data showing that platelets alter morphology, function and genetic expression of tumor cells inducing highly dynamic and aggressive phenotypes changes the paradigm of “tumor educated platelets” (TEPs) into “platelets educating tumors” (PETs).

Breaking down functional heterogeneity to immunotherapy using patient-derived organoids

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Introduction

Cellular Immunotherapy (CI) has revolutionized cancer treatment for blood cancers, yet translation of this success to solid tumors remains challenging. Heterogeneity in treatment response across and within patients contributes to this hurdle and complicates effective treatment. This demonstrates an urgent need for technology platforms that adequately model inter and intratumor heterogeneity to unravel key pathways involved in immunotherapy efficacy.

Material and Methods

Here we exploited a dynamic immuno-organoid 3D imaging-transcriptomics platform, BEHAV3D, to study patient and intra-tumor heterogeneity in CI response. By implementing subcloning methods we have generated a biobank of ~150 clonal organoid lines, derived from individual organoids of parental cultures from different breast cancer patients. We exposed individual clones to CIs, including B7H3-CAR T cells and an emerging metabolic-abnormality-targeting T-cell immunotherapy called TEGs. We mapped functional intra-tumoral heterogeneity by assessing clonal response to treatment and transcriptomic heterogeneity through RNA sequencing.

Results and Discussions

We detected a high variation of CI-mediated killing efficacy in cultures derived from 14 breast cancer patients and different targeting kinetics over time. Clonal analyses showed remarkable variation in treatment response in between individual clones and compared to the parental culture, which was retained over multiple passages, implying biological and intrinsic variation in responsiveness. RNA sequencing of top differentially responding clones revealed significant transcriptomic variability between treatment-sensitive and treatment-resistant clones. We thereby identified several candidate genes and pathways overrepresented in sensitive or resistant clones, some of which we validated for their involvement in CI response and others are currently being followed-up. Many of these have to date not been reported to have a role in CI effectiveness and therefore could lead to novel strategies to improve targeting of solid tumors.

Conclusion

By exploiting clonal organoid lines, we developed a scalable platform for unravelling inter- and intratumoral heterogeneity in immunotherapy response and identification of key genes and pathways involved. The resulting insight could be used towards improving cellular immunotherapy effectiveness and predicting solid tumor response.

Unbiased inference of cancer cell states reveals lepidic-to-solid transition as a major axis of transcriptomic heterogeneity in lung adenocarcinoma

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Introduction

Lung adenocarcinoma (LUAD) harbors extensive genetic, epigenetic and transcriptomic intra-tumor heterogeneity. In the clinic, the emergence of histologically distinct patterns of cells leading to spatial intra-tumor heterogeneity has been implicated in disease progression, with lepidic and solid patterns at the two extremes. The coexistence of these patterns in the same tumors prompts the question of how cancer cells progress from an indolent lepidic-like phenotype to the aggressive solid-like state, and which molecular features drive this transition.

Material and Methods

Here, we analyzed the transcriptomic features of 30 individual histologic patterns selected with histologically guided multi-regional sampling from 10 early stage and untreated lung adenocarcinomas. In parallel, we downloaded and re-processed 7 LUAD datasets of single cell RNA sequencing profiles from 87 patients, comprising about 70'000 malignant cells. Using non-negative matrix factorization, we unbiasedly inferred the major transcriptomic axes of heterogeneity between cancer cell states that recurred across patients, and we compared them with the features extracted from the histologic patterns of our cohort.

Results and Discussions

The cancer cell states emerging from the unbiased analysis included previously described axes of heterogeneity such as those related to cell cycle, stress response and interferon. Additionally, a LUAD-specific state characterized by alveolar type II markers was found to be predominant in the lepidic pattern, and progressively lost in more advanced patterns such as acinar and solid. This transition was accompanied by the downregulation of lung lineage transcription factors such as NKX2-1 and FOXA2. On the other hand, the cell cycle axis showed features of increased proliferation in the solid pattern, paralleled by higher activity of MYBL2 and of the stemness factor FOXM1. When tested in independent large-scale datasets such as TCGA, lepidic-driving and solid-driving states were associated with good and poor patient survival rates, respectively.

Conclusion

The histologic patterns of lung adenocarcinoma are characterized by tumor cells of diverse states; the transition from lepidic to solid involves de-differentiation from committed lung epithelial cells and increase in cell proliferation. The prognostic significance of this major axis of LUAD transcriptomic heterogeneity might suggest its blockage and reversion as therapeutic opportunities.

EACR22-0317

ORAL PRESENTATION

Expressed cellular barcoding system reveals differences in clonal growth of basal and luminal human breast tumours

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Introduction

Breast cancer progression and resistance to treatment are driven by a minority of cells that are capable of clonal propagation. However, differences in clonal growth between subtypes of human breast cancers have not been investigated in detail.

Material and Methods

To perform clonal tracking experiments on patient-derived tumour xenografts (PDXs), we have constructed an expressed cellular barcoding system comprised of a semi-random 27 base-pair sequence with an additional 4 base-pair library identifier. Upon lentiviral transduction, the barcode sequence that was inserted into the 3' untranslated region of the fluorescence reporter gene is permanently integrated into host cell DNA and transcribed into mRNA transcripts ensuring that the barcode sequence can be detected by both DNA and RNA sequencing methods.

Results and Discussions

DNA sequencing validation of barcode diversity in the lentiviral plasmid library reveals $\sim 2 \times 10^5$ unique barcode sequences, one-third of which is captured in human breast cancer cells following lentiviral transduction. The sensitivity of clone detection with our approach is 90% and 100% for clones containing 10 cells and 50 cells, respectively. The specificity of detecting clones above our defined signal-noise threshold is 100%. An initial 9 tumours derived from 3 PDX models have been analyzed in this way, demonstrating a clone-initiating cell (CIC) frequency of 1 in 1,000 to 30,000, with each CIC producing a clone of 10 to 60,000 cells. Interestingly, CIC frequency is lower in the basal/triple-negative models with only a few clones dominating the entire tumour compared to a higher CIC frequency and more equal clone representation in the luminal/ER-positive model. Distinct cell clusters were detected based on gene expression profiles obtained from single-cell RNA sequencing of barcoded tumours. Furthermore, distinct clone types were identified as differentially abundant in basal vs. luminal tumours based on their regeneration of cells that express or do not express integrin alpha-6, a cell surface marker known to enrich for cells containing stem cell activity.

Conclusion

In conclusion, we have developed a highly sensitive and quantitative clonal tracking system to integrate clonal growth activity with single cell transcriptomic profiles. We identify differences in clonal growth between basal and luminal subtypes with implications for how clonal propagation contributes to disease progression in these subtypes of human breast cancer.

EACR22-0174

The origin and evolution of pediatric secondary neoplasms

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Introduction

Pediatric cancer patients without known germline predisposing mutations that present with a second malignancy during their young age are rare. When this occurs it is often assumed that the second malignancy could be related to the treatment received for the first one. Other possible explanations for the second tumor include shared early driver mosaic mutations.

Material and Methods

In this study, we investigated the origin and evolution of second neoplasms from eleven pediatric patients at Sant Joan de Déu Barcelona Children's Hospital, through whole-genome sequencing of the primary and secondary tumors and normal blood samples. We investigated the timing of clonal expansion of the secondary neoplasms through treatment-related mutational signatures.

Results and Discussions

We have identified cases of secondary acute myeloid leukemia with clonal treatment-related mutations, pointing to an initiation of leukemogenesis posterior and likely related to the treatment. Solid secondary tumors showed no clonal treatment-related mutations, suggesting the possibility that the clonal expansion of the tumor occurred before the treatment. In a few cases we identified an early mosaic mutation shared by the two tumors that could explain the appearance of two tumors in a patient.

Conclusion

In conclusion, we show that mutational signatures can be used as barcodes to study the origin and evolution of secondary neoplasms. The study of driver mutations also sheds light onto this evolution.

EACR22-0428

OPTIMIZATION OF NONINVASIVE OXIMETRIC IMAGING OF ORTHOTOPIC GLIOBLASTOMA TUMORS

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Introduction

Tumor oxygenation has strong prognostic significance in the outcome of chemotherapy and radiotherapy. Thus, non-invasive methods to measure the partial pressure of oxygen (pO₂) in tumors may have enormous potential for evaluating the efficacy of cancer therapeutics. Electron paramagnetic resonance (EPR), using implanted probe microcrystals containing unpaired electron spins, have been used in preclinical tumor oximetry. The probes allow to obtain the repeated oxygen concentration from the small area around site of implantation. The aim of this study was to optimize the protocol for noninvasive imaging of orthotopic glioblastoma oxygenation using EPR and lithium octa-n-butoxynaphthalocyanine (LiNc-BuO)-based microspheres implanted together with cancer cells.

Material and Methods

In vivo EPR oximetric imaging was conducted with an L-band CW spectrometer (Bruker Elexsys-II E540) in orthotopic glioblastoma GL-261 growing in C57bl/6J mice (N = 20). 5x10⁴ cancer cells, together with 0,5 mg of either bare (N=10) or polydopamine modified (N = 10) LiNc-BuO-based microspheres were inoculated into the center of the mouse brain hemisphere. 2D spatial-spectral oxygen imaging was conducted twice a week since implantation. Additionally, once a week, anatomical MRI measurements were performed using a 9.4T system (Bruker, Germany) and RARE sequence. At the endpoint, tumors were collected and analyzed histologically.

Results and Discussions

The use of LiNc-BuO-based microspheres allows to image spatial heterogeneity of glioblastoma tumors oxygenation. It's a noninvasive way to access absolute concentration of oxygen in tumors not accessible to most oximetric methods. Implantation of a probe together with cancer cells makes the measurements not dependable on coregistration with other modalities because the signal comes only from the area of the tumor. Histology of tumors showed that probe is distributed within tumor tissue. No differences in probe distribution and imaging feasibility were seen between the hydrophobic and hydrophilic microspheres.

Conclusion

The evolution of intratumoral pO₂ heterogeneity in orthotopic glioblastoma tumors was assessed non-invasively with the use of LiNc-BuO-based microspheres and electron paramagnetic imaging.

EACR22-1132

Bevacizumab leaves a photoacoustic fingerprint in breast cancer mouse models

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Introduction

Photoacoustic tomography (PAT) provides a direct readout of tumour haemoglobin (Hb) concentration and oxygenation. This readout could be used to provide an early indication of response to anti-angiogenic drugs, thus optimizing the management of these therapies.

Material and Methods

Nude Balb/c were inoculated with estrogen-independent MDA-MB-231 (n=19) cells. Mice were randomly split into Control (Ctrl) and Bevacizumab (Bev, 10 mg/Kg, IP, weekly) groups. PAT was performed weekly. Oxy- and deoxy-Hb were quantified in the tumour and reference areas. Total Hb (THb) and O₂saturation (SO₂) were calculated. Blood samples and tissues were collected, hypoxia (Hypoxypoint and CA-IX) and vascular density / maturity (CD31 and ASMA) were analyzed by IHC. Circulating levels of human and mouse vascular endothelial growth factor (hVEGF and mVEGF) and Hb were measured.

Results and Discussions

Most of the mice from the Bev group (8/11) showed no improvement in survival. Non-responders (Bev-NR) showed an increase in hVEGF production (247 vs. 330). MDA-MB-231 responders (Bev-R) showed a significant decrease in hVEGF (247 vs. 80). These results indicate that VEGF was only successfully sequestered in a small subset of animals (3/11) and that VEGF overload might be a resistance mechanism.

At the final time point, PAT showed no difference between Ctrl and Bev-NR in THb (7.2 vs. 5.5). THb was found significantly increased in the Bev-R group by PAT (13.9 p-value^{vs.Ctrl}=0.0339; p-value^{vs.Bev-NR}=0.0072) and biochemical measurements. SO₂ showed a

slight but significant decrease between Ctrl and Bev-NR (0.58 vs. 0.48). A decrease was seen in the Bev-R group (0.31) and significantly different from Ctrl and Bev-NR. SO₂ was sensitive to early changes, SO₂ values increased in Ctrl and Bev-NR 48h after enrolment ($p^{\text{Ctrl}}=0.0134$; $p^{\text{NR}}=0.0268$) while Bev-R shows a decreasing trend ($p=0.0649$). Analysis of SO₂ over time shows a significant ($p<0.0001$) change in slope 3 weeks after enrolment in the Bev-R group compared to either of the Ctrl or Bev-NR groups. Our results indicate that PAT SO₂ can differentiate responders at very early stages of the treatment.

Histologically, we observed fewer blood vessels but better structured in Bev-NR than in Bev-R, which showed higher vascular density but also higher hypoxia.

Conclusion

Our results postulates PAT as a low-cost, label-free and non-invasive candidate to monitor response to Bevacizumab over time. Our results also indicate that tumours with functional vasculature may be resistant to Bevacizumab treatment.

EACR22-1232

Clarifying measures of tumour heterogeneity with insights into the phylogenetics of Barrett's Esophagus

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Introduction

Heterogeneity is a pervasive feature of many tumour types, with potential as a biomarker distinguishing low and high risk disease at an early stage. Phylogenetic trees are a key tool in resolving a tumour's heterogeneity and inferring its evolutionary history, allowing us to map the order of mutational events and the background on which they occur. Without phylogenies, we risk using overly simplified models which consider only shared and unique mutations. Barrett's Esophagus (BE) is a highly heterogeneous precursor to Esophageal Adenocarcinoma (EAC), ideal for demonstrating the range of characteristics of heterogeneity within and between tumours. There also exists a significant clinical need for profiling BE cases into risk categories for progression.

Material and Methods

We use 35 whole genome sequenced (WGS) biopsies from ten multi-sampled patients with varying disease stages of BE to (a) describe three levels of phylogenetic heterogeneity, and (b) provide novel insights into this disease state.

We infer multi-sample phylogenies using single nucleotide variants (SNVs) and copy number aberrations (CNAs) via calculation of SNV cancer cell fractions (CCF). Level 1 heterogeneity indicates the presence or absence of multiple phylogenetic trees within a sample set, describing multiple independent or single tumours respectively. Level 2 describes the shape and complexity of an individual phylogeny using a set of metrics - some borrowed from population genomics. Level 3 covers the spatial separation or intermixing of genomically distinct cell populations.

Results and Discussions

On top of known indicators of progression in BE (mutations in TP53 and increased chromosomal instability), our sample set highlights markers of progression in Levels 2 and 3. Level 2 shows significantly longer phylogenetic tree trunks in dysplastic over nondysplastic cases (potentially indicating selection) as well as an increased frequency of single-base substitution (SBS) 18 in dysplastic samples which increases over time. SBS17A and SBS17B are also seen significantly more frequently in subclonal than clonal cell populations

of non-dysplastic tumours. At Level 3, spatial mixing of genomically distinct cell populations is seen significantly more often in dysplastic than non-dysplastic samples.

Conclusion

Phylogenetic tree construction in BE describes three levels of heterogeneity and highlights novel indicators distinguishing dysplastic and nondysplastic cases within Levels 2 and 3. This classification system can be used across tumour types.

EACR22-1280

Characterising DCC subpopulations for impact on prognosis and understanding of NSCLC evolution

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Introduction

Lung cancer is still the most common cause of cancer-related death worldwide due to early dissemination of tumor cells and subsequent metastasis formation. We therefore tested the prognostic impact of disseminated cancer cells (DCCs) spread to regional lymph nodes (LN) and interrogated single cell genomic data for insights into systemic cancer progression.

Material and Methods

We collected LN samples from 122 NSCLC patients and compared three different approaches for the detection of early lymphatic cancer spread: (i) 2952 LNs was examined by routine histopathology (HP) based on H&E stained tissue sections; (ii) half a LN from 220 of these samples was disaggregated into single cell suspension and immunocytology (IC) using the anti-EpCAM antibody BerEP4 was performed; and (iii), 47 samples of these bisected LNs were also analyzed by double immunofluorescence (IF) against EpCAM and Cytokeratins 8/18/19 (CK). Positive cells were isolated and submitted to Whole Genome Amplification.

Results and Discussions

IC identified 22% positive patients as opposed to 4.5% by HP, revealing a much higher sensitivity of IC ($p < 0.0001$) using paired halves of LNs. Assessment of all available LNs of the same patients by HP uncovered a proportion of patients escaping detection of lymphatic tumor spread by IC alone. A combined LN status of IC and complete HP on a larger cohort of patients outperformed all risk factors in multivariable analysis for prognosis ($p < 0.001$). Upon IF double staining, we found a high correlation between EpCAM and CK positive cell numbers. In further analyses, both IF markers correlated with a reduced OS and PFS. Despite the low numbers of samples analyzed so far, first data indicate that double IF may further improve diagnostic precision. Molecular analysis revealed that DCCs differ from matched primary tumors and acquire genetic alterations during colony formation within the LN.

Conclusion

IC in single-cell approaches improves diagnostic accuracy in NSCLC patients when combined with HP, reduces workload, and enables molecular characterization of lymphatically spread cancer cells. Moreover, LN-IC enables investigation of multiple DCC subpopulations and gives insight into phenotypic plasticity of DCCs highlighting opportunities to better identify and treat patients at risk for metastatic recurrence. The prognostic suitability of double IF as well as of copy number alterations and mutations in single DCCs is currently under investigation.

EACR22-0787

Development of a clinically relevant multi-parametric 3D drug screening assay that enables differentiation of cytotoxic/cytostatic responses and treatment resistant subclones at single-organoid level.

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Introduction

Over the past years, it has been shown that the survival of treatment resistance subclones drives tumor relapse and metastasis in pancreatic ductal adenocarcinoma (PDAC). Recently, patient-derived tumor organoids were developed to study this clonal heterogeneity and inter-patient differences in response rate. So far, the CellTiter-Glo® 3D viability assay has been extensively used to quantify the viability of tumor organoids, however, it lacks the ability to distinct a cytotoxic from a cytostatic response. Moreover, this non-specific metabolic readout is not able quantify single-organoid responses, thereby missing the information of resistant organoid clones that could drive therapeutic resistance. Accordingly, there is a strong interest in developing more clinically relevant readouts since current assays only extract a fraction of information that organoids could potentially provide us.

Material and Methods

As a proof-of-concept, we included both treatment naïve (N=4) and pre-treated (resistant) (N=4) PDAC patients. For each patient, we seeded 200 organoids per well (in a 384-well plate) and subsequently treated them with clinically relevant concentrations of FOLFIRINOX, Gemcitabine/Nab-Paclitaxel and the corresponding monotherapies. In order to quantify single-organoid responses, we have further optimized our previously developed deep-learning-based organoid analysis platform (Orbits), which is able to kinetically distinguish cytotoxic from cytostatic responses from live-cell imaging data.

Results and Discussions

We observed clear differences in terms of cytostatic and cytotoxic effects between each individual patient, highlighting the heterogeneity of response. Moreover, these responses were in concordance with the clinical observations. For instance PDAC044 showed a durable clinical response to Gemcitabine/Nab-Paclitaxel for 3 years, which was recapitulated *in vitro* by a high cytotoxicity and absence of resistant subclones. On the other

hand, PDAC068 (FOLFIRINOX resistant) showed a high presence of non-responsive organoid clones (even at extremely high concentrations of FOLFIRINOX).

Conclusion

We have successfully developed a novel multi-parametric organoid readout that is able to take the subclonal variance (heterogeneity) of treatment response into account. Furthermore, our data addresses the need for including inter-clonal organoid responses, since this could result in a more accurate prediction of the patient-specific clinical outcome.

EACR22-1049

Is there the entire the message in the bottle? Clonal representativity of liquid biopsies in triple negative breast cancer revealed by genetic barcoding

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Introduction

The use of liquid biopsy to obtain clinically relevant information is emerging as a powerful source of diagnostic tools for precision medicine. Despite its huge potential, it is unclear whether detectable circulating biomaterials (CBMs) can efficiently depict the complete heterogeneity of a tumor rather than just few subclones. This is a primary concern, especially for tumors characterized by a marked intra-tumoral heterogeneity such as triple-negative breast cancer (TNBC). In this study we took advantage of genetic barcoding to investigate the clonal representativity of different CBMs - circulating tumor cells (CTCs), extracellular vesicles RNA (evRNA) and circulating tumor DNA (ctDNA) - in patient-derived TNBC xenografts.

Material and Methods

Primary cultures of patient-derived TNBC cells, obtained by dissociation of resected tumors, were transduced with a library of vectors carrying degenerated sequences (barcodes) to univocally label each cell. Barcoded cultures were xenotransplanted in immunodeficient mice. Once tumors reached a size of 300 mm², mice were sacrificed, tumor masses were explanted and CBMs were retrieved as follows:

CTCs) negative selection enrichment from blood followed by recovery with DEPArray.

evRNA) ultracentrifugation of plasma followed by RNA Purification.

ctDNA) direct purification from plasma.

Clonal analysis of each sample was performed by Next Generation Sequencing of barcodes using Illumina NextSeq 500 platform. Bioinformatic analyses were performed using in-house developed software.

Results and Discussions

Comparative clonal analysis of tumor masses and their respective liquid biopsies, obtained from 6 distinct TNBC patients, revealed that ctDNA faithfully renders the primary tumor heterogeneity: main clones are efficiently detectable, and even subclones as few as 2% of the tumor mass have a 50% probability of detection. Analysis of CTCs and evRNA is still ongoing, while preliminary results suggest these two CBMs as markedly less representative.

Conclusion

In this study, for the first time, clonal representativity of liquid biopsies has been systematically investigated by genetic barcoding. Thanks to our experimental design we were able to empirically measure the relationship between clone size and probability of detection in liquid biopsy with depth and precision levels almost impossible to obtain elsewhere. Our evidence suggests that small subclones are efficiently identifiable, and therefore ctDNA liquid biopsy can efficiently address the high intra-tumoral heterogeneity of TNBC.

EACR22-1223

Spatial transcriptomics as a powerful tool for studying glioblastoma heterogeneity

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Introduction

Glioblastoma (GBM) is the most common and deadliest brain tumor. The high tumoral heterogeneity and therapy resistance development result in a poor prognosis for GBM patients. Lately, it has been shown that the tumor microenvironment (TME) has an indispensable role in GBM progression. However, an exhaustive study of the patient samples is needed to get the most information about the tumor components and their interactions. We studied the intratumoral heterogeneity of one GBM sample using the spatial transcriptomics technique. It allowed us to visualize the distribution of gene expression on the tumor section and correlate it to the tissue structure.

Material and Methods

GBM patient tissue was analyzed by the Visium Spatial Gene Expression Kit (10x Genomics). Briefly, 10-µm sections of fresh frozen tissue were placed on the special slide, fixed with methanol, and stained with hematoxylin and eosin. After image acquisition, tissue was permeabilized to release the mRNA and capture it onto primers on the slide. After the reverse transcription, second strand DNA synthesis and denaturation, cDNA was transferred to the tube for amplification and library construction. Sequencing was done on a lane of a HiSeq 4000 (Illumina) flowcell. Raw FASTQ files and histology images were aligned against Human Reference Genome (GRCh38-2020-A) with the Space Ranger software, and downstream analysis was done in Seurat R package. Differentially expressed genes were extracted for each spatial cluster and used to make a functional analysis.

Results and Discussions

The histology of the studied GBM sample was homogeneous, except for one necrotic zone. However, spatial expression analysis revealed six transcriptional clusters and their distribution within the tissue. Functional analysis showed that different pathways were enriched among clusters, and their position in relation to the necrotic zone corresponded to the GBM progression. The technique also allowed the visualization of genes of interest on

the tissue, so the identification of GBM molecular subtypes was possible. Different subtypes existed within the sample, depending on the transcriptional cluster, suggesting the high heterogeneity of this tumor.

Conclusion

Spatial transcriptomics is a powerful technique for simultaneous studying of tissue structure and gene expression, contributing to a deeper understanding of TME. Since high molecular heterogeneity was observed among spatial clusters in one GBM sample, the bulk study of the tumor could mask relevant information about GBM disease.

EACR22-1281

Disseminated cancer cells isolated from bone marrow of DCIS patients harbor genomic aberrations

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Introduction

About 3% of patients with exclusively non-invasive in situ lesions of the breast will die from metastatic breast cancer. Furthermore, while increasing tumor size above 1 cm in diameter in invasive breast cancers is associated with increasing risk to die from breast cancer, this is not observed for lesions below 1 cm, generating questions about the molecular characteristics of metastatic cells from very early-stage lesions. Since we and others previously found that in ductal carcinoma in situ about 13-20% of patients harbor cytokeratin-positive cells, bona fide disseminated cancer cells (DCC), in bone marrow, we aimed to develop a method to detect, isolate and characterize such very rare cells. The challenge here was that although thousands of cytopins from past decades are available, no such method exists

Material and Methods

The optimized protocol comprised of a combination of laser-assisted ablation of surrounding cells, manual micromanipulation of the cell of interest, whole genome amplification, quality control and low coverage sequencing for copy number alterations. The protocol was validated on frozen cytopins prepared with MCF-7 cells spiked in blood mononuclear cells before application to bone marrow DCCs from DCIS patients. We then screened frozen archived bone marrow cytopins of DCIS patients treated at the University hospital in Tübingen between 2001 and 2016 for DCCs.

Results and Discussions

Out of 627 DCIS patients, 72 (11%) patients had detectable DCCs from which only 8 (1.2%) patients harbored 7-10 DCCs in their bone marrow, making these archived samples extremely precious. All 32 test cells, including MCF7 and T cells isolated by laser assisted ablation showed good DNA quality. CNV analysis showed balanced profile for T cells and expected aberrant profile for MCF7 cells. We found genomic aberrations in 15/18 (83%) DCCs isolated from 8 patients. Heterogeneity was observed in DCIS DCC genomes across patients and within a single patient. Additionally, DCCs shared only a few aberrations with their matched DCIS.

Conclusion

Cytokeratin-positive cells from patients with in situ carcinoma display multiple copy number alterations. The method presented here demonstrates that decade-old archived cytospin samples can be used to study putative metastasis founder cells. Application of the method to the complete cohort may enable detection of alterations that are linked with the rare lethal progression of DCIS.

EACR22-1111

ORAL PRESENTATION

Genomic hallmarks and therapeutic implications of cellular quiescence in cancer

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Introduction

Therapy resistance in cancer is often driven by a subpopulation of cells that are temporarily arrested in a non-proliferative, quiescent state, which has been recently linked with drug-tolerant persister cell phenotypes. Despite its crucial role, quiescence remains poorly characterised in cancer due to the lack of consensus markers and precise ways to measure this state. There is a great need to elucidate the drivers and context in which quiescence emerges, as this would help identify therapeutic vulnerabilities of such cells within the tumour.

Material and Methods

We present an integrated computational framework for evaluating tumour cell quiescence and its genomic triggers across a variety of cancers in bulk and single cell data. We have developed a transcriptomic signature of quiescence, which we have extensively validated in single cell datasets and cancer cell lines. Using this signature, we profiled proliferation/quiescence decisions in multiple bulk and single cell cancer datasets, and employed ensemble regression modelling to these decisions with the presence of mutational signatures and genomic alterations of cancer drivers.

Results and Discussions

We show that quiescence preferentially emerges in the context of more stable, less mutated genomes which maintain TP53 integrity and lack the hallmarks of DNA damage repair deficiency, while presenting increased APOBEC mutagenesis. Using machine learning, we uncover several novel genomic dependencies of this process that could be exploited to maintain or promote exit from this state. In particular, we propose and validate the role of the centrosomal gene *CEP89* as a modulator of proliferation/quiescence capacity. We also use single cell data to demonstrate quiescence is a key resistance mechanism to a wide range of cancer therapies. Finally, we discuss the implications that quiescence has to the broad reorganisation of the tumour tissue and interactions with the microenvironment, as inferred from tumour-immune interaction analyses at single cell level, as well as deep learning and graph reconstruction in histopathology slides.

Conclusion

Overall, our study provides, for the first time, a pan-cancer view of cellular quiescence and its evolutionary constraints. We highlight novel mutational dependencies that could potentially be exploited either for the long-term maintenance or for exit from quiescence. We propose a quiescence signature which can be robustly measured in bulk tissue or single cells, and could inform therapeutic strategies or risk of relapse.

Castration-induced down-regulation of SPARC in stromal cells drives neuroendocrine differentiation of prostate cancer

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Introduction

Fatal neuroendocrine differentiation (NED) of castration-resistant prostate cancer is a recurrent mechanism of resistance to androgen deprivation therapies (ADT) and anti-androgen receptor pathway inhibitors (ARPI) in patients. The design of effective therapies for neuroendocrine prostate cancer (NEPC) is complicated by limited knowledge of the molecular mechanisms governing neuroendocrine differentiation. The paucity of acquired genomic alterations and the deregulation of epigenetic and transcription factors suggest a potential contribution from the microenvironment. In this context, whether ADT/ARPI induces stromal cells to release molecules promoting neuroendocrine differentiation and the underlying molecular networks are undefined.

Material and Methods

Here, we utilized transgenic and transplantable mouse models and co-culture experiments to unveil a novel tumor-stroma crosstalk that is able to induce neuroendocrine differentiation under the pressure of androgen deprivation. Translational relevance of results was proved through analyses of human deposited data set and patient-derived tumor specimens.

Results and Discussions

We found that upon castration tumor cells triggers a cascade of molecular events that cause the down-regulation of SPARC in nearby stromal cells and, in turn, neuroendocrine differentiation of adenocarcinoma cells. In detail, castration induced the upregulation of GRP78 in tumor cells, which in turn triggers miR-29b production. Then, miR-29b released by tumor cells mediates down-regulation of the matricellular protein SPARC in the nearby stroma. Consequently, SPARC down-regulation enabled stromal cells to release IL-6, a known inducer of neuroendocrine differentiation. A drug that targets GRP78 blocked neuroendocrine differentiation in castrated mice. In a public, human NEPC gene expression data set we found that *Hspa5* (encoding for GRP78) positively correlates with hallmarks of neuroendocrine differentiation. Finally, we confirm (i) GRP78 up-regulation in tumor cells and (ii) SPARC down-regulation in stroma of prostate cancer specimens in patients developing focal NEPC after ADT.

Conclusion

We unveil a tumor-stroma crosstalk promoting neuroendocrine prostate cancer in resistance to hormone therapy, and identify GRP78 gain in tumor and SPARC loss in stroma as potential therapeutic target and diagnostic biomarker, respectively.

EACR22-0382

A mechanistic model captures the emergence and implications of non-genetic heterogeneity and reversible drug resistance in ER+ breast cancer cells

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Introduction

Resistance to anti-estrogen therapy is an unsolved clinical challenge in successfully treating ER+ breast cancer patients. Recent studies have demonstrated the role of non-genetic (i.e. phenotypic) adaptations in tolerating drug treatments; however, the mechanisms and dynamics of such non-genetic adaptation remain elusive. Here, we investigate coupled dynamics of epithelial–mesenchymal transition (EMT) in breast cancer cells and emergence of reversible drug resistance.

Material and Methods

We employ an integrated approach of mechanism-based mathematical modeling and transcriptomic data analysis from preclinical/clinical datasets to identify how EMT and resistance to tamoxifen dynamics can influence each other. Next, we decipher the role of tamoxifen-induced and stochastic cell-state switching in a population dynamics model, and propose combinatorial strategies to combat non-genetic heterogeneity in the context of tamoxifen resistance in ER+ breast cancer.

Results and Discussions

Our mechanism-based model for underlying regulatory network reveals that these two axes can drive one another, thus enabling non-genetic heterogeneity in a cell population by allowing for six co-existing phenotypes: epithelial-sensitive, mesenchymal-resistant, hybrid E/M-sensitive, hybrid E/M-resistant, mesenchymal-sensitive and epithelial-resistant, with the first two ones being most dominant. Next, in a population dynamics framework, we exemplify the implications of phenotypic plasticity (both drug-induced and intrinsic stochastic switching) and/or non-genetic heterogeneity in promoting population survival in a mixture of sensitive and resistant cells, even in the absence of any cell–cell cooperation. Finally, we propose the potential therapeutic use of mesenchymal–epithelial transition inducers besides canonical anti-estrogen therapy to limit the emergence of reversible drug resistance.

Conclusion

Our results offer mechanistic insights into empirical observations on EMT and drug resistance and illustrate how such dynamical insights can be exploited for better therapeutic designs.

EACR22-0740

Longitudinal analysis of recurrent Glioblastomas with long-term relapse emphasizes concerted contribution to Tumor Growth Factor β (TGF- β) pathway enrichment at multi-OMICS level

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Introduction

Glioblastoma (GBM, IDH wild type) remains a therapeutic challenge due its extensive plasticity, heterogeneity, limited therapeutic options, and inevitable relapse. Accordingly, elucidating underlying molecular mechanisms has been a major focus of research, with the hope of new therapeutic interventions overcoming resistance.

Material and Methods

Matched retrospective pairs of therapy-naïve and post-therapeutic recurrent GBM with more than one year of relapse period were included in a mono-centric study and whole exome sequencing combined with mRNA and microRNA expression profiling was performed with the aim to identify processes that are altered in recurrent GBM.

Results and Discussions

Somatic mutational analysis demonstrated that tumor relapse follows an early divergence evolution pattern that is distinct from initial tumor. Investigating the effect of therapy on the recurrent tumors showed evidence for increased SBS11 and decreased SBS1 consensus mutational signatures. mRNA and microRNA expression profiling, revealed high tumor plasticity. In particular, we found evidence for TGF- β pathway enrichment owing to upregulation of *ALK7* and *LTBP1* and downregulation of *LEFTY2* at the mRNA level, and enhanced Nuclear Factor Kappa B signaling at the microRNA level. *ALK7* and *BCL2* were both upregulated, as confirmed at expression level using our cohort's tissue micro array and immunohistochemistry. Importantly, we found that *ALK7* upregulation correlates positively with *BCL2* and negatively with pro-apoptotic proteins, *BAX* and *BAK* in TCGA-GBM dataset, suggesting that the canonical *ALK7* role in apoptosis induction is uncoupled in recurrent tumors; instead, *ALK7* drives the TGF- β cascade to fuel tumor stemness through EMT.

Conclusion

Integrated longitudinal analysis indicated that instead of one specific secondary genomic alteration to be responsible for resistance, differential clonal architecture of recurrent tumors contribute to enrichment of pathways that collectively drive tumor stemness and EMT, an incident that is essentially driven by TGF- β pathway. This supportive role of TGF- β signaling in tumor plasticity, and therapy resistance, supports direct clinical implication of TGF- β inhibitors and their potential use in combination therapy to interfere with chemoradiation resistance.

EACR22-0509

ADK-VR2, a cell line derived from a treatment-naïve patient with SDC4-ROS1 fusion primarily crizotinib-resistant NSCLC: a novel preclinical model for new drug development of ROS1-rearranged NSCLC

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Introduction

ROS1 fusions are driver molecular alterations in 1-2% of non-small cell lung cancers (NSCLCs). Several tyrosine kinase inhibitors (TKIs) have shown high efficacy in patients whose tumors harbour a ROS1 fusion. However, the limited availability of preclinical models of ROS1-positive NSCLC hinders the discovery of new drugs and the understanding of the mechanisms underlying drug resistance and strategies to overcome it.

Material and Methods

The ADK-VR2 cell line was derived from the pleural effusion of a treatment-naïve NSCLC patient bearing SDC4-ROS1 gene fusion. The sensitivity of ADK-VR2 and its crizotinib-resistant clone ADK-VR2 AG143 (selected in 3D culture in the presence of crizotinib) to different TKIs was tested in vitro, in both 2D and 3D conditions. Tumorigenic and metastatic ability was assessed in highly immunodeficient mice. In addition, crizotinib efficacy on ADK-VR2 was evaluated in vivo.

Results and Discussions

ADK-VR2 cell growth was partially inhibited by crizotinib in vitro. On the contrary, the treatment with other TKIs, such as entrectinib and DS-6051b, did not result in cell growth inhibition. Lorlatinib showed dramatically different efficacy, depending on the cell culture conditions. In 3D culture, ADK-VR2 growth was indeed almost totally inhibited by lorlatinib, compared to 2D culture, in which the TKI showed no efficacy. The clone ADK-VR2 AG143 showed higher resistance to crizotinib treatment in vitro, compared to its parental cell line, in both 2D and 3D cultures. Similarly to ADK-VR2, ADK-VR2 AG143 growth was strongly inhibited by lorlatinib in 3D conditions. Nevertheless, ADK-VR2 AG143 sphere formation was less affected by TKIs treatment, compared to the parental cell line. In vivo experiments highlighted the high tumorigenic and metastatic ability of ADK-VR2 cell line, which, once injected in immunodeficient mice, gave rise to both spontaneous and experimental lung metastases. In addition, ADK-VR2 tumor growth was significantly reduced but not eradicated by crizotinib. Additionally, the crizotinib-resistant clone ADK-VR2 AG143 showed a slower growth in vivo, compared to ADK-VR2, suggesting a role for crizotinib in modulation of stemness and growth.

Conclusion

The ADK-VR2 cell line is a promising NSCLC preclinical model for the study of novel targeted therapies against ROS1 fusions and the mechanisms of resistance to TKI therapies.

EACR22-0973

Isolation of radio- and chemo-resistant stem-like cells from human glioblastoma

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Introduction

Glioblastoma (GBM), the most aggressive and heterogeneous brain tumor, almost invariably relapses within a year, despite surgical resection and treatment with the alkylating agent temozolomide (TMZ) combined with ionizing radiations (IR). GBM stem-like cells (GSCs) have been recognised as responsible for GBM recurrence, but the mechanisms associated with their therapeutic resistance are poorly understood. Moreover, it is still debated whether, in the primary tumor, distinct GSCs can coexist and differently contribute to relapse. To tackle this problem, we developed a protocol for selecting GSCs resistant to conventional therapies that should represent those driving tumor recurrences.

Material and Methods

Conventionally, GSCs are isolated from a single GBM bioptic sample and grown as neurospheres (NS) in a standard medium containing EGF and bFGF. However, the limited sample size usually does not recapitulate the complex subclonal composition of the entire tumor. To obtain NS cultures representative of the different subclones coexisting in the original GBM and resistant to therapeutic pressures, we modified the derivation protocol by (i) starting from ideally whole GBM cell populations, removed by ultrasonic surgical aspiration; (ii) culturing cells in a standard medium supplied with PDGFBB and HGF, which promote cell survival and treatment tolerance; and (iii) applying therapeutic pressures with TMZ and/or IR. From the same GBM we thus obtained multiple cultures whose genetic, molecular and biological features were compared.

Results and Discussions

From a series of GBMs we derived 11 'NS families', each including an untreated control NS (NS-CTRL) and one or two NS selected by treatment with TMZ or IR (NS-TMZ and NS-IR respectively). All NS belonging to the same family showed self-renewal ability but displayed genetic and phenotypic differences, including distinct karyotypes, genetic alterations, transcriptional profiles, clonogenic and differentiative properties, proliferative requirements and response to conventional treatments. The causal relationships between NS features and therapeutic resistance are under investigation.

Conclusion

From the same GBM, we can isolate different GSC subclones characterized by genetically and phenotypically distinct features and displaying different sensitivity to treatment. These GSCs likely include those driving GBM recurrence.

EACR22-1249

Comparison of Patients' Parental and Osteosarcoma Stem Cells Treated in vitro with Ascorbic Acid – viability, differentiation and metabolic phenotype

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Introduction

Osteosarcomas are bone tumors of mesenchymal origin. They contain a subpopulation of osteosarcoma stem cells. To isolate osteosarcoma cells, the biopsy specimen is digested

and plated to start parental cell culture. Osteosarcoma stem cells are selected in restrictive conditions using low attachment dishes to grow spheroids for 2 generations. We wanted to characterize these 2 cell types and determine their sensitivity to ascorbic acid treatment. Therefore, we analyzed markers of stem cells and osteoblasts, viability and metabolic phenotype after ascorbic acid treatment.

Material and Methods

Tumor cells were isolated from patient for obtaining primary parental culture. The cells were passed through 2 generations of spheroids which we used to obtain cancer stem cells. We determined the markers of stemness and differentiation using the qPCR method. Parental OS and OS-CSC were treated with AA (24h) in conc. 1-30 µg/mL for the MTT assay. IC₅₀ value was calculated. Bioenergetic profiling was performed using Seahorse XFe24. 24 h prior to measuring, cells were exposed to 10mM AA, while controls were left untreated. Assay results were analysed with Wave.

Results and Discussions

The increase expression of stem cell marker SOX2 is detected in OS-CSC in comparison with parental. On contrary, the expression of differentiation markers alkaline phosphatase, beta-glycerophosphate and collagen type I is higher in parental than OS-CSC. Decrease in viability of parental cells was at concentration of 20mM, while IC₅₀ was at 17.1mM. Strongest effect of AA was at concentration 15 and 20 mM where OS-CSC cells show decrease in viability indicating slight sensitivity compared to parental cells. IC₅₀ for this OS-CSC cell was 15.5 mM. From 20 to 30 mM almost no live cells were recorded for either of the 2 groups. Parental cells phenotype map shows a decrease in stressed OCR and an increase in stressed ECAR values in treated comparing to the untreated cells. Baseline values were almost equal for treated versus untreated cells. OS-CSCs treated with 10mM AA caused slight decrease in baseline values in both OCR and ECAR and large decrease in stressed OCR values in treated comparing to untreated.

Conclusion

OS-CSCs showed the highest sensitivity to the AA treatment among tested cell types. Metabolic rate was reduced in both normal and tumor cells incubated for 24h in 10mM AA in vitro. Metabolic tested values showed that OS-CSCs can potentially use both glycolysis and oxidative phosphorylation, and can turn to glycolysis in unfavourable conditions.

Tumour Immunology

EACR22-0801

ORAL PRESENTATION

The glyco-code in colorectal cancer immunoediting: glycans as novel immune checkpoints

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Introduction

Colorectal cancer (CRC) is one of the major cause of morbidity and mortality worldwide, remaining in the top 3 of the most commonly diagnosed and deadly cancers in the world. Modifications of the cellular glycosylation signature are a hallmark of malignant transformation and cancer progression. Indeed, tumor growth has been described to be accompanied by dramatic changes in the cellular glycome, such as the aberrant expression of *MGAT5*-mediated β 1,6GlcNAc branched *N*-glycans structures (Pinho&Reis, Nature Rev Cancer 2015). Some mechanisms used by tumor cells have been described to elude immunosurveillance and to escape antitumor immune response. However, the role of changes in the cellular glycome in cancer immune-editing, particularly the role of the pro-tumoral branched *N*-glycan structures in cancer immune evasion remains to be clarified.

Material and Methods

A large cohort of CRC samples were glycophenotyped using specific lectin histochemistry and glycogene transcription. Further, human/murine gastrointestinal cancer cell lines were glycoengineered by *MGAT5* gene overexpression or knockout (CRISPR-Cas9) and co-cultured with human/mice-derived PBMC; DCs and T cells, being evaluated the immune profile and cytokine release. CRC mouse models (*Apc*^{Min/+}; *VCrMsh2*^{LoxP}) were crossed with *Mgat5*^{-/-} mice and the tumor immune response were characterized by FACS and ELISA.

Results and Discussions

Our results showed that along colorectal carcinogenesis there is an abnormal overexpression of β 1,6GlcNAc branched *N*-glycans by cancer cells that follow the same tendency as FoxP3-expressing cells. *Ex vivo/in vivo* experiments showed that branched *N*-glycans structures are used by CRC cells to escape immune recognition, instructing the creation of immunosuppressive network through inhibition of IFN- γ production. Glycoengineered mouse models that spontaneously develop CRC and with absence of *Mgat5*-mediated branched *N*-glycans (*Apc*^{Min/+} and *VCrMsh2*^{LoxP} *Mgat5*^{-/-} mice) developed a lower number of tumor lesions with lower size when compared with controls. The removal of this abnormal “glycan-mask” was demonstrated to expose immunogenic glycans that potentiate immune recognition, resulting in an effective antitumor immune response.

Conclusion

We revealed a glycoimmune checkpoint in CRC, highlighting the therapeutic efficacy of its deglycosylation to potentiate immune recognition and, thus, improving cancer immunotherapy (Silva&Fernandes *et al*; Cancer Immuno Res 2020).

EACR22-1161

ORAL PRESENTATION

Multi-omic characterisation of B cell immunosurveillance in metastatic breast cancer

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Introduction

B and T cells are key components of the adaptive immune system and play an important role in anti-tumour immunity. We aimed to characterise the nature of B cell immunosurveillance in metastatic breast cancer, as well as the relationship between tumour-infiltrating B cell populations with tumour clonal architecture.

Material and Methods

27 metastatic tumour samples obtained from 8 patients with metastatic breast cancer at the time of a warm autopsy were molecularly profiled by whole exome, RNA, B cell (BCR) and T cell receptor (TCR) sequencing and their histological architecture analysed using digital pathology. The mutational and neoantigenic landscapes of these tumours were characterised and correlated with spatio-migratory mapping of B and T cell clones across metastatic sites.

Results and Discussions

BCR and TCR α /TCR β clonal structures were significantly correlated across different metastatic sites, indicating shared determinants of B and T cell infiltration, selection and activity within tumours. BCR and TCR VDJ phylogenetic trees across metastatic sites recapitulated tumour mutational phylogenetic trees, indicating that both B cell and T cell responses co-evolve with metastatic genomes, a finding which has not been previously reported. Notably, the tumour BCR clonal structure was highly correlated with the tumour MHC class II neoantigen landscape.

BCR clones performing immunosurveillance across metastatic sites were distinct from site-specific private clones and were clonally expanded with high rates of class switch recombination (CSR) and somatic hypermutation (SHM), indicating that these clones were antigen experienced. Higher levels of hypoxia within the tumour were associated with decreased B cell CSR and SHM, indicating that hypoxia is a mediator of B cell activation.

Finally, through *in silico* BCR spatio-migratory mapping and network analysis, we observed that lymph nodes are key sites of B cell clonal diversification and expansion. Only a small selection of these clonal BCR variants perform immunosurveillance across other sites which can be predicted from the centrality of the BCR with respect to the overall clonal structure. There is minimal additional diversification of BCRs in non-lymph node sites.

Conclusion

We present an integrated BCR, TCR and tumour genome analysis in metastatic breast cancer that shows that the adaptive immune response co-evolves with metastatic tumours and sheds light into the dynamics and predictability underlying B cell metastatic immunosurveillance in breast cancer.

EACR22-0913

ORAL PRESENTATION

Increased proteasome activity defines exhausted T cells in the tumour microenvironment

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Introduction

Tumour infiltrating lymphocytes (TILs) must exert their anti-tumour activity in the tumour microenvironment (TME), which in turn promotes T cell exhaustion (T_{EX}) limiting the success

of cancer immunotherapies. The identification of novel biomarkers of exhausted T cells would offer genuine therapeutic opportunities, broadening the scope of cancer immunotherapy in solid tumours.

Material and Methods

Comprehensive single-cell transcriptomic analyses of tumour-infiltrating T cells from 17 different tumour types (n=77 patients) revealed a strong upregulation of proteasomal genes in exhausted T cells. Multi-colour flow cytometry was used to validate these findings *in vitro* in human T cells, and *in vivo* in mouse models. Proteasome modulators (bortezomib, TWS119) were used to explore the pharmacologic potential of this novel biomarker.

Results and Discussions

Single-cell transcriptomics and bioinformatic analyses revealed an enrichment in genes encoding several subunits of the proteasome in exhausted T cells compared to other TILs. T_{EX} is characterized by accumulation of damaged proteins as a result of pathological levels of reactive oxygen species (ROS) and hypoxia in the TME. We generated a proteasome score that strongly correlates with signatures of T_{EX} and ROS in TILs. The ubiquitin-proteasome system is a pathway that maintains proteostasis and cellular fitness through the clearance of ROS-induced damage in the T cell proteome. A variety of assays demonstrated increased proteasome activity in human and mouse exhausted T cells. In this context, pharmacological activation of the proteasome might contribute to alleviate the proteotoxic stress suffered by exhausted T cells and preserve the effector function of TILs.

Conclusion

We found that exhausted T cells in the TME are characterised by increased expression and activity of the proteasome. Modulation of proteasomal activity is a novel strategy to prevent exhausted T cell dysfunction promoted by ROS and hypoxia in the TME of solid tumours.

EACR22-0180

Fibroblast-regulated immune clearance of pancreatic cancer cells

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Introduction

Cancer associated fibroblasts (CAFs) are one of the main components of the tumor microenvironment in pancreatic cancer. CAFs have been classically linked to a tumor promoting effect by increasing the tumor growth, causing immunosuppression and promoting metastasis. However, genetic and pharmacological intervention studies have revealed the existence of both tumor promoting and suppressive CAF functions. We have recently described two fibroblast subpopulations with distinct functions. Specifically, whereas one of the fibroblast populations was tumor permissive, the other population was highly tumor suppressive, an effect that depends on functional innate and adaptive immunity. However, the specific mechanism whereby CAF populations coordinate permissive and restrictive functions needs further characterization.

Material and Methods

Here we identify transcriptional regulators of tumour restrictive fibroblasts. We used causal network analysis of different RNA sequencing datasets to identify upstream regulators of inflammatory mediators. Potential hits were validated by mining scRNAseq datasets and fibroblast knockout clones for the individual transcription factors were generated afterwards.

Finally, *in vitro* assays and mouse models were used to assess the importance of these transcription factors for inflammatory signaling and tumor development.

Results and Discussions

Ingenuity pathway analysis indicated that CCAAT-enhancer-binding proteins (CEBP) are key upstream regulators of inflammatory gene expression in tumor restrictive fibroblasts. CEBP transcription factors displayed bimodal expression in fibroblasts of different scRNAseq datasets and CEBP^{pos} fibroblasts showed an increased expression of inflammatory mediators. CRISPR/Cas9-mediated knockouts followed by qPCR analysis reveal that CEBP-transcription factors control inflammatory gene expression in fibroblasts *in vitro*.

Subcutaneous co-injection of pancreatic cancer cells together with control CRISPR manipulated tumor restrictive fibroblasts efficiently control tumor growth. However, co-injection of tumor cells and CEBP-KO fibroblasts altered immune cell infiltration to formed immune cold tumors, with concomitant loss of tumor control.

Conclusion

This study demonstrates that inflammatory regulation of tumour restrictive fibroblasts is critical for immune cell recruitment and restriction of tumor growth. CEBP-signalling is one of the main drivers for this signaling in fibroblasts and it is necessary for the tumor suppressive effect of fibroblasts.

EACR22-0268

EXPLORING THE APPLICABILITY OF CX3CR1 ANTAGONISM TO PROMOTE NK CELL MIGRATION TOWARDS THE TUMOUR IN OBESITY ASSOCIATED CANCER.

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Introduction

Oesophageal adenocarcinoma (OAC) is an aggressive obesity-associated cancer, with a dismal 5-year survival rate of <20%. OAC patients face poor treatment response rates of <30% and urgently require new therapeutics. Our group have previously shown fractalkine drives natural killer (NK) cell migration to omentum in OAC patients, where their viability and function is altered. We propose that targeting the CX3CR1:Fractalkine pathway holds therapeutic potential in OAC.

Material and Methods

NK cells were treated with OAC patient-derived adipose conditioned media (ACM) +/- CX3CR1 antagonist E6130 or fractalkine and/or IL-15. Their chemotaxis towards tumour conditioned media (TCM) was measured. NK cells were treated with fractalkine and/or IL-15 and their death receptor ligand and adhesion molecule expression profiles were analysed by flow cytometry. TRAIL, FasL, $\alpha 4\beta 7$ and L-selectin expression was profiled on NK cells from OAC patient blood, omentum and tumour.

Results and Discussions

Culture in ACM significantly decreased NK cell migration towards TCM ($p=0.02$). Pre-treatment with E6130 reversed the modulatory effects of ACM on NK cell migration towards TCM ($p=0.01$). Fractalkine had opposing effects on NK cell migration towards TCM when used alone, significantly decreasing migration ($p=0.02$), however when it is combined with IL-15, migration is significantly increased ($p=0.03$). Furthermore, fractalkine had inhibitory

effects on IL-15-mediated stimulation of death receptor ligand TRAIL ($p < 0.0001$) and FasL ($p = 0.0009$) and adhesion molecule $\alpha 4\beta 7$ ($p = 0.0008$) expression. There were unique profiles TRAIL, FasL, $\alpha 4\beta 7$ and L-selectin expressing NK cells in OAC patient blood, omentum and tumour. Interestingly, the frequency of $\alpha 4\beta 7$ ($p = 0.01$), TRAIL ($p = 0.0044$) and FasL⁺ ($p = 0.0003$) NK cells were significantly lower in blood and omentum of obese OAC patients compared to non-obese counterparts.

Conclusion

Our data demonstrate that OAC patient-derived ACM significantly decreases the capacity of NK cells to migrate towards TCM and this can be reversed by CX3CR1 antagonism. Fractalkine and IL-15 have opposing effects on NK cell migration, phenotype and function. Differential NK cell phenotypes exist in obese and non-obese OAC patients, highlighting the detrimental systemic effects of visceral obesity on NK cell tumour surveillance. Our data provide novel insights into the therapeutic utility of CX3CR1 antagonism to enhance NK cell movement towards tumour in OAC.

EACR22-0304

Prognostic significance of the secretory phenotype of macrophages and tumor cells in cutaneous melanoma

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Introduction

Cutaneous melanoma is characterized by its heterogeneous metastatic behavior and robust biomarkers are still needed to identify those patients with increased risk for distant metastasis, in order to guide new adjuvant treatments. Tumor-associated macrophages (TAMs) constitute a large fraction of infiltrating immune cells in melanoma tissues, but their significance for clinical outcome remains unclear. Here we explored the role of key cytokines that are involved in the interaction of TAMs with melanoma cells.

Material and Methods

Melanoma cells were cocultured with GM-CSF (Granulocyte Macrophage Colony-Stimulating Factor) or M-CSF (Macrophage Colony-Stimulating Factor) differentiated macrophages to determine the expression of secreted proteins and separated cells lysed for qPCR analyses. Melanoma cells were also used to perform proliferation, tumor 3D invasion and survival assays to elucidate the functional roles of upregulated chemokines and cytokines in vitro; and a murine model with subcutaneously inoculated chemokines was used for the in vivo experiments. To analyze the differential expression of these cytokines in human tissues, multicolor fluorescence was performed in a paraffin-embedded primary cutaneous melanomas collection.

Results and Discussions

We found that the expression of the chemokines CCL8, CCL15 and CCL20, and the cytokines TNF (Tumor necrosis factor) and VEGFA (Vascular Endothelial Growth Factor A) were highly upregulated in melanoma/macrophage co-cultures. These chemokines induced invasion and proliferation of melanoma cells, and promoted tumor growth and lung metastasis in murine models. Blockade of the corresponding chemokine receptors was performed to study the functional axis operating in vitro. Remarkably, significant higher expression of the mentioned chemokines and cytokines was quantified in metastasizing primary melanomas when compared with non-metastasizing tissues.

Conclusion

The chemokines CCL8, CCL15, and CCL20 display pro-tumoral and pro-metastatic roles in human melanoma. These chemokines, together with the cytokines TNF and VEGFA, constitute a potent biomarker signature with prognostic value in cutaneous melanoma patients.

EACR22-0426

Immune determinants of response to neoadjuvant chemo-radiation in esophageal adenocarcinoma

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Introduction

The standard therapy for locally advanced esophageal adenocarcinoma (EAC) is pre-operative neoadjuvant chemo-radiation (NACR) and surgery. Less than 30% of patients achieve a pathological complete response (pCR) and increased 5-year survival after NACR. Understanding the mechanisms of response to NACR is pivotal to better stratify patients and inform the design of more efficacious therapies. Cancer progression partly depends on defective immune surveillance. We hypothesize that NACR induces immunogenic cell death that stimulates immune responses against tumor neoantigens (TNAs), derived from cancer somatic mutations, in the responders. We thus aim at comparing responders versus non-responders to NACR for tumor immune contexture, immune molecular pathways, circulating immune markers and TNAs-specific T cells responses.

Material and Methods

Prospective pre-NACR tumor biopsies from EAC patients (n=32) were subjected to DNA exome sequencing (WES) and RNAseq, high dimensional (HD) multiparametric flow cytometry and immune contexture analysis by histology and by proteome digital spatial profiling (n=53 targets), while their peripheral blood mononuclear cells and plasma collected pre- and post- NACR were analyzed by HD flow cytometry and cytokines/chemokines n=30 multiplexing assay.

Results and Discussions

Treatment-naïve EACs of NACR responders showed higher TNAs load and enriched immune signatures compared to non-responders. pCR was found also associated with a ferroptosis vulnerability gene signature. Pre-treatment EACs of responders had augmented

effector CD8+ T cells infiltration and reduced Tregs and M2-like pro-tumoral macrophages, while low M2-like macrophages score associated with higher overall survival. Increased frequencies of circulating myeloid suppressor populations were detected both at baseline and after NACR in non-responder patients. These observations were associated with circulating levels of stimulatory cytokines/chemokines and with spatial expression of immune markers within treatment-naïve EAC biopsies of NACR responders.

Conclusion

Collectively, these results support the hypothesis that NACR stimulates a pre-existing anti-tumor immune contexture in the responder EAC patients, suggesting possible ways to stratify the patients based on a predictive immune signature and direct them to more tailored neoadjuvant and/or adjuvant combination regimens, incorporating immunotherapy and targeting tumor ferroptosis vulnerability.

EACR22-0522

Discerning Poly(IC) effects in distinct cell types composing a cancer tissue

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Introduction

Elevated viral response and double-stranded RNA (dsRNA) signalling have recently been proposed by our group as a novel therapeutic option to reduce relapse rates in patients with colorectal cancer (CRC). Using *in silico* and *in vivo* studies, we identify increased signalling of STAT1 and NFkB/REL, following treatment with poly(IC), as prerequisites for activation of the antigen processing and presentation (APP) machinery, which in-turn results in a reduced metastatic burden in a model of aggressive CRC. However, the lineage-specific effects of poly(IC), in epithelial, immune and stromal components of a bulk tumour remain unclear. Therefore, the aim of this study is to investigate the lineage-specific nature of this signalling, and their dynamics, in distinct cell lines upon poly(IC) treatment.

Material and Methods

Human colorectal cancer (CRC) HCT116 and HT29 and human monocytic THP-1 cells were treated with the long synthetic analog of dsRNA [Poly(I:C) HMW] (#tlrl-pic). Samples then underwent assessment of major components of the NFkB/REL and STAT1 pathway by Western blot, followed by whole transcriptome profiling of each lineage. These were matched with assessments of APP, using *in vitro* assays.

Results and Discussions

Poly(IC) treatment in epithelial cells induced a rapid but short-lived elevation in p65 and IKBα phosphorylation levels, in the absence of significant STAT1 activation, suggesting that TLR3 triggering provokes mainly NF-KB activation in CRC cell lines. Contrarily, STAT1 phosphorylation, but not IKBα and p65, underwent a significant and sustained increase following poly(IC) treatment in THP-1 cells. Matched transcriptional analyses revealed distinct pathway-level activation in discrete lineages, aligned to the antigen processing and presentation phenotype.

Conclusion

Numerous downstream signalling pathways have been attributed to poly(IC), however there is limited data to show the extent of activation of each pathway in the cell types that make up the colorectal tumour microenvironment. We show in this study that poly(IC) treatment

induces distinct lineage-specific signalling. Our transcriptomics analyses provide the basis for a better comprehension of the interplay between the cells inside the cancer tissue and how each of them contributes to the global effect of anti-tumour immunity upon poly(IC) treatment.

EACR22-0574

Characterisation of the Colon Adenocarcinoma microenvironment with a focus on Natural Killer Cells

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Introduction

Colorectal Cancer (CRC) is the third most common cancer and the fourth cause of death worldwide. In patients with advanced CRC, standard treatment strategies have shown low effectiveness; thus, it is critical to develop novel therapies. Adoptive Cell Therapy is an immunotherapy approach that relies on the use of autologous T-cells to target tumour cells. More recently, Natural Killer (NK) cells have appeared as an alternative to T-cells due to their lesser secondary effects, strong cytotoxicity and TME regulatory capacity. The **main goal** of this project is to evaluate the immune infiltrates in CRC tissue specimens, with a focus on NK cells, using multiplex immunofluorescence (Mplex IF).

Material and Methods

Formalin-fixed paraffin-embedded (FFPE) tumour specimens from 60 patients who underwent surgery for CRC were used to characterise the TME. Multispectral microscopy was used to quantify and analyse the distribution of NK cells, their subtypes and functionality, using antibodies from different animal sources (e.g., mouse, rat, and rabbit). Some primary antibodies were labelled with fluorescence fluorophores and others were detected by fluorescently labelled secondary antibodies, distributed along the 420-700 nm spectrum of light. After initial Mplex optimization, 10 regions of interest were acquired for each specimen using a Nikon 90i fluorescence microscope with a multispectral camera. A spectral library was built, the images were unmixed with the Nuance software and later analysed using the Fiji/ ImageJ software.

Results and Discussions

Two different Mplex were developed: a) Mplex1 to evaluate the activation state of NK cells, with the following markers: CD56 (NK Cells), CD16 (cytotoxic NK cells), NKG2D (activated NK cells) and PD1 (exhausted NK cells); b) Mplex2 to determine the functional activity of NK cells, composed by CD56, CD226 (activated NK cells), CD107a (NK cell degranulation) and HLA Class I (all nucleated cells). In both Mplex, Pan-Cytokeratin was used to label tumour cells, which allowed us to localize the immune cells with respect of the neoplastic nests.

Conclusion

We expect to quantify and functionally characterise the NK cells present in the different tumour compartments (stroma on the periphery of the tumour, stroma between the tumour glands and intraepithelial) and correlate with patient's clinicopathological characteristics such as stage, molecular characteristics, and survival, to better understand NK cells anti-tumour activity.

EACR22-0738

Resistance to interferon-gamma (IFN- γ) in HER2-positive breast cancer cells

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Introduction

A mediator of immune check points inhibitors, by unleashing cytotoxic T cells, is the secretion of IFN- γ , causing tumor cell cycle arrest and/or apoptosis. To what extent IFN- γ acts on HER2+ breast cancer, with or without anti-HER2 therapy, is poorly characterized. Here we investigated the ability of IFN- γ to regulate PD-L1 and MHC class I expression and its anti-proliferative functions on HER2+ breast cancer.

Material and Methods

The activity of IFN- γ in presence of anti-HER2 therapy (trastuzumab and pertuzumab) was tested on six HER2+ breast cancer cell lines by assaying cell growth (MTS viability) and apoptosis (flow cytometry). IFN- γ pathway, MHC class I, and PD-L1 expression were analyzed by qPCR, western blot, and flow cytometry on breast cancer cells. Direct PBMC-mediated cytotoxicity against cancer cells was also determined.

Results and Discussions

IFN- γ increased surface PD-L1 and MHC class I expression in five out of six HER2+ breast cancer cells tested, supporting that IFN- γ signaling was functional. BT474 cells have a deletion of *CD274* and in these cells IFN- γ effects on PD-L1 were not detected. Trastuzumab plus pertuzumab generally enhanced MHC class I induction by IFN- γ . Despite all cell lines having a functional response to IFN- γ , its effects on cell viability were not uniform. At 72h post IFN- γ treatment, an inhibition of cell growth was observed in only two cell lines. After 7 days of exposure to IFN- γ , four cell lines were growth-inhibited and two cell lines were deemed as resistant (< 10% inhibition). In the panel of cell lines tested, adding IFN- γ to cells exposed to trastuzumab plus pertuzumab was more active than either agent alone in those cells sensitive to both agents. In contrast, in cells resistant to IFN- γ cellular effect, no benefit was detected for the combination. The effects of recombinant IFN- γ also appeared to predict the sensitivity of cancer cell lines to PBMC exposure. IFN- γ sensitive HCC-1954 cells were more sensitive to activated PBMCs-induced growth inhibition than the IFN- γ resistant SK-BR-3 cells, despite in both cell lines IFNGR1 was expressed and IFN- γ exposure resulted induced pSTAT1 and IRF1.

Conclusion

HER2+ breast cancer cells might be either sensitive or resistant to recombinant IFN- γ , a surrogate of NK or cytotoxic T cell mechanism of action. Identifying HER2+ breast cancers with an IFN- γ resistant phenotype might help to identify resistance to immune checkpoint inhibitors alone or, particularly when combined with anti-HER2 agents.

EACR22-0770

Interleukin-11 dysregulates tumor immunity, promoting the tumor progression

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Introduction

Tumor growth and progression are normally favored by a dysregulated cytokine signaling. Tumorigenic actions of tumor-growth cytokines are elicited through an autocrine signalling loop on cancer cells and through a paracrine cross-talk between cancer cells and tumor microenvironment (TME). Our group has demonstrated that increased levels of interleukin 11 (IL-11) significantly drive the progression of lung adenocarcinoma (LUAD). IL-11 is involved in cancer progression of others inflammation-related cancers such as colorectal cancer or gastric cancer. Despite the relevant role of the IL-11 pathway on cancer progression, it is not still clear the implication and interaction of IL-11 within TME in the LUAD

Material and Methods

We have explored the role of IL-11 in a tobacco-exposure LUAC *in vivo*, a model characterized for high tumor inflammation. A/J mice were exposed to mainstream tumor smoke (MTS) for 3 alternate months in presence or absence of rh-IL11. Characterization of pulmonary immune cell populations of A/J mice were analysed by flow cytometry. We have also analysed LUAD patient samples by RNASeq and Digital Spatial Profiling (DSP). RNASeq using the Oncomine Immune Response Research Assay panel (OIRRA) was employed in RNA extracted from frozen 32 tumors from LUAD patients. Digital Spatial Profiling (DSP) was performed on FFPE samples from 20 LUAD patients. Visualization markers for pan-cytokeratin (PanCK. tumor) and CD45 (immune), together with a cocktail of 44 unique photocleavable oligonucleotide-labelled primary antibodies targeting immuno-oncology markers were used.

Results and Discussions

We examined the immune modulatory effects of IL-11 in a mouse model of tobacco carcinogen-induced of LUAD, and we found a significant decrease of CD3, CD4 and CD8 lymphocyte infiltration, favoring an immunosuppressive microenvironment. We further explored cancer-associated immune evasion in tumor samples from LUAD patients, where we found two patterns of gene expression determined by IL-11 expression levels. Interestingly, we found that patients with high expression levels of IL-11 showed a downregulation of genes involved in anti-tumor immune response whereas genes involved in tumor progression and immune evasion were overrepresented. By DSP, we confirm that LUAD patients with high expression levels of IL-11 express lower levels of some immune markers.

Conclusion

Collectively, our data demonstrate that IL-11 dysregulates tumor immunity towards an immunosuppressive microenvironment favoring cancer progression.

EACR22-0844

The glycosylation profile of T cells along Colorectal Cancer progression

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Introduction

Colorectal cancer (CRC) is the second leading cause of cancer related death worldwide, remaining a serious public health problem in developed regions. Despite the clinical success of immunotherapy, only a minority of CRC patients benefit from this therapeutic modality, highlighting the urgent need for effective CRC therapies.

Changes in glycosylation are a hallmark of cancer. Glycans play a pivotal role in each pathophysiological step of malignant transformation (Pinho & Reis, Nat Rev Cancer 2015) including in cancer immunoediting. Evidences from our group highlight the importance of glycans as relevant immune-checkpoints in cancer immunosurveillance and immunoediting (Silva & Fernandes *et al.* Cancer Immunol Res 2020). Moreover, glycans have been shown to be key players in the regulation of T cell-mediated immune response. In fact, in inflammatory bowel disease, we demonstrated that mucosal T cells display deficient branched *N*-glycans, being associated with decreased threshold of T cell activation and a pro-inflammatory response (Dias *et al.* PNAS 2018; Pereira & Alves *et al.* Frontiers Immunol 2018).

In this project, we are studying the glycosylation signature of different T cell populations along CRC progression, from pre-malignant lesions to full-blown CRC.

Material and Methods

Through *in silico* approaches using transcriptomics data, we assessed the correlation between *MGAT5* glycogene and *FOXP3* as well as immune checkpoints on T cells. In parallel, we performed molecular biology techniques, such as flow cytometry and immune assays, to characterize the glycosignature of infiltrating T cell from colonic biopsies, using a cohort of CRC patients.

Results and Discussions

The correlation between *MGAT5* glycogene and *FOXP3* revealed a significant positive correlation between the expression of complex branched *N*-glycans and regulatory T cells (Tregs) in the tumor microenvironment, which suggests the impact of *N*-glycan in defining T cell properties and an immunosuppressor/regulatory microenvironment imposed by Tregs. Additionally, we found that the expression of *MGAT5* glycogene is correlated with the expression of immune checkpoints on T cells. Moreover, a unique glycosignature was observed in infiltrating T cells isolated from fresh colonic biopsies along CRC development.

Conclusion

Taken together, these results suggest the key role of glycans in cancer immunosurveillance, supporting the importance of T cell glycosylation as a promising target for novel immunotherapeutic strategies.

EACR22-0970

Knockout of Collagen Type I Impedes Tumor Growth and Supports Immune Cell Infiltration in a Murine Model of Pancreatic Cancer

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Introduction

A key characteristic of cancer progression is remodeling of the extracellular matrix (ECM). This remodeling results in a tumor-specific ECM which is often of high density and rich in collagen. The high collagen density of the tumor ECM correlates with a poor prognosis in many types of cancer and with low infiltration of immune cells. The direct effects of increased intratumoral collagen on tumor progression are not yet fully understood. In this study, the role of collagen on tumor growth and immune cell infiltration were directly investigated *in vivo* using transgenic murine models allowing for manipulation of collagen levels.

Material and Methods

Two transgenic murine models were used to study the effects of the level of intratumoral collagen type I; a conditional knockout mouse model with decreased collagen deposition and a collagenase-resistant mouse model with increased collagen deposition. Changes in the level of collagen type I synthesis and content were demonstrated using qRT-PCR, ELISA, and histological staining. Collagen-mediated effects on immune cell infiltration were investigated using flow cytometry analyses, qRT-PCR, and immunohistochemistry. All animal studies were following institutional guidelines and were approved by the Danish Animal Experiments Inspectorate.

Results and Discussions

Collagen type I expression and deposition was significantly decreased in the conditional knockout mouse model. Knockout of collagen led to impeded growth of Pan02 pancreatic carcinomas. This was accompanied by increased infiltration of immune effector cells, including an increase in the amount of NK cells and an increased CD8/CD4 ratio. These results were backed up by employing the collagenase-resistant mouse model with increased collagen deposition. In these mice, tumor growth was increased and there was a higher CD4/CD8 ratio and an increased amount of immunosuppressive myeloid cells.

Conclusion

The results of this study demonstrate pronounced effects of collagen on tumor progression and on the tumor immune microenvironment. The potential immunosuppressive role of collagen type I provides a rationale for extending future research in cancer immunotherapy to include focus on the ECM and in particular collagen type I.

EACR22-1062

Lipid-loaded macrophages support melanoma progression

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Introduction

Macrophages represent the main infiltrating immune subset in most cancer types and tumor-associated macrophages (TAMs) sustain tumor growth, promote invasion and mediate immune suppression. Importantly, cellular metabolism is implicated in the education of macrophages and dictates their phenotype. We identified a population of lipid-loaded macrophages that infiltrates prostate cancer and sustains therapy resistance. Interestingly, macrophages engulfed with lipids have been recently described in melanoma. However, the mechanisms that drive lipid accumulation in macrophages and the functional impact of lipid-loading still need to be uncovered.

Material and Methods

We set up a murine model of melanoma and we profiled the cancer immune infiltrate by mean of single cell-based approaches, including multiparametric Flow Cytometry and single cell RNA sequencing. We applied in vitro co-culture assays to dissect the interplay between macrophages and melanoma cells and we took advantage of Flow Cytometry and immunofluorescence analysis to follow the dynamics of lipid accumulation in tumor-conditioned macrophages.

Results and Discussions

We confirmed that lipid accumulation is a feature of tumor-infiltrating macrophages in melanoma and we unveiled a significant increase in the amount of lipid droplets in tumor-infiltrating neutrophils and dendritic cells. Notably, lipid-accumulation on immune cells correlates with the expression of markers that regulate immunosuppression, including Arginase and PD-L1. Importantly, the abundance of lipid-loaded macrophages correlates with tumor size and administration of immunotherapy reduces the accumulation of lipids in TAMs. In vitro, cancer cells shift the polarization of macrophages toward a pro-tumoral state and augment the capability of macrophages to accumulate lipid droplets when triggered with lipid species.

Conclusion

Here we show that lipid engulfment is a feature of TAMs in melanoma and that accumulation of lipid droplets confers to macrophages pro-tumorigenic features. Still the source of lipids and the consequences of lipid accumulation in TAMs in the context of immunotherapy need to be further explored. We believe that being able to target lipid metabolism in TAMs may represent a promising opportunity in cancer therapy.

EACR22-1172

Elucidating the Immunogenicity of Non-Thermal Plasma Combination Therapy in a 3D Tumour Model of Head and Neck Squamous Cell Carcinoma

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Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide. Patients in advanced stages of the disease often face relapse or metastasis (R/M HNSCC), frequently with detrimental outcome. The current first-line treatment strategy of immunotherapy alone or in combination with platinum-based chemotherapeutics (PLAT) has limited benefits due to low response rates and severe side effects in already weakened patients.

Non-thermal plasma (NTP), an ionised gas containing reactive oxygen and nitrogen species, has been reported to induce immunogenic tumour cell death (ICD). Therapeutic ICD inducers, like NTP, are clinically interesting as they can engage the patient's natural cancer immunity cycle and provide long-lasting anti-tumour immunity. Therefore, the study aim was to investigate a novel combination strategy of NTP with current first-line therapies of R/M HNSCC to improve treatment efficacy and response. Since two-dimensional monolayer models are inadequate in simulating the complex processes in HNSCC and its tumour microenvironment, we performed our study using three-dimensional tumour models.

Material and Methods

We optimised a micro-tissue spheroid model for several HNSCC cell lines. All experiments were performed using a microsecond-pulsed dielectric barrier discharge plasma system. Combination treatments of NTP and PLAT (cisplatin) were analysed in the Spark® Cyto, monitoring tumour growth and cell death rates, via real-time imaging and the CellTiter-Glo 3D endpoint assay. In addition, the expression and release of several ICD-related damage-associated molecular patterns (e.g. ecto-calreticulin) were assessed to measure ICD induction.

Results and Discussions

Initial results indicated that NTP-monotherapy increased ecto-calreticulin up to 24 hours post treatment. Next, combining NTP with PLAT, we were able to show that reduction in tumour viability was significantly higher in the NTP-PLAT combination compared to PLAT monotherapy at 24h, and both monotherapies at 48h post treatment. These results highlight the potential of NTP to enhance treatment efficacy and tumour immunogenicity. This data was further validated in HNSCC tumour organoids, our patient-derived model that uniquely mimics the phenotypic and genotypic characteristics from the original tumour.

Conclusion

This all together will accelerate clinical translation of the obtained results and is the first step towards a rationally designed combination strategy with NTP to improve current first-line HNSCC therapies.

EACR22-0059

A Standardized Analysis of Tertiary Lymphoid Structures in Human Melanoma: Disease Progression- and Tumor Site-Associated Changes With Germinal Center Alteration

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Introduction

There is increasing evidence that tertiary lymphoid structures (TLS) control not only local adaptive B cell responses at melanoma tumor sites but also the cellular composition and function of other immune cells. In human melanoma, however, a comprehensive analysis of TLS phenotypes, density and spatial distribution at different disease stages is lacking.

Material and Methods

Here we used 7-color multiplex immunostaining of whole tissue sections from 103 human melanoma samples to characterize TLS phenotypes along the expression of established TLS-defining molecular and cellular components. TLS density and spatial distribution were determined by referring TLS counts to the tissue area within defined intra- and extratumoral perimeters around the invasive tumor front.

Results and Discussions

We show that only a subgroup of primary human melanomas contains TLS. These TLS rarely formed germinal centers and mostly located intratumorally within 1 mm distance to the invasive tumor front. In contrast, melanoma metastases had a significantly increased density of secondary follicular TLS. They appeared preferentially in stromal areas within an extratumoral 1 mm distance to the invasive tumor front and their density varied over time and site of metastasis. Interestingly, secondary follicular TLS in melanoma often lacked BCL6+ lymphatic cells and canonical germinal center polarity with the formation of dark and light zone areas.

Conclusion

Our work provides an integrated qualitative, quantitative and spatial analysis of TLS in human melanoma and shows disease progression- and site-associated changes in TLS phenotypes, density and spatial distribution. The frequent absence of canonical germinal center polarity in melanoma TLS highlights the induction of TLS maturation as a potential additive to future immunotherapy studies. Given the variable evaluation strategies used in previous TLS studies of human tumors, an important asset of this study is the standardized quantitative evaluation approach that provides a high degree of reproducibility.

EACR22-0139

Uncoupling CD4+ T-cell-mediated tumor killing from JAK-signaling

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Introduction

A significant proportion of patients present with primary or acquired resistance to immune checkpoint inhibitors. Impaired MHC Class I-presentation and insensitivity to immune

effector molecules such as IFN γ , often associated with loss of β 2-microglobulin (B2M) or alterations in the Janus kinase (JAK) signaling pathway respectively, have been reported in multiple cases of immune resistance. In addition, recent evidence shows that CD4 $^{+}$ T cells can exert cytotoxic functions to tumors, but it is unclear whether CD4 $^{+}$ T cell responses can be exploited to improve the clinical outcomes of patients affected by immune-checkpoint-blockade resistant tumors.

Material and Methods

Here, we exploited CRISPR/Cas9 gene editing to reproduce immune-resistant tumor phenotypes via gene knockout (^{KO}). Using real-time cell-cytotoxicity technologies, we characterized the CD4 $^{+}$ T-cell-mediated tumor killing in matched pairs of autologous melanoma cell lines and tumor-infiltrating lymphocytes (TILs), used as a model of patient-specific immune-tumor interaction. Around 50% of melanomas express MHC Class II molecules constitutively; hence melanomas with or without natural constitutive MHC Class II expression (MHCII $^{const+}$ or MHCII $^{const-}$) were employed.

Results and Discussions

CD4 $^{+}$ T-cell-mediated tumor killing was not affected by *B2M* loss but was strictly dependent on the expression of *CIITA*, the master regulator of MHC Class II expression. MHCII $^{const+}$ melanomas were killed by tumor-specific CD4 $^{+}$ TILs even in the absence of IFN γ -mediated MHC Class II induction; in contrast, IFN γ -mediated upregulation of MHC Class II was necessary to kill MHCII $^{const-}$ melanomas. Notably, while tumor-specific CD4 $^{+}$ TILs did not kill *JAK1* ^{KO} MHCII $^{const-}$ melanomas even after IFN γ stimulation, *JAK1* ^{KO} MHCII $^{const+}$ melanomas maintained sensitivity to CD4 $^{+}$ T-cell-mediated tumor killing.

Conclusion

In conclusion, our data indicate that CD4 $^{+}$ T-cell-mediated tumor killing can help overcome resistance to immune-checkpoint-blockade mediated by JAK-signaling loss in MHCII $^{const+}$ melanomas, highlighting the potential of personalized immunotherapeutic strategies aiming to boost the response of cytotoxic CD4 $^{+}$ T cells in tumors with constitutive expression of MHC Class II molecules.

EACR22-0395

Comparison of tumor infiltrating lymphocytes (TILs) in ovarian cancer and associated lymphocytes (TALs) in ascites – an exploratory analysis

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Introduction

Ovarian cancer is the 5th most frequent cancer among women and the 8th most lethal worldwide. Most women will relapse after surgery and first line chemotherapy. New treatments are therefore needed. Several studies have attested the prognostic significance

of tumour infiltrating lymphocytes (TILs) in advanced ovarian cancer and current research focus on their therapeutic role. The study of TILs alongside the disease course is however hampered by the difficulty to collect tumor samples as women who relapse undergo rarely surgery. Tumour associated lymphocytes (TALs) present in the ascites are more easily accessible. Thus, to facilitate the study of the micro-environment evolution during the disease course, we wondered if TALs reflect TILs.

Material and Methods

In this interventional, monocenter, non-randomized study among patients with an advanced ovarian or tubal epithelial cancer, with International Federation of Gynecology and Obstetrics stage IIIC or IV, eligible to laparoscopy for diagnosis, we collected samples from ascites and peritoneal carcinomatosis (PC). We performed flow cytometry analysis to compare TILs and TALs. Statistical analysis was done using Wilcoxon signed-rank test (for paired data). This trial is registered on ClinicalTrials.gov, number NCT03922776.

Results and Discussions

We included 21 patients in TILsOV-1805 study. We collected samples from all patients before chemotherapy. We found a difference in the proportion of CD4+ T cells ($p=0.002$) and CD4+/CD8+ ratio ($p=0.002$) in ascites compared to PC. In the subpopulations of T cells, we found a difference with more regulatory T cells ($p=0.003$) and less memory T cells in ascites compared to PC. The proportion of naïve B cells was more important in ascites compared to PC ($p=0.03$). We didn't find differences in the proportion of T helper cells nor T follicular helper cells between the two tissues. We found a difference in TILs PD1+ expression with less expression in ascites than in PC. No difference was observed in CTLA4+ lymphocytes.

Conclusion

Our results suggest differences between TILs and TALs, especially for pro-tumoral T-cells and naïve cells. Thus, TALs from ascites can't be considered as a reliable substitute for TILs from peritoneal carcinomatosis in ovarian cancer.

EACR22-0437

Highly multiplexed, single-cell spatial phenotyping of Epstein-Barr virus infected cancer tissues.

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Introduction

The Epstein-Barr virus (EBV), a group 1 carcinogen, is a causative factor in nine different cancers that cause ~165,000 deaths globally each year. Almost all adults carry EBV asymptomatically with the virus persisting primarily in rare memory B cells for the lifetime of the host. However, in some infected individuals, for reasons that are unknown, EBV may contribute to the development of cancer. The immune microenvironment in which infected cells reside may influence or trigger cancer development, but we know very little about how this occurs. We have begun to address these critical questions by deploying a spatial biology assay for comprehensive study of the immune microenvironment of EBV-infected tissues.

Material and Methods

The PhenoCycler™ technology provides a comprehensive solution for spatially-resolved, highly multiplexed biomarker analysis with single cell resolution. This approach uses single step staining and an iterative multicycle process of tagging and imaging of antibodies to record highly multiplexed biomarker expression data in situ. Using the PhenoCycler™ assay, we thus developed an antibody panel that can detect EBV infected cells in addition to 40+ immune-system and cancer related biomarkers.

Results and Discussions

The data presented here provide a comprehensive account of the immunology and spatial biology of EBV infected tissues and their microenvironment. By employing single-cell spatial phenotyping and deep bioinformatic analyses, we have examined how the immune microenvironment of virus-infected cells influences tissue pathology and, ultimately, may partake in the development of cancer.

Conclusion

We provide a custom panel of more than 40 biomarkers that can be used to study the tissue microenvironment on EBV infected tissues. The results from our study provide deep insight of the complexities of the tumor microenvironment and role of EBV infection in carcinogenesis.

EACR22-0469

ICOS and ICOSL are novel biomarkers for multiple myeloma

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Introduction

Multiple myeloma (MM) is a plasma cell neoplasia, still incurable disease, resulting from monoclonal expansion of long-lived plasma cells (myeloma cells) in the bone marrow. The ICOS/ICOSL interaction plays a role not only in the immune response and T/B cell interaction, but it is capable also to trigger a bidirectional signal acting on the ICOSL-expressing cells. Previously we showed that ICOS-Fc, a soluble recombinant form of ICOS, inhibits adhesiveness and migration of endothelial cells and tumor cell lines in vitro, and metastases in vivo. Moreover, when loaded into nanoformulations ICOS-Fc was capable to inhibit also tumor growth of established subcutaneous B16-F10 tumors. The aim of this project was to evaluate the ICOS-Fc therapeutic effect in vitro with migration assay and the ability of ICOS-Fc loaded in PLGA to inhibit tumor growth of myeloma cells.

Material and Methods

The cell Migration Assay was performed in order to evaluate the migration effect of different MM cells, after ICOS-Fc treatment. An in vivo MM model was generated by subcutaneous injection of MOPC21^{ICOSL} cells into NGS female mice. The PLGA nanodelivery system was chosen as nanocarrier for ICOS-Fc therapeutic administration, due to the biocompatibility and biodegradability of this polymer. Tumor masses development was measured in order to

evaluate the ICOS-Fc/PLGA therapy effect. Immunofluorescence and immunohistochemistry assays were performed to study angiogenesis and proliferating cells markers, respectively.

Results and Discussions

This work shows that ICOS-Fc might exert direct effects on both human and mouse myeloma cells since it inhibits their migration in vitro in an ICOSL-dependent manner. This effect was supported by our in vivo experiments showing that treatment with ICOS-Fc/PLGA substantially inhibits the growth of MOPC21^{ICOSL} in subcutaneous MM mouse model, with significantly lower tumor vascular density and proliferation rate of ICOS-Fc/PLGA treated group compared to the others.

Conclusion

Results suggests that ICOS and ICOSL may be novel markers and therapy targets for MM. Finally, the fact that ICOS and ICOSL are expressed not only on myeloma cells, but also on several cell types of the myeloma niche suggests that these molecules may be targeted in MM therapy.

EACR22-0565

Teneurin-4 as novel immune-target and biomarker in triple negative breast cancer.

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Introduction

Triple negative breast cancer (TNBC) is one of the most aggressive types of human breast cancer with a high incidence of recurrences and metastasis. This is attributable to the lack of targetable molecules and to the low efficacy of current therapies to eliminate cancer stem cells (CSC). Therefore, the aim of this work is to find a novel therapeutic target associated to TNBC stem cells suitable for immunotherapy.

Material and Methods

RNA-seq was used to identify differences in gene expression between tumorspheres and epithelial cells. TNBC cells were silenced through RNAi, and knock-out (KO) mouse TNBC cells were obtained with the CRISPR/Cas9 technology. TENM4 role in self-renewal and migration, was tested in vitro performing tumorsphere-forming ability assay and transwell invasion assay. To evaluate TENM4 as TNBC biomarker TENM4 presence in the sera and in the exosomes of tumor bearing mice and of breast cancer patients was tested through ELISA and Western blot, respectively. Finally, the immunogenicity of a DNA-based vaccine against TENM4 was tested *in vivo* by Flow cytometry and ELISPOT assay.

Results and Discussions

TENM4 was selected among the overexpressed transmembrane proteins in murine and human TNBC stem cells-enriched tumorspheres. Its silencing significantly impairs the tumorsphere forming potential and cell ability to migrate, accompanied by a significant decrease in FAK phosphorylation. This suggests a role of TENM4 in cancer stem-like features and metastatic potential. Indeed, while no differences in the tumor take and growth was observed between TENM4-KO and TENM4-WT cells, a lower number of lung metastasis was found in TENM4-KO tumor-bearing mice. Interestingly, we detected the presence of TENM4 in the blood of tumor-bearing mice and of some breast cancer patients, as well as in the exosomes from cancer cell supernatants and from breast cancer patients' plasma, suggesting its potential use as biomarker. Finally, DNA-based vaccination of BALB/c mice with plasmids coding for mouse or human TENM4 resulted safe and effective

in the induction of an anti-TENM4 antibody response as well as specific T-cell response. Future experiments will be held to evaluate the impact of anti-TENM4 vaccination on TNBC tumorigenesis.

Conclusion

Overall, these data identify TENM4 as overexpressed in TNBC stem cells with a role in stemness and migration and as possible novel biomarker. Moreover, the immunogenicity of the vaccination against TENM4 point out the possibility to use TENM4 as a valuable immune-target in TNBC.

EACR22-0607

Alveolar macrophages modulate cancer-associated fibroblasts in a preclinical model of NSCLC.

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Introduction

Fibroblasts in tumors are associated with hallmarks of cancer malignancy, including tumor invasion and metastasis, angiogenesis, and immune evasion. While the contribution of cancer-associated fibroblasts (CAFs) to cancer progression is well-established, very little is known about the factors that regulate fibroblast activation in the early stages of tumorigenesis and how we can take advantage of early interventions to modify the tumor microenvironment of lung cancer.

Our laboratory has identified a population of macrophages that while residing in the lung (tissue-resident macrophages or TRMs, also known as alveolar macrophages) they are found in NSCLC tumors. TRMs promote epithelial-mesenchymal transition in cancer cells and boost regulatory T cells in incipient lung KP-tumors. Yet, TRMs closely interact with fibroblasts, leading us to hypothesize whether TRMs can contribute to CAFs malignant conversion.

Material and Methods

Using multi-parameter flow cytometry, confocal imaging, and transcriptomics from *in vivo* purified KP-fibroblasts, we showed that TRMs ablation is sufficient to control PDGFRa and podoplanin-expressing fibroblasts.

Results and Discussions

Interestingly, TRM-depleted tumors showed increased activation and infiltration of T cells, suggesting that TRMs mediate fibroblast activation which in turn, shielded T cells and hindered immune surveillance.

Conclusion

Our results uncover a novel immunomodulatory axis in NSCLC, and open new venues for stroma modulation by a unique subset of macrophages in lung cancer.

EACR22-0807

SAMHD1 expression determines proinflammatory signalling and immune infiltration capacity in 3D breast cancer in vitro models

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Introduction

SAMHD1 is a dNTP triphosphohydrolase involved in the regulation of intracellular dNTP pool and in the modulation of inflammatory responses although the exact mechanisms remain unclear. Its involvement in cancer onset and disease outcome is being extensively investigated. Previous results from our team showed that SAMHD1 expression is a prognostic and predictive factor in breast cancer patients. Here, we developed a 3D heterotypic culture model of breast cancer, including tumor cells and primary mononuclear cells to evaluate SAMHD1 influence in tumor progression and antitumor immune response.

Material and Methods

SAMHD1 knockout (KO) cells were generated by CRISPR/Cas9 in T47D cells. Spheroids were generated by seeding T47D cells on low adherent plates. At day 4, cocultures were established by adding peripheral blood mononuclear cells (PBMCs) from healthy donors and stained with CellTracker™ Green CMFDA Dye. Cell infiltration into the spheroids was evaluated by multipanel flow cytometry. Imaging of spheroid composition and cell invasion was performed by microscopy. Cytokine expression and immune signalling was assessed by qPCR, western blot and ProQuantum.

Results and Discussions

SAMHD1 WT and KO breast cancer spheroids showed no significant differences in growth rate, viability or spheroid integrity. Evaluation of immune signalling pathways showed significant decrease of proinflammatory cytokines (IL6, IL8, IL1a) in KO spheroids, both at mRNA ($p=0.001$, $<.0001$ and $.0475$, respectively) and protein level (IL8, $p=.0277$), in contrast with 2D cultures. Moreover, in depth evaluation of immune pathways confirmed the reduction in proinflammatory signalling in KO spheroids, overall suggesting that SAMHD1 depletion affects tumor immune infiltration capacity. Breast tumor spheroids cocultured with PBMCs presented decreased immune infiltration in KO spheroids compared to WT ($p=.0014$). Immunophenotypic characterization of infiltrated cells identified significant changes in myeloid APCs, monocytic cells and high cytotoxic NKs ($p=.0156$, $.0053$ and $.0025$, respectively), whereas CD4+ and CD8+ T lymphocytes did not differ.

Conclusion

SAMHD1 expression influences proinflammatory signalling potential of breast cancer *in vitro*, leading to changes in chemoattraction and subsequent immune cell infiltration. These results highlight the importance of SAMHD1 as a key factor in modulating antitumor immune response in breast cancer, pointing towards its use as a prognostic factor in breast cancer, as suggested from *in vivo* cohort studies.

EACR22-0819

Continuous and sequential T cell receptor stimulation in the presence of mediators of inflammation as a novel protocol to generate terminally exhausted CD8+ T cells

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Introduction

T cell exhaustion is a dysfunction of antigen-specific T cells that occurs during chronic viral infections and cancer. In the context of cancer, prolonged exposure to antigen and inflammatory conditions in the tumor microenvironment (TME) results in continuous loss of T cell effector functions, concomitant upregulation of immune checkpoint receptors, metabolic changes, as well as acquisition of antigen-independent memory responsiveness. Procurement of sufficient numbers of exhausted human tumor-infiltrating lymphocytes (TIL) is one of the key limiting factors for translational immune-oncological *in vitro* studies. We developed a novel *in vitro* T cell exhaustion protocol that implements mediators of inflammation in sequential and continuous stimulations.

Material and Methods

Here we performed an *in vitro* CD8⁺ T cell stimulation using anti-CD3/CD28 beads followed by continuous stimulations in the presence of various mediators of inflammation. CD8⁺ T cell exhaustion was measured by flow cytometry, ELISA and Seahorse real-time cell metabolic analyses.

Results and Discussions

The protocol allows for generation of functional and metabolic T cell exhaustion as shown by elevated immune checkpoint receptor expression, decreased cytokine and perforin production, low oxygen consumption and extracellular acidification rates and maintain exhaustion when TCR stimulus is removed. Using this method, we were able to generate both reversible and non-reversible CD8⁺ T cell exhaustion to mimic various activation states detected within the TME.

Conclusion

This protocol is an easily reproducible way of generating exhausted T cells that could lessen the need for TIL for complex immune-oncological *in vitro* experimental models. This study suggests that efforts to produce *in vitro* exhausted cytotoxic T lymphocytes may represent an important research methodology with which to evaluate potential novel immunotherapies.

EACR22-1063

Pharmacological and genetic screenings as powerful tools to unveil mechanisms of macrophage polarization in the tumor microenvironment

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Introduction

Macrophages are innate immune cells with variable functionality based on external stimuli. This plasticity can lead to more inflammatory (M1) or anti-inflammatory (M2) phenotypes. It is known that cancer cells can deeply affect macrophage behavior. The pro-tumoral role (M2-like activation) that macrophages can acquire in the tumor microenvironment (TME) is object of studies for therapeutic possibilities since many years. This work aims to investigate

the molecular pathways driving macrophage polarization in the TME through different approaches, thus providing strong candidates to be targeted in therapy.

Material and Methods

A broad pharmacological screening was carried out by treating murine macrophages with 147 FDA-approved drugs commonly used in anti-cancer therapies. The aim was to identify drugs able to prevent macrophages from acquiring typical M2-like features (upregulation and down-regulation of CD206 and MHC-II markers, respectively) after exposure to culture medium derived from cancer cells. Flow cytometry analyses were performed to define macrophage phenotype. For selected drugs, both *in vitro* and *in vivo* validation experiments were added to better characterize their impact on macrophages. Concomitantly, a genome-scale CRISPR-Cas9 knockout screening was performed on tumor-conditioned macrophages, followed by cell sorting and next-generation sequencing, in order to identify key genes for their polarization.

Results and Discussions

The pharmacological screening identified specific families of drugs like taxanes, hormone-related compounds and some kinase inhibitors as effective in preventing tumor-induced phenotype, highlighting the importance of these pathways in macrophage activation. As for taxanes, comprehensive validation experiments showed a more complex influence on macrophage behavior, with subsequent need for a deep dissection of the selected drugs-related pathways. A strong support to this research is provided by the genetic screening, whose analyses confirmed the key role of specific genes in macrophage polarization.

Conclusion

The crossed-analysis from pharmacological and genetic screenings provides a powerful platform for the investigation of tumor-induced activation of macrophages and the identification of new molecular targets for their re-education against tumor development.

EACR22-1135

Evaluating the potential of targeting the TIGIT axis for the treatment of pancreatic ductal adenocarcinoma

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a devastating disease, characterized by a 5 year overall survival below 10%. This dismal outcome is mainly due to its high resistance to all current therapies. Therefore, novel and effective treatment options are urgently needed for these patients. The emerging field of immune checkpoint inhibitors targeting programmed death (PD)-1/ programmed death-ligand 1 (PD-L1) and Cytotoxic T lymphocyte antigen-4 (CTLA-4) resulted in sustained anti-tumor response in several cancer types. However, PDAC patients enrolled in these clinical trials demonstrated no clinical benefit, leaving PDAC patients in desperate need of new treatment options. Hence, new and effective targets for novel immunotherapy combinations need to be sought. In this regard, our preliminary data, as well as literature (Steele et al, *Nature Cancer* 2020), prompted TIGIT as an emerging therapeutic target for immunotherapy. In a pilot experiment, where we compared TIGIT

expression on T cells in matched tumor and peripheral blood samples of ten PDAC patients, we demonstrated that a significant higher percentage of TIGIT⁺ CD3⁺ T cells were present within the tumor, which ties with the CyTOF data from Steele et al. However, the complex TIGIT axis includes multiple inhibiting (e.g. TIGIT and CD96) and activating receptors (e.g. DNAM-1) expressed on various subtypes of immune cells, as well as different ligands (e.g. CD112 and CD155) expressed on both tumor cells and immune cells.

Material and Methods

In this study, we will unravel the complete TIGIT axis in PDAC patients by analyzing the expression of all involved receptors and ligands of this axis on different immune cells of interest, including natural killer cells, regulatory T cells, cytotoxic T cells, and dendritic cells. Additionally, co-expression of PD-1 and PD-L1 will be checked. Therefore, tumor tissue and matched peripheral blood samples of PDAC patients will be analyzed using a multicolor flow cytometric panel. Additionally, immune histochemistry staining on tumor tissue will provide insight into the ligand expression on different cell types and their spatial distribution within the PDAC tumor microenvironment. Eventually, these data will be correlated with the patients' clinicopathological data.

Results and Discussions

Together, these data will provide the necessary evidence for a rational-based combination immunotherapy.

Conclusion

Eventually, this might pave the way towards clinical benefit for PDAC patients, who are in dire need of new treatment options.

EACR22-0036

Na⁺ transport: a novel immune checkpoint regulator in breast cancer

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Introduction

Immune checkpoint proteins have been widely studied as therapeutic targets in cancer. Another particular aspect of cancer dynamics is the study of ionic disruptions associated with several types of tumours, especially in breast cancer. The role of ionic dynamics in regulating immune checkpoint protein activity is, however, currently unknown. In this study we show that increasing intracellular Na⁺ has an inhibitory effect on the immune checkpoint protein and tryptophan catabolic enzyme, indoleamine pyrrole 2', 3' – dioxygenase 1 (IDO1), in MDA MB 231 breast cancer cells.

Material and Methods

The impact of 31 ion transport-targeting compounds on IDO1 function was screened using a spectrophotometric assay that measures kynurenine levels, a downstream catabolite of tryptophan. IDO1 gene and protein expression were quantified via qPCR and western blot. A Na⁺-binding ratiometric dye, sodium-binding benzofuran isophthalate acetoxymethyl ester (SBFI-AM) was used to measure intracellular Na⁺ levels.

Results and Discussions

Ouabain and digoxin, two cardiac glycosides which inhibit the main regulator of intracellular Na⁺ levels, the Na⁺/ K⁺ ATPase (NKA), downregulated IDO1 activity and expression in a concentration-dependent manner, also decreasing protein levels of the main IDO1

expression regulator, phosphorylated signal transducer activator of transcription 1 (pSTAT1). siRNA downregulation of the $\alpha 1$ subunit of the NKA (ATP1A1) combined with cardiac glycoside treatment enhanced IDO1 and pSTAT1 inhibition. The IDO1 inhibitory effect of ouabain and digoxin was paralleled by an elevation in intracellular Na^+ levels. Our results suggest that intracellular Na^+ levels might modulate IDO1 activity, potentially playing a role in cancer immune evasion.

Conclusion

We, therefore, propose a novel association between increases in intracellular Na^+ levels and immune checkpoint regulation, as a potentially targetable regulatory mechanism of tumour immune evasion.

EACR22-0190

Myeloid-derived suppressor cells regulate metastatic dissemination in triple-negative breast cancer

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Introduction

The prognosis for patients with triple-negative breast cancer (TNBC) remains poor, with newly developed immunotherapies failing to produce the benefits seen in other cancers. This failure is often associated with the presence of myeloid-derived suppressor cells (MDSCs): an immune population with high plasticity and potent immunosuppressive capacity that plays an active role in the development of metastasis, the primary cause of death in TNBC patients. Targeting MDSCs is an attractive therapeutic strategy, however the specific molecular features that define their identity, and the inflammatory mechanisms these cells employ to drive metastatic spread, remain elusive.

Material and Methods

Using a single-cell multi-omic approach, we profiled the tumour microenvironment and lung cellular ecosystem before and after metastatic colonization in the highly metastatic 4T1.2 model of TNBC. Comparatively, we used the non-metastatic 67NR model as a baseline to identify the pro-metastatic MDSCs and define the specific mechanisms that contribute to cancer dissemination.

Results and Discussions

We have created a multi-omic spatiotemporal atlas of inflammation associated with breast cancer metastasis in TNBC. Our scRNAseq approach revealed the composition and functional diversity of inflammatory cells in tumours and metastatic sites, identifying a subclass of granulocytic MDSCs involved in the formation of the pro-metastatic niche and progression to metastatic disease. We further characterized this pro-metastatic MDSC subclass integrating the scRNAseq with chromatin accessibility (scATACseq) and a high-dimensional (100+) cell surface antibody panel (CITE-seq) to reveal molecular targets with potential therapeutic value.

Conclusion

Our multi-omic definition of pro-metastatic MDSCs is a step forward for designing new strategies to efficiently reprogram immunosuppressive mechanisms and stimulate antitumor immunity, ultimately paving the way for the development of the next generation of immunotherapy for metastatic breast cancer.

EACR22-0398

Autotaxin impedes anti-tumor immunity by suppressing chemotaxis and tumor infiltration of CD8⁺ T cells

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Introduction

To improve the efficacy of immunotherapy, it is essential to better understand how cytotoxic CD8⁺ T cells infiltrate into tumors. Here, we examine a role for autotaxin (ATX) in this process. ATX (encoded by *ENPP2*) is a secreted phospholipase that produces the lipid mediator lysophosphatidic acid (LPA) to regulate multiple biological functions via specific G protein-coupled receptors, termed LPAR1-6. ATX/LPA promotes tumor cell migration via LPAR1 and T-cell motility via LPAR2, yet its actions in the tumor microenvironment remain unclear. Here, we show that tumor-intrinsic ATX suppresses T-cell infiltration to impede anti-tumor immunity, and identify LPAR6 as a T-cell migration inhibitory receptor. Hence, ATX inhibition may show clinical benefit for patients with cancer.

Material and Methods

We apply a variety of molecular, cell biological and biochemically techniques for *in vitro* and *ex vivo* studies, as well as single-cell RNA-seq analysis of human tumors. In addition, we use a murine anti-cancer vaccination model to monitor ATX-dependent T-cell responses and tumor infiltration, and apply single-cell RNAseq analysis of human tumors.

Results and Discussions

We find that ATX secreted by melanoma cells is a chemo-repellent for *ex vivo* expanded tumor-infiltrating lymphocytes (TILs) and peripheral CD8⁺ T cells, overruling chemokine activity. Mechanistically, T-cell repulsion is mediated by G_{12/13}-coupled LPAR6, which is highly expressed in immune cells. Contrary to prevailing notions, secreted ATX is bioactive at physiologically insignificant steady-state LPA levels, revealing its secondary function as an LPA carrier or chaperone. Upon anti-cancer vaccination of tumor-bearing mice, tumor-intrinsic ATX does not affect the induction of systemic T-cell responses but, importantly, suppresses tumor infiltration of cytotoxic CD8⁺ T cells and thereby impairs tumor immune control. Moreover, *ENPP2* expression in melanoma tumors – in both malignant and stromal cells – is associated with reduced T-cell infiltration, as inferred from single-cell

transcriptomics. These findings highlight an unexpected role for the pro-metastatic ATX-LPAR axis in suppressing CD8⁺ T cell infiltration and anti-tumor immunity.

Conclusion

By opposing T-cell infiltration while activating tumor cells via different LPA receptors, the ATX/LPA complex exerts dual synergistic actions in the tumor immune microenvironment. ATX inhibition could benefit patients with cancer, depending on their tumor ATX expression profile.

EACR22-0598

Deciphering prognostic potential of purinergic enzymes in colorectal cancer

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Introduction

Purinergic signaling has been in the focus of tumor immunology studies due to its potential role in immunosuppression. High expression of purinergic ecto-enzymes was indicated in numerous cancers including breast, ovarian, lung and colorectal. Therefore, the aim of this study is to characterize tumor cell specific expression of purinergic ecto-enzymes CD39 and CD73 and to associate prognostic significance of these expression patterns in colorectal cancer (CRC) patients.

Material and Methods

Gene expression of the target genes in various CRC cell lines were assessed via WB, RT-PCR and flow cytometry. Additionally, tumor vs stromal cell expression of the target genes was analyzed from publicly available patient expression datasets. Finally, the correlation between CD39 and CD73 expression with patient prognosis was analyzed via TCGA datasets.

Results and Discussions

In CRC cell lines, CD39 found to be not expressed at all while CD73 was expressed extensively in most cell lines via WB, RT-PCR and flow cytometry analyses. Patient microarray expression data confirmed the results from CRC cell lines that CD39 expression was very low in epithelial/tumor cells relative to other stromal cell types yet CD73 was expressed abundantly in every cell type within patient tumor samples. Interestingly, CD39 expression in patient tumors was correlated with favorable prognosis while CD73 expression was associated with worse prognosis.

Conclusion

Although CD39 and CD73 are related enzymes involved in extracellular purinergic signaling, their expression patterns in tumor cells and prognostic effects in patients show opposing outcomes. Therefore, better insights in understanding the functional involvement of purinergic ecto-enzymes in colorectal tumor development is needed via further mechanistic studies.

EACR22-0658

Uncovering a new regulatory $\gamma\delta$ T cell subpopulation in human solid cancers

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Introduction

$\gamma\delta$ T cells contribute to the anti-tumor immunity within the tumor microenvironment (TME) in various cancers. Despite their well-described effector functions, recent studies correlated their presence in the TME with solid tumor progression suggesting that $\gamma\delta$ T cells may display pro-tumor activities. My project aims to characterize those pro-tumoral or regulatory $\gamma\delta$ T cells and decipher their role in cancer.

Material and Methods

For flow cytometry analysis, $\gamma\delta$ T cells were isolated from the blood of healthy donors or from fresh breast and ovarian tumors. For imaging analysis, breast and ovarian tumor samples were from paraffin-embedded tissue or tissue microarray collections.

Results and Discussions

We demonstrated *in vitro* that inflammatory signals promote the development of a regulatory $\gamma\delta$ T cell sub-population characterized by the expression of CD73 and displaying immunosuppressive functions through the production of immunosuppressive molecules such as IL-10, adenosine and the angiogenic and chemotactic factor IL-8. The challenge associated with the characterization of CD73+ $\gamma\delta$ T cell resides in assessing their existence *in vivo* as well as their relevance in human cancers. We showed in human breast cancer that ~20% of $\gamma\delta$ tumor infiltrating lymphocytes (TILs) expressed CD73 and displayed the same immunosuppressive functions as described *in vitro*, suggesting that they could promote tumor development via these mechanisms. In line with these observations, we showed that the presence of $\gamma\delta$ TILs is associated with late tumor grades in breast cancer. We extended such observations to ovarian cancer and showed that the density of CD73+ $\gamma\delta$ TILs negatively correlates with patient survival, suggesting that CD73+ $\gamma\delta$ TILs density could be used as a prognosis factor. Using Imaging by Mass Cytometry, we are now investigating the cellular networks of regulatory $\gamma\delta$ TILs (CD73+) and their effector counterpart (CD73-) in breast and ovarian tumors to better understand their role in cancer. Our data show different immediate ecosystems for CD73+ compared to CD73- $\gamma\delta$ TILs, with more blood vessels and cancer-associated fibroblasts in contact with CD73+ $\gamma\delta$ TILs, while CD73- $\gamma\delta$ TILs interact more with activated $\alpha\beta$ TILs reinforcing the idea that CD73+ and CD73- $\gamma\delta$ T cells are functionally different.

Conclusion

Altogether, these data improve our knowledge on human $\gamma\delta$ T cell immunobiology during cancer development, with the in-depth characterization of the new regulatory $\gamma\delta$ T cell subset, their localization and their functions within the TME.

EACR22-0678

B7-H4 NON-IMMUNOLOGICAL ROLE IN INCREASING ENDOMETRIAL CANCER AGGRESSIVENESS

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Introduction

Endometrial cancer (EC) represents the most diagnosed gynaecological malignancy. Based on histopathology and clinical behavior, EC is divided in estrogen-dependent endometrioid type I and the most aggressive estrogen-independent non-endometrioid type II. Despite treatment options are used successfully for Type I subtype, limited options remain if tumors relapse or metastasize. Overall, the 5-year overall survival rate for high stage cancer is

around 15%-17% and mortality has increased by approximately 1.4% per year. For these reasons, identify a novel biological marker could help to design a better therapeutic approach target for patients with advanced or recurrent disease. Its high immunogenicity and increased CD3+ and CD8+ tumor-infiltrating lymphocytes, makes it an ideal target for immunotherapy. B7 family, a cell-surface protein ligands, binding to receptors on lymphocytes, is intensely studied for their potential clinical impact in human malignancies. Its member, B7-H4, play a role in inhibition of both CD4+ and CD8+ T cell. Additionally, its expression has been found in different cancer types and associated with poor clinical outcomes.

Material and Methods

Analysing TCGA data, it has been performed a molecular profiling in a cohort of 507 patients, both types I and II: Using RT-PCR and western blot B7-H4 expression was analyzed in different primary human EC cell lines. In vitro, it was investigated the cell autonomous role of B7-H4 in controlling cell survival and migration.

Results and Discussions

Preliminary results show that B7-H4 is more expressed in Type II EC patients compared with Type I. Similarly, it has been found in EC primary cell lines with a high expression in the most aggressive mixed type I/II cells. Additionally, patients with high B7-H4 expression show a shorter disease-free survival. These findings support a possible role of B7-H4 in increasing the aggressiveness of cancer cells. Furthermore, silenced B7-H4 cells show a reduction in migration capability and cell proliferation. To support B7-H4 immunological role, preliminary data obtained by in vitro co-culture with macrophages and Natural Killer cells, showed that B7-H4-silenced cells influence immune cells recognition.

Conclusion

These preliminary results may suggest that B7-H4 could have an additional non-immunological role supporting EC malignancy and could be useful as prognostic marker and potential new biological target for immunotherapy.

EACR22-0936

High levels of Cartilage Oligomeric Matrix Protein relate to a higher density of collagen fibers and exclusion of the immune cells in periampullary adenocarcinoma

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Introduction

Cartilage Oligomeric Matrix Protein (COMP) is a large pentameric protein predominantly expressed in the cartilage under physiological conditions. Previously, we showed that COMP can be expressed by the cancer cells and in the stroma of breast cancer patients. Expression of COMP by the cancer cells was an independent prognostic marker of poorer prognosis. Further investigation of the molecular mechanism in vivo and in vitro revealed that COMP alters cancer cell metabolism, chemoresistance, tumor volume and metastases. Following studies established that COMP is expressed in tumors from patients with colon cancer, prostate cancer, and hepatocellular carcinoma.

Material and Methods

The current study evaluated COMP expression using immunohistochemistry in TMA from 175 patients with periampullary adenocarcinoma. Biopsies from the primary tumors and corresponding lymph node metastases were included in the TMAs. Collagen was evaluated with Sirius Red-Fast Green staining. Expression of COMP and collagen density was co-analyzed with detailed data of tumor-infiltrating immune cells collected in previous studies.

Results and Discussions

Tumors from approximately 50% of the patients expressed high COMP levels in the cancer cells and stroma. A strong correlation between elevated COMP levels and pancreatobiliary-type tumors morphology was detected. A relationship between high levels of COMP expression and exclusion of immune cells was detected. In particular, COMP expression led to the exclusion of T cells from the cancer cell compartment of the tumor. Such exclusion phenomenon is observed in patients with tumors that are resistant to immune-checkpoint inhibitors. Finally, COMP expression was associated with more dense collagen fibers, which also correlated with the exclusion of the cancer compartment's immune cells.

Conclusion

High levels of COMP expression in the cancer cell and stroma are associated with pancreatobiliary type and correlate with dense collagen fibers, which may be preventing the infiltration of T-cells into the cancer cells compartment of the tumor.

EACR22-0141

Exploiting the Immune-modulatory Effects of PI3K/mTOR inhibitors to Remodel the Tumor Microenvironment in Uterine Leiomyosarcoma

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Introduction

Uterine leiomyosarcomas (uLMS) are aggressive tumors with poor prognosis and high risk of hematologic spread. Currently, no effective therapeutic agents are available for metastatic and relapsed cases. Despite indications that uLMS might benefit from immune checkpoint blockade (ICB), a clinical trial investigating single-agent nivolumab proved unsuccessful with no responses. The lack of response to ICB in uLMS can be attributed to T-cell exclusion from the tumor microenvironment (TME), which represents a major mechanism of intrinsic resistance to ICB. Recent research has focused on identifying feasible approaches to inflame these 'immune-desert' tumors to prime them for ICB. Here we show that PI3K/mTOR inhibitors are able to substantially remodel the TME of uLMS with high S6 phosphorylation levels (pS6^{high}), reflecting PI3K/mTOR pathway over-activation, which could open new horizons for combination treatment strategies with ICB.

Material and Methods

Humanized (Transcure) and immunodeficient uLMS patient-derived xenografts (PDXs) were treated with a combination of sapanisertib (0.3 mg/kg/day) and alpelisib (25 mg/kg/day) or vehicle. Tumor punch biopsies were collected pre- and post-treatment and subjected to bulk and single-cell RNA sequencing. Data analysis was performed in R using DESeq2 or Seurat based pipelines.

Results and Discussions

Transcriptional profiling of the pre- and post-treatment biopsies collected from pS6^{high} uLMS PDXs, revealed that PI3K/mTOR inhibitors induce the expression of pro-inflammatory chemo- and cytokines. In humanized pS6^{high} uLMS PDXs, treatment with PI3K/mTOR inhibitors elicits an anti-tumor immune response, leading to tumor growth stabilization vs. resistance development and regrowth in immunodeficient PDXs. Treated tumors in humanized PDXs display clonal expansion of the tumor-infiltrated T-cell population and increased expression of activation and cytotoxicity markers and co-inhibitory ligands.

Conclusion

Here, we show that PI3K/mTOR inhibitors drive T-cell activation and trigger an anti-tumor immune response in humanized pS6^{high} uLMS PDXs. The tumor-infiltrated T-cells display the transcriptional profile of antigen-experienced T-cells with polyfunctional effector properties. Based on our results, we believe that PI3K/mTOR pathway over-activation confers an immunosuppressive phenotype and may be driving primary ICB resistance in a fraction of uLMS (pS6^{high}). Thus, targeted inhibition of this pathway could be a potential strategy to make these tumors susceptible to ICB.

EACR22-0153

A more active immune infiltrate delineates aggressiveness of rhabdomyosarcomas in adolescent and young adults

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Introduction

Adolescents and young adults (AYA) with rhabdomyosarcoma (RMS) form a subgroup of patients whose optimal clinical management and best possible access to care remain a challenge and survival lacks behind that of children while diagnosed with histologically similar tumors. We previously reported a modulation of the immune infiltrate present in AYAs-RMS suggesting a compromised/suppressed immune profile possibly driving the age-related aggressiveness of these tumors.

Material and Methods

A careful immunohistochemical (IHC) characterization of the immune infiltrate of the tumor parenchyma and also the modulations of the immune response in adjacent normal tissue samples was performed on a cohort of pediatric (PED) and AYA RMS. A panel of immune markers was evaluated both in tumor stroma and in non-neoplastic tissue surrounding the

tumor, with particular interest in possible presence of Tertiary Lymphoid Structures (TLSs). GEP data obtained from our RMS clinical series was analyzed with the perspective to stratify patients based on the composition of their tumor microenvironment, immune contexture and age.

Results and Discussions

IHC analysis unveiled 5 distinct pattern of expression of immune cells: Diffuse, Aggregate, Early-TLS, TLS, and Negative. A much more compromised and active immune infiltrate consisting of increased T cells, and in many cases also B, located mainly in the stroma and around the vessels was observed in the AYA-RMS, with 3 cases presenting TLSs. PEDS-RMS revealed a lower involvement by the immune contexture with the majority of cases classified as negatives and only one case presented structures suggestive of early TLS. Interestingly, MHC I was reported always negative (or poorly expressed) by the tumor, on the contrary always very expressed by the infiltrate and by the endothelial cells, both in PEDS and AYA. GEP analysis stratified RMS in the 5 immunological classes (immune desert, vascularized, immune inflamed and 2 intermediates), and revealed an enrichment of expression of endothelial genes leading to a better prognosis for pediatrics and a worse prognosis for AYA-RMS.

Conclusion

Our preliminary GEP analysis and IHC findings describe an enrichment of endothelial genes and a more active immune infiltrate in AYA-RMS, with presence of tertiary lymphoid structures, providing a possible rationale for aggressiveness of the AYA-RMS.

EACR22-0359

Differential Molecular Mechanisms Regulating the Expression of the Immune Checkpoint Protein VISTA in Human T Lymphocytes, Cancer and Embryonic Cells as well as Keratinocytes

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Introduction

Immune checkpoint proteins play crucial roles in human embryonic development, yet increasing evidence shows that this biochemical machinery is used by cancer cells to escape immune surveillance. These proteins and biochemical pathways associated with them form complex networks, which are capable of affecting the ability of cytotoxic immune lymphoid cells to attack and kill cancer cells. One such recently discovered immune checkpoint proteins is V-domain Ig-containing suppressor of T cell activation (VISTA). The

biochemical mechanisms regulating VISTA expression remain unknown, their investigation was the aim of this work.

Material and Methods

We used human Jurkat T cells, TALL-104 cytotoxic T lymphocytes, THP-1 acute myeloid leukaemia (AML), MCF-7 breast cancer, WT3ab Wilms tumour cells, HaCaT keratinocytes, K562 chronic myelogenous leukaemia cells. Primary AML samples, primary T cells and primary human embryonic cells were also investigated. Western blot analysis, ELISA, on-cell Western, qRT-PCR, and chromatin immunoprecipitation (ChIP) assay were used as main instruments to conduct the study.

Results and Discussions

We discovered for the first time that VISTA expression is controlled by the TGF- β – SMAD3 signalling pathway. Smad3 was found to directly bind VSIG (VISTA encoding gene) promoter region. However, the responses observed were differential. Granzyme B expressing cytotoxic T cells responded by downregulation of VISTA expression when exposed to TGF- β . Other types of T cells, cancer cells expressing VISTA, primary human embryonic cells and keratinocytes showed clear TGF- β -dependent upregulation of VISTA. The only exception were human acute myeloid leukaemia (AML) monocytes, which, despite absence of cytotoxic activity, responded by downregulation of VISTA expression. More premature AML cells, in line with other types of cancer cells studied, showed clear TGF- β -dependent upregulation of VISTA. Further experiments suggested that nuclear compartmentalisation most likely determines the ability of Smad3 to access VSIG, leading to the expression being upregulated.

Conclusion

Our study revealed the self-supporting biochemical mechanism responsible for induction of VISTA expression. We demonstrated the differential biochemical control of VISTA expression in human T cells and various types of rapidly proliferating cells, including cancer and foetal cells as well as keratinocytes. We hypothesized that this phenomenon is most likely determined by differential nuclear compartmentalisation of VSIG containing loci.

EACR22-0485

Transcriptomic studies provide new queues in tumor immune escape

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Introduction

The importance of understanding how intra-tumor heterogeneity limits natural and therapy-induced anti-tumor immune responses is very much appreciated by today's oncologists and researchers. While the impact of genetic heterogeneity in the success of immunotherapies has been well established, some studies show that immune-driven selective pressure does not affect genetic heterogeneity. Thus, we put our efforts in exploring the phenotypic adaptations of immunogenic tumors that may be implied in the escape to immunosurveillance.

Material and Methods

MC38 (colorectal adenocarcinoma) and Panc02 (pancreatic adenocarcinoma) cell lines were subcutaneously implanted in wild type or immunodeficient (Rag2^{-/-} Il2rg^{-/-}) mice. After

monitoring tumor growth kinetics, bulk tumor DNA was used for exome sequencing, and transcriptome analysis was performed in enriched tumor samples after CD45⁺ cell sorting.

Results and Discussions

Tumor growth kinetics comparing both immune settings confirmed immunogenic (MC38) and non-immunogenic (Panc02) behaviors, in the sense that MC38 cells grow better in an immunodeficient background, and Panc02 cells grow equally well in immunodeficient and immunocompetent backgrounds. Interestingly, mutation load analysis showed an excess of synonymous and non-synonymous mutations in Panc02 tumors growing in the absence of adaptive immunity, while no difference was observed in MC38 tumors. Growth under the presence or absence of adaptive immunity led to different tumor cell transcriptomes. Interestingly, non-immunogenic Panc02 tumors showed the strongest changes, with significant alterations in mRNA levels of several genes, suggesting potential roles for chemokine signaling and cell adhesion in evasion and/or subversion of the immune system.

Conclusion

Contrary to our expectations, signs of immunoediting are observed in tumors with a non-immunogenic behavior but not in immunogenic tumors. Our results suggest that the reason why Panc02 cells grow with equal kinetics in immunodeficient and immunocompetent backgrounds is not their lack of immunogenicity, but rather their capability of efficiently subverting the immune response. This study supports the potential of applying transcriptomic studies in apparently non-immunogenic tumor models to screen for new queues involved in tumor immune escape. Ultimately, we hope to help improve the efficacy of existing and new immunotherapy approaches, as these can be directed to subsets of cancer cells that play a defining role in the immunomodulatory actions of the tumor.

EACR22-0757

Interferon-independent STING activity in Non-Small Cell Lung Cancer translates into cancer progression and aggressiveness.

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Introduction

Canonical STimulator of INterferon Gene (STING) signaling in tumor infiltrated immune cells leads to a potent induction of type I interferon (IFN) and interferon-stimulated genes, governing the establishment of a more efficient anti-tumor immune response. However, recent publications suggest that some type of tumors may rely on STING activation to fuel their growth. In this study, we evaluate the contribution of STING expression on Non-Small Cell Lung Cancer (NSCLC) progression.

Material and Methods

Using the CRISPR/Cas9 system, we investigated the impact of endogenous STING expression through different in vitro and in vivo approaches based on cancer cell lines derived from primary tumors of the Kras^{LSL-G12D}/WT;p53^{fl/fl} (KP) mouse model of lung cancer.

Results and Discussions

Direct stimulation of STING pathway revealed an IFN-deficient STING signaling in our model. Monitoring the immune infiltrates in tumors showed that STING expression can sustain tumor promoting neutrophil infiltration and immune-suppression. Coherently with these results, when treated with anti-Programmed cell death protein 1 antibody (a-PD1)

immunotherapy, STING proficient tumors were more resistant to therapy compared to STING knockout. RNA sequencing analysis on cancer cells sorted from ex vivo tumors confirmed these results and highlighted a contribution of STING in the differential expression of the sex determining region Y-box 2 (SOX2) transcription factor, known to orchestrate the recruitment of tumor promoting neutrophils and cancer progression in squamous lung cancer. Consistently, we noticed that the absence of STING in the cancer cells significantly reduced their ability to engraft and form tumors in vivo. We performed bioinformatics analysis highlighting a link between STING expression and the Epithelial to Mesenchymal Transition (EMT) program across multiple human lung cancer cells. Migration assays and expression profiles of EMT markers in our models confirmed these findings. These results imply an original role of STING in the EMT program that could influence tumor progression and shape the tumor microenvironment.

Conclusion

The evidences presented above delineate a complex role of STING in cancer cells. Our results are opening intriguing new scenarios to investigate the biological processes linked to the STING pathway, in a context where STING agonists are seen as an extremely attractive option to increase immune checkpoint blockade efficacy in NSCLC patients.

EACR22-0849

Predictive value of immune parameters for the response to induction chemotherapy in laryngeal and hypopharyngeal squamous cell carcinoma

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Introduction

The role of the immune response to cancer is of increasing importance, showing a determining role in the response to treatment and prognosis of patients. The identification of new biomarkers related to the immune response may allow a better selection of treatments and the identification of potential therapeutic targets.

Our goal was to assess the correlation of immune parameters with the response to induction chemotherapy (ICT) in laryngeal and hypopharyngeal squamous cell carcinoma patients.

Material and Methods

Pretreatment biopsies from 64 patients with laryngeal and hypopharyngeal squamous cell carcinoma that received ICT were assessed for PD-L1 protein expression and density of CD8+ and FOXP3+ tumor infiltrating lymphocytes (TIL). In addition, the neutrophil-lymphocyte ratio (NLR) was calculated from pretreatment whole blood counts.

Results and Discussions

In total, 55% of cases exhibited PD-L1 combined proportion score (CPS) positivity ($\geq 1\%$ stained cells). PD-L1 CPS positivity correlated with a high density of both CD8+ ($p = 0.01$) and FOXP3+ ($p < 0.001$) TILs. There was no correlation between PD-L1 expression or TIL

density and NLR values. In univariate analyses, the absence of PD-L1 CPS expression ($p = 0.042$) and a high NLR ($p = 0.034$) were significantly correlated with response to ICT. Neither CD8⁺ TIL ($p = 0.99$) nor FOXP3⁺ TIL densities ($p = 0.71$) were associated with response to ICT. In multivariate analysis, only a high NLR was associated with response to ICT (HR = 4.06, 95% CI = 1.06–15.5, $p = 0.04$). In addition, a high NLR was also independently associated with lower disease-specific ($p = 0.03$) and overall survival rates ($p = 0.04$), particularly in the subset of patients who received definitive surgical treatment

Conclusion

These results suggest that NLR could emerge as a predictive biomarker of response to ICT.

EACR22-0889

Diesel particles exposure favors lung cancer progression through induction of an immunosuppressive microenvironment.

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Introduction

Air pollution, and more specifically fine particulate matter, is a major health problem. These particles are characterized by a small diameter and a deep penetration into the respiratory track. Indeed, several epidemiological studies have reported a significant association between exposure to fine particles and the development of both lung cancer and chronic respiratory/cardiovascular diseases. However, the impact of fine particles on the pathogenesis of lung cancer progression and the underlying mechanisms remain unclear.

The aim of this study was to determine whether exposure to fine particles may create a lung microenvironment suitable for lung cancer progression.

Material and Methods

To this purpose, C57BL/6 mice were exposed to diesel exhaust particles (DEP) by intratracheal instillations. After 2 instillations of DEP (200µg/mL), lungs were collected and the immune microenvironment was analyzed by flow cytometry using a set of specific antibodies. Among neutrophils, polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs) are of particular interest for their role in cancer progression. To evaluate whether the inflammatory microenvironment conditioned by DEP had an impact on tumor progression, mice were injected orthotopically with Lewis Lung Carcinoma (LCC) cells.

Results and Discussions

DEP-instilled mice display an increased lung tumor progression as compared to sham-treated mice. Tumor microenvironment neutrophil counts and FACS analysis have revealed increase neutrophil counts. Our results indicate that CD14^{int} and CD14^{high} PMNs are increased in the lung of DEP instilled mice.

Conclusion

Exposure to DEP contributes to the creation of a supportive lung microenvironment for tumor progression in which recruited neutrophils are probably involved. We will perform systematic T-cell proliferation assays to characterize PMN-MDSCs immunosuppression capacity.

EACR22-1127

Immune activity in tumors is regulated by collagen

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Introduction

The extracellular matrix (ECM) is a complex network of fibrillar proteins and proteoglycans, which in addition to forming a physical scaffold that holds tissues together can affect numerous biological processes. However, little attention has been given to the possibility that the ECM can have immune modulatory functions. During cancer progression, the ECM is extensively remodeled and the generated tumor-specific ECM is known to have pro-tumorigenic functions. Here, we have investigated if collagen type I, which is the main ECM component in solid tumors, can affect the activity of immune cells.

Material and Methods

T cells or macrophages were cultured in 3D systems allowing investigations of the effects of different collagen densities on cellular function. The effects of collagen on T cells and macrophages were evaluated through a combination of whole-transcriptome analysis and a number of functional assays (e.g. proliferation and cytotoxicity assays). The effects of collagen on tumor progression and the tumor immune environment in vivo was investigated using two different transgenic mouse strains that allowed for specific manipulation of collagen type I levels. These mice were combined with studies of the syngeneic pancreatic cancer model PanO2.

Results and Discussions

In a 3D cell culture system, a high collagen density led to reduced proliferation of primary T cells compared to culture in a low-density collagen matrix. A high collagen density also induced a distinct gene expression profile, which clearly indicated that the T cells became less cytotoxic and more immune regulatory. This effect of collagen on T cells was confirmed using tumor-infiltrating T cells from melanoma patients, which after culture in high-density collagen compared to low-density collagen became less efficient at killing autologous cancer cells. A high collagen density also changed the phenotype of macrophages towards a more immune suppressive type. In vivo, a reduction of collagen levels using inducible conditional collagen type I knockout mice led to reduced tumor growth accompanied by increased infiltration of NK cells and CD8 T cells. The opposite effects on tumor growth and on immune infiltration were observed in transgenic mice, which accumulated higher levels of collagen in the tumors.

Conclusion

Our studies identify collagen-induced T cell suppression as a novel and conceptually different immune modulatory mechanism, which could play an important role in cancer and in other pathological conditions.

EACR22-0322

Toll-like Receptor 2 promotes breast cancer progression and resistance to chemotherapy

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Introduction

Toll-Like Receptor 2 (TLR2) is a fundamental player of innate immunity that recognizes molecules associated with pathogen invasion and tissue damages, promoting inflammation and immune responses. However, TLR2 expression in breast cancer is associated with poor prognosis. We have demonstrated that TLR2 is overexpressed in breast cancer stem cells (CSCs), responsible for cancer progression, therapy resistance and invasion, and promotes their self-renewal through an autocrine pathway initiated by high mobility group box 1 (HMGB1). To evaluate the potential benefits of TLR2 targeting, here we further investigated the role of TLR2 in breast cancer progression and resistance to chemotherapy in preclinical models.

Material and Methods

We generated TLR2^{WT}-neuT and TLR2^{KO}-neuT mice expressing the rat HER2-neu oncogene, which spontaneously develop mammary tumors. We compared tumor progression and performed FACS analysis of CSCs and immune cells in tumors and other compartments. We derived cancer cell lines from primary tumors of TLR2^{WT} and TLR2^{KO} mice in order to characterize *in vitro* the TLR2-mediated cancer cell intrinsic pro-tumor effects. Moreover, we analyzed the effect of TLR2 inhibitors in combination with chemotherapy either *in vitro* or *in vivo*.

Results and Discussions

TLR2^{KO}-neuT mice showed a delayed tumor onset and increased survival as compared to TLR2^{WT}-neuT mice, which was accompanied by a reduction in CSCs and T regulatory cells. Transplantation experiments demonstrated that TLR2 acts mainly through cancer cell intrinsic mechanisms. The *in vitro* characterization of tumor-derived TLR2^{WT} and TLR2^{KO} cell lines demonstrated that TLR2 affects cancer cell proliferation and CSC self-renewal. Furthermore, TLR2 conferred resistance to chemotherapy in mouse and human breast cancer cell lines. This effect was mediated by chemotherapy-induced release of the TLR2 ligand HMGB1, which through TLR2 signaling promotes breast CSC self-renewal and proliferation. Treatment with a TLR2 inhibitor impaired viability and induced apoptosis of breast cancer cell lines, and an enhanced effect was observed when combined with chemotherapy *in vitro* and *in vivo*.

Conclusion

Taken together, our results enlightened the pro-tumorigenic role of TLR2 in breast cancer progression and chemoresistance in preclinical models of breast cancer. Moreover, we demonstrated that TLR2 silencing or inhibition impair breast tumor progression and restore sensitivity to therapy, opening new perspectives in breast cancer treatment.

EACR22-0907

Translation initiation at the non-canonical start codon `CUG` is negatively regulated by Elongin B (alias Tceb2)

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Introduction

Therapeutic cancer vaccines aim to induce cytotoxic T-lymphocytes (CTL) responses that can eliminate malignant cells. CTLs recognize peptide antigens present on major histocompatibility complex (MHC) class I molecules. Ideally, vaccine formulations should target cancer-specific antigens that are not expressed in healthy tissues. Mutation-dependent neoantigens therefore appear as ideal candidates, but low frequencies of immunogenic targets and labor-intensive procedures challenge this personalized approach.

More recently, atypical or 'cryptic' protein translation events are discussed as mutation-independent neoantigens in cancer. Translation of non-coding gene elements or alternative reading frames could be targets for immunotherapy, if cancer specific. Translation initiation at non-canonical start codons can lead to cryptic expression, but the mechanisms involved remain poorly understood. Here, we aim to study the processes involved in non-canonical translation initiation.

Material and Methods

Murine MC57 fibroblasts were transduced to express a green fluorescent protein (GFP) driven by the non-canonical start codon 'CUG' (CUG-GFP). Next, we performed a genome-wide CRISPR-Cas9 screen to identify knockout (KOs) events that induced cryptic GFP expression. The top 5% GFP-positive cells were enriched by fluorescence-activated cell sorting (FACS) and subsequently sequenced to identify negative regulators of non-canonical translation initiation. Validation of hits was performed using three independent cryptic reporter systems.

Results and Discussions

We identified the transcription elongation factor B (Elongin B, alias Tceb2) as a negative regulator of CUG-initiated translation. Tceb2 KO cells showed increased translation of CUG-GFP and a CUG-nanoluciferase reporter. Also, Tceb2-deficiency induced the MHC class I presentation of the H-2K^b-restricted model antigen SIINFEKL (from chicken ovalbumin) when expressed as CUG-SIINFEKL minigene. As Tceb2 is known as a key component of specific Cullin E3 ubiquitin ligases, we currently investigate if deactivation of these enzymes can induce cryptic translation. To this end, we could exclude a role for the E3 ligase Cullin 2 in this process.

Conclusion

Together, we identified Tceb2 as a negative regulator of non-canonical translation initiation, and we currently study the underlying mechanisms of action. Our results contribute to the understanding of cryptic translation and may provide mechanistic insights relevant for the generation of mutation-independent neoantigens in cancer.

EACR22-1267

B1-cells of Innate Immunity in the Bone Marrow in Breast Cancer

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Introduction

In recent years more attention has been paid to the study of the innate immune system, which includes B1-lymphocytes. They produce pentameric M antibodies, which play an important role in the induction of apoptosis in tumor cells. The Study of lymphocyte populations can help to reveal the phenomenon of persistence of disseminated tumor cells in the bone marrow (BM) of breast cancer (BC) patients.

Material and Methods

This study included BM punctuates from 64 BC patients and 10 women with benign processes. The study was carried out by two methods: morphological and immunological. Calculation of the myelogram under light microscopy was performed by two expert morphologists. Multiparameter flow cytometry (FACSCanto II cytometer) has been used to

assess the populations of BM lymphocytes. Antibodies CD20, CD5, CD19, CD38, CD22, CD45 were used.

Results and Discussions

It was found that with luminal B-Her2 "+" breast cancer, the content of CD5+ cells is higher than with B-Her2 "-": 10.2% (n=10) versus 4.0% (n=20), p=0.032. The highest levels of CD5+ cells were observed in stage IIA: 12.4±10.7%. Also increased levels of these cells were observed with 2 affected lymph nodes and their maximum size: 16.0±10.2% (n=5) and 5.8±1.6% (n=29), p=0.07. Tumor size and stage were correlated with the levels of promyelocytes and metamyelocytes. The average levels of B1-lymphocytes were significantly higher with a normal or reduced relative content of segmented neutrophils in BM in BC in comparison with cases of increased segmented neutrophils: 8.1±1.8% (n=39) and 2.0±0.5% (n=19); p=0.002. A correlation was found between CD5+lymphocytes and the number of monocytes in benign processes: R= -0.953; p=0.002; n=9. The content of B1-lymphocytes correlated with eosinophilic myelocytes (R=0.365; p=0.011; n=48), plasma cells (R=0.409; p=0.004; n=48) in BC. Her2/neu«+» BC is associated with lower levels of neutrophilic myelocytes (8.3±0.4% versus 9.6±0.5, p=0.043) and the maturation index of granulocytic cells (0.4±0.02% versus 0.5±0.03, p=0.005).

Conclusion

We believe that the determination of the level of B1-lymphocytes in the BM can serve as an additional marker of the molecular subtype of BC. It is described that an increase in the content of plasma cells takes place with DTC in the bone marrow. Based on this it can be assumed an increase in the level of B1-lymphocytes is associated with a high probability of metastases in the BM.

EACR22-0623

ORAL PRESENTATION

Pancreatic cancer subtype-specific secreted factors determine the immunosuppressive tumor microenvironment

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a complex disease displaying genetic heterogeneity along with a tumor microenvironment (TME) unique in its composition. Whereas the PDAC TME is known to be highly immunosuppressive, it remains elusive how distinct genetic alterations affect TME cell type recruitment and how this, in turn, influences progression and treatment outcome. Therefore, we systematically investigated the TME composition and tumor-immune crosstalk in molecular PDAC subtypes.

Material and Methods

Here, we describe the TME landscape of a comprehensive tumor cohort derived from a *Kras*-driven PDAC mouse model by integrating single-cell RNA-sequencing (scRNA-seq) with histopathological analysis, tissue RNA-seq followed by deconvolution and flow cytometry immunophenotyping. In order to dissect tumor-immune crosstalk, we performed secretome analysis of mouse *Kras*-driven as well as human PDAC cell cultures representing the two major transcriptional PDAC subtypes (classical and mesenchymal) and integrated secretome data (ligand expression from tumor cells) with scRNA-seq data (receptor expression of TME cells) to decipher cell-cell communication networks within the PDAC TME.

Results and Discussions

Our results show that distinct molecular PDAC subtypes recruit divergent subpopulations of infiltrating immune cells, most notably evidenced by major differences in frequency and phenotype of macrophages and myeloid-derived suppressor cells (MDSC). Functional secretome analysis of well-characterized *Kras*-driven mouse and primary human PDAC cell lines revealed subtype-specific factors which differ between the two main transcriptional subtypes of PDAC (classical and mesenchymal). Accordingly, crosstalk between tumor and immune cells inferred by ligand-receptor interaction analysis from scRNA-seq data nominates the CSF signaling network as a key player in mesenchymal PDAC, whereas CXCL signal hubs mediate higher infiltration of immunosuppressive MDSC in classical tumors.

Conclusion

Our findings contribute to the understanding of PDAC subtype-specific biology and aid the identification of potential immunotherapeutic vulnerabilities thereof.

EACR22-0835

ORAL PRESENTATION

Therapy-induced senescence triggers viral mimicry and promotes anti-tumour immunity in acute myeloid leukaemia

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Introduction

Acute myeloid leukaemia (AML) is an aggressive haematological malignancy treated with high-dose chemotherapy. However treatment failure is frequent and mainly due to the development of chemo-resistant tumours, thus underscoring the need to investigate the molecular events occurring immediately after chemotherapy. Therapy-induced senescence (TIS) is a state of irreversible growth arrest triggered by several stressors including anticancer compounds. Senescent cancer cells may elicit immune-mediated responses contributing to senescent cell eradication or favouring tumour relapse. Our work aims at functionally interrogate the crosstalk between senescent leukemic cells and the immune system with the final long-term goal to unveil mechanistic insights into the factors dictating AML response to chemotherapy and immunotherapy

Material and Methods

Multiparametric flow cytometry analysis integrated with quantitative imaging was used to measure several senescent markers and human leukocyte antigen (HLA) expression. Mixed lymphocyte reaction assay was adopted to evaluate T cells activation. Immunological synapses were analysed by ImageStreamX technology. Viral mimicry via transposable elements reactivation was identified by RNA-seq and validated by qPCR.

Results and Discussions

We established *ex-vivo* cultures of primary AML samples or cell lines undergoing senescence by chemotherapy or Ibrance (Palbociclib®) treatment. RNA sequencing and flow cytometry analyses of treated samples revealed upregulation of senescence-associated genes and immune-related interferon gene categories with concomitant induction of several HLA class I and class II molecules, suggesting a causal link between TIS and blasts immunogenicity. Mechanistically, we found that TIS cells activate distinct endogenous retroviral elements (TEs) families converging on NFkB or IFN-related gene programs depending on the initial senescence inducer. Senescent blasts triggered an enhanced CD4⁺ and CD8⁺ T cell activation and promoted an increased immunological synapses formation, leading to leukaemia eradication. *In vivo* profiling of T cell subsets in senescent-competent AML primary samples revealed increased percentage of T cells with an activated phenotype and reduced exhaustion markers upon chemotherapy compared to diagnosis.

Conclusion

Our findings uncover a novel link between senescence induction and leukemia immune recognition via viral mimicry, which may rapidly translate into innovative senescence-based strategies to cure or prevent AML post-therapy relapse.

EACR22-1180

ORAL PRESENTATION

SMAC Mimetics and Endocrine Blockade Enhance Antigen Presentation and T-cell mediated Cytotoxicity in Estrogen Receptor Positive Breast Cancer.

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Introduction

Immuno-oncology has revolutionized cancer care, particularly for cancers characterized by high tumor mutational burden, high levels of tumor-infiltrating lymphocytes (TILs) and high expression of PDL-1. Currently, efficacy of immunotherapy in ER+ breast cancer (BC) is very limited and was demonstrated in selective patients only, underscoring the importance of identifying mechanisms to harness the immune system for therapeutic utility for a broader population of patients with ER+BC.

Material and Methods

We used TCGA and METABRIC data to investigate the association between ER expression and immune related transcriptional signatures. We also measured the response to IFNg stimulation in established ER+ BC cell lines cultured in hormone deprived (HD) conditions

and in the presence of estradiol (E2). We performed RNA, ChIP and ATAC sequencing of ER+ cells in HD and E2 conditions at different time points of IFN γ stimulation. We used an in vitro co-culture system between ER+ BC cells and primary human T cells to test T-cell mediated cytotoxicity and migration.

Results and Discussions

We found that in ER+ Luminal BC, the expression of immune related genes were significantly higher in the ESR1-low compared to ESR1-high tumors indicating a link between high ER expression and a lower immune phenotype. MHC-I and PD-L1 induction by IFN γ was significantly attenuated by the presence of E2 compared to HD conditions. RNAseq and ATACseq results suggested that the activation of immune related gene sets after downregulation of ER signaling is orchestrated by key transcription factors, including NF κ B, STAT3 and AP1. Since NF κ B signaling can be pharmacologically enhanced by the SMAC mimetics, we studied the effect of the combination of Birinapant with Fulvestrant on IFN γ response, cytokine secretion, T cell mediated cytotoxicity and migration. We found that this combination increased the secretion of cytokines associated to antitumoral immune response and enhanced T cell mediated cytotoxicity and migration of T cells toward ER+ cancer cells.

Conclusion

Here we describe a potential role of ER signaling in modulating the antitumoral immune response. Our studies indicate that the transcriptional activity of NF κ B is augmented by silencing of ER signaling, suggesting that the crosstalk between ER and NF κ B has a key role in the diminished response to IFN γ mediated by ER. These results suggest that the combination of endocrine treatment and a SMAC mimetic is a potential novel therapeutic approach to leverage immune based therapies in ER+ BC.

EACR22-0516

Epigenomic and mutational profiling of primary and metastatic colorectal cancers: exploring epigenetic determinants of tumor lymphocytic infiltration

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Introduction

Immunotherapy offers promising therapeutic strategies complementary to conventional chemotherapy. Immunotherapy is mostly based on stimulating the host immune response against cancer cells. Tumor-infiltrating lymphocytes (TILs) are essential for this response: CD8+ lymphocytes must first infiltrate the tumor mass and recognize the tumor-specific neoantigens exposed by the cancer cells. In metastatic colorectal cancer (CRC), the efficacy of immunotherapy is currently limited to tumors with microsatellite instability (MSI). This is partially explained by the much higher mutational burden and potentially higher generation of neoantigens of MSI tumors, favoring higher immunogenicity. However, MSI tumors account for less than 5% of all metastatic CRCs. Moreover, not all MSI CRCs exhibit high TILs levels and, conversely, a small proportion of MSS CRCs exhibit very high levels of TILs. Thus, there must be factors other than the mutational burden modulating tumor immunogenicity. Uncovering those factors is necessary to improve the response to immunotherapy, especially in non-MSI CRC patients who comprise the vast majority of metastatic CRC cases.

We hypothesize that, in addition to genetic mutations, epigenetic alterations alter the tumor

microenvironment potentially affecting lymphocytic infiltration. We are investigating the interplay between genetic and epigenetic alterations and their association with the level of TILs in a collection of 200 primary and 80 metastatic CRCs.

Material and Methods

We profiled mutations in *TP53*, *KRAS*, *BRAF*, *BAX*, and MSI by direct sequencing of their mutational hot-spots, generated epigenomic profiles with Illumina Methylation Arrays HM450K/EPIC and methylation-sensitive QPCR of repetitive DNA elements, and assessed tumor-infiltrating lymphocytes by H&E staining and immunostaining of CD8 and CD3 antigens.

Results and Discussions

Our preliminary data is in agreement with previous observations: *KRAS* mutations were associated with MSS CRCs ($p < 0.001$), *BRAF* mutations with MSI CRCs ($p < 0.001$) and hypermethylation of CpG islands ($p < 0.001$), and high levels of TILs were more frequent in MSI than in MSS CRCs. We present a preliminary integration of mutational, epigenomic, and immunogenicity information in a large collection of CRCs.

Conclusion

The integration of mutational and epigenomic profiles might reveal novel factors underlying CRC immunogenicity independently of their tumor mutational burden, facilitating future therapeutic interventions to improve the response to immunotherapy in CRC patients.

EACR22-0612

Targeting the MYC transcriptional repressor complex reactivates CD8 T-cell infiltration in an immunogenic mouse model of Lung Adenocarcinoma

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Introduction

Modelling dynamic immune responses in genetically engineered mouse models has proven challenging, primarily due to low mutation burden and consequent lack of immune visibility of most allelic combinations. APOBEC family enzymes are overexpressed in several cancers and drive mutagenesis by deaminating Cytidine residues, with the resulting Uracil replaced by base excision or an equivalent DNA repair mechanism. Mutations in coding sequences or aberrantly expressed transcripts may result in generation of neo-epitopes, with consequent recognition of the affected cell as non-self by the adaptive immune system. However, we and others have shown that common driver oncogenes MYC and KRAS actively suppress immunosurveillance, in part via repression of the Type I Interferon pathway. Alongside MYC's role as transcriptional activator, it also forms repressive complexes with MIZ1 and the histone methyltransferase G9A, suppressing expression of key regulators of Type I Interferons, amongst other genes. Here we examined the potential for pharmacological targeting of the MYC repressor complex, via G9A inhibition, to restore critical immune signalling pathways within the tumour microenvironment of Lung Adenocarcinoma.

Material and Methods

Lung tumours were induced in mice carrying conditional alleles *Lsl-KRas^{G12D}*, *Rosa26^{DM.lsl-MYC}*, +/- *Rosa26^{DS.lsl-APOBEC3B}*, (KMA or KM, respectively) by intranasal installation of Adeno-SPC-CRE. Tumours were harvested at defined times post induction and analysed by IHC, RNA-SEQ, & ScRNA-SEQ. Tumour bearing mice were treated with anti-PD1 +/- a novel G9A inhibitor, HKTM-01-005, and examined acutely or monitored for survival benefit.

Results and Discussions

Inclusion of the APOBEC3B allele drove a transiently effective CD8 T-cell mediated anti-tumour immune response, resulting in extended survival of KMAs compared with KMs. Temporal analysis showed pronounced CD8 tumour infiltration at 8 weeks in KMA lungs but immune exclusion by 12 weeks post induction. PD1 blockade alone was ineffective whereas HKTM-01-005 treatment strongly elevated Type I Interferon signalling and restored CD8 infiltration in a subset of tumours. Survival benefit studies are ongoing.

Conclusion

Pharmacological targeting of the MYC repressor complex can potentially enhance immunotherapy in LuAd. Heterogeneity of immune responses to G9A inhibition requires further investigation.

EACR22-0894

Patient-derived 3D microfluidic models to assess glioblastoma tumor microenvironment immunomodulation

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Introduction

Glioblastoma (GBM) is the most frequent and malignant brain tumor in adults, with a 5-year survival rate lower than 5% that has been largely unchanged for decades. High intratumor heterogeneity and a strongly immunosuppressive microenvironment are some of the features that make the search for treatment a real challenge. In recent years, groundbreaking advances in immunology have moved immunotherapies to the first-line treatment for several cancers. However, immunotherapy efficacy in GBM remains limited. Organ-on-chip technology allows biomimetic simulation of the physiological environment of organs and tissues in vitro, making it a powerful tool for studying more effective treatments in the context of personalized medicine.

Material and Methods

To assess the immunomodulatory effect exerted by the GBM tumor microenvironment, patient-derived spheroids were cocultured with allogeneic immune cells (CD4+ and CD8+ T cells) in a 3D collagen matrix. The suppression exerted by the tumor on T cells was

characterized by RT-qPCR. Next, macrophages were added to the GBM tumor environment, and the role of this population in modulating the immune response was also assessed. The phenotypic outcome of the interaction between both populations and the underlying mechanisms were analyzed by cytokine detection via ELISA and intracellular cytometry. Immunohistochemical and microscopy techniques were performed.

Results and Discussions

T cell gene expression was altered in the presence of GBM. Furthermore, macrophages were shown to play a key role in regulating the anti-tumor immune response of T cells in the scope of a hypoxic GBM microenvironment. Finally, the distinct localization of T cells and macrophages in a tumor microenvironment generated in vitro with cells derived from GBM patients was determined. As a remarkable result, hypoxia was shown to regulate the behavior of these immune populations in GBM.

Conclusion

GBM microenvironment dampens the immune response by a net of complex mechanisms that involves several cell types such as macrophages and T cells. A better understanding of the mechanisms underlying tumor-induced immunosuppression will allow us to improve clinical outcomes. Moreover, microfluidics and organ-on-chip allow a better representation of tumor microenvironment complexity, as well as controlled monitoring of processes such as cell communication or cell tracking. Our work has paved the way to bring us closer to precision medicine, allowing us to tailor immunotherapy treatment in a patient-specific manner.

EACR22-1236

Methylglyoxal regulates the recruitment of tumor-infiltrating immunosuppressive cells in breast cancer

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Introduction

Cancer metabolism, and in particular the Warburg glycolytic switch, is an essential aspect of tumorigenesis that points to methylglyoxal (MG) as a novel oncometabolite which accumulation sustains specific pro-cancer functions. MG is a very reactive dicarbonyl with high protein glycation capacity notably leading to the formation of advanced glycation end products (AGEs). We have previously demonstrated that MG stress triggers enhanced breast tumour growth and metastasis *in vivo*.

Material and Methods

MMTV-PyMT spontaneous breast cancer mouse model was used in order to assess the accumulation of MG adducts during disease progression. Immunohistological staining was performed to target MG adducts during adenoma, early and late carcinoma progression steps. MMTV-PyMT mice were treated with carnosine, a potent MG scavenger dipeptide, in order to assess the immune landscape in primary tumors and lung metastases using FACS screening. To explore the specific recruitment of immune cells to the primary tumor upon MG

treatment, we performed a cytokine array for the simultaneous detection of several cytokines and chemokines in conditioned medium of 4T1 murine breast cancer cell line.

Results and Discussions

Using immunohistochemistry, we showed a significant cytoplasmic accumulation of MG AGEs from adenoma to late carcinoma lesions indicating the occurrence of MG stress during spontaneous tumour progression in MMTV-PyMT model. In good accordance, we observed a significant reduction of lung metastatic foci upon mice treatment with carnosine. FACS screening and characterization of the whole immune cell population in MMTV-PyMT experimental tumors demonstrated a significant decrease of specific immunosuppressive cell subtypes upon carnosine treatment. These changes in immune landscape upon MG stress were notably linked to CXCL5 and CXCL1 increase as revealed by cytokine array analysis.

Conclusion

Taken together, our data point to a novel function of MG in the recruitment of immunosuppressive cells, that could be reversed using carnosine and potentially targeted to enhance the susceptibility of breast tumors to available therapeutic regimen.

EACR22-0739

ETV7- a novel regulator of breast cancer immunity

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Introduction

ETV7 is an oncoprotein, up-regulated in breast cancer (BC). We have recently demonstrated that the ETV7 is induced in the response to the DNA damaging agents and that this increased expression directly promotes resistance of BC cells to doxorubicin. Furthermore, ETV7 is widely known to be an Interferon-stimulated gene (ISG). It has been shown that ETV7 is involved in the viral immune response by suppressing a subset of ISGs. However, the role of ETV7 in BC immune processes is still to be studied. For a better application of immune therapies, we should identify novel regulators of tumor immunity and uncover the strategies used by cancer cells to evade host immune responses. In this study, we propose ETV7 as a novel regulator of tumor immunity, involved in several immune evasion mechanisms in BC.

Material and Methods

To decipher the transcriptional networks regulated by ETV7, we performed RNA-seq on BC cells stably over-expressing ETV7 (MCF7 and T47D). The results were validated using molecular and cellular techniques such as Western blot, qPCR, ChIP-qPCR, Gene reporter assays, mammosphere formation assay and FACS.

Results and Discussions

Gene ontology analyses on the RNA-seq data from BC cells over-expressing ETV7 vs Empty vector showed that ETV7 is involved in the repression of pro-inflammatory and

Interferon-stimulated genes. After confirming the down-regulation of target genes, we focused on unveiling the potential biological consequences. Firstly, we demonstrated that the ETV7-mediated repression of the ISGs could lead to an increased subpopulation of breast cancer stem cells. We confirmed that upon ETV7 over-expression, there was a significant increase in the population of CD44⁺/CD24⁻ cells (representing breast cancer stem cells). Moreover, ETV7 directly represses TNFRSF1A (encoding for TNFR1) by binding to its Intron I, leading to reduced activation of NF-κB pathway. The repression of ISGs, as well as, the reduced activation of NF-κB is known to cause the down-regulation of antigen-presenting – one of the major mechanisms of immune evasion in cancer.

Conclusion

Here we present two novel ETV7-regulated molecular mechanisms involved in immune response in BC. Firstly, we propose a novel role for ETV7 as a regulator of breast cancer stem cell-like plasticity, which is mediated by the repression of ISGs. Secondly, we show that ETV7 directly down-regulates TNFRSF1A reducing the activation of NF-κB signaling. Both mechanisms suggest, that ETV7 can modulate tumor immunity and potentially promoting immune evasion.

EACR22-0785

Role of the cystine/glutamate antiporter xCT in the interaction between mammary cancer and the immune system

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Introduction

The cystine/glutamate antiporter xCT is upregulated by cancer cells to afford protection against oxidative stress and represents a relevant target for cancer treatment. xCT activity is also required for the activation and proliferation of some immune cells. However, whether the overall outcome of xCT depletion in the tumor and in the immune system results in promotion or suppression of tumor growth, and whether xCT has a role in the interplay between cancer and immune cells, is unknown. The aim of this study is to investigate how xCT deficiency in mammary cancer cells affects tumor progression in an immunocompetent mouse model, and if systemic xCT deficiency positively or negatively affects tumor growth by modulating the antitumor immune responses.

Material and Methods

We generated xCT-KO mouse mammary cancer cells (4T1) and tested their malignant properties *in vitro* and *in vivo*. We generated xCT^{null} BALB/c mice to investigate the role of xCT in the immune system and xCT^{null} / ErbB2-transgenic BALB-neuT mice to study the contribution of xCT to tumor initiation and progression in a mammary cancer-prone model.

Results and Discussions

Despite xCT depletion in 4T1 reduced clonogenic potential and sensitized cancer cells to oxidative stress *in vitro*, it did not prevent transplantable tumor initiation and growth *in vivo*. It however reduced migration *in vitro* and lung metastasization *in vivo*, possibly with the contribution of altered cytokine secretion and immune cell recruitment. Systemic depletion of xCT prevented activation/polarization of immune cells cultured *ex vivo* but did not impair the proper mounting of both humoral and cellular immune responses *in vivo*. Finally, xCT depletion in mammary cancer-prone mice did not alter autochthonous tumor initiation.

However, when cultured *in vitro*, cancer cells isolated from these mice showed proliferation and redox balance defects compatible with the lack of xCT.

Conclusion

While xCT is required for both cancer cell malignancy and immune cell functionality *in vitro*, it is partially dispensable *in vivo*. The mechanisms underlying this discrepancy need to be identified. Nevertheless, xCT depletion in cancer cells may sensitize otherwise normally growing tumors to oxidative stress-inducing therapies, opening possibilities for combinatorial approaches involving xCT targeting. Our results also suggest that therapies targeting xCT would have an anti-metastatic effect while preserving antitumor immunity.

EACR22-0597

Multiparametric characterization of early-stage Small Cell Lung Cancer human tumors reveals novel patient subgroups based on specific molecular to immune landscape associations.

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Introduction

Small cell lung cancer (SCLC) is a lethal malignancy for which more therapies are urgently needed. SCLC tumors are rarely resected, and the limited sample to study has complicated the emergence of new treatments. Genetic heterogeneity in SCLC tumors preclude patient subgrouping although recent findings established groups of SCLC patients with similar transcriptomic profiles (SCLC-A, SCLC-N, SCLC-P, SCLC-I), and specific treatment susceptibilities. We hypothesize that SCLC patients can also be defined by distinct molecular and immune landscapes, which might have impact on clinical outcome and therapy selection.

Material and Methods

We employed samples from a cohort of 119 early-stage surgically resected SCLC patients. We comprehensively described these samples by immunohistochemistry (immune landscape), RNA-sequencing targeted panel of immune related genes (onco-immune transcriptomics) and by exome sequencing (genomics). All data, including clinical annotations, were integrated by multiparametric computational analysis.

Results and Discussions

Clustering of the onco-immune transcriptomic data identified two major groups of patients with proimmunogenic and protumorigenic profile with better and worse survival, respectively. The proimmunogenic group showed higher expression of genes related to TCR co-expression and lymphocyte infiltrate, and higher infiltration of immune cells assessed by IHC. In detail, we observed an overexpression of B cell regulation (MS4A1, CXCR5, FCGR2B) and immune checkpoint genes (TIGIT). In contrast, the protumorigenic group presented higher expression of proliferation and tumor marker genes, and less immune infiltrate. Patients in the proimmunogenic group might be better candidates to receive immunotherapies than the protumorigenic group. This novel distribution of patients did not relate to either any genomic features nor any of the previously described transcriptomic profiles, since most of the samples were described as SCLC-A by the predominant expression of ASCL1.

Conclusion

Here we present a novel classification of early-stage SCLC patients according to their proimmunogenic and protumorigenic transcriptomic profiles. This condition significantly impacts immune infiltrate and disease outcome, and it is not related to either previously described transcriptomic subtypes nor genetic alterations. These new subgroups can contribute to find effective biomarkers of immunotherapy response, which will require validation in independent cohorts of advanced disease.

EACR22-0508

Integration of miRNA:mRNA co-expression revealed crucial mechanisms modulated in immunogenic cancer cell death

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Introduction

Immunogenic cell death (ICD) in cancer is a functionally unique regulated form of stress-mediated cell death that activates innate and adaptive immune system response against tumor cells. ICD is characterized by the sequential release and/or exposure of certain damage-associated molecular patterns by dying cells. MicroRNAs (miRNAs) play critical roles in the regulation of both cell death and immune-associated mechanisms in cancer, while evidence of their potential involvement in ICD is limited. Therefore, we aimed to explore the participation of miRNAs and their putative targets in the context of ICD.

Material and Methods

B cell lymphoma (Mino) and breast cancer (MDA-MB-231) cell lines were exposed to two different ICD inducers: RA/IFN α and doxorubicin, and to non-ICD inducer: gamma irradiation. Then, miRNA and mRNA profiles were studied by next-generation sequencing. miRNA predicted targets were analyzed by real-time PCR and flow cytometry.

Results and Discussions

Co-expression analysis identified 16 miRNAs differentially modulated in cells subjected to ICD. Integrated miRNA-mRNA functional analysis revealed candidate miRNAs, mRNAs, and modulated pathways associated with Immune System Process (GO Term). In this sense, ICD induced a distinctive transcriptional signature hallmarked by regulation of antigen presentation, a crucial step for a proper immune system antitumor response activation. Interestingly, the major histocompatibility complex class I (MHC-I) pathway was upregulated whereas class II (MHC-II) was downregulated. Analysis of MHC-II associated transcripts and HLA-DR surface expression validated the inhibition of this pathway by ICD on lymphoma cells. miR-4284 and miR-212-3p were the strongest miRNAs upregulated by ICD associated with this event. It is well known that MHC-II expression on tumor cells facilitates the recruitment of CD4+ T cells. However, the interaction between tumor MHC-II and the inhibitory coreceptor LAG-3 on tumor-associated lymphocytes could provide an immunosuppressive signal that directly represses effector cytotoxic activity. In this context, MHC-II downregulation by ICD could enhance antitumor immunity.

Conclusion

We found that the miRNA profile was significantly altered during ICD. Several miRNAs are predicted to be involved in the regulation of Class I and II MHC pathways, whose implication in ICD was demonstrated herein for the first time, which could eventually modulate tumor recognition and attack by the immune system.

EACR22-1058

A common genetic variant of the gene encoding Granzyme B associates with cancer risk in patients with Lynch syndrome

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Introduction

Lynch syndrome (LS) is a frequent cancer predisposition syndrome caused by the germline heterozygous mutations of key genes of the DNA mismatch repair machinery (*MLH1*, *MSH2*, *EPCAM*, *MSH6*, *PMS2*). LS-associated tumors are characterized by high levels of microsatellite instability (MSI-H), resulting in high tumor mutation burden, neoantigen exposure, immune infiltration and favorable response to immune checkpoint inhibition therapies. At-risk individuals with germline pathogenic mutations in *MLH1*, *MSH2* or *EPCAM* genes have 40-60% lifetime risk for colorectal (CRC) and uterine cancers, leaving room for disease-modifying genetic and environmental factors. Granzyme B is the most abundant serine protease present in the granules of cytotoxic T cells mediating apoptosis in target cells in a perforin-dependent manner, contributing to anticancer immune response. Rs8192917, a common missense single nucleotide polymorphism (SNP) of the gene encoding Granzyme B (*GZMB*) has been associated with nonsegmental vitiligo, a frequent dermatologic condition of autoimmune pathomechanism, presumably due to the differential cleavage of autoantigens of key pathogenic relevance. Our aim was to investigate if rs8192917 has any association with cancer risk in individuals with LS.

Material and Methods

145 individuals with germline pathogenic mutations in *MLH1*, *MSH2* or *EPCAM* genes (mean age: 42.9 ± 15.2 years) were included in this study. DNA was isolated from peripheral blood. Rs8192917 was genotyped by TaqMan Allelic Discrimination Assay on an Applied Biosystems 7500 real-time PCR instrument. The effect of the SNP on cancer risk was analyzed using log-rank test. Granzyme B cleavage sites of shared neoantigens of MSI-H tumors were analyzed by the PROSPEROUS online tool.

Results and Discussions

Homozygous carriers of the minor allele of rs8192917 compared to heterozygous or homozygous carriers of the major allele were associated with later occurrence of LS-related cancers ($p = 0.0050$) or CRC in particular ($p = 0.0188$). High probability Granzyme B cleavage sites were found in 41.3% of the shared neoantigens of MSI-H tumors.

Conclusion

Rs8192917 might exert a protective role regarding carcinogenesis in patients with LS. Granzyme B might be involved in the modification of several MSI-H associated neoantigens.

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EACR22-0643

Eliciting Anti-tumor Immunity by Reprogramming Cancer Cells to Type 1 Conventional Dendritic Cells

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Introduction

An important hallmark of cancer is escaping the immune system. Despite advances in immunotherapy, only a subset of patients experiences clinical benefits. It was shown that adoptive T cell or checkpoint inhibition therapy rely on the presence of conventional dendritic cells type 1 (cDC1). cDC1 excel in recruiting and priming protective CD8⁺ T cells through cross-presentation. However, in tumors cDC1 are often impaired in function. Recently, we demonstrated that overexpression of PU.1, IRF8 and BATF3 (PIB) imposes a cDC1 fate in fibroblasts by direct cell reprogramming. As such, we hypothesise that a similar combination of transcription factors would reprogram cancer cells into tumor-antigen presenting cells (tumor-APCs) and set in motion antigen-specific immunity.

Material and Methods

30 mouse tumor lines were selected to evaluate reprogramming into tumor-APCs. Reprogramming was induced by overexpression of PIB via lentiviral transduction. The phenotype was profiled by flow cytometry for cDC1 markers CD45, MHC-II, CLEC9A, XCR1 and APC markers MHC-I, CD80/86. Population mRNA-seq was applied to assess transcriptional changes. To assess cDC1 functions, cytokine secretion, cross-presentation and T cell cytotoxicity assays were performed. *In vivo*, ovalbumin expressing tumors were established and treated by adoptive transfer of tumor-APCs. Tumor growth and animal survival were monitored.

Results and Discussions

Upon transduction with PIB, 26 solid tumor and 4 leukemia lines initiated expression of CD45, MHC-II, at efficiencies ranging from 0.5-57.7%. Reprogramming was accompanied by CLEC9A, XCR1 and MHC-I, CD80/86 upregulation. Transcriptomic analysis of low immunogenic lines B16 and LLC, reveals that PIB overwrites the cancer transcriptome and imposes antigen presentation and cDC1 gene signatures. Importantly, tumor-APCs present endogenous antigens on MHC-I and become prone to T cell mediated killing. Functionally, reprogrammed tumor-APCs secrete inflammatory cytokines such as IL12p70 and strikingly, acquire the ability to cross-present antigens and prime naïve CD8⁺ T cells. *In vivo*, adoptive

transfer of cross-presenting tumor-APCs delays tumor growth and extends survival of animals.

Conclusion

This approach combines cDC1 antigen presentation abilities with endogenous generation of tumor antigens. The induction of a cDC1 identity in tumor cells sets in motion T cell responses and makes them target for T cell mediated killing. Our study represents a pioneering contribution merging cell reprogramming with immunotherapy.

EACR22-0445

Bacterial lipopolysaccharide influences the tumour-immune microenvironment in colorectal cancer

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Introduction

The immune microenvironment in colorectal cancer (CRC) has a huge impact on cancer progression. Among the consensus molecular subtypes (CMS) of CRC, CMS1 and CMS4 are both characterized with immune infiltrates, yet CMS1 is considered to have good prognosis while CMS4 is the opposite. Although CRC subtyping has been a useful way of characterizing manifestations of this heterogeneous disease, contributions of the microbiome to the subtypes has seldom been discussed. We aimed to determine the contributions of the microbiome to the paradoxical effects we see in the immune-infiltrated subtypes, CMS1 and CMS4.

Material and Methods

We carried out RNA-sequencing on 260 CRC tumour samples, subtyped samples into respective CMS, and obtained human gene expression and microbial abundance profiles. We then identified enriched gene sets and corresponding relatively abundant microbes in CMS1 and CMS4 subtypes. Based on the relationships between enriched gene sets and abundant bacteria, we identified lipopolysaccharide (LPS) from particular bacterial species as potential modifiers of the tumour-immune microenvironment. Using peripheral blood mononuclear cells (PBMCs) as an in vitro model, we sought to investigate the effects of different LPS molecules on secreted cytokine expression.

Results and Discussions

In addition to previously described immune characteristics of CMS1 and CMS4 tumours, “lipopolysaccharide binding” was identified as an enriched gene set, suggesting a relationship with bacterial LPS. We identified *Fusobacterium* and *Bacteroides spp.* in CMS1 and *Porphyromonas spp.* in CMS4 as bacteria with LPS that may affect immune activation in the tumour microenvironment. Treatment of PBMCs with LPS from these bacteria showed opposing immunomodulatory effects on cytokine secretion.

Conclusion

Different microbes in the CRC tumour microenvironment not only increase immune activity but may also have an immunosuppressive effect. The balance of tumour-resident microbes may influence molecular subtypes and have important implications for CRC progression.

Translational Research

EACR22-0175

ORAL PRESENTATION

Oxidative phosphorylation is a metabolic vulnerability of treatment resistant ER positive metastatic breast cancers

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Introduction

Treatment resistance and development of metastasis are associated with metabolic reprogramming in breast cancer (BC). Evidence of a metabolic rewiring in clinical studies is mainly based on transcriptomic changes, while metabolite concentration analyses are less prevalent due to the scarce amount of tissue in metastases biopsies. The aim of this study was to identify metabolic vulnerabilities in treatment resistant metastases of ER+ BC.

Material and Methods

We developed PDX of metastatic ER+ BC from bone metastases (BM) of patients progressing on endocrine therapy and CDK4/6 inhibitors. Gene expression and global metabolomics analyses of matched primary tumors and BM-derived PDX were performed by RNAseq and UPLC-MS, respectively. PDX were treated with an inhibitor of the oxidative phosphorylation (OXPHOS), IACS -010759 (IACS). A pharmacodynamic metabolomics analysis was performed to identify metabolic pathways driving response or resistance to IACS treatment. Finally, the prognostic relevance of different OXPHOS associated genes was assessed by RT-PCR in a cohort of 503 BC.

Results and Discussions

At the transcriptomic level, BM samples showed enrichment of oxidative phosphorylation (OXPHOS) and respiratory electron transport gene sets. Metabolomics analyses identified enrichment in different pathways, including the TCA cycle, pentose pathway, nucleotide synthesis and glutathione metabolism. IACS treatment of PDX resulted in strong anti-tumour activity in multiple endocrine resistant models, including a PDX derived from a patient resistant to the CDK4/6 inhibitor palbociclib. Moreover, IACS treatment strongly prolonged mice survival in a PDX with acquired resistance to palbociclib, and characterized by an enrichment of different mitochondria associated pathways. The metabolomics analysis revealed that glutathione and cysteine metabolism were down-regulated in IACS responder PDX as compared to IACS resistant models, both at the basal level and in treated tumours, suggesting a decreased capacity to counteract oxidative stress. PDX treatment with an inhibitor of glutathione synthesis decreased resistance to IACS, further supporting the role of glutathione metabolism in determining the response to the OXPHOS inhibitor. Finally, high expression of different OXPHOS genes was associated with a shorter metastases-free survival in ER positive BC patients.

Conclusion

These results demonstrate that OXPHOS is a metabolic vulnerability of treatment resistant metastatic ER positive BC.

EACR22-0038

LIRAGLUTIDE PLUS CALORIE RESTRICTION PREVENTS THE SPONTANEOUS DEVELOPMENT OF TYPE 1 ENDOMETRIAL CANCER IN BDII/HANS RATS

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Introduction

Glucagon-like 1 peptide (GLP-1) based therapies may hold potential for the prevention and arrest of early-stage Type 1 endometrial cancer (EC1) We investigated the impact of the GLP-1 analogue liraglutide in combination with caloric restriction on tumor dynamics in the BDII/Han rat model of spontaneous EC1.

Material and Methods

Twelve-month-old BDII/Han rats (n=38) were randomised to either *ad libitum* access to a standard rodent chow diet (STD n=17) or Liraglutide (1mg/kg/d) therapy plus 50% calorie (LIR-diet n=21). After 3 months, animals were euthanised and uterine horns retrieved to record tumour incidence and assess both tumour type and grade. Oestrus cycle stage was established via assessment of ovarian and endometrial histology.

Results and Discussions

The LIR-diet regimen resulted in 15% weight loss. Tumour incidence was 58% (10 of 17) in STD and 19% (4 of 21) in the LIR group (p = 0.0184). In the STD group, 7 of the 8 tumours assessed histologically were EC1 type, with one animal developing a serous type tumour (EC2). All tumours in the LIR-diet group were EC2 type, as confirmed by p16 immunohistochemistry and were immune poor/excluded and CD8+ lymphocyte negative. LIR rats were predominantly arrested in diestrus (64%) and proestrus (29.4%), with follicular atresia frequently observed.

Conclusion

Liraglutide in combination with dietary restriction prevented the development of type 1 endometrial cancer-like disease in BDII/Han rats. Evidence suggests that the treatment also selected for the development of serous type, hormone-independent cancer in a smaller number of animals.

EACR22-0286

Impedance of autophagy improves the potency of utroside B, a US FDA-approved 'orphan drug' against hepatocellular carcinoma

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Introduction

Our previous study has demonstrated that uttroside B (Utt-B), a saponin isolated from the leaves of *Solanum nigrum* Linn, is ten times more cytotoxic towards liver cancer cells, than sorafenib, the standard anti-HCC drug approved by U.S. FDA. The compound induces apoptosis in hepatic cancer cells and exhibits a remarkable growth inhibition of Hepatocellular Carcinoma (HCC). Our innovation has been granted a patent from the US (US 2019/0160088A1), Canada (3,026,426.), Japan (JP2019520425) and South Korea (KR1020190008323) and the technology have been transferred commercially to Q Biomed, a leading US-based Biotech company. Recently, the compound received approval as 'Orphan Drug' against HCC from US FDA, which reveals the clinical relevance of evaluating its antitumor efficacy against HCC.

Material and Methods

Human liver cancer cell lines, HepG2 and Hep3B were employed as HCC models and the therapeutic efficacy of Utt-B against HCC was evaluated *in vivo* in NOD.CB17-Prkdcscid/J mice bearing HepG2-induced HCC xenografts.

Results and Discussions

In the present study, we report that Utt-B is more potent than sorafenib, *in vivo*. Utt-B also promotes pro-survival autophagy in hepatic cancer cells as evidenced by the increased expression of autophagy-related proteins, including LC3-II, Beclin1, ATG 5, and ATG 7, as well as a rise in the autophagic flux. We investigated whether Utt-B-induced autophagic response is complementing or contradicting its apoptotic program in HCC. Inhibition of autophagy using the pharmacological inhibitors, Bafilomycin A1(Baf A1), and 3-methyl adenine (3-MA), and the biological inhibitor, Beclin1 siRNA, significantly enhances the apoptosis of hepatic cancer cells and hence the cytotoxicity induced by Utt-B. We also found increased expression of autophagy markers in Utt-B-treated xenografts derived from HCC. Our study revealed that inhibition of autophagy using the antimalarial drug, Chloroquine (Cqn), a well-known autophagy inhibitor, significantly enhances the antitumor efficacy of Utt-B *in vitro* and *in vivo*, in NOD SCID mice bearing HCC xenografts.

Conclusion

Our results suggest that Utt-B is a more potent anti-HCC drug than sorafenib and the antitumor effect of Utt-B against HCC can be further enhanced by blocking autophagy. Hence, Utt-B in combination with Cqn, a clinically approved drug, if repurposed and used in a combinatorial regimen with Utt-B, can further improve the therapeutic efficacy of Utt-B against HCC.

The phyto-saponin, Uttroside B, displays an augmented therapeutic index over sorafenib in a pre-clinical model of hepatocellular carcinoma

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Introduction

We previously reported the potency of *Solanum nigrum*-derived uttroside B (Utt-B) against HCC. Recently Utt-B is flagged as an 'orphan drug' against hepatocellular carcinoma (HCC) by the United States Food and Drug Administration (U.S. FDA). The current study aims to validate *in vivo* the enhanced efficacy of Utt-B over sorafenib, the first-line treatment option against HCC using murine model.

Material and Methods

Human liver cancer cell line, HepG2 was employed as an HCC model and the comparison between Utt-B vs sorafenib therapeutic efficacies against HCC were evaluated *in vivo* in NOD.CB17-Prkdcscid/J mice bearing HepG2-induced HCC xenografts. Acute and sub-chronic toxicological studies were conducted in Swiss albino mice.

Results and Discussions

Our data indicate that Utt-B is a more potent anti-HCC drug than sorafenib, *in vivo*. Apart from the superior therapeutic benefit over sorafenib, Utt-B is pharmacologically safer *in vivo*, and owing to this virtue, the drug-induced side effects are largely alleviated in the context of HCC chemotherapy.

Conclusion

Our data demonstrate the superior therapeutic index of Utt-B over sorafenib against HCC. Clinical studies in HCC patients utilizing Utt-B, which now holds the U.S. FDA approval as an 'orphan drug' is an essential step to promote this drug from bench to bedside.

Response to genomic-driven targeted therapies as compared to standard of care treatments in PDX models of advanced ER positive breast cancer

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Introduction

A significant number of patients with estrogen receptor positive (ER+) breast cancer (BC) display intrinsic or acquired resistance to endocrine treatment (ET) and CDK4/6 and mTOR inhibitors. Recently, investigations of the genomic landscape of metastatic BC have provided additional insight into metastatic BC biology and revealed potential therapeutic targets. The aim of this study was to evaluate response to genomic-driven targeted therapies as compared to standard of care treatments in patient-derived xenografts (PDX) of ER+ metastatic BC.

Material and Methods

PDX were generated from ER+ metastatic BC patients progressing under ET or CDK4/6 inhibitors. Molecular characterizations using SNP-arrays and targeted exome sequencing analysis were performed to identify targetable genomic alterations. The anti-tumor efficacy of PI3K, MEK, mTOR, PARP, FGFR1 and AKT1 inhibitors were tested *in vivo* as compared to the ET fulvestrant combined to the CDK4/6 inhibitor palbociclib or the mTOR inhibitor everolimus. The best response and the best average response were calculated to classify treatment response as progressive disease, stable disease or response.

Results and Discussions

Twelve PDX (11 from bones and 1 from liver metastases) were established. Targetable genomic alterations were identified in 10/12 PDX (83%) including pathogenic mutations in AKT1, BRCA2, PI3KCA, mTOR and NF1 genes and focal amplifications of FGFR1 and FGFR2. In 8 PDX, targeting these alterations resulted in tumor regression or stable disease: two AKT1 mutated models responded to the AKT1 inhibitor AZD5363, 3 PIK3CA mutated PDX responded to the PIK3CA inhibitor alpelisib, the NF1 mutated tumor responded to a MEK inhibitor trametinib and 2 FGFR1 amplified models to a FGFR1 inhibitor. Treatment with palbociclib and everolimus associated with fulvestrant resulted in tumor regression or stable disease in respectively 80% and 100% of PDX. In 78% of PDX, responses to targeted therapies combined to fulvestrant was greater than responses to palbociclib or everolimus associated with ET, including 2 PDX models established in the post-CDK4/6 setting.

Conclusion

In summary, we report the establishment of different PDX that recapitulate the genomic landscape of advanced ER+ BC and allowed to investigate the anti-tumor activity of different biomarker-driven targeted therapies as compared to standard of care.

EACR22-0368

ERBB2 exon 20 insertions are rare in Brazilian Non-small Cell Lung Cancer

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Introduction

Lung cancer is the deadliest cancer around the world. Targeted therapies have revolutionized the treatment of lung cancer patients. Recently, agents targeting *ERBB2* oncogene were developed and can be an alternative for lung cancer patients, particularly those harboring exon 20 insertions. Therefore, we aimed to evaluate the frequency of *ERBB2* exon 20 insertions and their clinicopathological and molecular features in a large series of Brazilian NSCLC patients.

Material and Methods

We analyzed 722 Brazilian non-small cell lung cancer (NSCLC) patients from Barretos Cancer Hospital that were indicated to routine lung cancer molecular testing. *ERBB2* exon 20 insertions were evaluated by a targeted panel using next-generation sequencing (NGS). Clinicopathological and molecular data were collected from patients' medical records.

Results and Discussions

From 722 NSCLC patients, most of them were lung adenocarcinomas (85.2%), male (53.9%), self-declared white skin color (59.6%), quitter or current smokers (66.8%), and diagnosed in an advanced stage of the disease (63.2%). We identified 6/722 (0.8%) *ERBB2* mutated patients harboring the insertion p.(Tyr772_Ala775dup) at exon 20. All mutant *ERBB2* patients were lung adenocarcinoma, never smoker, and wild-type for *EGFR*, *KRAS*, *BRAF*, and *ALK*.

Conclusion

We observed that *ERBB2* exon 20 insertions are rare in Brazilian NSCLC, yet these patients could benefit from anti-*ERBB2* drugs.

EACR22-0438

Patient-derived models of brain metastases are useful tools for the preclinical evaluation of anticancer therapies

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Introduction

Brain metastases (BM) are the most common brain tumor in adults. Patients with BM have a poor outcome, with a median survival rate of 8–16 weeks even after standard of care treatment. Despite recent medical advances, knowledge about the mechanism underlying BM development is limited and no effective therapies exist to treat these patients. Thus, there is an unmet need to develop translational platforms that are appropriate for therapeutic development. We have established patient-derived cultures (PDCs) and xenografts (PDXs) using BM collected from patients with diverse primary tumors and observed that these models recapitulate the biology of human disease. Here, we aimed to validate the utility of our patient-derived models for drug testing.

Material and Methods

PDCs were isolated from BM derived from melanoma, lung, endometrium and colon cancer patients. PDCs were treated with buparlisib (pan-PI3K inhibitor) and everolimus (mTOR inhibitor). We assessed downstream signaling inhibition by western blot and drug effect on proliferation by MTS. Subcutaneous and intracranial xenograft models using NSG mice were developed with the PDCs. Mice were randomized into three groups (vehicle, buparlisib and everolimus), and received three cycles of drug administration by oral gavage. Tumor samples were collected at necropsy and submitted to histopathological analysis. mRNA sequencing analysis was performed in human BM and the corresponding subcutaneous tumors of xenografts.

Results and Discussions

The *in vitro* drug effect was variable according to each tumor type. Buparlisib inhibited pAkt in all PDCs, while everolimus inhibited the downstream target pS6 in PDCs from endometrium and colon cancer. The inhibition of cell proliferation was stronger for buparlisib, across all PDCs. Everolimus was effective in inhibiting proliferation of PDCs from endometrium and lung cancer. *In vivo*, both compounds effectively reduced subcutaneous and intracranial tumor growth and everolimus was also effective in decreasing leptomeningeal dissemination. Finally, mRNA sequencing analysis revealed that paired human BM and their corresponding PDX tumors have similar transcriptional profiles.

Conclusion

These observations are a proof-of-concept that our patient-derived models are useful tools for the preclinical testing of anticancer therapies.

EACR22-0474

Disruptive and truncating TP53 mutations are associated with African-ancestry and worse prognosis in Brazilian patients with lung adenocarcinoma.

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Introduction

TP53 is the most mutated gene in lung tumors. However, its prognostic role remains unclear, mainly in admixed populations. We aimed to evaluate the frequency and clinicopathological impact of TP53 mutations in Brazilian non-small cell lung cancer (NSCLC) patients.

Material and Methods

We analyzed 446 NSCLC patients from Barretos Cancer Hospital. TP53 mutational status was evaluated by targeted NGS and biologically classified as disruptive/nondisruptive, and as truncating/non-truncating. Genetic ancestry was assessed using 46-ancestry informative

markers. Analysis of lung adenocarcinomas from the cBioportal dataset was performed. *TP53* mutations were associated with patients' clinicopathological features.

Results and Discussions

TP53 mutations were detected in 64.3% (287/446) of NSCLC cases. For lung adenocarcinomas, *TP53* mutations were detected in 60.4% (n=221/366) of cases. *TP53* mutations were associated with brain metastasis at diagnosis, tobacco consumption, and higher African ancestry. Disruptive and truncating mutations were associated with younger age at diagnosis. *TP53* mutations were associated with younger age and Black skin color in cBioportal datasets. Patients harboring disruptive/truncating *TP53* mutations had worse overall survival than nondisruptive/non-truncating and wild-type patients.

Conclusion

TP53 mutations are common in Brazilian lung adenocarcinomas, and their biological characterization as disruptive and truncating mutations is associated with African ancestry and shorter overall survival.

EACR22-0534

The expression levels of methylated markers for endometrial carcinoma detection were compared in cytological samples of the uterine cavity, cervical canal, and cervix

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Introduction

The invasive procedure of obtaining endometrial tissue in histology is the gold standard for the diagnosis of endometrial cancer (EC). The difference of endometrial tissue sampling may lead to possible pathological missed diagnosis and continuous follow-up diagnosis. The methylation DNA analysis of EC detected by cytological specimens is very promising in minimally invasive detection.

Material and Methods

Six surgical women were enrolled in the study, including 2 patients with endometrial cancer (45 and 56 years old), 3 (48,50, and 49 years old) with myoma and 1 with adenomyosis of uterus (50 years old). Four cytological samples of endometrium (diseased and non-diseased), cervical canal, and cervix of each patient were collected for methylation detection. The analysis of methylation level of CDO1 were determined by using CisEndo methylation real-time system (CISPOLY Co., China). The positive results were determined at ΔCt -CDO1<8.4.

Results and Discussions

The methylated CDO1 was positive in 4 parts of EC samples, but the endometrium (disease) was at the border line for the patients with non-EC and the other 3 parts of samples were negative. The ΔCt range of methylated CDO1 were endometrium (diseased and non-diseased) were at ΔCt =1~2, cervical canal at ΔCt =1.5~3, cervix at ΔCt =6~7, and the endometrium (disease) of non-EC at ΔCt =7.8~9.94, respectively. In tissue samples of EC, methylated CDO1 was positive in endometrium (disease) at ΔCt =1~4, and other 3 were negative.

The data show that methylated CDO1 gene is a biomarker to identify EC in cytological samples from cervix to uterine cavity. The methylated CDO1 gene has regional specificity in

tissue samples, but there is no regional specificity in cell samples. Exfoliated EC cells may adhere to different parts of the uterine cavity, or accumulate in the cervical canal and cervix, resulting in the methylation results of 4 cytological samples being positive, but only positive in the EC area-tissue sample. This is the first time it has been confirmed.

Conclusion

The methylation test of cytological samples may be a new detection method to prevent missed diagnosis of hysteroscopy and this new discovery need more data for future validation.

EACR22-1012

Development of a hoRizontal data intEgration classifier for NOn-invasive early diAgnosis of breast cancer: the RENOvATE clinical trial

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Introduction

Early diagnosis of breast cancer (BC) is suboptimal in terms of accuracy and often requires invasive and expensive tests such as tissue biopsy. The assessment of circulating biomarkers to differentiate benign lesions from BC, together with radiomics, is of great potential in BC management.

Material and Methods

The RENOvATE clinical trial (NCT04781062) is a prospective translational study investigating the accuracy of the combined assessment of multiple circulating analytes together with radiomic variables for the non-invasive differential diagnosis of early BC. Patients are recruited at their presentation at the Diagnostic Senology Unit of Ospedale Policlinico San Martino (Genoa, IT) for the execution of a diagnostic biopsy after the detection of a suspect breast lesion with radiological size ≤ 2 cm (cT1N0). Patients are asked to donate one K₂EDTA tube (BD Vacutainer™), one Tempus™ Blood RNA tube (Applied Biosystems™) and two PAXgeneBlood ccfDNA tubes (BD Vacutainer™), together with a 40 ml urine bottle cup. ctDNA, cf-methylated DNA and circulating proteins are assessed in the collected samples together with those collected from patients with histologically confirmed benign lesions of similar size. A cohort of healthy controls (negative mammograms for two consecutive years) is also collected.

Results and Discussions

The trial started on January 18th 2021 and is currently open. After one year recruitment 193 patients undergoing BC biopsy were recruited. Of these, 98 presented a benign lesion, 60 presented a malignant lesion. 35 patients were not considered due to prespecified exclusion criteria. A cohort of 105 healthy patients with negative mammography were collected as well as healthy controls.

Conclusion

The overall goal of the present study is to develop a horizontal data integration classifier for the early differential diagnosis of BC. Such aim would be practice-changing in the management of such disease. Besides the planned analyses, our study has generated a unique sample biobank to benchmark novel, transformative liquid biopsy methods.

EACR22-1196

Drug screening on patient GBM cell cultures identifies omacetaxine mepesuccinate as a potent anti-glioma agent with the ability to cross the blood-brain-barrier

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Introduction

Glioblastoma (GBM) is one of the most aggressive and lethal type of brain tumors. The median overall survival of GBM patients receiving the standard of care treatment is approximately 15 months. Little progress has been made in the development of effective new therapies against GBM the past decades. This may in part have resulted from the use of sub-optimal cell line-based preclinical models. Patient-derived GBM cell culture models have therefore now become the gold standard for GBM drug discovery and development. Another major obstacle in identifying novel drugs against GBM remains the blood brain barrier (BBB). Therefore, it is crucial to select drugs able to cross BBB and reach the tumour region in therapeutically effective concentrations. In current drug repurposing approach, we evaluated available anti-cancer agents in our patient-derived drug screening platform for effective drug candidates against GBM.

Material and Methods

The FDA Oncology Drug Set II library containing 107 compounds was tested on 45 primary GBM cell cultures. We developed a drug shortlisting pipeline combining efficacy data with PD/PK characteristics of each compound. These include *in-vitro* IC50 values in relation to reported Cmax plasma values, physicochemical properties, substrate properties for efflux pumps, %plasma protein binding, literature research on CNS penetration. The therapeutic efficacy of the selected agent was assessed in orthotopic mouse PDX models, while penetration into the CNS was assessed by LC/MS/MS.

Results and Discussions

Omacetaxine mepesuccinate (OMA) was ranked as one of the most promising candidates after applying our drug selection approach. *In-vitro*, OMA revealed anti-tumour activity at IC50 values well-below reported Cmax plasma values in approximately 80% of GBM cultures. NanoString nCounter analysis, revealed DNA damage repair as the main pathway involved in OMA's anti-tumour effect. Activation of caspase 3/7 activity and decrease of glioma cell invasiveness were also linked to its anti-tumour effect. *In-vivo*, 1mg/kg dose of OMA was found to reach the brain tumour tissue in concentrations similar to the reported IC50 values *in-vitro*. No adverse reactions were noted and a survival benefit was observed in a proportion of the treated mice.

Conclusion

OMA reaches the tumour brain tissue in therapeutically effective concentrations in mice while a moderate therapeutic benefit was observed. Consequently, additional experiments are ongoing investigating increasing the dosages and treatment duration of OMA.

EACR22-1264

Ovarian carcinosarcoma genomics and preclinical models enable identification of novel mechanisms of action of eribulin

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Introduction

Ovarian carcinosarcoma (OCS) is the most lethal gynaecological cancer. Evidence-based treatment is limited to first-line platinum-based chemotherapy and most patients develop lethal, relapsed disease within one year of treatment. Therefore, identifying new therapeutic strategies is vital. OCS are heterogeneous, with both epithelial (carcinoma) and mesenchymal (sarcoma) components proposed to exist in a state of stable epithelial-to-mesenchymal transition (EMT).

Material and Methods

We dissected the carcinoma and sarcoma components from 18 OCS and carried out targeted DNA and whole transcriptome sequencing. We characterised a cohort of molecularly annotated OCS patient-derived xenograft (PDX) models and compared the efficacy of standard treatments and eribulin *in vivo*. We carried out RNA sequencing (RNAseq), western blotting and immunohistochemistry on tumours following eribulin treatment. We also undertook cholesterol assays on treated tumours and cell lines.

Results and Discussions

Molecular analysis indicated that OCS are monoclonal. RNAseq analysis indicated that the carcinoma components of OCS are more mesenchymal than pure ovarian carcinomas, indicating these tumours may have been primed to undergo sarcomatous transformation early in carcinogenesis via EMT.

Anti-microtubule agents, including eribulin (Halaven®) which is known to reverse EMT, were more effective than platinum chemotherapy in reducing the growth of most PDX tumours. Importantly, after a single dose of eribulin, a decrease in the expression of HMGA2 and other mesenchymal markers was observed. Genes involved in the mevalonate (MVA) pathway were also significantly down-regulated and genes involved in activation of immune responses were significantly up-regulated. We demonstrated that eribulin treatment resulted in an accumulation of intracellular cholesterol, prior to MVA pathway down-regulation. With respect to immune activation, eribulin treatment induced infiltration of CD8+ T-cells into tumours.

Conclusion

We have identified completely novel mechanisms of action of eribulin; inducing intracellular cholesterol accumulation. We have also validated previous findings that eribulin can induce an immune response. These findings not only supporting eribulin for the treatment OCS, but suggesting eribulin/immunotherapy combinations may demonstrate even greater efficacy. As

such, we have designed the international EPOCH trial, Eribulin and Pembrolizumab in Ovarian Carcinosarcoma, to improve treatment of this aggressive cancer.

EACR22-1019

8-miRNAs signature defines prognostic clusters in NENs and identifies key oncogenic pathways involved in NENs biology

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Introduction

Neuroendocrine neoplasms (NENs) are malignancies of great biological heterogeneity as they arise in multiple primary tumor location and display very different clinical behaviors and prognosis depending on their classification. NENs, and particularly Neuroendocrine Tumors, are genetically quiet, harboring relatively few somatic mutations compared to other malignancies. Therefore, epigenetic mechanism such as miRNA and DNA methylation dysregulation which can modulate expression of genes and pathways may be involved in the natural history of this tumors. However, a better characterization of these mechanisms is required to better understand NEN's biology and the clinical outcome of patients. Our aim is to perform a molecular characterization of non-mutational alterations in a cohort of NEN patients and identify key oncogenic pathways involved in NEN biology.

Material and Methods

MiRNA expression (n = 86), gene expression (n = 63) and DNA methylation (n = 30) data was obtained from patients with lung and gastroenteropancreatic NENs. Association with OS was assessed to select miRNAs involved in the biology and clinical outcome of NENs. To understand downstream mechanism underlying miRNA dysregulation, correlation analysis was performed between the expression of these miRNAs and the expression of their in silico predicted target genes. Similarly, correlation analysis was assessed between the expression of the selected miRNAs and methylation levels of CpG sites within regulatory regions to characterize potential upstream mechanism driving miRNA alterations. Finally, validation of target genes was performed using transient transfections in vitro and using public TCGA data.

Results and Discussions

We identified a signature of 8 miRNAs that are involved in the biology and clinical outcome of NENs. Moreover, 75 target genes were significantly correlated with their miRNA and were enriched in oncogenic pathways related to the Hallmarks of Cancer. These target genes were validated *in vitro* in H727 and BON-1 NEN cell lines and by analyzing TCGA data.

Finally, methylation analysis led to the identification of 5 CpG sites involved in the epigenetic regulation of the 8 miRNAs.

Conclusion

A comprehensive molecular characterization of NENs led to the identification of key epigenetic mechanism, genes and pathways involved in the biology and prognosis of NEN patients. Further analyses are underway to explore the role of these miRNAs/targets in NEN's biology and the potential of novel therapeutic strategies.

EACR22-0027

Pre-clinical inhibition of the PI3-K pathway to reduce secondary bone metastases in breast cancer

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Introduction

The bone is the main site for metastases in breast cancer. These manifest as painful osteolytic lesions resulting in reduced bone density and pathological fractures. This represents an unmet clinical need as they are incurable. Previous research has demonstrated an increase in osteoclasts (bone resorbing cells) as a driving mechanism for osteolytic lesions. The bone marrow is a naturally hypoxic environment and hypoxia and its associated signalling pathways have been correlated with tumour progression and invasion. The Phosphoinositide 3-Kinase (PI3-K) pathway has been suggested as key in this niche as it is a known regulator of Hypoxia Inducible Factor 1 (HIF1) which itself controls cellular responses to hypoxia. The inhibition of PI3-K is already established in the treatment of breast cancer but could also offer a targeted approach against osteoclasts and establishment of osteolytic lesions.

Material and Methods

Primary osteoclast cultures were differentiated from murine bone marrow. Osteoclasts were treated with PI3-K inhibitor GDC-0941 and/or co-cultured with 4T1 cells (murine triple negative breast cancer cell line). Changes in protein and gene expression of osteoclast markers were assessed. In vivo, the impact of GDC on tumour growth and metastatic markers via IHC and intra-tumour RNA analysis was assessed in orthotopic 4T1 breast tumours. Changes in bone marrow were analysed by FACS.

Results and Discussions

Osteoclasts co-cultured with 4T1 presented an increase in bone resorbing protease Cathepsin K. This was significantly decreased with GDC treatment. The pattern of osteoclast differentiation and activation followed that of PI3-K pathway activation, suggesting a link between these pathways and a key role in osteoclast function. GDC treatment delayed primary tumour growth and reduced downstream gene targets of HIF1 linked with angiogenesis (VEGF) and prognosis for bone metastasis (OPN). A decrease in monocytes in the bone marrow, which are precursor cells to osteoclasts, was observed in GDC-treated mice.

Conclusion

Targeting PI3-K has pleiotropic effects, that include the potential to influence response to a hypoxic microenvironment, both in primary tumours and metastatic sites, such as the bone marrow where it could counteract the osteoclast over-differentiation and activation observed in osteolytic lesions. PI3-K pathway inhibition by GDC-0941 is an effective tool to elucidate

the interaction between tumour cells and the bone microenvironment as well as driving mechanisms of breast-to-bone metastases.

EACR22-0041

Human metastatic cholangiocarcinoma patient-derived xenografts and tumoroids for preclinical drug evaluation

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Introduction

Cholangiocarcinoma (CCA) is the second-most common hepatobiliary malignancy. About 75% of CCA cases are diagnosed in advanced metastatic stages with limited therapeutic options. To date, adequate animal models for developing rational treatments for advanced CCA are still unavailable. Identified in approximately 15-20% of intrahepatic CCA patients, mutations in the genes encoding for isocitrate dehydrogenase 1 and 2 (*IDH1/2*) are becoming a promising therapeutic target. Indeed, it has been demonstrated that several types of tumor cells harboring *IDH1/2* mutations (*IDH1/2^{mut}*) exhibit a homologous recombination (HR) deficiency phenotype, and showed thus enhanced sensitivity to Poly(ADP-ribose) polymerase (PARP) inhibitors (PARPi). Nonetheless, the effect of PARPi has not yet been examined in preclinical CCA models with *IDH1/2^{mut}*.

Material and Methods

A unique collection of patient-derived xenografts (PDXs) has been established by subcutaneously implanting biopsy samples from advanced metastatic CCA patients into mice. Standard H&E staining, IHC of CCA markers, and targeted exome sequencing were used for histological and genomic characterizations. Moreover, we also optimized a protocol to generate CCA tumoroids, allowing fast *ex vivo* drug screens. The effects of PARPi were examined both in CCA tumoroids *ex vivo* and CCA_PDXs *in vivo*. Finally, a clinically used RAD51 assay was employed to evaluate the HR status of CCA tissues.

Results and Discussions

Our collection of CCA_PDXs faithfully recapitulates the histopathological and molecular features of the original patient tumor. Importantly, we observed that PARPi treatments do not inhibit the growth of *IDH1^{mut}* CCA tumoroids *ex vivo* and CCA_PDXs *in vivo*. In line with these findings, both CCA_PDX and advanced CCA patient biopsy samples with *IDH1/2^{mut}* showed RAD51 scores compatible with an HR proficiency profile, which could explain the absence of response to PARPi.

Conclusion

This collection of CCA_PDXs provides new opportunities for evaluating drug response and understanding the biology of advanced CCAs. Our results suggest that the mutations of *IDH1/2* should not be used as a general biomarker to predict PARPi response in advanced CCA patients, who are unlikely to benefit from PARPi therapy.

EACR22-0397

Transplant of human mesenchymal stem cells modulates tumor microenvironment

and delays glioma growth in mice

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Introduction

Malignant gliomas are usually very aggressive and can affect people of any age, including children. Standard treatment for glioma consists of maximal surgical resection, radiotherapy and chemotherapy. Despite intensive efforts to find more effective therapeutic options, survival time of patients with gliomas has not increased significantly in the last decades and the prognosis remains poor. Therefore, there is an urgent need to develop new treatment strategies for malignant gliomas. In this context, mesenchymal stem cells (MSCs)-based therapy has emerged as a promising option for the management of this devastating disease and its sequelae. However, it is crucial to understand how MSCs modulate cellular and molecular processes in the tumor and its environment before boosting the therapeutic use of MSCs in cancer therapy.

Material and Methods

Glioma U87 cells were subcutaneously transplanted into the flank of nude mice (U87 group). A cohort of mice was co-injected with glioma U87 cells and MSCs (U87+MSC group) or glioma U87 cells and MSCs pre-treated with the cytoprotective molecule melatonin (MT) (U87+MSC^{MT} group). Tumor growth was monitored for 30 days and then tumors were harvested for further studies using histology, ultrastructure and immunological techniques.

Results and Discussions

Our results indicated that MSCs delay tumor growth. Interestingly, the cellular processes induced by MSCs differed from those induced by MSC^{MT}, with changes in collagen deposition, vascularization and immunological parameters.

Conclusion

The understanding of the mechanisms by which MSCs exert their therapeutic effects in malignant glioma will provide valuable information to design safe MSC-based therapies for the management of cancer treatment.

EACR22-0421

Uterine leiomyoma organ culture as a preclinical model for controlled-release drug testing

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Introduction

Uterine leiomyoma (LM) is the most common benign tumor in women of reproductive age. A recent epidemiological study determined that 85% of women aged 35 and 50 had fibroids in Spain. Ovarian steroids play a pivotal role in LM growth, so the pharmaceutical treatment

has mainly focused on blocking the pituitary-gonadal axis to cease sex steroids production. This causes substantial fibroid reduction but undesirable systemic effects.

Most of the effects of these drugs have been tested using traditional 2D cultures, where tumor cells are cultured on a flat monolayer. However, 3D culture better represents in vivo conditions, and LM cells maintained viability and responded to environmental cues in organ culture of tissue slices placed onto alginate scaffolds. Our main goal was to analyze the effect of a drug encapsulated in PLGA microspheres.

Material and Methods

Drug PLGA-MPs were prepared by double emulsion method and then incorporated into an alginate scaffold that supports tissue slice culture. MPs were characterized in terms of size distribution and morphology. To determine the encapsulation efficiency, we used ¹²⁵I-drug as a tracer, and release assays were performed at different time points. Slices were cultivated for 7 days, with drug-alginate MPs (AlgMPs-G), drug MPs (MPs-G), Supplemented drug solution (S-G), and control group. H&E staining was used for histological characterization of tissue cells. To quantify the percentage of apoptotic cells, we carried out a Click-It TUNEL assay

Results and Discussions

The mean volume diameter of microspheres was 68,9 μ m (10% < 25,9 μ m, 90% > 131,8 μ m). No differences were observed between MPs-G and AlgMPs-G. The encapsulation efficiency was 55 $\pm$ 0.76 % and 49.93 $\pm$ 5,9% for MPs-G and AlgMPs-G, respectively. Tissue histological characterization showed increased apoptotic nuclei and vacuolated cells in slices treated with ganirelix. A statistically significant increase in the percentage of apoptotic cells was observed in slices treated with AlgMPs-G (p < 0,01), MPs-G (p < 0,01), or supplemented with ganirelix in culture medium (p < 0,01) compared to control slices. Additionally, we observed an increase of apoptotic cells in highly fibrotic LM, suggesting that an excess of ECM favors drug diffusion.

Conclusion

Drugs encapsulated in PLGA microspheres induced a significant apoptosis rate in LM organ culture. This result opens the possibility of a new therapy to treat fibroids.

EACR22-0945

Antitumor activity of the novel selective PARP1 inhibitor AZD5305 as monotherapy or in combination with carboplatin or an ATR inhibitor in BRCA1/2-mutant patient-derived tumor xenografts

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Introduction

Tumors with *BRCA1* or *BRCA2* (*BRCA1/2*) mutations are defective in DNA repair by homologous recombination (HRR) and highly sensitive to PARP inhibitors (PARPi). All the current clinical PARPi target both PARP1 and PARP2. AZD5305 is a highly potent and selective PARP1 inhibitor in clinical development as single agent or in combination with

chemotherapy. Here, we aimed to characterize the activity of AZD5305 alone or in combination with carboplatin or an ATR inhibitor (ATRi) in PDX models from germline *BRCA1/2* (g*BRCA*) mutation carriers, and to identify mechanisms of resistance including the restoration of functional HRR measured by RAD51 nuclear foci.

Material and Methods

The antitumor activity of AZD5305 (1 mg/kg) was tested alone or in combination with carboplatin (37.5 mg/kg) or the ATRi ceralasertib (AZD6738, 25 mg/kg), or olaparib alone (100 mg/kg) in a panel of 12 g*BRCA* PDX from breast and ovarian cancer. We selected 6 olaparib-sensitive and 6 olaparib-resistant PDX, of which 4 were matched to an olaparib-sensitive model. Treatment response was evaluated as the capacity of the therapies to induce tumor regression and prevent disease progression. Baseline and post-treatment scores of RAD51 were determined by immunofluorescence (IF) to assess the HRR functionality. To further study mechanisms of acquired-resistance, DNA sequencing was performed.

Results and Discussions

All 6 olaparib-sensitive PDX responded to AZD5305, showing potent antitumor activity either by inducing a pronounced response in 3 models (PDX474.2, -99%; PDX173, -96%; PDX124, -100%) or preventing disease progression (PDX474.2, 1/11; PDX124, 2/10; PDX230, 0/8). Upon olaparib treatment, PDX474.2, PDX173 and PDX124 showed a tumor reduction of 86%, 98% and 2%, and 5/13, 0/6 and 9/11 tumors progressed, respectively; PDX230 showed 5/8 tumors progressing. Among the olaparib-resistant models, PDX341 presented 27% of tumor regression and PDX127 showed 77% of tumor growth inhibition upon treatment with AZD5305. All acquired-resistant tumors showed restoration of HRR (high RAD51 levels). AZD5305 also showed profound and durable regressions when combined with carboplatin or AZD6738 in a PDX with primary resistance to olaparib (PDX127), but not in 2 others with preclinical/clinical acquired-resistance to PARPi (PDX474.2OR and PDX474.7).

Conclusion

The novel PARP1 inhibitor AZD5305 yields a potent antitumor response in g*BRCA* mutated breast and ovarian cancer PDX in terms of tumor regression and preventing tumor regrowth.

EACR22-1207

Innovative Public Procurement in Liquid Biopsy: A public initiative for the implementation of new health technologies in the clinical practice.

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Introduction

Traditional Public Procurement is in the process of changing towards a new scenario. The Innovative Public Procurement (IPP) offers the Andalusian Public Health System (APHS) a new approach to acquiring new solutions. This instrument is intended to encourage public procurement of SMEs, entrepreneurs, and/or innovative companies, turning the APHS into a

favorable environment to work and develop new technological products and services. Then, "PRECISION DIAGNOSIS AND TREATMENT IN INFECTIOUS DISEASES AND CANCER" was a project co-financed 80% by the European Regional Development Fund and whose main objective is the development of innovative solutions in the APHS. Thus, this IPP focused on liquid biopsy, which detects a biomarker in a body fluid obtained non-invasively to monitor the patient in real-time. The most common and validated is blood, and among the most common and analyzed biomarkers are circulating tumor DNA (ctDNA) and circulating tumor cells (CTCs). The project's main objectives are obtaining innovative platforms that allow the identification and characterization of biomarkers in the blood (CTCs and ctDNA) within an automated and simultaneous platform to monitor the patient in real-time, predicting potential relapses early and tracking the response to treatment of a custom shape.

Material and Methods

The device must analyze a minimum of ten genes with five mutations each, including RAS family, EGFR, HER2, PI3K, and distinguish between high and low mutation frequencies. The detection of allelic frequencies must be in a range of 0.1 to 0.5.

Results and Discussions

Two prototypes of new technologies were performed to identify and characterize biomarkers in the blood (CTCs, exosomes, and free DNA) in an automated and simultaneous way for early diagnosis, personalization, and monitoring of cancer patients. The time to obtain a diagnosis/prognosis per case was reduced, improved cost-effectiveness over current techniques. The device must undergo a technical validation which included the analysis of a representative sample of patients with breast, colon, and lung carcinoma (15 patients/tumor/stage).

Conclusion

The IPP acted as an instrument to promote the commercial market. The grant model was moved towards the public contract model as the first client with the IPP. The Liquid Biopsy project developed two prototypes that allow the identification and characterization of blood biomarkers to personalize and monitor patient treatment.

EACR22-1245

Exploiting genetic vulnerabilities of BAP1 loss by HDAC inhibitors

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Introduction

BAP1 is a powerful tumor suppressor gene that is frequently mutated in several cancer entities, such as renal cell carcinoma, uveal melanoma and cholangiocarcinoma. We previously discovered that BAP1 loss is associated with aggressive tumors, metastasis and poor patient survival in renal cell carcinoma and developed an immunohistochemistry test for stratifying patients. Unfortunately, there are no specific treatments for tumors with mutations in BAP1.

Material and Methods

The aim of this project is the identification of synthetic lethal targets of *BAP1*-mutated tumor types using a large-scale RNA interference screen and validate the results *in vitro* in cell lines from renal cell carcinoma, uveal melanoma and cholangiocarcinoma and *in vivo* in mice.

Results and Discussions

Through bioinformatics analyses, we found a histone deacetylase (*HDAC*) as one of the most prominent hits in the synthetic lethality screen. After knockdown of *HDAC*, a decrease of proliferation and colony formation in *BAP1*-deficient cell lines from renal cell carcinoma, uveal melanoma and cholangiocarcinoma but not in *BAP1*-competent cell lines was found. Besides, by treating cells with the *HDAC* inhibitor quisinostat, we detected a stronger decrease of cell viability of *BAP1*-deficient cell lines compared to wild-type *BAP1* cell lines. Additionally, the knockdown of the histone deacetylase had a higher efficiency suppressing the migration of *BAP1*-null cells compared to *BAP1*-competent cells. Characterizing the role of *HDAC*, a G1 arrest in *BAP1*-deficient cell lines could be uncovered, which was not associated with an increase of apoptosis. Furthermore, *in vivo* studies in mice revealed a significant depletion of tumor growth after knockdown of the histone deacetylase in the context of *BAP1* loss, strongly suggesting this *HDAC* as a true synthetic lethal interactor with *BAP1* loss.

Conclusion

HDAC inhibitors might be effective for treating patients of renal cell carcinoma, uveal melanoma and/or cholangiocarcinoma with mutations in *BAP1*.

EACR22-1121

Understanding The Role Of Prostate Zones In Cancer Progression

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Introduction

The prostate is composed of four main areas: anterior fibrous septal area, transition zone (TZ), peripheral zone (PZ) and central zone (CZ), with more than 95% of tumours located in PZ and TZ. Each zone has variable tumour incidence, with patients diagnosed with TZ tumours having a better prognosis than PZ and CZ tumours. Recent molecular and histopathological studies have shown that unique zonal features may contribute to the diverse patient outcome carrying tumours arising from each prostate zone. However, there is

still very limited understanding of the precise cellular composition of the zones and their functional contribution to tumour progression.

Material and Methods

We here define the cellular composition of the prostate zones, focusing on the epithelial tumour cells and their microenvironment. We analysed more than 100,000 single cells from seven patients with either PZ or TZ localised prostate cancer using single cell RNA sequencing.

Results and Discussions

Our analyses showed a higher proportion of epithelial cells in the PZ than the TZ, both in histologically normal (63% vs 51%) and tumour (40 vs 34%) regions, which affects predominantly luminal prostate cells. Interestingly, analysis of the spatial prostate microenvironments revealed unique immune and endothelial landscapes in the different prostate zones. This spatial diversity highlights the existence of tumour-promoting and tumour-restrictive zonal environments within each individual prostate.

Conclusion

We identified spatial cellular diversity in the peripheral and transitional zones of the prostate evolving during tumorigenesis. These hitherto unappreciated differences are likely highly relevant for tumour progression and may open new treatment options, based on the zonal presentation of prostate cancer.

EACR22-0003

POSTER IN THE SPOTLIGHT

Exportin 1 inhibition as a novel therapeutic target for small cell lung cancer

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Introduction

Small cell lung cancer (SCLC) is an aggressive disease comprising 13% of all lung cancer cases. With few treatment options, typically resulting in only transient responses, SCLC is responsible for approximately 250,000 deaths globally per year. The backbone of SCLC treatment over the past several decades has been platinum-based doublet chemotherapy, with the recent addition of immunotherapy to first-line chemotherapy showing limited benefit in a small subset of patients. Major hurdles to improving SCLC treatment include development of rapid chemoresistance and ineffective second line therapies. The identification of more durably effective therapeutic strategies is a major unmet clinical need.

Material and Methods

We performed an *in vitro* CRISPR screen in SCLC cell lines from all major SCLC subtypes, including short-term cultured cells from patient-derived xenografts (PDXs), to identify potential therapeutic targets to sensitize to chemotherapy. Candidate hits were validated genetically and pharmacologically with *in vitro* synergy assays, *in vivo* clonal competition assays and pharmacologic assessments in PDX models. Signaling pathways were studied by RNA sequencing and western blot, and toxicity studies were performed *in vivo* to assess the safety of the agents at pharmacologically effective doses.

Results and Discussions

Our CRISPR screen revealed the nuclear exporter exportin 1 (encoded by the *XPO1* gene) as a promising target sensitizing to chemotherapy, independently of the SCLC subtype. We found that *XPO1* mRNA expression was higher in SCLC than in any other solid tumor or hematological malignancy, and demonstrated consistently high protein expression by IHC in clinical TMAs. A potent and selective exportin 1 inhibitor, selinexor, is approved for use in hematological malignancies. Combination of selinexor with cisplatin or irinotecan demonstrated synergy *in vitro* and efficacy *in vivo* in an array of chemo-naïve and chemoresistant SCLC PDXs, including all major SCLC subtypes. The combinations were well tolerated in mice. The chemo-sensitizing effects of selinexor were associated with suppression of chemotherapy-induced AKT activation.

Conclusion

Exportin 1 inhibition strongly enhances sensitivity of SCLC tumors to cisplatin and irinotecan, used in first line and second line treatment of SCLC tumors, respectively. These results provide preclinical rationale for the combination of selinexor with cisplatin or irinotecan in naïve and relapsed SCLC.

EACR22-0204

Pervasive biological misinterpretations of transcriptional signatures in tumour samples can unknowingly undermine mechanistic understanding and therapeutic development

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Introduction

Gene expression-based signatures (GESs) provide valuable prognostic and predictive information in cancer research. Precise mechanism-based GESs have been developed in appropriate *in vitro* and *in vivo* model systems, to identify important cancer-related signalling, where complete control of lineage purity can be achieved. However, if the genes within GESs can be expressed in stromal components to any extent, the signatures may, unbeknownst to the end-user, reflect tumour stroma percentage (TSP) rather than the intended mechanistic signalling or biological cascades they have been based on and interpreted to represent.

Material and Methods

To address the consequences of variations in TSP on sample classification using GES, we provide a comprehensive characterisation of the tumour transcriptome, stratified primarily into epithelium and stroma using LCM and spatial transcriptomics, alongside a more granular assessment of individual lineages using FACS and scRNAseq. These findings were validated within a bulk clinical trial cohort (FOCUS) to confirm the applicability of our findings to true biological samples.

Results and Discussions

Analysis interrogating the stromal transcriptome's contribution to molecular subtyping, phenotypic characterisation and ontology enrichment has shown that heterogeneity of

stromal composition in bulk tumours can skew the interpretation of the most used GESs in cancer research. A number of individual signatures are consistently associated with the strongest confounding effects of stromal content, as an exemplar the HALLMARK EPITHELIAL MESENCHYMAL TRANSITION (EMT) signature is strongly correlated with histologically assessed desmoplastic stromal percentage ($p=0.69$, $p<2.2e-16$). Multiregional biopsies from primary resection material ($n=7$) demonstrates that the HALLMARK EMT signature correlates with stromal content ($p=0.69$, $p<2.2e-16$) rather than patient of origin, highlighting potential clinical implications due to biological misinterpretation. Spatially transcribed regions across a whole-face tumour section, overlaid with Cytokeratin IHC, highlights the non-epithelial origin of this signature, and others found to be confounded within this study.

Conclusion

While we do not challenge the prognostic and predictive value of transcriptional GESs, data presented here highlights pervasive issues related to stromal influence in bulk tumours data and demonstrates the clear potential for misinterpretation of the results.

EACR22-0518

Molecular risk stratification in patients with T1 colorectal cancer

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Introduction

Reduction in colorectal cancer (CRC) mortality will largely involve prevention, earlier detection and treatment optimisation. Bowel cancer screening (BCS), detects asymptomatic tumours much earlier, with stage I CRC (T1/2, N0, M0) now accounting for 48% of BCS-detected tumours, compared to 9% outside screening. Histological assessment of resected T1 tumour tissue is unable to reliably identify aggressive lesions, therefore some patients have an unidentified risk of metastatic spread.

We hypothesise that within early lesions detected by BCS, there are highly aggressive early-disseminating tumours that would otherwise be detected at later stage, but due to BCS they are being caught earlier while potentially curable. We propose that molecular profiling can discriminate between lesions based on their probability of developing lymph node (LN+) and/or distant metastasis (dM+).

Material and Methods

To identify factors associated with early-dissemination, we have collected and have begun comprehensive histological and molecular characterisation of a cohort ($n=300$) of T1 CRC, enriched for aggressive tumours (LN+ and/or dM+ T1 CRCs). Profiling includes 3' and whole exome sequencing for RNA, whole exome sequencing for somatic variant detection, low pass whole genome sequencing for copy number alterations, alongside molecular and digital pathology assessments. Using these data, a series of unsupervised and supervised analyses will be performed.

Results and Discussions

Following clinical review, a subset of T1 tumours with definitive nodal status following full resection were identified, from within the Dutch T1 CRC Working Group cohort. The first n=251 samples, which include n=206 T1/N0/M0 and n=45 T1/N+/M0 cases at diagnosis, were selected for initial analysis. This resulted in n=3495 sections undergoing macrodissection and dual RNA/DNA extraction. Nucleic acids have been quantified, with no samples excluded due to low yield. Molecular profiling is underway, with data anticipated to become available in Spring 2022.

Conclusion

Distinguishing between lethal early-stage cancers that require more extensive treatment and non-lethal cancers that do not is currently a key priority for early detection research. This project aims to generate a molecular risk stratification tool for patients diagnosed with T1 tumours, alongside providing a new understanding of the biology underpinning aggressive disease.

EACR22-0594

Optimization of GPA33-targeted immunotherapy against colorectal cancer

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Introduction

Glycoprotein A33 (GPA33) is a membrane protein almost exclusively expressed in intestinal epithelia and in more than 95% of colorectal cancers (CRCs). This renders GPA33 a promising target for immunotherapeutic approaches. In order to develop an optimized treatment protocol for anti-GPA33 immunotherapy, we investigated GPA33 regulation and the potential for pharmacological induction of GPA33 expression *in vitro* and *in vivo*.

Material and Methods

We assessed intratumoral heterogeneity of GPA33 in tissue specimens of 223 stage II CRCs using immunohistochemistry and immunofluorescence, and obtained scRNAseq transcriptomic analysis from 12 primary CRCs. We then manipulated WNT and PPAR γ signaling pathways and transcription factor expression in colon cancer cells using specific inhibitors, siRNAs or inducible dominant negative TCF4 (dnTCF4), and analyzed the effects on GPA33 protein and RNA expression. Furthermore, we investigated the effects of WNT inhibition on GPA33 *in vivo* in NOD/SCID xenografts.

Results and Discussions

In the CRC cohort we found that GPA33 expressing cells had a differentiated phenotype and low WNT activity. GPA33-negative subpopulations were located at the infiltrative tumor edge and showed increased nuclear β -Catenin. Downregulation of WNT pathway activity using inhibitors, siRNAs or inducible dominant negative TCF4 (dnTCF4), as well as activation of PPAR γ signaling induced GPA33 expression *in vitro*. Forced expression of dnTCF4 also enhanced GPA33 expression *in vivo* in previously GPA33-negative tumor cell subpopulations in colon cancer xenografts. siRNA mediated knockdown of CDX1 and KLF4, two transcription factors that were previously implicated in GPA33 regulation, only moderately reduced GPA33 expression levels *in vitro*, indicating a complex regulation in colon cancer.

Conclusion

Our analysis highlights a heterogeneous expression of GPA33 *in vivo* as a limiting factor for anti-GPA33 therapy. WNT inhibition or PPAR γ activation induced GPA33 expression *in vitro* and *in vivo*. We suggest that pharmacological induction of GPA33 expression may increase the efficacy of anti-GPA33 targeted immunotherapy in patients with CRC.

EACR22-0630

Modeling Vulnerability of KRAS and BRAF mutant Colorectal Cancer towards Interference with DNA Damage Response

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Introduction

Colorectal Cancer (CRC) accounts for about 10% of cancer related mortalities and ~50% of all CRC patients harbor oncogenic mutations in KRAS, NRAS or BRAF. An important function in cancer is the control of DNA repair and DNA damage response (DDR) pathways as tumor cells often suffer from elevated replication stress. Activation of MAPK signaling by RAS/RAF oncogenes is an important inducer of replication stress. Targeting the DNA repair machinery in KRAS^{mut} cancer cells was previously suggested to offer therapeutic potential. Yet, DDR checkpoint inhibitors failed in clinical trials for lack of understanding of molecular processes involved in DDR checkpoint signaling and the mechanistic link between RAS/MAPK-effector networks and DDR in CRC is minor. Our project aims to investigate functional dependencies between MAPK and DDR regulatory networks, and to uncover mechanisms mediating checkpoint inhibitor sensitivity in CRCs.

Material and Methods

We tested sensitivity of KRAS and BRAF^{V600E} mutant CRC cell lines towards checkpoint inhibition and analysed MAPK and DDR regulatory networks by CyTOF (Cytometry by time of flight) and quantitative Western Blot analysis. In addition, synthetically lethal targets for Chk1/Mk2 inhibitors were identified by a targeted CRISPR-i screen and possible resistance mechanisms uncovered.

Results and Discussions

Survival and metabolic analysis of 21 CRC cell lines using Chk1/2 and Mki2 inhibitors revealed a strongly diverse response. Interestingly, the majority of BRAF^{V600E} mutant CRC lines stood out to be overly sensitive towards Chk1 inhibition. Analysis of checkpoint inhibitor response using a CyTOF panel and quantitative Western Blot analyses revealed differential regulation of checkpoint effectors and of the p38 MAPK, mTOR and NFkB pathways between sensitive and resistant lines. A CRISPR-i screen in the Chk1i sensitive cell line HT29 suggested the involvement of replication fork regulators either directly interacting with Chk1 or as Chk1 effectors. Investigation of TCGA RNA sequencing data from tumor cohorts revealed upregulation of DDR-related signatures in BRAF mutant CRC indicating increased dependency on functional DDR signaling. In contrast, the presence of oncogenic KRAS does not indicate sensitivity towards checkpoint interference.

Conclusion

Our results suggest that a group of BRAF^{V600E} mutant CRC might be overly sensitive to checkpoint inhibition. This phenotype of BRAF^{V600E} might be the result of a deregulated DDR network in these tumors.

EACR22-0978

INTEGRATIVE TRANSCRIPTOME ANALYSIS FOR THE DEFINITION OF ROBUST INTRINSIC SUBTYPES IN LUNG ADENOCARCINOMA

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Introduction

Lung adenocarcinoma (LUAD) is clinically and genetically heterogeneous. Current diagnostic practices based on histological and molecular characteristics do not completely recapitulate this heterogeneity, leading to unsuccessful treatment performance. Transcriptional profiling has emerged as a promising tool for further patient stratification and treatment guidance. However, intrinsic technical and analytical limitations have hindered the use of expression signatures in the clinical setting. We aimed to develop a robust classification of LUAD based on signature-based transcriptional tumour profiling that could improve our ability to discriminate the different LUAD subtypes and implement specific treatment strategies.

Material and Methods

The activity of 50 canonical pathways in 4573 LUAD patients from 56 gene expression datasets was inferred using GSVA algorithm. LUAD molecular subtypes were defined based on the joint behaviour of the studied pathways. Once LUAD transcriptional subtypes were defined, we sought to characterize these tumors at different clinical and molecular levels.

Results and Discussions

Seven molecular subtypes were defined for LUAD based on the activity of the 50 molecular pathways: AD1 (17%), AD2 (19%), AD3 (10%), AD4 (13%), AD5 (21%), AD6 (17%), AD7 (3%). While the classification seems to be strongly guided by the expression of cell-cycle and immune system related genes, other more specific pathways also contribute to further refine the identified groups. AD1, AD4 and AD5, were associated with better overall survival compared to AD2, AD3, AD6 and AD7, which show higher cell cycle-related pathways expression. AD2 and AD6 were found to be associated with higher genomic instability and were enriched for TP53 mutations, higher copy number alteration rates and increased homologous recombination deficiency. Finally, concerning the immune characterization, the results show that these subtypes present specific infiltration patterns. Also, we found a consistently higher expression of CD274 (PD-L1) in AD3 and AD6 LUAD subtypes, which could suggest a potential benefit from anti-PD-L1 immunotherapy treatment.

Conclusion

We have defined a robust classification of seven LUAD subtypes based on the differential expression of landmark pathways. These subtypes differ in survival and molecular characteristics. Our classification may pave the way for novel and more precise therapeutic strategies beyond current DNA-based targeted therapies, especially in patients without actionable genomic alterations.

EACR22-0992

A complex pattern of PTEN protein expression in cancer: a predictive study using novel anti-PTEN mAb

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Introduction

Nonsense and frameshift mutations creating premature termination codons (PTC) are frequent PTEN somatic mutations in tumors and in the germline of PTEN Hamartoma Tumor Syndrome (PHTS) patients. These mutations cause the synthesis of truncated PTEN protein variants displaying diminished protein stability and function, in a manner dependent on the location of the PTC in the protein amino acid sequence. A diversity of translational and splicing PTEN isoforms have been recently identified, but how PTEN PTC mutations present in PHTS patients limit the cellular expression and function of PTEN protein isoform variants has not been investigated in detail.

Material and Methods

Using novel anti-PTEN mAb against distinct PTEN protein regions and recombinant PTEN variants, we have performed a predictive study on the mutation-specific expression and subcellular localization patterns of PTEN protein isoforms in patients carrying PTEN PTC mutations.

Results and Discussions

We have found that the stability of the PTEN long translational isoforms is less affected by PTC than canonical PTEN. Accordingly, the PTEN D splicing isoform is also more stable in the background of PTEN long isoforms, when compared to PTEN, and differential subcellular localization is observed. In addition, we show that PTC or frameshift mutations creating PTC upstream to the Met35 PTEN residue drive the translation of a PTEN M35 N-

terminal truncation which lacks PIP3 phosphatase activity in cells and does not accumulate in the nucleus.

Conclusion

A complex pattern of expression and subcellular localization of functionally distinct N-terminal and C-terminal PTEN truncations is envisioned depending on the specific PTEN isoform expression and PTC mutations present in patients. The availability of well-defined anti-PTEN mAb recognizing distinct PTEN protein regions will help to unveil the complexity of PTEN protein expression in cancer and in PHTS patients

EACR22-0035

Contribution of heparan sulphate binding domain of chemokine CCL21 to trans-endothelial migration of breast cancer cells

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Introduction

Lymph node metastasis constitutes a key event in Breast Cancer progression. Chemokines are small proteins, which can promote metastatic spread by inducing cancer cell migration and invasion. Chemokine function is dependant upon their binding to both cell surface heparan sulphate (HS) molecules and to their specific receptor. We hypothesized that a non-HS binding forms of chemokine CCL21 can disrupt the normal response to CCL21, therefore reducing the metastasis of CCR7-expressing cancer cells

Material and Methods

Truncated CCL21 chemokine (Δ 98-134 c-terminal basic extension), was synthesised to investigate a possible linkage between chemokine binding capacity and cell activation. Wild type (WT) and mutant-CCL21 were tested for their ability to stimulate a dose-dependent increase in intracellular-free calcium in PBMC and MDA-MB-231. A murine model was used to assess the potential of mut-CCL21 to prevent lymph node metastasis in vivo

Results and Discussions

A further series of experiments were performed to compare the potential of WT and mutant-CCL21 to stimulate the migration of cells across endothelium. In contrast to results for trans-filter migration, it was found that the non HS-binding mutant stimulated no increased in transendothelial cell migration above the background at each of the tested concentrations, 10, 30 and 50 nM respectively ($P > 0.05$). However, WT-CCL21 stimulated significant increased PBMC migration at each of the tested concentration (all $P < 0.001$). Mice were injected with 4T1-Luc cells in the mammary fat pad and treated daily for a week with 20 g mut-CCL21. Mice were imaged weekly with IVIS and sacrificed on day 18. Luciferase expression was significantly reduced in lymph nodes from mice that had been treated with mut-CCL21 compared to the control ($p = 0.0148$)

Conclusion

The evidence from this study suggests that mut-CCL21 shows decreased binding to the GAGs present in the endothelial cells, and thus fails to create the chemotaxis gradient necessary for trans-endothelial migration and extravasation. However, mut-CCL21 can still bind and activate CCR7 as demonstrated by calcium flux, confirming that GAGs are not necessary for the binding and activation of the chemokine receptors in vitro. This was also observed in vivo, where mut-CCL21 could reduce the number of metastatic lymph nodes in a breast cancer murine model. This highlights the therapeutic potential of disrupting GAG–chemokine interactions

Identification of Maml1 as a novel negative regulator of Itch E3 ubiquitin ligase activity: new insights in cancer biology

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Introduction

In mammals, Maml1 belongs to a family of proteins, which act as transcriptional coactivators for Notch signalling, an evolutionarily conserved pathway. Maml1 has been recently shown to act as coactivator in other signalling pathways, such as p53, Wnt and Hippo in a Notch-independent manner. More recently, we have demonstrated the involvement of Maml1 in the Hedgehog pathway, behaving as a novel co-activator of Gli1/2 transcription factors.

Hedgehog and Notch signalling pathways are directly involved in the onset/development of several cancers and are both regulated at post-translational level by Itch/E3 ubiquitin ligase protein. Itch activity is enhanced by the adaptor Numb that is able to bind Itch-WW domains to induce ubiquitination and degradation of Gli1 and Notch1.

Material and Methods

We utilized different experimental approaches: immunoprecipitation and ubiquitination assays in both *in vitro* and *ex vivo* (*Maml1*^{-/-} murine model) cell lines; Maml1 silencing with CRISPR/Cas9 technology; analysis of Itch post-translational modification; siRNA-mediated depletion of Maml1 in breast and colon cancer cell lines; wound healing assay; proliferation assays.

Results and Discussions

Here, we demonstrate that Maml1 can control the expression levels of Gli1 and Notch1 proteins at post-translational level, by directly inhibiting Itch catalytic activity. For the first time, we identify the functional role of the Maml1 C-terminal domain as a post-translational regulator of target proteins. Moreover, we pinpoint the molecular mechanism through which Maml1 acts as negative regulator of Itch, by inducing auto-ubiquitination events. Therefore, Maml1 increases the expression levels of Gli1 and Notch1 oncogenic proteins, by switching off Itch activity. Accordingly, in pathological contexts, such as breast and colon cancers, Maml1 silencing impinge on Notch1 and Gli1 protein levels, hindering proliferation and epithelial-mesenchymal transition events.

Conclusion

Overall, our data suggest a protective role mediated by Maml1 on Itch-target proteins involved in cancer biology. Indeed, the ability of Maml1 to negatively regulate Itch activity could have an impact in the activation of oncogenic pathways, such as Hedgehog and Notch. The identification of Maml1 as a novel negative regulator of Itch adds a piece in the understanding of tumour biology and could help to set out new therapeutic approaches based on the dual role of Maml1.

Establishment and characterization of a unique tumoral cell line derived from a sinonasal teratocarcinoma

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Introduction

Sinonasal teratocarcinomas (SNTCS) are rare but highly aggressive tumors that mainly originate in the nasal cavities and the skull base. These tumors show a complex histology characterized by mixed epithelial, mesenchymal, and neuroendocrine components. Recent studies highlight the role of SMARCA4, SALL-4 and β -catenin expression as differential markers for SNTCS diagnosis. No effective pharmacological therapy is currently available. This is partly due to a lack of experimental models for studying the pathobiology and drug responsiveness of this tumor. We present the first SNTCS cell line and describe growth, pathological and genetic characteristics.

Material and Methods

Cell line TCS627 was established from a 68-year-old man with a previously untreated, primary stage T4 SNTCS originating in the ethmoid sinus with invasion into the brain. Both primary tumor and cell line were histopathologically characterized using diagnostic immunochemical markers, and genetically by next-generation sequencing (NGS) and PCR. The growth rate of TCS627 was determined and the karyotype was evaluated by conventional cytogenetics.

Results and Discussions

The histological features and the immunochemical staining profile with positivity for epithelial, mesenchymal, neuroendocrine and germ cell markers including CK8, S-100, enolase and SALL4 showed that the primary tumor and the derived cell line is representative of SNTCS. However, we did not observe the previously described loss of SMARCA4 expression or nuclear staining or β -catenin. Population doubling time of TCS627 was 48 hours. Cytogenetic analysis showed a predominant tetraploid karyotype with copy number loss at 1p and gains at 1q and 12p, in accord with previous findings in SNTCS. NGS and PCR revealed somatic mutations in STAG2, NOTCH3 and CDKN2A, both in tumor and cell line.

Conclusion

Cell line TCS627 appears representative of its original primary tumor and of SNTCS in general, although further studies are needed. This is the first SNTCS cell line established to date, constituting a suitable model for studying cellular and molecular mechanisms involved in the tumorigenesis and behavior of this type of tumors as well as a tool for testing new therapeutic approaches for SNTCS.

EACR22-0867

N-glycomic profiling of microsatellite unstable colorectal cancer

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Introduction

Approximately 15% of colorectal cancers (CRC) display microsatellite instability (MSI), caused by defective DNA mismatch repair and leading to hypermutation. MSI tumors with distant metastases (stage IV) have a worse prognosis than local or locally advanced (stage II-III) MSI tumors. Among all CRC tumors, stage II-III MSI tumors have a better prognosis than corresponding microsatellite stable (MSS) tumors, whereas stage IV MSI tumors have a worse prognosis than corresponding MSS tumors. The molecular mechanisms behind these differences are unclear. Our aim here was to analyze the N-glycan profiles of MSI CRC tumors to identify possible reasons for their differing behavior.

Material and Methods

We compared MSI CRCs subdivided into stage II and stage IV subgroups, both further divided into BRAF V600E immunohistochemistry negative and positive cases (n=10 in each group). As a control, we analyzed pools of paired normal samples for each tumor subgroup. In addition, we compared the stage II MSI tumors to our previously analyzed stage II MSS tumors. For glycomic profiling, neutral and acidic N-glycans were extracted from formalin-fixed, paraffin-embedded tissue specimens and the glycans were analyzed by MALDI-TOF mass spectrometry.

Results and Discussions

In line with previous CRC reports, MSI CRC tumors showed significantly higher relative abundance of paucimannosidic N-glycans, especially H2N2F1, than control tissues. Sulfated N-glycans, on the other hand, showed the opposite behavior, reported to be increased in CRC but found to be decreased in MSI CRCs as compared to the controls. Most interestingly, no sulfated N-glycans were identified in any of the stage II MSI tumors containing BRAF V600E mutation, the subgroup associated with the best prognosis.

Conclusion

In contrast to previous CRC reports, MSI CRC tumors display highly decreased N-glycan sulfation compared to normal colon tissue. Lack of N-glycan sulfation, e.g. in the form of sulfated selectin ligands, may partly explain the better prognosis of BRAF mutated stage II MSI CRC.

EACR22-0947

TNBC Profiling Based on Protein Expression of Biomarkers

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Introduction

Triple-negative breast cancers (TNBCs) are the most aggressive breast cancer subtype, lacking hormone receptors and human epidermal growth factor receptor (HER2) expression. TNBC patients show higher recurrence and metastasis, with no available targeted therapy. TNBC is clinically challenging, molecularly heterogeneous, and more prevalent in India compared to the west. Hence, this heterogeneity needs to be unravelled to propose personalised treatment decisions accordingly. To understand the heterogeneity amongst TNBC tumours, molecular markers such as EGFR and CK5/6 to identify basal tumours and Androgen Receptor (AR) expression were evaluated in treatment naïve tumour samples. The Association of the IHC expression of these markers with the disease progression and long-term patient survival is assessed here.

Material and Methods

A retrospective cohort of 81 TNBC patients from a single onco-surgeon breast cancer clinic treated with a uniform treatment strategy is evaluated for EGFR, CK5/6, and AR expression. FFPE tissues of the primary tumour of invasive breast carcinoma were collected with patient consent and ethical approvals. Immunohistochemistry was standardised for all markers, and all the TNBC samples were stained for EGFR, CK5/6, and AR. Scoring was done for membrane expression of EGFR, cytoplasmic and membrane expression of CK5/6, and nuclear expression of AR by a pathologist. Scores were binned and correlated with disease outcome, clinical parameters, and stromal tumour-infiltrating lymphocytes (sTILs) scores.

Results and Discussions

Based on positive EGFR and CK5/6 expression, 87.7% of basal tumours were identified in TNBC patients in our cohort. Basal TNBC tumours showed association with poor disease-free and overall survival. In comparison, patients with AR-positive tumours (36% with 10% cut-off) were associated with small tumour size and presented in late age patients, with poor survival compare to that of AR-negative tumours. Higher AR scores were negatively correlated to sTILs scores.

Conclusion

TNBC patients showed a higher proportion of basal tumours, which were associated with poor survival. AR expression was seen in patients with late disease onset and smaller tumours but was associated with poor survival and lower sTILs scores. The observed trend will be validated with a more extensive data set and associated with response to treatment.

EACR22-1147

Effect of melatonin treatment on doxorubicin-induced skeletal muscle atrophy in an *in vivo* model of breast cancer

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Introduction

Skeletal muscle atrophy is a major concern in patients suffering with malignancy. Chemotherapeutic agents, such as doxorubicin (DOX), can further exacerbate this loss of skeletal muscle. There are no therapies routinely used to effectively treat muscle atrophy. The aim of the study was to investigate whether melatonin (MLT) can attenuate doxorubicin-induced skeletal muscle and myotube atrophy in an *in vivo* rodent model of breast cancer as well as in an *in vitro* model of DOX-induced myotoxicity.

Material and Methods

Female Sprague-Dawley rats were inoculated with LA7 cancer cells and were randomly assigned to six groups: Control, Tumour control (TCN), Vehicle control (VEH), MLT, DOX and DOX + MLT (DM). Prophylactic treatment of MLT (6 mg/kg) was administered in drinking water daily and rats received three intraperitoneal injections of DOX (4 mg/kg, 3 times at 3-day intervals). Following sacrifice, blood samples (whole blood counts) and skeletal muscle tissue were collected for histological, immunoblot, antioxidant capacity and immunofluorescence analyses.

Results and Discussions

DOX treatment significantly reduced animal weight as well as *gastrocnemius* muscle weight and cross-sectional area (CSA) compared to DM treated animals. Markers of muscle degradation were also significantly increased in the DOX treated group compared to MLT

treated groups. Serum myoglobin levels were significantly elevated in the DOX group compared to the DM group; while white and red blood cell counts (WBC and RBC) were significantly decreased in the DOX group compared to the MLT treated groups respectively. MLT treatment significantly increased intramuscular antioxidant capacity, mitochondrial biogenesis and satellite cell number. The improvement in animal weight, muscle to body weight ratio, muscle CSA as well as the reduction in myoglobin levels in the treatment groups compared to the DOX group indicate that MLT protects against DOX-induced atrophy. Moreover, MLT pre-treatment improved circulating levels of WBC and RBC compared to the DOX only group and attenuated skeletal muscle atrophy by reducing cell apoptosis and increasing satellite cell number suggesting that MLT assists with muscle repair. The *in vitro* study indicated that DOX-induced myotube atrophy was preceded by increases in mitochondrial ROS.

Conclusion

Pre-treatment with exogenous MLT protects against skeletal muscle wasting induced by DOX in a pre-cachectic tumour-bearing rat model.

EACR22-1247

Discovery of novel treatment options for head and neck squamous cell carcinoma based on a multi-modal approach

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Introduction

The molecular, anatomical, histological, and clinical heterogeneity of head and neck squamous cell carcinomas (HNSCC), the sixth most common cancer worldwide, pivotally contributes to the lack of prognostic markers for personalized therapies. Efforts of personalized patient care, such as EGFR blockade, showed limited success due to the heterogeneous nature of HNSCC. We aim to find novel multimodal theragnostic markers for the treatment guidance of HNSCC.

Material and Methods

In the present study, we combined whole exome sequencing (WES) and hybrid *in vivo* imaging of patients with primary HPV-unrelated HNSCC tumors to discover prognostic genetic traits and to explore their associations to quantitative imaging markers. For the classification of somatic variants, the combined annotation dependent depletion (CADD) scores on a pathway-level basis were used. The most prognostic pathway was dissected in *in vitro* and *in vivo* models to validate its importance for HNSCC carcinogenesis, these investigations included routine molecular techniques, such as Western blotting and RNA profiling, but also μ PET / μ CT analyses of the xenografted mice.

Results and Discussions

Our multi-level approach revealed that the genetic state of Hedgehog (Hh) signaling is not only a relevant marker for patient survival ($p=0.029$, high CADD score patients surviving shorter) but also associates to distinct imaging traits from CT and PET, such as “CT: Histogram ih. skewness”. Moreover, an upregulation of the Hh pathway could be identified in primary tumor samples (compared to adjacent normal tissue) by RNAScope® technology.

Mechanistically, we revealed that blockade of Hh activity by JK-184 (GLI1 specific inhibitor) effectively dampens the proliferation of HNSCC cell lines (IC₅₀: 6-200 nM) and treated cells showed higher apoptosis indices (cleavage of PARP [$p<0.05$] and Annexin V positivity [$p<0.05$]) and were arrested in the G1 cell cycle phase, underlining the importance of Hh signaling in HNSCC cells. In addition, genetic perturbation of the Hh pathway by GLI1 knockdown replicated these observed phenotypes.

Conclusion

Our proposed interdisciplinary approach uncovered a previously weakly-acknowledged pathway (Hh signaling) for HNSCC patient survival. Moreover, the ability to stratify the patients based on Hh signaling as well as the potent effect after its *in vitro* / *in vivo* modulation proved its clinical and functional relevance. This study shows on multiple levels that Hh signaling might be relevant for HNSCC management in the future.

EACR22-0610

ORAL PRESENTATION

Multiplex organoid-based 3D live imaging platform to screen probes for Fluorescence Guided Surgery

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Introduction

Achieving complete tumor resections without major complications remains challenging. Fluorescence guided surgery (FGS) with tumor-specific probes can visually assist the surgeon in discriminating tumorous from healthy tissue. However, tumor-specific probes are currently screened one-by-one and per tumor type. Predictive platforms to develop FGS probes in a tumor-specific and even patient-specific way are lacking. Here, we combine tumor organoid biobanks with 3D imaging technology to present a screening platform for FGS probes.

Material and Methods

Both an adult and pediatric tumor organoid biobank were cultured, respectively breast cancer and neuroblastoma. Surface markers that are upregulated in neuroblastoma and/or breast cancer were identified based on RNA profiling, the human protein atlas and literature review. Probes targeting these surface markers were conjugated to six different fluorophores ranging between 488nm and 647nm. Together with the general marker efluor, they were used for seven-color 3D multispectral live imaging using confocal microscopy on living organoids. Using segmentation analysis by parallelization of 3D datasets (STAPL-3D) organoids were segmented and their fluorescent signals of the respective probes quantified. This high-throughput *in vitro* screening was validated by testing the three most promising FGS probes for neuroblastoma *in vivo* using a mouse xenograft model with tumors originating from neuroblastoma organoids.

Results and Discussions

Our multiplex organoid-based 3D live imaging platform was able to simultaneously screen six different probes on two full organoid biobanks. Segmentation and quantification using STAPL-3D showed heterogeneous expression of markers on the organoid lines. The screening capability of this platform was validated by testing the three most promising probes for neuroblastoma (GD2, L1cam and NCAM-1) *in vivo*. These probes resulted in tumor-to-background ratios above 2.0, indicating good intraoperative visibility. Therefore,

these probes could be tested for clinical purposes. For heterogeneous tumor types that are not covered with only one probe, this platform also offers the chance to screen most potential combinations of probes.

Conclusion

We present a novel multiplex organoid-based 3D live imaging platform to screen FGS probes in a patient-specific manner with the potential to develop personalized FGS probes or probe combinations.

EACR22-0026

TOP ABSTRACT

Defining the behavioral-transcriptomic landscape of engineered T cells provides insight into cancer immunotherapy

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Introduction

T cell immunotherapy is fast evolving and applied for several malignancies, but has shown minimal efficacy against solid tumors. One of the challenges to overcome this issue is the development of predictive platforms for modeling their mode of action.

Material and Methods

To unravel the behavioral and underlying molecular mechanisms of solid tumor targeting we developed a dynamic immuno-organoid 3D imaging-transcriptomics platform: BEHAV3D. BEHAV3D combines organoid and 3D imaging technology with a computational pipeline for analysis of functional single cell behavior integrated with transcriptomic profiling to decipher and manipulate the solid tumor-targeting strategy of engineered immune cells. We applied this platform to an emerging cancer metabolome-sensing immunotherapy: ab T cells engineered to express a Vg9/Vd2 T cell receptor (TEGs) and a group of human breast cancer derived organoid lines with distinct genetic dysregulations.

Results and Discussions

BEHAV3D revealed a high level of heterogeneity in targeting efficacy and functional behavior of the tested T cells. By using live-tracking of over 150,000 TEGs, targeting breast cancer organoids, we identified a 'super engager' behavioral cluster, comprising potent serial killing TEGs. Inference of single-cell behavior with transcriptomics unraveled gene signatures linked to these specific behaviors. The most notable signature was specific to 'super engager' killer TEGs with 27 genes with no previously described T cell function. Furthermore, guided by a dynamic type 1 interferon (IFN-I) signaling module induced by high TEG-sensitive organoids, we showed that IFN-I can prime resistant breast cancer organoids for TEG-mediated killing.

Conclusion

BEHAV3D combines organoid, imaging and sequencing technology to offer a comprehensive platform that integrates multiple single-cell readouts, including tumor death dynamics, single-cell behavior and transcriptomic profiling. Using BEHAV3D we

characterized behavioral-phenotypic heterogeneity of TEG cellular immunotherapies against breast cancer, holding promise for improving cancer targeting in a patient-specific manner.

EACR22-0237

Ex vivo screening and analysis of novel effective treatments for ovarian cancer

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Introduction

Ovarian cancer (OVC) is the most lethal gynecological cancer. The 5-year survival rate of OVC in Europe is 47% and in Denmark it is only 41%, indicating need for identifying novel efficient treatments. More than 75% of ovarian cancers are characterized as high-grade serous ovarian cancer (HGSOC), and almost all HGSOC patients experience recurrence and development of platinum-taxane treatment resistance. Here we established a personalized drug screening and evaluation platform using HGSOC patient samples and drugs approved for treatment of different types of cancers.

Material and Methods

Tissues collected from OVC patients are grown as 3-dimensional (3D) ex vivo long-term tumor organoid cultures as part of the DECIDER project (<https://www.deciderproject.eu/>, ClinicalTrials.gov identifier: NCT04846933). In addition, four HGSOC cell lines – OVCAR3, OVCAR4, OVSAHO and Kuramochi – are grown as 3D tumor spheroid cultures. Cancer stemness and classification of these samples are determined by immunofluorescent staining of pax8, Ki67, acetylated tubulin, and p53 followed by high-throughput imaging using the ImageXpress Micro Confocal microscope (Molecular Devices). Image analysis is performed using MetaXpress software. Organoid/spheroid responses to selected drugs are tested by invasive growth and live/dead assays, to assess resistance towards cisplatin and carboplatin and responsiveness to new potential treatments. These drugs have been selected according to a screening of the NCI Approved Oncology Drugs library on MCF7, LR-MT2, and SKOV3 cells, analyzing for change in lysosomal localization.

Results and Discussions

20 patient tumor samples received from Turku University Hospital, Finland (JH) have been cultured in four different culture media to obtain highest possible survival rates. Spheroids of OVC cell lines are established from rederived cancer cells from xenotransplant tumors in NOG mice (Permission no. 2018-15-0201-01391, C1). Organoid/Spheroid analysis protocols and screening conditions have been set up for high-throughput microscopy assessing growth, survival, and invasiveness.

Conclusion

We are currently set up for testing the nine most promising drugs emerging from previous drug screening experiments in our ex vivo 3D experimental design, and the results from this

will be presented here. This study is supported by Fabrikant Chas. Otzen's Fond and by Horizon2020 project DECIDER.

EACR22-0307

Evaluation of the therapeutic efficacy of a bifunctional antibody in combination with chemotherapeutic drugs, in a mouse model of pancreatic adenocarcinoma (PDAC) generated by eco-guided imaging.

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Introduction

PDAC is one of the cancers with worst prognosis^{1,2}. This occurs because PDAC is often diagnosed at advanced stages, and because of an intrinsic resistance to the most common chemotherapeutic drugs. Advanced stage PDAC is treated primarily with gemcitabine³, known to be metabolically unstable. To compensate, it is administered in high doses, which generates serious side effects. To overcome these problems, we generated an orthotopic xenograft mouse model in which we tested the antineoplastic efficacy of a new engineered bifunctional antibody (scDb, Patented by MCK therapeutics, n°: PCT102017000083637) which has been proven to affinity target PDAC cancers, in combination with Gemcitabine (GEM).

Material and Methods

The orthotopic PDAC mouse model was developed by USGI of 1 X 10⁶ PANC-1 cells on the pancreas of athymic nude mice. A 20 µL bolus of cells suspended in PBS was injected directly into the pancreas using Hamilton syringe with a 27 g needle previously placed in the mechanical syringe holder and lined up parallel to the US-transducer and perpendicular to the body. VevoLAZR-X system was used for USGI and for monitoring the tumor development. 4 groups of treatment were made: 1) CTRL (saline); 2) GEM 0.5 mg/mouse; 3) scDb (bifunctional antibody directed against the molecular target hERG1/β1 integrin complex) 320 mg/mouse; 4) GEM 0.1 mg/mouse + scDb 320 mg/mouse. GEM was administered i.p. 3 times per week; scDb was administered i.v. every day.

Results and Discussions

The development of the PDAC has been characterized and monitored by US imaging. The treatment started after 2 weeks from the cell injection and continued for the next 3 weeks. In terms of tumor growth, we observed a similar effect between scDb and GEM at the lower dose (subtherapeutic), but the most relevant result was obtained in the group treated with scDb+GEM 0.1 mg/mouse. A similar reduction of tumor growth was indeed observed in the group treated with the combination scDb+GEM and the group treated with GEM 0.5 mg/mouse.

Conclusion

The orthotopic PDAC model, derived from US-guided injection, showed a slower growth rate of tumor developed that is preferable because allows to better monitor a therapeutic effect over time. In this preliminary set of experiment was observed an interesting antitumoral effect of the combination therapy scDb + GEM. This allows to reduce the dose of the GEM maintaining the anti-neoplastic effect overcoming the side effects of chemotherapy.

Changes in metabolome levels of breast cancer patients treated with neoadjuvant therapy

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Introduction

Breast cancer is the most diagnosed and the leading cancer death cause in women worldwide. Nowadays, neoadjuvant treatment (before surgery) is increasingly used, and these therapies affect the organism in many ways, including modifying the expression of biomarkers associated with breast cancer (ER, PR, HER2+, ki67), as well as physiological changes affecting patients' metabolic profiles (such as BMI, glucose levels and triglycerides blood levels, and bone mineralization index). Metabolomics allows the quantification of the entire set of metabolites in blood samples. The main aim of this study is to identify temporal metabolic patterns relevant to the clinical response to neoadjuvant treatment in breast cancer.

Material and Methods

Blood plasma samples from 103 patients with breast cancer treated with neoadjuvant chemotherapy were used to perform metabolomics experiments. Three samples per patient were recollected and analyzed (one sample pre-treatment (basal), one sample before surgery (pre-surgery), and another after (post-surgery)). Clinical data regarding response to treatment (complete response or partial response) along with other clinical parameters were collected. Metabolomics experiments were performed using UPLC-MS/MS. Generalized Linear Mixed Models (GLMM) were used to establish temporal metabolic patterns.

Results and Discussions

GLMM identified metabolic pathways that suffered changes through time. Changes are found in metabolites related to the metabolism of amino acids, peptides, polyamines, steroid hormones, corticosteroids, primary bile acids, sterols, ceramides, sphingomyelins, fatty acids, monoacylglycerols, plasmalogens, lysophospholipids, carnitine, and acylcarnitines. Major changes are found between the basal and pre-surgery temporal points. Some changes remain post-surgery, possibly related to the disappearance of the tumor. Besides, there are also changes related to response to treatment in sterol, purines, pyrimidines, and glycerolipid metabolism pathways.

Conclusion

Results point out that treatment causes major changes in metabolite levels regardless of the pathological response achieved by patients. Some minor changes remain post-surgery, and these could be related to the disappearance of the tumor. By identifying metabolic pathways and physiological processes impacted by neoadjuvant treatment, tentative dietary supplements could be established to improve patients' metabolic profiles, thus increasing their quality of life.

MmCMS: Consensus molecular subtypes of colorectal cancer mouse models

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Introduction

Genetically engineered mouse models (GEMMs) aim to faithfully mimic the complexity of human colorectal cancer (CRC) and, when appropriately aligned, represent ideal pre-clinical systems to test new drug treatments. Therefore, stratification of mouse CRCs according to human subtypes is a critical step to improve disease-positioning of models and translation of findings from the pre-clinical setting. Despite its importance, dual-species classification has been limited by the lack of a reliable and standardised approach. Here we present a set of options for human-to-mouse consensus molecular subtypes (CMS) classifications, using various levels of biological information.

Material and Methods

We have employed TCGA data, as used for the original human CMS classifier, to develop and compare the performance of new dual-species CMS classifiers, within a collection of 51 GEMMs across 6 genotypes. The nearest template prediction method was employed to predict the proximity of each GEMM expression profile to CMS subtypes.

Results and Discussions

Classifier development resulted in three approaches; Option A, an individual gene-level classifier, Option B, an ontology-level approach and Option C, a combined pathway system encompassing multiple biological and histological signalling cascades. Initial testing in human and mouse tumour samples indicated that Option B and C, given their broad biological knowledge-based approaches, were less influenced by non-biological factors such as batch effect and normalization methods, leading to more robust CMS classifications compared to the gene-based approach in Option A. When applied to our GEMM collection, Option A results in more “unclassified” calls (31%) compared to Option B (6%) and C (17%). Although all options could identify tumours associated with stromal-rich CMS4-like biology, the Option A classifier was unable to accurately classify epithelial-like subtypes (CMS2/3), as gene set enrichment analysis indicates that samples classified as CMS2 by Option A were associated with activated metabolic signalling, a well-defined feature of CMS3. Both Option B and C classified CMS2 and CMS3 GEMM tumours with biology more aligned to human tumours.

Conclusion

These analyses indicate that when applying human-based classifiers in mouse tumour models, a pathway-level classifier, rather than an individual gene-level system, is optimal. Our R package provides all 3 options to help researchers to select suitable mouse models of human CRC subtype for their experimental testing.

EACR22-1088

MYC is a clinically significant determinant of mTOR inhibitor resistance in breast cancer

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Introduction

The PI3K/AKTmTOR signaling pathway is frequently activated in breast cancer. Accordingly, blockade of this signaling axis using small-molecule compounds has been widely investigated, ultimately resulting in PI3K and mTOR targeted therapeutics as standard-of-care treatments for breast cancer. However, the factors determining therapeutic responses

to PI3K/mTOR targeted therapies remain poorly understood, which hampers optimal treatment choices. Here, we aimed to identify mTOR inhibitor (mTORi) resistance factors with clinical potential for patient stratification in breast cancer.

Material and Methods

Tumors sensitive or resistant to the mTORi AZD8055 obtained from the *K14-Cre;Cdh1^{F/F};Trp53^{F/F}* (KEP) mouse model of invasive lobular breast carcinoma (ILC) were subjected to multi-omics profiling. Primary cell cultures derived from AZD8055-resistant tumors and somatic mouse modeling were used to decipher mechanisms of acquired resistance and identify drug targets synergizing with mTOR blockade. These findings were validated using data from patients with ER+/HER2- metastatic breast cancer from the Hartwig Medical Foundation and the Everolimus Biomarker Study.

Results and Discussions

Multi-omics analysis of KEP-tumors revealed recurrent activation of MYC in tumors with acquired resistance to AZD8055. MYC activation was associated with biological processes linked to mTORi response, such as immune evasion and glutamine-fueled oxidative phosphorylation. MYC expression facilitated resistance to AZD8055, both *in vitro* in KEP cell lines and *in vivo* using tamoxifen-inducible *Myc^{ERT2}* in ILC models. Combined treatment with mTOR and MYC inhibitors synergistically inhibited the growth of AZD8055-resistant KEP cell lines. Importantly, MYC status was significantly associated with poor clinical outcomes during everolimus-based therapy in metastatic breast cancer patients. Together, these findings highlight MYC as a central mediator of mTORi resistance. Importantly, our study demonstrates that MYC status may predict clinical response to mTORi, thus potentially guiding cancer patient selection for indications where mTORi targeted therapies such as everolimus are standard of care, e.g., ER-positive/HER2-negative breast cancer.

Conclusion

Our study demonstrates that MYC is a clinically significant mTORi resistance factor in breast cancer, and MYC status could potentially be used to select patients for mTORi treatment.

EACR22-0596

Characterization and Clinical Application of Bladder Cancer Patient-Derived Organoids for Personalized Medicine

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Introduction

Failure to standard-of-care (SOC) and the frequent relapses are the main clinical problems of bladder cancer (BLCa), indicating that tailored methods are needed. Genomic showed a limited role in identifying therapeutic options, thus patient-derived organoids (PDO) have been tested for functional assays to predict drug response in many tumors. Here, we aimed to explore the ability of BLCa PDO to retain cancer features and to

determine patient drug sensitivity to tailor therapy accordingly by linking PDO-drug responses to parental tumor (PT) genomic profiles.

Material and Methods

To derive PDO, tumors were digested into single cells and cultured in suspension. PDO drug sensitivity was evaluated after 48 hours of treatment by viability assay. Whole exome sequencing was done on genomic DNA (PDO, PT and blood). Histological evaluation of PDO and PT marker expression and morphology was done.

Results and Discussions

We successfully derived PDO from 11 non-muscle invasive BLCA (NMIBC) and 6 muscle invasive BLCA (MIBC). PT and PDO genomic analysis showed a good matching of mutational burden with copy-number variations (CNV) and single nucleotide variants (SNVs). SNVs clonality showed that PDO conserved similar level of PT subclonal mutations, hence preserving PT heterogeneity. Moreover, PDO and PT genomic profiles showed typical BLCA mutations (*Tp53* loss in MIBC and *FGFR3* mutations in NMIBC). PDO marker expression and morphological analysis was linked to PT in respect to its histopathological features. PDO were tested for SOC and FDA-approved drugs. 50% of NMIBC and 33% of MIBC PDO were resistant to SOC, underling the impact of interpatient heterogeneity in response to chemo. Non-SOC drugs were variably effective, and analysis of drug response showed correlations with mutational profiles, showing that PDO retain PT response by preserving druggable mutations. *FGFR3* CNV was associated with erdafitinib sensitivity, *TUBB1* CNV with docetaxel sensitivity, and *MAP4* SNV with paclitaxel resistance. Finally, PDO longitudinally derived from one patient captured the variation caused by the therapy earlier used on the patient.

Conclusion

We set up a protocol highly efficient on generating BLCA PDO that cover a wide range of disease stages and maintain the PT histopathological and molecular heterogeneity in terms of tumor content, deleterious SNVs and cell subpopulations. We showed the clinical relevance and feasibility of a PDO-based drug assay on stratifying patients according to drug sensitivity that can be used to improve their

EACR22-0722

Identification of tumor heterogeneity and drug sensitivity in primary prostate cancer

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Introduction

Prostate cancer (PCa) is a highly heterogeneous disease with multiple and distinct cancer foci within the same prostate gland. We hypothesize that heterogeneous lesions, in terms of genomic aberrations and tumour staging, may be linked to different functional properties e.g. differential drug response sensitivity. Here we investigate which histopathological and molecular properties are associated with ability to maintain PCa cells as ex vivo organoid cultures and specific drug responses.

Material and Methods

To simultaneously assess the histopathology, genetic profile and organoid drug sensitivity, we employed mirror biopsies for FFPE and fresh tissue preparations. Four cores of prostate tissue from radical prostatectomies (N=13) were used. Patient-derived organoids (PDOs) were characterized for morphology, number, and diameter. Targeted genomic sequencing was performed on PDOs and their parental FFPE tissues. Drug response was evaluated by ATP-based viability assay after treatment with standard-of-care (AR inhibitors), and approved compounds for other malignancies (DNA synthesis, receptor tyrosine kinase (RTK) and mTOR inhibitors).

Results and Discussions

Heterogeneous cores with different Gleason score range 6 to 9 per prostate were found in 76% of cases (N=10/13). Regarding formation efficiency, 78% of tumor-containing cores (N=22/28) and 66% of benign cores (N=16/24) showed organoid formation. Solid, cystic, and mixed morphologies were overall observed; tumor-containing cores formed higher number of mixed structures and solid structures of larger diameter ($p<0.05$, $p<0.01$, vs normal cores, respectively). PCa somatic mutations were found in 19 cores with 11 cores sharing mutations with the deriving PDOs. Different cores from the same prostate showed different drug response, while RTK inhibitors, Crizotinib and Ponatinib showed efficacy in the majority of cases ($p<0.01$ over vehicle, N=29/32 and N=30/32, respectively).

Conclusion

The majority of primary PCa cases showed genetic and histopathological multifocality, representing a highly heterogeneous cohort. PDOs recapitulated the genetic signature of the parental tissue and formed solid, adenocarcinoma-like morphology. Drug responses of PDOs did not follow patient-specific patterns but rather revealed high intra-patient heterogeneity. Overall low response to AR and mTOR inhibitors and strong response to RTK inhibitors was observed, possibly providing new options for PCa treatment.

EACR22-0489

The spectrum of Second Primary Malignancies Among Patients with Penile cancer in the United States: A Population-Based Study

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Introduction

Cancer of the penis is uncommon in the United States (US), accounting for less than 1 percent of cancers in males. It has an annual incidence of 1-2 per 1,00,000 men in the US. Of these, nearly 95% are squamous cell carcinomas. The most common etiology is human papillomavirus infection, and other risk factors are smoking, phimosis, lack of circumcision, warts, condylomas, and ultraviolet irradiation. Data is lacking on the incidence of second primary malignancies (SPMs) in patients with penile cancer in the recent era. So, this study aimed to identify the spectrum and discover the time period to the occurrence of SPMs after the penile cancer diagnosis.

Material and Methods

The incidence of SPMs among two-month survivors of penile cancer was estimated using the National Cancer Institute's Surveillance, Epidemiology, and End Results (SEER)-13 registries from 1992-2018. We estimated the risk of SPMs using standardized incidence ratios (SIRs).

Results and Discussions

We included a total of 3,731 patients with penile cancer in this study. The median age at diagnosis was 69 years (range 17-85 years). The median follow-up duration was 42 months (range 0-322 months). A total of 411(11%) patients developed 464 SPMs at the last follow-up. The SPM risk for the cohort was significantly elevated (SIR 1.38, 95% confidence interval [CI] 1.26 – 1.51) compared with general population. The median latency period for SPM development was 50 months (range 2-292 months). The SIR according to age groups were as follows age < 40 years (SIR 2.79, 95% CI 1.02 – 6.08), age 40-60 years (SIR 1.72, 95% CI 1.42 – 2.05), and age > 60 years (SIR 1.28, 95% CI 1.15 – 1.43). Specific sites with the highest risk of SPM were thyroid (SIR 8.50, 95% CI 1.03 – 30.71), anus (SIR 5.38, 95% CI 1.46 – 13.76), kidney (SIR 5.26, 95% CI 1.93 – 11.45), lung and bronchus (SIR 1.95, 95% CI 1.58 – 2.39), and oral cavity and pharynx (SIR 1.82, 95% CI 1.04 – 2.96). The risk of malignancies of the thyroid, kidney, lung and bronchus was higher within the first year, while the oral cavity and pharynx malignancies were highest within 1 to 5 years of penile cancer diagnosis. Finally, the risk of anal cancer was highest at 5 to 10 years of penile cancer diagnosis.

Conclusion

Our study reveals that the risk of developing SPMs is elevated in patients with penile cancer than in the general population, especially in patients under 40 years of age. During their follow-up, penile cancer patients will benefit by screening for thyroid, kidney, anus, lung, and oral cavity cancers.

EACR22-0228

Investigation of the MIR-379 locus for synergistic treatment of aggressive breast cancer

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Introduction

MiRNAs play a critical role in the cancer setting through the negative regulation of tumor suppressor genes and oncogenes. Understanding miRNA-mediated downregulation of tumor progression has resulted in immense interest in the therapeutic application of these sequences. Members of the miR-379 locus located on the C14 gene megacluster have been shown to have tumour suppressor effects in cancer. The current work focuses on the potential anti-cancer synergism of combining multiple miRNAs from the miR-379 gene family for treatment of aggressive breast cancer.

Material and Methods

Invasive human and murine breast cancer cell lines (MDA-MB-231, HCC-1954, 4T1) were stably transduced with RFP-tagged lentiviral constructs for miR-379-5p and miR-758-3p, either individually or in combination. Virus transduction success was confirmed by RQ-PCR and fluorescent imaging. The impact of elevated miRNA expression on cell metabolism was analysed using CellTiter 96® Aqueous One Solution Assay. Cell conditioned media (CCM) was harvested from transduced cells and the ability to stimulate tubule formation determined using Human Umbilical Vein Endothelial Cells (HUVECs). CCM was also analysed on a 20-target angiogenesis array. Corning Transwell Inserts with 8µm pores were used to quantify

migration. Gene targets of miR-379-5p and miR-758-3p were analysed by immunofluorescence, western blot and gene expression PCR.

Results and Discussions

Significant elevation of miR-379-5p and miR-758-3p was confirmed in all cell lines after transduction. There was no change in cell metabolism in the presence of one or both miR-379-5p and miR-758-3p. HUVECs incubated with CCM from cells expressing miR-379-5p and miR-758-3p alone or in combination had a reduced ability to form tubules. A decrease in migration was observed in MDA-758 and MDA-379 cells but the greatest decrease was observed in MDA-MB-231 cells engineered to express both miRNAs simultaneously. Protein array analysis revealed leptin and angiogenin, important players in breast cancer progression, were secreted from control cells but not from MDA-379 and MDA-379/758. VEGF-C, a potent stimulator of tumour angiogenesis, was also significantly decreased in MDA-379 and MDA-379/758 CCM compared to controls.

Conclusion

This preliminary data showcases the combined effect of miR-379-5p and miR-758-3p for treatment of breast cancer by targeting genes involved in cancer progression and angiogenesis. The synergistic effects observed may have important implications for development of miRNA therapy.

EACR22-0350

Towards sustained release of therapeutic EVs for breast cancer therapy via 3D biocompatible hydrogels

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Introduction

Extracellular vesicles (EVs) have emerged as potentially promising and versatile vehicles for the delivery of therapeutic cargo to metastatic breast cancer. However, a local sustained delivery system for EVs at the target site that will ensure prolonged exposure, maximum efficacy and dose, and minimal toxicity is required to minimize rapid clearance from the body. Biocompatible hydrogels hold immense promise as tunable delivery systems for localised and controlled delivery of EVs at the target site. The aim of this study is to investigate the potential of tyramine modified hyaluronic acid (HA-TA) hydrogels for incorporation and sustained delivery of therapeutic EVs.

Material and Methods

HA-TA hydrogels (1% & 2%) with varying concentrations of hydrogen peroxide (H₂O₂) and horse radish peroxidase (HRP) crosslinkers, were formed using a benchtop hydrogel mixer device. Analysis of hydrogel swelling and degradation was performed at various time points from 5-72 hours. EVs were isolated from plasma samples using size exclusion chromatography (SEC) and characterized using Nanoparticle Tracking Analysis (NTA) and western blot. The hydrogel concentrations with well-defined and intact structures were then loaded with varying concentrations of EVs. The rate of release of EVs from hydrogels was

assessed using NTA and protein assay. The uptake of EVs by recipient breast cancer cells was then analyzed.

Results and Discussions

NTA revealed small EVs in the size range of 30- 200 nm were successfully isolated from plasma samples. Western blot of the EV protein extract confirmed expression of EV markers CD63, TSG101 and CD81. The hydrogel swelling and degradation assay revealed a requirement for higher concentrations of HA-TA and crosslinkers to maintain suitable hydrogel structure and ensure continued swelling. Plasma EVs were successfully incorporated into the hydrogels with varying release patterns observed over time, depending on EV concentrations loaded and hydrogel formulation employed. Efficient transfer of hydrogel encapsulated/released EVs to recipient breast cancer cells was also demonstrated *in-vitro*.

Conclusion

These preliminary data highlight the potential of HA-TA hydrogels for incorporation and subsequent release of therapeutic EVs. Further research is warranted to optimise the tunable characteristics of the system. Biocompatible hydrogels are employed clinically for multiple applications and hold immense promise in the cancer setting.

EACR22-0492

microRNA gold-nanobeacons for selective lung cancer gene therapy

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Introduction

Lung cancer (LC) is a leading cause of death worldwide (Conde et al., JAMA Oncology 2021). Standard treatment shows severe side-effects and poor drug accumulation at the target site. Effective LC therapies should rely on specific targeting and localized delivery of payloads capable of disrupting malignant cells (Sousa et al., Trends in Cancer 2021). microRNAs (miRNAs) regulate gene expression at the post-transcriptional level. Upregulated miRNAs participate in tumorigenesis by deregulating cellular processes. Gold nanoparticles (AuNPs) are suitable vehicles for gene therapy due to the lack of undesirable immune responses or off-target effects and ease of functionalization with moieties (Conde et al., Nat. Commun. 2016). We expect that AuNPs combined with molecular beacons specifically bind to upregulated microRNAs, inducing their silencing and decreasing LC progression, resulting in an improved alternative to currently available therapies (Conde et al., PNAS 2015).

Material and Methods

LC cell lines were analyzed using miRNA and whole genome arrays to address which miRNAs and miRNA-related genes were altered. AuNPs were functionalized with polyethylene glycol and molecular beacons, targeting the human miRNA-31. The nanobeacons were characterized in terms of variation of the surface plasmon resonance (SPR) peak by UV-Visible spectroscopy; size and surface charge by dynamic light scattering; stability with temperature, pH, in reducing conditions, and with RNase, and ability to hybridize with targets, by fluorescence spectroscopy. Their toxicity was evaluated in a lung cell line using the MTT assay.

Results and Discussions

Anti-miRNA-31 5p and 3p nanobeacons showed SPR peaks of 519 nm, hydrodynamic diameters of 51.3 and 136.4 nm and zeta potentials of -27 and -28 mV, respectively. The anti-miRNA-31 5p was stable in different pHs and reducing conditions and showed instability with increasing temperatures and after incubation with RNase, while hybridizing correctly with its target. On the other hand, the anti-miRNA-31 3p was stable in different pHs and reducing conditions, more stable with increasing temperatures and less stable after incubation with RNase, and failed to hybridize with its target.

Conclusion

We have developed and characterized a nanotechnology-based approach for controlled and efficient silencing of endogenous miRNAs involved in LC. We are evaluating its in vitro effects in LC cells, regarding miRNA expression, cellular localization and cell cycle, among others.

EACR22-0744

Tumor penetrating utorubicin-loaded polymersomes for anti-cancer therapy

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Introduction

For over 30 years, anthracyclines (e.g., doxorubicin) have been widely used to treat many types of cancer. The fact that therapeutic application of anthracyclines is limited by side effects, especially by dose-limiting cardiotoxicity, has led to extensive efforts - like the development of less toxic anthracycline derivatives, their coupling to and encapsulation in various nanoparticles - to achieve their improved safety profile and broader therapeutic index. The brightest example, liposomal doxorubicin (Caelyx®), is clinically approved for the treatment of several types of cancer. However, compared to the free drug, the anti-cancer efficacy of liposomal doxorubicin is not significantly higher, and, despite an improved safety profile, it still elicits side effects. Thus, there is an urge for more potent, effective, and safe anthracyclines and their delivery systems for cancer therapy.

Material and Methods

In collaboration with the Estonian company TBD-Biodiscovery LLC, we synthesized and encapsulated a novel anthracycline, utorubicin (UTO), in biocompatible polymeric nanovesicles (polymersomes, PS). The PS were functionalized with tumor-penetrating peptides (TPPs) to selectively target and kill tumor cells overexpressing the peptide receptor. We performed a comparative cytotoxicity study using TPP-functionalized UTO-loaded PS (TPP-UTO-PS) on cultured cancer cells, studied the biodistribution of the TPP-UTO-PS in triple-negative breast tumor (TNBC) xenografts, and evaluated the anti-tumor effect of our UTO-nanopatform in the treatment of mice bearing peritoneal carcinomatosis.

Results and Discussions

In cultured tumor cells, free UTO showed significantly higher anti-cancer activity than clinically used doxorubicin proving the rationale of UTO development. TPP-UTO-PS showed selective internalization and killing of cultured peptide-receptor positive cells and preferential accumulation in TNBC xenografts implanted in mice upon systemic administration without accumulation in the heart. Moreover, experimental treatment of mice bearing peritoneal carcinomatosis of gastric carcinoma origin (MKN45P) with UTO-PS resulted in significant suppression of tumor growth.

Conclusion

We developed a tumor-specific nanoplatform that selectively delivers a novel drug candidate UTO to breast and peritoneal tumors and has an anti-cancer effect in a model of peritoneal carcinomatosis. Our study encourages further preclinical and clinical studies on UTO as a nanocarrier payload for precision cancer therapy with reduced drug side effects.

EACR22-1276

Bioengineered humanised models of bone metastatic prostate cancer

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Introduction

The bone metastatic niche is a complex microenvironment with various cell populations that have different roles in cancer cell migration, survival, and therapy resistance. While osteoblasts and osteoclasts fuel metastasis progression, bone marrow adipocytes (BMAs) account for 70% of the marrow and have been identified as a potential contributor. To date, there is however a lack of relevant models to study the complex relationship between cancer cells and adipocytes, as most models either employ murine cell lines, high fat diet mice, and white adipocytes instead of bone marrow adipocytes, impairing to truly dissociate the role of human BMAs in cancer progression in bone. We have thus developed sophisticated bioengineered humanised models with a high human adipose content that better replicate the complexity of the marrow microenvironment, *in vitro* and *in vivo*.

Material and Methods

We developed a humanised fatty marrow niche by co-culturing primary human osteoblasts, human bone-marrow derived mesenchymal stem cells differentiated into adipocytes and androgen-sensitive human prostate cancer (PCa) cells (LNCaP and C4-2B), encapsulated into Gelatin Methacryloyl (GelMA) hydrogels for *in vitro* and *in vivo* investigation. After four weeks of osteogenic differentiation and mineralization *in vitro*, the bioengineered mineralized tissues were subcutaneously implanted into male NSG mice. Six weeks later, adipose/cancer constructs were implanted in the same subcutaneous pocket to create a fatty niche, as to assess the effects of human adipocytes on cancer progression. *In vivo* bone formation was followed by computed tomography (CT) and cancer progression by bioluminescence imaging. After five weeks of co-culture, the humanized niche was characterized *ex vivo* by microCT and histology.

Results and Discussions

Construct optimization allowed to culture each cell type independently or concomitantly in GelMA hydrogels for up to six weeks *in vitro*, resulting in appropriate adipogenic differentiation, cancer spheroids formation and osteoblast mineralization. *In vivo* data showed bone formation with high mineralization overtime and the morphology of human native bone. *In vitro* and *in vivo*, cancer progression was observed for the most aggressive cancer cell line (C4-2B) and was enhanced by the presence of human adipocytes.

Conclusion

The bioengineered models presented here offer an advanced platform for bone metastatic cancer research, such as for the screening of novel therapies on the bone tumour microenvironment.

Targeted nanovaccine as a powerfull platform to improve immune modulation and checkpoint therapies in colorectal cancer

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Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the fourth cause of cancer death worldwide. This work is focused on the development of a multifunctional CRC immunotherapy based on a nanoplatform rationally designed to deliver combinations of neoantigens and immune regulators to modulate host immunity against CRC.

Material and Methods

Poly(lactic-co-glycolic) (PLGA) nanoparticles (NP) prepared by the double emulsion solvent evaporation method, were modified with: 1) mannose to target CD206 at dendritic cells (DC) and 2) a peptide motif to target $\alpha_v\beta_3$ receptors at tumor cells. NP physico-chemical properties were characterized and the loading of CRC neoantigens, adjuvants, immune modulator was quantified by fluorescence. The immunotherapeutic potential of our multifunctional nanovaccine, isolated and in combination with immune modulators and/or a tumor-associated macrophages (TAM) inhibitor, was assessed in the immune-competent MC38 CRC mouse model. The added value of combining an immune checkpoint inhibitor was also evaluated.

Results and Discussions

NP presented a mean diameter close to 200 nm, low polydispersity index, neutral surface charge, spherical shape, and high loadings for neoantigens, adjuvants, and immune modulators. A noteworthy tumor remission with the modulation of immune subsets within tumor microenvironment (TME) was observed in MC38-bearing mice treated with the double or triple combination therapies including the nanovaccine, the TME-targeted NP, and/or the TAM inhibitor. Our nanovaccine in combination with TAM inhibitor also sensitized CRC to immune checkpoint therapy, significantly delaying tumor burden.

Conclusion

This innovative approach discloses the synergy among the targeted nanovaccine and immune modulatory and checkpoint therapies within TME, which overall outcome may constitute a real hope for patients with metastatic CRC disease.

Unraveling the therapeutic potential of CEA-targeted nanoparticles in colorectal cancer cells and to modulate its tumor microenvironment

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Introduction

Colorectal cancer (CRC) is one of the deadliest cancers worldwide. Its tumor microenvironment (TME) is known to dictate the treatment outcome and has been proposed to stratify patients with microsatellite unstable (MSI) and stable (MSS) tumors. Despite the well-established 5-FU treatment regimes, their severe toxicity and low tumor-specific selectivity renders them unsuccessful. To provide an effective and targeted therapy, nanomedicine constitutes a promising alternative. Carcinoembryonic antigen (CEA), an overexpressed molecule in CRC, is a promising candidate to target CRC cells and to modulate the immune response. This work intends to develop an innovative nanosystem to target CEA-expressing CRC cells, delivering 5-FU in a more effective manner.

Material and Methods

Poly (lactic-co-glycolic acid) poly (ethylene glycol) (PLGA-PEG) co-polymer was chemically conjugated with the anti-CEA ScFv antibody. PLGA/PLGA-PEG NPs were produced by double emulsion and loaded with 5-FU. Physical-chemical properties were assessed by DLS and LDA. Morphology, drug loading (DL) and conjugation efficiency (CE) were evaluated by TEM, NMR and HPLC, respectively. CEA expression in different MSI and MSS CRC cell lines was evaluated by flow cytometry and confirmed by western blot. Targeting ability of the NPs was evaluated in CEA-expressing and non-expressing cell lines by immunofluorescence and ImageStream.

Results and Discussions

The conjugated polymer revealed anti-CEA scFV CE efficiency of 80%. Produced spherical NPs were 150 nm in size and presented a Pdl of 0.2, confirming the monodisperse population, and neutral surface charge (-3 mV). Furthermore, a 4% of 5-FU DL was successfully attained. Caco-2 (MSS cell line) and SW-48 (MSI cell line) showed high expression levels of CEA, while SW480 (MSS cell line) and HCT15 (MSI cell line) did not express CEA. NPs uptake were around 30% in CEA-expressing cells and 6% in non-expressing cells, confirming the high specificity of the nanosystem, 24h after NPs administration. To confirm the binding affinity, competitive assays were performed. Currently, in vitro spheroids models are being established to assess the binding efficiency of the nanosystem and to evaluate the impact on cancer and immune cells viability.

Conclusion

This work validates the development of CEA-targeted NPs loaded with 5-FU. This system will be further exploited to evaluate its impact on cancer and immune cells crosstalk, challenging the TME to improve chemotherapy outcome.