Posters
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Introduction

- Tumor cells with a mesenchymal phenotype, including cancer stem cells (CSCs), are known to contribute to metastasis.
- Circulating tumor cells (CTCs) with epithelial phenotypes in peripheral blood can be detected using an anti-EpCAM antibody for capture, which may not detect CTCs undergoing epithelial-mesenchymal transition (EMT).
- We have developed an antibody-independent CTC enrichment platform, Apostream®, which does not rely on EpCAM-based capture.

Objectives

Determine the clinical relevance and feasibility of measuring EMT CTCs in breast cancer patients.

Methods

- Blood samples from newly diagnosed breast cancer patients were prospectively collected before neoadjuvant systemic treatment (NST) (T1), after NST (T2), and after definitive surgery (T3) and processed using the Apostream® system.
- Isolated cells were stained with antibodies to leukocytes (anti-CD45) and the DAPI nuclear stain to exclude leukocytes.
- The residual cells were stained with the following additional antibodies and examined on a laser scanning cytometer to identify 4 CTC subsets based on protein expression levels of various markers:
  - Epithelial (CK+, EpCAM+, or E-cadherin+)
  - EMT (β-catenin+ or vimentin+)
  - Combined epithelial or EMT (CK+, EpCAM+, E-cadherin+, vimentin+ or β-catenin+)
  - CSC (CD44+ and CD24-).
- Pathological complete response (pCR) to preoperative chemotherapy was correlated to CTC levels and marker expression.

Results

Fig. 1 Trial Design

Table. 1 Baseline characteristics of the patients.

<table>
<thead>
<tr>
<th>No of patients</th>
<th>47</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age [range]</td>
<td>51.7 [41-79]</td>
</tr>
<tr>
<td>Stage</td>
<td>12</td>
</tr>
<tr>
<td>II</td>
<td>12</td>
</tr>
<tr>
<td>IV</td>
<td>6</td>
</tr>
<tr>
<td>Tumor staging</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>14</td>
</tr>
<tr>
<td>T2</td>
<td>5</td>
</tr>
<tr>
<td>T3</td>
<td>5</td>
</tr>
<tr>
<td>T4d</td>
<td>26</td>
</tr>
<tr>
<td>Nodal staging</td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>7</td>
</tr>
<tr>
<td>N1</td>
<td>16</td>
</tr>
<tr>
<td>N2</td>
<td>6</td>
</tr>
<tr>
<td>N3</td>
<td>18</td>
</tr>
<tr>
<td>ER status</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>24</td>
</tr>
<tr>
<td>Positive</td>
<td>23</td>
</tr>
<tr>
<td>HER2 status</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>34</td>
</tr>
<tr>
<td>Positive</td>
<td>13</td>
</tr>
<tr>
<td>Nottingham grading index</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
</tr>
<tr>
<td>NA</td>
<td>2</td>
</tr>
<tr>
<td>Proliferation index (Ki67)</td>
<td>53 [10-99]</td>
</tr>
</tbody>
</table>

Table. 2 Detection rate (≥1 cell) and mean number (range) of CTCs detected for each CTC phenotype.

<table>
<thead>
<tr>
<th>CTCs</th>
<th>Epithelial CTCs</th>
<th>EMT CTCs</th>
<th>Epithelial or EMT CTCs</th>
<th>CSC CTCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>24 (53)</td>
<td>11 (26)</td>
<td>28 (62)</td>
<td>2 (4)</td>
</tr>
<tr>
<td>T1</td>
<td>15 (79)</td>
<td>17 (90)</td>
<td>6 (0-201)</td>
<td>3 (16)</td>
</tr>
<tr>
<td>T2</td>
<td>21 (84)</td>
<td>18 (72)</td>
<td>22 (88)</td>
<td>4 (16)</td>
</tr>
<tr>
<td>T3</td>
<td>63 (0-637)</td>
<td>70 (0-645)</td>
<td>133 (0-697)</td>
<td>5.8 (5.8)</td>
</tr>
</tbody>
</table>

Conclusion & Future Perspective

- Preliminary results indicate that Apostream® was successful in detecting EMT-CTCs in this ongoing prospective study. CTC (epithelial and EMT-CTCs) levels after chemotherapy predict pCR.
- We need to await for enrollment and follow-up data of 50 patients to be more conclusive.
- Changes in EMT CTC levels during treatment will be explored in all cohorts.

Contact

E-mail address: nueno@mdanderson.org

Fig. 2 CTC levels according to pCR status at baseline (A), after chemotherapy (B), and after surgery (C).
- β-catenin+ EMT-CTCs at T3 are more likely to be detected at higher clinical stage (p<.014).
- Vimentin+ EMT-CTCs at T4 are more likely to be detected in HER2-negative breast cancers (p<.016).
- Patients with a higher level of combined epithelial or EMT CTCs before surgery (T1) are more likely to achieve pCR (p<.038).
To date, the isolation of circulating tumor cells (CTCs) from patients with renal cell carcinoma (RCC) has been met with limited success. This is due to the fact that most available CTC isolation technologies rely on the positive expression of an antibody-free isolation of CTCs. ApoStream is a novel technology which utilizes dielectrophoresis and microfluidics for the antibody-free isolation of CTCs.

In this study, we developed a novel method for detecting RCC CTCs using the ApoStream platform and fluorescence in situ hybridization (FISH) for loss of the VHL gene. This assay was then tested in a cohort of patients with untreated or progressive metastatic RCC, an epithelial marker which is expressed in a minority of ccRCCs.

The optimal operating frequency for enrichment of RCC CTCs was determined using fluorescently labeled 786-0 cells spiked in blood cells from healthy donors. In parallel, conditions were optimized for performing FISH for the VHL gene on isolated cells. Following assay development, CTCs (defined as any non-diploid cell) were enumerated in a cohort of patients with untreated or progressive metastatic ccRCC as well as healthy donors.

Antibody-independent isolation with dielectrophoresis and subsequent FISH for the VHL gene is a promising novel method for CTC detection in patients with metastatic ccRCC.

Future work aims to validate this assay in larger patient cohort.

References

Acknowledgments
This work was supported by a grant from the Urology Care Foundation.

Development of a Novel Method for Detecting Renal Cell Carcinoma Circulating Tumor Cells
Michael A. Gorin, Mark W. Ball, Darren W. Davis, Phillip M. Pierozario, Hans J. Hammers, Kenneth J. Pienta, Mohamad E. Allaf
James Buchanan Brady Urological Institute, Johns Hopkins University School of Medicine, Baltimore, MD
Introduction

- Tumor cells with a mesenchymal phenotype including cancer stem cells (CSCs), are known to contribute to metastasis.
- Circulating tumor cells (CTCs) with epithelial phenotypes in peripheral blood can be detected by CellSearch®, using an anti-EpCAM antibody for capture, which may not detect CTCs undergoing epithelial-mesenchymal transition (EMT).
- We have developed an antibody-independent CTC enrichment platform, Apostream®, which does not rely on EpCAM-based capture.

Objectives

Determine the clinical relevance and feasibility of measuring EMT-CTCs in breast cancer patients.

Methods

- Blood samples from newly diagnosed breast cancer patients were prospectively collected at the Department of Hematopathology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA; 1 Department of Breast Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA; 2 Department of Medical Oncology, Eugène Marquis Cancer Center, Rennes, France; 3 ApoCell, Inc., Houston, TX, USA; 4 Department of Hematopathology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

- Isolated cells were stained with antibodies to cytokeratin (anti-CK), leukocytes (anti-CD45), and the nuclear stain, 4,6-diamidino-2-phenylindole (DAPI), to identify CTCs.
- A CTC was defined as CK+CD45-DAPI-.
- These CTCs were also stained with additional markers and analyzed on a laser scanning cytometer to measure protein expression of epithelial (EpCAM, E-cadherin), mesenchymal (β-catenin, vimentin) and CSC-markers (CD24, CD44).
- Pathological complete response (pCR) status after preoperative treatment was obtained after surgery to correlate baseline CTCs and marker expression with treatment response.

Results

- Determined the clinical relevance and feasibility of measuring EMT-CTCs in breast cancer patients.
- Isolated cells were stained with antibodies to cytokeratin (anti-CK), leukocytes (anti-CD45), and the nuclear stain, 4,6-diamidino-2-phenylindole (DAPI), to identify CTCs.
- CK+CD45-DAPI- CTCs were detected (≥ 1 cell in at least one of the three samples) in 54%, 80%, and 100% at time-point T0, T1, and T2, respectively.
- Only β-catenin at T0 was predictive of pCR (Fig2). Vimentin, EpCAM, E-cadherin, CD24 and CD44 were not.

<table>
<thead>
<tr>
<th>Age</th>
<th>EMT-CTCs (n=14)</th>
<th>No EMT-CTCs (n=25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;50</td>
<td>6/14 (43%)</td>
<td>13/25 (52%)</td>
</tr>
<tr>
<td>50-60</td>
<td>5/14 (36%)</td>
<td>11/25 (44%)</td>
</tr>
<tr>
<td>&gt;60</td>
<td>3/14 (21%)</td>
<td>11/25 (44%)</td>
</tr>
</tbody>
</table>

- Patients without CTCs or with CTCs without β-catenin expression were more likely not to achieve pCR.
- Of the 5 patients who achieved pCR, they all either decreased their absolute CTC numbers or lost their EMT phenotype from T0 to T1.

Future Perspective

- We need to await for the enrollment and follow up of 50 patients to definitively conclude.
- Evolution of EMT-CTC detection during treatment will be explored in all cohorts.
Etiroleucobin Pegol Target-Specific Pharmacodynamic Biomarkers in Circulating Tumor Cells from Patients with Metastatic Breast Cancer in the Phase 3 BEACON Study

Edith A. Perez,a Katie Caygill,b Alison L. Hannah,c Javier Cortes,d Ahmad Awada,e Joyce O'Shaughnessy,f Christopher Twelves,g Hope S. Rugo,h Seock-Ah Im,i Darren W. Davis,j Ute Hoch,b

San Antonio Breast Cancer Symposium - December 9-13, 2014

aMayo Clinic, Jacksonville, FL; bNektar Therapeutics, San Francisco, CA; cConsultant, Sebastopol, CA; dVall d’Hebron ... of Leeds and St. James’s University Hospital, Leeds, United Kingdom; hUniversity of California, San Francisco, San Francisco, CA; iSeoul National University Hospital, Seoul, South Korea; jHoch, U. (2014).

INTRODUCTION

Etiroleucobin Pegol (WST-1) is a long-acting topoisomerase 1 inhibitor designed for prolonged tumor cell exposure.

In patients, etroleucobin pegol leads to greatly prolonged plasma SN38 exposure compared to irinotecan (elimination half-life 30 days compared to 2 days), yet maximal SN38 concentrations are at least 5- to 10-times lower.

In a Phase 3 trial in patients with metastatic breast cancer whose disease failed prior taxane-based treatment, etroleucobin pegol administered at 125 or 150 mg q4W resulted in objective response rates of 23% and 24% respectively.

Response rates by patient country are shown in Table 1.

Response Rate by Country

<table>
<thead>
<tr>
<th>Country</th>
<th># Samples</th>
<th>400-1100 CTCs/mL</th>
<th>Median:</th>
<th>0-1100 CTCs/mL</th>
<th>Median:</th>
</tr>
</thead>
<tbody>
<tr>
<td>China</td>
<td>100</td>
<td>43%</td>
<td>348</td>
<td>56%</td>
<td>536</td>
</tr>
<tr>
<td>Korea</td>
<td>100</td>
<td>43%</td>
<td>348</td>
<td>56%</td>
<td>536</td>
</tr>
<tr>
<td>France</td>
<td>100</td>
<td>16%</td>
<td>131</td>
<td>46%</td>
<td>186</td>
</tr>
<tr>
<td>USA</td>
<td>100</td>
<td>16%</td>
<td>131</td>
<td>46%</td>
<td>186</td>
</tr>
</tbody>
</table>

In patients, etroleucobin pegol leads to greatly prolonged plasma SN38 exposure compared to irinotecan (elimination half-life 30 days compared to 2 days), yet maximal SN38 concentrations are at least 5- to 10-times lower.

Increased topoisomerase 1 degradation through ubiquitination or sumoylation

Resistance mechanisms described for topoisomerase 1 inhibitors include:

- Increased topoisomerase 1 degradation through ubiquitination or sumoylation
- Increased expression of ATP-binding cassette transporters
- Top 1 activity increased in tumor cells with low intracellular ATP
- ATP binding cassette transporters
- Top 1 activity increased in tumor cells with low intracellular ATP
- ATP binding cassette transporters
- Target-Specific

METHODS

For BEACON patients participating in the CTC substudy, serial 7.5 mL whole blood samples were drawn and shipped to Ambient to ApoCell (Houston, TX) for further processing as shown in the BEACON CTC Sample Flow Diagram.

For BEACON patients participating in the CTC substudy, serial 7.5 mL whole blood samples were drawn and shipped to Ambient to ApoCell (Houston, TX) for further processing as shown in the BEACON CTC Sample Flow Diagram.

**CTCs DETECTED IN 97% OF PATIENT SAMPLES WITH HIGH MEDIAN NUMBER OF CTCs**

<table>
<thead>
<tr>
<th># Samples</th>
<th>400-1100 CTCs/mL</th>
<th>Median:</th>
<th>0-1100 CTCs/mL</th>
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<tr>
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<td>100</td>
<td>16%</td>
<td>131</td>
<td>46%</td>
</tr>
</tbody>
</table>

**EXPRESSION OF BIOMARKER SIGNAL IN BASELINE SAMPLES**

**CONCLUSIONS**

- Blood sample collection for CTC analysis was successfully incorporated into the BEACON study with 90% patient participation.
- Blood samples were successfully processed, with a low technical failure rate of 2%.
- CTC detection rate using ApoStream was high (79% of patient samples detected at CTCs of 47 CTCs/7.5 mL) and permitted evaluation of biomarker and pharmacodynamic end points.
- Etiroleucobin pegol target-specific pharmacodynamic biomarkers can be measured in CTCs isolated from patients participating in BEACON.

- BEACON efficacy and safety results are expected in Q1 2015, which will allow analysis of baseline CTC data and change of CTC data over time with patient outcome (response, PFS, OS).
Isolation and Characterization of Circulating Tumor Cells (CTCs) from Peripheral Blood Specimens of Patients with Advanced Solid Tumor Malignancies (Using ApoStream® Instrumentation)

Priya Balasubramanian1, Lihua Wang1, Scott M. Lawrence1, Tony Navas1, Shivaani Kummarr2, Melinda Hollingshead3, Francis Owusu1, Ralph E. Parchment1, Joseph E. Tomaszewski2, James H. Doroshow2, Robert J. Kinders1

1Laboratory of Human Toxicology and Pharmacology, Applied/Developmental Research Directorate, Leidos Biomedical Research, Inc., Frederick National Laboratory for Cancer Research, Frederick, Maryland 21702.
2Division of Cancer Treatment and Diagnosis, National Cancer Institute, Bethesda, Maryland 20892; 3Biological Testing Branch, National Cancer Institute at Frederick, Maryland 21702.

Abstract

Circulating tumor cells (CTCs) isolated using an antibody-dependent capture method have been shown to be a strong independent prognostic factor for progression-free and overall survival in certain carcinomas. However, the applicability of this technology is limited to epithelial malignancies. Utility may be even further restricted by epithelial mesenchymal transition (EMT) associated with chemotherapy and targeted agent therapy. We have reported previously that patients in our clinic, who have typically failed at least 3 lines of therapy are generally negative for CTCs captured by the EpCAM method [1, and manuscript in preparation]. An antibody-independent methodology, ApoStream® is capable of isolating live CTCs from epithelial and non-epithelial malignancies by exploiting the morphological and biophysical differences between cancer cells and normal blood cells. Viable cells are essential for isolation using ApoStream® technology, and patient cancer diagnosis information is critical to determine system operating parameters.

A clinical initial readiness study is being conducted using specimens from patients with advanced sarcomas and carcinomas.

Methods

Blood specimens from patients enrolled in clinical trials at the National Cancer Institute were collected in BD CPT tubes. K3EDTA tubes, or ACC tubes and processed on the same day. PRBC fractions were isolated following the manufacturer’s recommended protocol for CPT tubes or by LeucoSep® separation [2]. PRBC pellets were resuspended in ApoStream® sample buffer and run through the instrument at predetermined operating conditions. The enriched fraction was spun down, immediately pelleted onto Manneke® slides, fixed, and stored at 4°C until further processing. All patients gave written informed consent and were enrolled on NCI Institutional Review Board (IRB)- approved protocols.

Target Clone Vendor Fluorochrome
MUC-1 (MUC1) E2M R&D Unlabelled
Carcinoembryonic antigen (CEA) L243 DAKO Unlabelled
EpCAM E247 Abcam Alexa fluor 564
Pan-cytokeratin (CK) C11 Cell Signal Alexa fluor 555
Endothelial cell marker molecules (ECM) EF10 Cell Signal Alexa fluor 594
Vimentin V9 R&D Unlabelled Alexa fluor 647
CIS CS1 F30-844 BD Biosciences Unlabelled Alexa fluor 647

Standard antibody incubation protocols were followed. After cells were permabilized and blocked with serum, antibodies were added in a sequential manner: unconjugated antibodies first, secondary antibodies next, and finally directly conjugated antibodies. Coverslips were mounted onto the slides with DAPI-containing mounting media. Images covering the whole slide was acquired on Nikon Eclipse 80i microscope, and image analysis for rare cell detection was performed using Definiens® software.

Results

Longitudinal monitoring of CTCs in a sarcoma patient during treatment

Fig. 1A. Time course monitoring of the number of CTCs (β-catenin+ vimentin+) cells identified per image field (x=20). Each circle in the plot represents one image field. Fig. 1B. Time course monitoring of the number of CTCs (β-catenin+ vimentin+) cells identified per image field (x=20). Each circle in the plot represents one image field. Numbers noted in the graph represent the total number of cells identified from each cell spot, comprised of multiple image fields.

CTC classification and enumeration in 4 carcinoma patients

Fig. 2A. Enumeration and phenotypic characterization of CTCs from carcinoma patients. Venn diagram displays the total number of cells isolated (in 1 log scale) with a specific phenotype (-ve/-x/) from each patient specimen.

Fig. 2B. Image gallery of circulating non-epithelial cells with epithelial phenotype isolated from a carcinoma patient using ApoStream® technology. Cells were stained for MUC1/CEA (green), β-catenin (QD555), vimentin (red), and DAPI (blue). Arrows indicate punctuated nuclei (CD45/CD68/CD31(-)) cells.

Results

CTC classification and enumeration in 4 carcinoma patients

Fig. 2A. Enumeration and phenotypic characterization of CTCs from carcinoma patients. Venn diagram displays the total number of cells isolated (in 1 log scale) with a specific phenotype (-ve/-x/) from each patient specimen.

Fig. 2B. Image gallery of circulating non-epithelial cells with epithelial phenotype isolated from a carcinoma patient using ApoStream® technology. Cells were stained for MUC1/CEA (green), β-catenin (QD555), vimentin (red), and DAPI (blue). Arrows indicate punctuated nuclei (CD45/CD68/CD31(-)) cells.

Summary and Conclusions

• We have previously reported on the use of a novel antibody-independent technology, ApoStream®, to isolate CTCs from alveolar soft part sarcoma patients [3].
• Here, we report preliminary results on the initial clinical readiness testing using specimens from patients with advanced sarcoma and carcinomas using this technology.
• A multiplex phenotyping assay (CD45, β-catenin, and vimentin) was developed to characterize circulating tumor cells isolated from patients with advanced sarcomas.
• Carcinoma CTCs were identified using an array panel consisting of tumor-specific markers (MUC1 and CEA) and EMT markers (CK, EpCAM, and β-catenin).
• Definiens® software was used to develop an interim, user-defined analysis algorithm for rare cell detection, classification, and enumeration.

Our current efforts are focused on evaluating the utility of ApoStream®-isolated CTCs for assessing the pharmacodynamic effects of anticancer agents on DNA damage response in patients with refractory solid tumors.

References

doi:10.1002/1551-7046.20070012252


Acknowledgments

This project has been funded in whole or in part with federal funds from the National Cancer Institute, National Institutes of Health, under Contract No. HHSN23220100001E.

This research was supported in part by the Developmental Therapeutics Program in the Division of Cancer Treatment and Diagnosis of the National Cancer Institute. Frederick National Laboratory for Cancer Research is accredited by AALAC International and follows the Public Health Service Policy for the Care and Use of Laboratory Animals. Animal care was provided in accordance with the procedures outlined in the Guide for Care and Use of Laboratory Animals (National Research Council, 1996; National Academy Press, Washington, D.C.).
**Characterization of Circulating Tumor Cells Isolated from Bladder Cancer Patients Using ApoStream® Reveals Heterogeneity and Biomarkers of Epithelial-Mesenchymal Transition**

Collin P. Dinney¹, Vladia Melnikova², Weiguo Wu³, Miguel Garza⁴, Shanna M. Pretzsch¹, Darren W. Davis², and Kenna Andersè

¹The University of Texas MD Anderson Cancer Center, Houston, TX, ²ApoCell, Inc., Houston, TX

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**Abstract**

Background: Evaluating the metastatic phenotype that drives the progression of metastatic bladder cancer (BC) from local disease to metastatic may provide insights into prognosis and potentially treatment. The frequency of detection of circulating tumor cells (CTCs) in metastatic BC patients is low, using ApoStream®. The enrichment of CTCs for CD45−/CD146+ cells over CD45+/CD146− cells may provide an advantage for the further non-clonal characterization and enumeration of CTCs as a means to comprehensively evaluate (CME) CMT by a panel of epithelial and mesenchymal markers. The current study utilized CMT panel methodology to evaluate soluble markers, CMT signatures, and DNA aneuploidy in BC CTCs.

Methods: CTCs were isolated from metastatic BC patients with metastatic disease using ApoStream®. The isolated CTCs were enriched for CD45−/CD146+ cells and subjected to a panel of CMT markers, including vimentin (VIM), twist, uroplakin II/IV (URO, II/IV), and CD45 and characterized by Flow Cytometry (FCM) and Hybridization Microscopy (HM). Where applicable, the CTCs were also evaluated for altered DNA content using FISH with Urovysion® for chromosomal abnormalities.

Results: The CTCs isolated from the metastatic patients demonstrated a high percentage of vimentin-positive Mesenchymal (M) cells, while the Epithelial cells (E) were negative. The CTCs also demonstrated characteristic CMT signatures, including Twist (TWIST), which was detected in all CTCs. DNA aneuploidy was detected in a significant number of CTCs, indicating a potential correlation with disease progression. The CTCs also showed evidence of altered DNA content, as detected by FISH with Urovysion®.

Conclusion: The CMT panel methodology used in this study provides a means to comprehensively evaluate CTCs for their metastatic phenotype and potential for therapeutic targeting. The CTCs isolated from metastatic BC patients demonstrated a high percentage of vimentin-positive Mesenchymal cells, characteristic CMT signatures, and DNA aneuploidy, indicating potential correlation with disease progression. The CMT panel methodology used in this study provides a means to comprehensively evaluate CTCs for their metastatic phenotype and potential for therapeutic targeting.

**Study Design**

- **CTC Analysis Schematic**
  - Blood Collection
  - CTC Isolation
  - ApoStream®
  - CTC Staining
  - iCys Laser Scanning Cytometer
  - FISH Analysis

- **CTC Enumeration & Analysis**
  - Panel 1
    - CK
    - CD45
    - Uroplakin
    - Twist
  - Panel 2
    - CD31, CD68, Vimentin, E-cadherin
  - Panel 3
    - CD45, CD146
  - MFI = Mean Fluorescence Intensity.

- **Biomarker Expression in CTCs**
  - ▪ ApoStream® isolated heterogeneous populations of CTCs from 13 bladder cancer patients.
    - 31% (4/13) patients with CK/CD45/DAP® cells.
    - 62% (8/13) patients with CD45/DAPI/DAP® cells.
  - ▪ Urovysion® FISH testing on ApoStream® enriched blood samples was performed on 11 samples. Chromosomal abnormalities were detected in:
    - 3/6 (50%) metastatic bladder cancer patients.
    - 1/5 (20%) non-metastatic bladder cancer patients.

- **References**
Fluorescent In Situ hybridization Confirmation of Circulating Alveolar Soft Part Sarcoma Cells Isolated From Peripheral Blood Specimens Using ApoStream™ Instrumentation

Priya Balasubramaniam1, Lihua Wang1, Shivani Kummar2, Melinda Hollingshead2, Francis Owusu1, Ralph E. Parchment1, Joseph E. Tomaszewski2, James H. Doroshow2, Robert J. Kinders1

1Laboratory of Human Toxicology and Pharmacology, Applied/Developmental Directorate, Leidos Biomedical Research, Inc., Frederick National Laboratory for Cancer Research, Frederick, Maryland 21702; 2Division of Cancer Treatment and Diagnosis, National Cancer Institute, Bethesda, Maryland 20892; 3Biological Testing Branch, Frederick National Laboratory for Cancer Research, Frederick, Maryland 21702

Abstract # C197

Fluorescent In Situ hybridization Confirmation of Circulating Alveolar Soft Part Sarcoma Cells Isolated From Peripheral Blood Specimens Using ApoStream™ Instrumentation

Introduction

The study describes a novel method for the detection and isolation of Circulating Alveolar Soft Part Sarcoma (ASPS) cells from peripheral blood specimens using the ApoStream™ technology. The method involves the enrichment of ASPS cells from blood samples, followed by fluorescent in situ hybridization (FISH) to confirm the presence of ASPS cells.

Methods

Cell line preparation: ASPS-1 cells were developed at the Frederick National Laboratory for Cancer Research and were a kind gift from Dr. Melinda Hollingshead. Cells were cultured at 37°C in 5% CO2 in DMEM:F-12 growth medium supplemented with 10% FBS and penicillin/streptomycin with regular media changes. Cells at a predetermined confluence were harvested and washed with growth medium prior to prelabeling with fluorescent cellular dye (QiF tracker Green, Invitrogen, Carlsbad, CA). Spike-recovery experiments were performed using ASPS-1 cells spiked into sample buffer or Ficoll-isolated peripheral blood mononuclear cells (PBMCs).

ApoStream™ spike-recovery experiments: Spiked cell experiments were carried out in the ApoStream™ instrument at three different operating parameters to determine the optimal system parameters for efficient recovery of ASPS cells. Three aliquots of the enriched fraction were counted under a fluorescent microscope to enumerate the recovered cells. Recovery was calculated as the number of cells harvested divided by the total number of cells that were sent through the instrument.

Sample processing for CTC isolation: Briefly, blood specimens from patients with ASPS at the National Cancer Institute were collected in BD CPT tubes and processed on the same day. PBMC fractions were isolated following the manufacturer’s recommended protocol. PBMC pellets were resuspended in ApoStream™ sample buffer and run through the instrument at predetermined operating conditions. The enriched fraction was spun down, fixed in Carnoy’s fixative, and stored at -20°C until further processing. All cells were hybridized with probes for TFE3 break-apart FISH. Live cell recovery of EpCAM positive and negative cell lines under the same operating conditions.

ApoStream™ Performance

Cross-over frequency range of cancer cell lines from different histologies and normal blood cells measured at 30 mS/m is shown. The difference in the cross-over frequencies between cancer cells and blood cells is critical for CTC isolation using the ApoStream™ device.

Conclusions

• We have employed a novel antibody-independent microfluidic technology to isolate live CTCs from ASPS patient specimens.
• We have developed a method to unambiguously identify ASPS CTCs by TFE3 break-apart FISH.
• CTC image acquisition by automated slide scanning and image analysis for enumeration using Dassserini® software is currently in development.
• Development of assays for measuring drug target effects in ApoStream™-isolated CTCs is currently ongoing.

Acknowledgements

Funded by NCI Contract No. HHSN261200800001E.
ApoStream™ Isolated Circulating Tumor Cells from Primary Breast Cancer Patients Reveals Heterogeneous Phenotypes Related to Epithelial-Mesenchymal Transition and Stem Cell Markers

Insiya Jafferji1, Kenna Anders2, Vlada Melnikova1, Darren W. Davis3, Summer A. Jackson4, James M. Reuben2 and Naoto T. Ueno2
1ApoCell, Inc., Houston, TX, 2The University of Texas MD Anderson Cancer Center, Houston, TX

Abstract

Background: Detection of circulating tumor cells (CTCs) is an indication of poor prognosis in patients with metastatic breast cancer and not in ovarian breast cancer (BRCA). The classical phenotypic definition of a CTC is to be epithelial (CK) and rare in ovarian cancer (OC) primary and BRCA negative. Immunoassays have shown that epithelial cell adhesion molecules (EpCAM) based methods detect only a fraction of the CTC population using cells enriched by a combination of CD44, CD24, and CD45 expression. In addition, the paucity of epithelial molecules on CTCs with loss of aggressive phenotype with features of metastasis and capacity for epithelial to mesenchymal transition (EMT) and stemness properties. CSCs in breast cancer are included among CTCs and can be either CD44+/CD24- (a hallmark of cancer stem cells). Recent studies have shown that CSCs and breast cancer stem cells (BCSCs) contribute to EMT and CTC escape and spread. CSCs expressing the EMT marker CD44+/CD24- cells (BCSCs) and CRPC are highly enriched in CSCs in breast cancer. We aimed to further enrich CTCs using a multiplexed method that targets both EpCAM and CK to detect CSCs in breast cancer patients with high CD44+/CD24- and stromal markers.

Materials and Methods: Breast tumor samples were obtained from 14 breast cancer patients. The correlation between breast cancer and CTCs was established using The Cancer Genome Atlas (TCGA) database. CTCs were isolated from peripheral blood using the MagCellect CTC enrichment kit. The enriched CTCs were stained with CD44, CD24, and EpCAM antibodies. Confocal microscopy was also used to detect CTCs using specific methods that were independent of EpCAM. In this study, we used ApoStream™ technology to enrich the CTC population that is not only enriched in epithelial circulating tumor cells but also in breast cancer cells. The enriched CTCs were stained with a panel of antibodies, including CD44, CD24, and EpCAM.

Results: In this study, we identified breast cancer CTCs that were enriched by ApoStream™ technology. These CTCs were characterized by their expression of CD44+CD24- and EpCAM. We also used confocal microscopy to detect CTCs using specific methods that were independent of EpCAM. These enriched CTCs were further characterized by their expression of CD44+CD24- and EpCAM. These results indicate the potential use of ApoStream™ technology in the detection of breast cancer CTCs and CSCs.

Conclusions: This study suggests that the use of ApoStream™ technology in the detection of breast cancer CTCs and CSCs could provide valuable insights into the clinical relevance of CSCs in breast cancer.

Summary

- CTCs (CK+CD45+/CD44+/CD24-) cells were detected in 71% (10/14) primary breast cancer patients prior to receiving preoperative therapy.
- EMT and stem cell markers range of expression and frequency of detection in PBC patients.
  - β-Catenin* range 6-37% in 21 (31%) patients
  - EpCAM Vimentin* was 3% in 14 (21%) patients
  - CK+CD45+/CD44+/CD24- range in 50% (8/14) patients
- In this ongoing clinical trial, we will test the hypothesis that low EMT-CTC and CSCs in baseline blood samples is correlated with a higher pCR rate compared to patients with high EMT-CTC and CSC counts.

References

Results

A novel, antibody-independent platform ApoStream™ successfully isolated CTCs from the blood of patients with advanced NSCLC. In a side-by-side comparison, ApoStream™ isolated more CK+/CD45- NSCLC CTCs compared to the CellSearch® platform in 3 out of 5 NSCLC patients sample with detectable CK+/CD45- cells; neither system-detected CTCs in 1 patient sample.

Phenotypic immunofluorescent analysis of cells isolated by ApoStream™ revealed the presence of CD45#, CK+, and EpCAM+. Using an immuno-PCR assay, analysis of CK+/CD45- CTCs was detected in NSCLC samples as compared to 0 in healthy donor blood. Additional samples are being acquired to establish CTC enumeration clinically.

Percent cells expressing EpCAM varied from 0% to 10% in CK+/CD45- cells, from 0-10% in CK+/CD45+ cells, and from 10-100% in CK-/CD45- cells, thus confirming that ApoStream™ separates EpCAM+ CTCs that could be identified by EpCAM-based technologies.

The use of ICE COLD-PCR coupled with standard Sanger sequencing allowed detection of EGFR Exon 19 mutations in CTCs isolated by ApoStream™. Method modifications led to the sensitivity of detecting EGFR Exon 19 mutations in CTCs from tissue set at ~5%, which is well below that of standard Sanger sequencing (4%). In conclusion, ICE COLD-PCR is sensitive and specific for EGFR exonic mutations.

For EGFR Exon 21, two mutations were observed in the tumor tissue from this set of patients. Using standard ICE COLD-PCR followed by Sanger sequencing on the amplicons DNA extracted from the CTCs isolated by ApoStream™, both mutations were found in addition for the tissue analysis was used EGFR Exon 19-150bp exon.

Analysis for BRAF-V600E and RET from isolated CTCs is in-progress.

In summary, successful isolation of NSCLC CTCs and detection of EGFR mutations by an integrated ApoStream™-ICE COLD-PCR approach verifies exploration of the clinical utility of CTCs as an alternative to tissue biopsy. Complete isolation analysis will be presented.

Summary & Clinical Significance

ICE COLD-PCR Sequencing

Methodology: A total of 5 NSCLC patient samples were isolated with ApoStream™, COLD-PCR tested on 4 NSCLC samples (samples failed due to low cell yield). Standard COLD-PCR testing was performed on 20% of CTC DNA species.

ICE COLD-PCR: (A) Modified ICE COLD-PCR was performing 40% of CTC DNA species. (B) Modified pre-amplification PCR combined with the standard ICE COLD-PCR. (C) Nodetect, % mutation detected, *** no data collected.

Step 1: Amplification of DNA from the CTC sample.

Step 2: ICE COLD-PCR amplification of the DNA.

Step 3: Analysis using a DNA sequencer.

Step 1: Initial to detect in a total of the wild cell and mutated sequence 100% of the sequence.

Step 2: ICE COLD-PCR amplification of the DNA.

Step 3: Analysis using a DNA seque
**Abstract**

Background: The current standard of care involves monitoring circulating tumor cells (CTCs) for early detection and identification of cancer. However, the detection of CTCs is limited by differences in cell size and morphology, which often result in false positives. Moreover, CTCs usually exhibit a heterogeneous population, indicating the need for precise and reliable detection methods.

ApoStream™ is a highly sensitive and accurate platform for detecting CTCs. It utilizes a combination of dielectrophoresis and immunofluorescence to detect CTCs with minimal false positives. The platform is designed to detect CTCs with differences in size, morphology, and expression of specific markers.

Methods: The performance of ApoStream™ was evaluated using a panel of breast cancer cell lines and normal human cells. The system was shown to detect CTCs with high sensitivity and specificity, with minimal false positives.

Results: The results demonstrated that ApoStream™ is a highly sensitive and accurate platform for detecting CTCs. The platform was shown to detect CTCs with high sensitivity and specificity, with minimal false positives.

Conclusion: ApoStream™ is a highly sensitive and accurate platform for detecting CTCs. It is a valuable tool for the early detection and identification of cancer.

**ApoStream™ Technology**

The ApoStream™ platform utilizes a combination of dielectrophoresis and immunofluorescence to detect CTCs with minimal false positives. The system is designed to detect CTCs with differences in size, morphology, and expression of specific markers.

**ApoStream™ Prototype Device**

The ApoStream™ prototype device is shown in the image. It consists of a dielectrophoresis chamber and an immunofluorescence system. The device is designed to detect CTCs with high sensitivity and specificity, with minimal false positives.

**Comparison of CTC Enrichment Methods**

The figure compares the performance of different CTC enrichment methods. The ApoStream™ method is shown to have the highest sensitivity and specificity, with minimal false positives.

**Heterogeneous CTC Phenotypes in BrCa Patients**

The table lists the heterogeneous CTC phenotypes in BrCa patients. The data shows that ApoStream™ is able to detect a wide range of heterogeneous CTC phenotypes.

**Summary & Clinical Significance**

- **ApoStream™ CTC isolation can be applied to all cancer types, including non-epithelial derived tumors because the basis for isolation is independent of antibodies to cell surface antigens like EpCAM.**
- **ApoStream™ instrument performance and recovery data are robust and reproducible.**
- **Antibody-independent rare cell isolation by ApoStream™ combined with phenotypic characterization allows identification of previously undetectable CTCs and enables insight into CTC population heterogeneity.**
- **A comparison between CellSearch® and ApoStream™ showed greater CTC counts with ApoStream™.**
- **EpCAM and EpCAM + CD45 + DAPI - CTCs were detected.**
- **Vimentin expression was detected in 30% of EpCAM + and 50% in EpCAM cells.**
- **Understanding heterogeneity of CTCs will be key to achieving clinical utility as biomarkers.**

**References:**

2. Sangal Shem et al. Dielectrophoresis has broad applicability to marker-free isolation of tumor cells from blood by microfluidic systems. Biomicrofluidics, 7, 016508, 2013.

**Funding:** This project has been funded in part with Federal funds from the National Cancer Institute, National Institutes of Health, under Contract No. N01CA00220901E.
Molecular characterization of circulating tumor cells recovered from metastatic pancreatic cancer patients using ApoStream™, a new antibody-independent dielectrophoretic device

Gaurn Varadhachary1, James Abbzzudese1, Rachna Shroff1, Vladislava Melenikova2, Dzifa Duose2, Vishal Gupta3, Miguel Garza4, Kenna Andreses, Darren W. Davis1 and Robert A. Wolff5.

1 MD Anderson Cancer Center, Houston, TX; 2 Department of Gastrointestinal Medical Oncology; 3 ApoCell, Inc., Houston, TX

Abstract

Background:
Inherent differences in the recovery of circulating tumor cells (CTCs) are well-known among various diagnostic tools including CellSearch® and EpCAM-like antibodies. In this study, we evaluated a novel dielectrophoretic platform called ApoStream™ to characterize CTCs in two pancreatic cancer patient samples.

Methods:
Cell Recovery:
- Circulating tumor cells were isolated from metastatic pancreatic cancer patient samples using ApoStream™, and the enumeration of circulating tumor cells (CTCs) was performed with an EpCAM-like antibody. As a positive control, CTCs were isolated from metastatic pancreatic cancer patient samples using CellSearch®

Conclusions:
- Comparable between CellSearch® and ApoStream™ in terms of CTC recovery and morphology. Advances in differential diagnosis of CTCs using ApoStream™

Heterogeneous CTC Phenotypes in PAC Patients

Table 1

<table>
<thead>
<tr>
<th>CTC Count</th>
<th>EpCAM-like Antibody</th>
<th>Cells</th>
<th>CD45</th>
<th>CK+/CD45</th>
<th>Plectin</th>
<th>Cytokeratin</th>
<th>CD45</th>
<th>CK+/CD45</th>
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Table 2

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<th>CTC Count</th>
<th>EpCAM-like Antibody</th>
<th>Cells</th>
<th>CD45</th>
<th>CK+/CD45</th>
<th>Plectin</th>
<th>Cytokeratin</th>
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<th>CK+/CD45</th>
<th>Plectin</th>
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</table>

Figure 1: CTC isolation with CellSearch® vs ApoStream™

Summary & Clinical Significance

- Antibody-independent new cell isolation by ApoStream™ combined with phenotypic characterization allows identification of previously undetectable CTCs and enables insight into CTC population heterogeneity.

- Plectin-1 has been shown to be a specific biomarker for pancreatic cancer while the specificity of CellSearch® as a pancreatic CTC biomarker will require further investigation.

- Metastasis was evaluated as a biomarker for pancreatic CTCs. Low antibody signal to background ratio and lack of specificity suggest it is not fit for this purpose.

- Inclusion of potential tumor associated markers like CA19-9 and plectin-1 may enable the expansion of the classical phenotypic definition of CTCs and monitoring of PAC patients.

- ApoStream™ CTC isolation can be applied to all cancer types, including non-epithelial derived tumors because the basis for isolation is independent of antibodies to cell surface antigens like EpCAM.

References:
- Sameer Singh et al. Dielectric characterization of circulating tumor cells from blood: Biofunctionalities. 2013
- S. A. Varadhachary et al. Should CTCs be measured as a GC patient's 12-14 h blood sample? Biofunctionalities. 2013

Figure 2: Representative images identify classic CTCs (CK+/CD45−) with associated pancreatic cancer markers CA19-9 and plectin-1 and normal PBMC (CK−/CD45−).
**INTRODUCTION**

Topoisomerase 1 (Topo I) is a nuclear enzyme that plays an essential role in DNA replication, transcription, recombination, and repair. In patients, Topo I is overexpressed in cancer cells due to resistance to SN38 in colorectal cancer, and the resulting pathway can be targeted by Topo I inhibitors such as irinotecan. CTCs, on the other hand, are an attractive minimally invasive alternative to tumor biopsies for clinical applications.

**ASSAY DEVELOPMENT AND QUALIFICATION**

**Assay Details:**
- **Phase:** Preclinical
- **Application:** Protein quantification, FISH analysis, genetic mutation
- **Sampling Schedule:**
  - Continuous Exposure
  - Holiday Exposure
  - Holiday Exposure
  - Holiday Exposure
- **Representative Images of Biomarker Staining on CTCs isolated from BEACON Patients**

**METHODS**

Primary and secondary antibodies were obtained from commercial sources. Control (0.1% DMSO) and drug treated (100 nM SN38, 10 nM irinotecan) HCT116, MCF7, A549, SKBr3, and PBMCs were incubated with patient-derived CTCs isolated from BEACON patients. Before staining, SN38 exposed cells were subjected to DNAse I treatment in order to exclude cell death resulting in high expression of apoptosis marker 

**Biomarkers**
- **ABCG2:** TOPOII+ CTC
- **AB63801:** TOPOII+ CTC
- **BAM995:** TOPOII+ CTC
- **ROS1:** TOPOII+ CTC
- **NAB9002:** TOPOII+ CTC
- **PA55022:** TOPOII+ CTC
- **JBW301:** TOPOII+ CTC
- **AB28432:** TOPOII+ CTC
- **Fluor 647:** TOPOII+ CTC
- **DAPI:** TOPOII+ CTC

**End of Treatment**

- **Patient 1:**
  - Non-Apoptotic CTCs
  - TOPOII+ CTC
  - KI67+ RAD51
  - KI67+ RAD51

**RESULTS**

- **Table 3.** NKTR-102 Target Specific Biomarker Qualification
- **Table 4.** Patient 5 (Screening)

**DISCUSSION**

- **Specific pharmacodynamic biomarkers can be reliably assessed.**
- **Staining panels for Etirinotecan pegol target specific biomarkers.**

**CONCLUSIONS**

- **Staining panels for Eliotinotecan pegol target-specific pharmacodynamic biomarkers have been successfully developed and qualified.**
- **CTC collection was successfully integrated into the BEACON study.**
- **CTC substudy patient participation is high.**
- **Eliotinotecan pegol target-specific pharmacodynamic biomarkers can be reliably measured in CTCs isolated from patients participating in BEACON.**

**TABLE 3.** NKTR-102 Target Specific Biomarker Qualification

<table>
<thead>
<tr>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Patient 4</th>
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<td>Staining Panel 5</td>
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**Phase III BEACON Study in Patients with Metastatic Breast Cancer (mBc)**

- **Ute Hoch, Dennis G. Fry, Yen Lin Chia, Katie Caygill, Alison L. Hannah, Edith A. Perez, Javier Cortez, Ahmad Awada, Joyce O’S...**

**Table 1.** Objective Response Rate (ITT Population)

<table>
<thead>
<tr>
<th>Objective Response Rate (%)</th>
<th>Topo II+ CTC</th>
<th>Topo II- CTC</th>
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<tr>
<td>CR, PR, SD≥6 months</td>
<td>13 (37.1%)</td>
<td>8 (22.9%)</td>
</tr>
<tr>
<td>Stable</td>
<td>10 (28.6%)</td>
<td>17 (48.6%)</td>
</tr>
<tr>
<td>Progressive</td>
<td>7 (19.4%)</td>
<td>5 (13.9%)</td>
</tr>
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</table>

**Table 2.** Etirinotecan pegol Target Specific Biomarkers

<table>
<thead>
<tr>
<th>Antibody</th>
<th>MFI Negative Control</th>
<th>Patient 1</th>
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<tr>
<td>DAPI</td>
<td>95; 5%</td>
<td>95; 5%</td>
<td>95; 5%</td>
<td>95; 5%</td>
<td>95; 5%</td>
<td>95; 5%</td>
</tr>
</tbody>
</table>
Characterization and identification of specific EGFR mutations in circulating tumor cells (CTCs) isolated from non-small cell lung cancer patients using an antibody independent method, ApoStream™

H. T. Tran1, V. O. Melnikova1, A. S. Taso1, F. V. FossaSella1, F. M. Johnson1, V. Papadimitrakopoulou1, M. Garzgi2, C. Neal2, D. Hasegawa2, A. Kruempel1, G. Wu1, K. Richardson1, M. E. Lewis1, B. L. Legendre Jr1, K. L. Andersen1, D. W. Davis1, J. Heymach1

1 MD Anderson Cancer Center, Houston, TX; Department of Thoracic, Head & Neck Medical Oncology; 2Apcell, Inc., Houston, TX; 3Transgenomic, Inc.

Abstract

Recent advances in cancer stem cell research have revealed that native cancer cells lose their dependence on oxygen by adopting a hypoxic and mesenchymal phenotype that is critical for cancer growth and dissemination. While numerous strategies have been developed to detect cancer cells (CTCs), the majority use antibodies to identify cancer cells based on surface markers that are expressed in a limited number of cases. Therefore, there is an unmet need for an antibody-independent method to detect CTCs. ApoStream™, a novel dielectrophoretic antibody-independent method, uses dielectrophoretic or electric field forces to identify CTCs. We have successfully isolated CTCs from patients with non-small cell lung cancer (NSCLC) and performed EGFR mutation analysis on these cells using an antibody-independent ICE-COLD PCR method.

Methods

All patients and healthy volunteers provided signed, written informed consent for this laboratory-based research study approved by UTMB/MD Anderson Cancer Center IRB (LAB11-0460). ApoStream™ Technology

ICE COLD-PCR Sequencing

CTC Analysis:

• Immuno-seq on Histo-statics
• Staining for CK+/CD45− and EpCAM+
• Analysis of 10% of CTC DNA specimen for EGFR mutations by ICE COLD-PCR
• Detection of EGFR TK domain mutations, including EGFR exon 19 deletion and EGFR exon 21 G719C
• Detection of EGFR Amoeba™ COLD-PCR
• Sequencing Method 1, 2A and 2B

CTC Harvest:

• CellSearch® tube
• CTC isolation and enumeration (CK+/CD45−)
• ApoStream™

Blood Collection:

• CellSearch® tube
• CTC isolation and enumeration (CK+/CD45−)

Study Design

Step 1: All DNA is deproteinized to single-strands.
Step 2: The R5-end is replaced to each strand of the epidermal growth hormone receptor messenger RNA (EGFR mRNA) in the PCR primer.
Step 3: The reaction is initiated at 50°C; the modified primer longer than the wild-type primer by 3 nucleotides.
Step 4: The PCR primer contains the forward and reverse PCR primers will be used to isolate the wild-type side of the wild-type DNA sequence. The reverse PCR primer is used to amplify the wild-type and wild-type DNA sequences. The mutant DNA is used as a primer for the PCR primer.
Step 5: Perform standard Sanger sequencing reaction.
Step 7: Analyze using a DNA sequence.

Results

<table>
<thead>
<tr>
<th>Study Design</th>
<th>CellSearch® Tube</th>
<th>CTC Isolated</th>
<th>CTC Enumeration</th>
<th>EGFR Exon 19 ICE-COLD PCR</th>
<th>EGFR Exon 21 ICE-COLD PCR</th>
<th>Method 1</th>
<th>Method 2A</th>
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Summary of CTC Enumeration and Mutation Analysis Results

• A novel, antibody-independent platform ApoStream™ successfully isolated CTCs from the blood of patients with advanced NSCLC. In a side-by-side comparison, ApoStream™ isolated more CK+/CD45− NSCLC CTCs compared to the CellSearch® platform in 3 out of 5 NSCLC patient samples with detectable CK+/CD45− cells, while System-detected CTCs in 1 patient sample.

• Phenotypic immune-reactive analysis of cells isolated by ApoStream™ revealed the presence of CK+/CD45− CTCs. As well as CK+/CD45− and CK−/CD45− cells. Median of 20% of CK+/CD45− CTCs was detected in NSCLC samples compared to 0% in healthy donor blood.

• Percent cells expressing EpCAM varied from 0% to 7% in CK+/CD45− cells, and from 20% to 50% in CK−/CD45− cells, that confirmed that ApoStream™ isolated EpCAM+ cells that could be analyzed by EpCAM-based technologies.

• The use of ICE COLD-PCR coupled with standard Sanger sequencing allowed detection of EGFR Exon 19 & 21 mutations in CTCs isolated by ApoStream™. Median modifications include low increases in the sensitivity of detecting EGFR Exon 19 and 21 mutations. CTCs in tissue-positive patients were 0% with standard ICE-COLD-PCR (Method 1) to 5% and 7% with Methods 2A and 2B respectively. Note that if only a portion of the extracted DNA was used per pre-amplification PCR, therefore, the entire sample population of the sample was not sequenced with each assay, this could lead to some discordant results.

• For EGFR Exon 21: no mutations were observed in the tumor tissue from this set of patients. Using a standard ICE-COLD-PCR followed by Sanger sequencing on the template DNA extracted from the CTCs isolated by ApoStream™, no mutations were found. Thus, the results from the ICE-COLD-PCR analysis were 100% concordant with the tumor samples with the ICE-COLD-PCR (Method 2) for EGFR Exon 21 CTC mutation analysis is ongoing.

• In summary, successful isolation of NSCLC CTCs and detection of EGFR mutations by an integrated ApoStream™ ICE-COLD-PCR approach enables exploration of the clinical utility of CTCs as an alternative to tissue biopsy.

References:


Dielectrophoretic forces biomimetically mimic diversity of tumor cells from blood by microfluidic systems. Biomicrofluidics, 7, 011108, 2013.
**Abstract**

ApoStream™, a new dielectrophoretic device for antibody-independent isolation and recovery of circulating tumor cells from blood of patients with metastatic pancreatic adenocarcinoma

Gaun Varathachary, James Abbazuzzese, Rachna Shroff, Vladislava Melnikova, Vishal Gupta, Chris Neal, Miguel Garza, Claire Reeves, Insiya Jafferji, Carmen Lam, David K. Hasegawa, Kenna Andresen, Darren W. Davis and Robert A. Wolff. MD Anderson Cancer Center, Houston, TX. Department of Gastrointestinal Medical Oncology, Apcell Inc., Houston, TX.

Current prototype design shipped to National Cancer Institute & Massey Cancer Center, VA, in Dec 2012

**Methods**

**Side-by-Side Comparison of Pancreatic CTC Isolation with ApoStream™ vs CellSearch®**

**ApoStream™ Prototype Device**

**ApoStream™ Technology**

**ApoStream™ Performance**

**Representative Images of Cells Isolated by ApoStream™ from Patient 1**

**Frequency of CTC-Positive Pancreatic Cancer Patients**

**Summary & Clinical Significance**

- ApoStream™ CTC isolation can be applied to all cancer types, including non-epithelial derived tumors, because the basis for isolation is independent of antibodies to cell surface antigens (EpCAM).
- Antibody-independent selection used by ApoStream™ allows phenotypic characterization of previously inaccessible CTCs and enables insight into patient population heterogeneity.
- CellSearch®-specific biomarker for pancreatic cancer while CA 19.9 is less specific.
- Inclusion of specific tumor-associated markers like Plac-tin will enable the expansion of the classical phenotypic definition of CTCs and monitoring of PAC patients.

References:
Expanded phenotypic and biological characterization of rare cells isolated from cancer patient blood using ApoStream™

Chris Neal, Vladislava Melnikova, Vishal Gupta, Insiya Jafferji, David K. Hasegawa, Kenna Anderes and Darren W. Davis
ApoCell, Inc., Houston, TX

Abstract

Background: Cancer patients often have small numbers of circulating tumor cells (CTCs) in their blood that may reflect the presence of tumors and serve as a mechanism of disease progression. CTCs can be isolated from patient blood using multiple methods, including the CellSearch® System, a gold-standard assay. ApoStream™ technology isolates CTCs from a broad range of cancer types.

ApoStream™ Technology

(A) Dielectric properties (polarizability) of cells are dependent upon many biophysical features.

(B) Dielectrophoretic, hydrodynamic and sedimentation forces are balanced to attract CTCs and repel normal cells from the chamber floor. CTCs are collected through a port located in the chamber floor while normal cells flow into a waste port.

(C) Cross-over frequencies from different tumor cell types including breast, colon, ovarian, lung and melanoma cell lines and from peripheral blood mononuclear cells (PBMCs) were determined. The differences in cross-over frequencies between normal cells enable ApoStream™ to separate CTCs from normal cells.

ApoStream™ Device

ApoStream™ = Industrial Design Sketch

Protein Quantification

- Phenotyping
- PD Biomarkers
- Prophylaxis
- RTks

Pathology

- EMT

Gene Expression

- Real-time quantitative RT-PCR
- Gene Expression Profiling

ApoStream™ CTC Enrichment, Identification and Enumeration

(A) CTCs from NSCLC patients captured by ApoStream™ were identified by immunofluorescent staining using standard DAPI/CD45 phenotypes.

(B) H&E staining of CTC clusters isolated from the blood of NSCLC patients.

ApoStream™ Performance

(A) Inter-day Precision

(B) Device Linearity

Multiple Cancers Types

Table 1. Distribution of EpCAM/Vimentin phenotypes in CK+/CD45−/DAPI− cells isolated from metastatic breast cancer patient blood by ApoStream™. NA=CellSearch™ not performed on these samples.

<table>
<thead>
<tr>
<th>Protein Cancer</th>
<th>EpCAM+/Vimentin−</th>
<th>EpCAM+/Vimentin+</th>
<th>NA</th>
<th>EpCAM−/Vimentin+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer patient 1</td>
<td>87%</td>
<td>0%</td>
<td>NA</td>
<td>0%</td>
</tr>
<tr>
<td>Cancer patient 2</td>
<td>90%</td>
<td>0%</td>
<td>NA</td>
<td>0%</td>
</tr>
<tr>
<td>Cancer patient 3</td>
<td>92%</td>
<td>0%</td>
<td>NA</td>
<td>0%</td>
</tr>
</tbody>
</table>

Table 2. Percent and expression of CK+/CD45− cells isolated from castrate resistant prostate cancer patient blood by ApoStream™.

<table>
<thead>
<tr>
<th>Protein Cancer</th>
<th>CK+/CD45−</th>
<th>NA</th>
<th>CK+/CD45−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer patient 1</td>
<td>87%</td>
<td>NA</td>
<td>0%</td>
</tr>
<tr>
<td>Cancer patient 2</td>
<td>90%</td>
<td>NA</td>
<td>0%</td>
</tr>
<tr>
<td>Cancer patient 3</td>
<td>92%</td>
<td>NA</td>
<td>0%</td>
</tr>
</tbody>
</table>

Table 3. Number of cytokeratin positive and negative cells isolated from pancreatic cancer patient blood by ApoStream™ NA=CellSearch™ not performed on this sample.

<table>
<thead>
<tr>
<th>Cytokeratin Type</th>
<th>Cytokeratin Expression</th>
<th>Cytokeratin Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer patient 1</td>
<td>87%</td>
<td>0%</td>
</tr>
<tr>
<td>Cancer patient 2</td>
<td>90%</td>
<td>0%</td>
</tr>
<tr>
<td>Cancer patient 3</td>
<td>92%</td>
<td>0%</td>
</tr>
</tbody>
</table>

Conclusions & Clinical Significance

- ApoStream™ CTC isolation can be applied to patients of all cancer types, including non-epithelial derived tumors.
- ApoStream™ isolates CTCs from a greater number of patients than currently available technologies.
- Antibody-independent selection used by ApoStream™ allows phenotypic characterization of previously inaccessible CTCs and enables insight into population heterogeneity.
- The increased numbers of CTCs isolated by ApoStream™ enable more robust molecular and genetic analyses to help guide individual treatment decisions.

References:

Comparison of Dielectrophoretic Field Flow Fractionation (DEP-FFF) with ApoStream™, an Antibody Independent Platform, with Immunomagnetic Capture Using CellSearch® for Enumeration of Circulating Tumor Cells in Patients with Metastatic Prostate Cancer.

Andrew Poklepovic¹, Vladislava Melnikova², Kenna Andersen², Weiguo Wu², Darren Davis²
¹VCU Massey Cancer Center, Richmond, VA; ²ApoCell, Inc., Houston, TX

Background

Development of circulating tumor cell (CTC) detection platforms is a rapidly advancing field. The CellSearch® technique relies on cell surface expression of EpCAM to select for rare tumor cells in whole blood specimens. Consequently, the use of EpCAM-based enrichment platforms to recover CTCs is limited to patients expressing EpCAM and is poorly suited for recovery of EpCAM negative cells such as melanoma or those having undergone epithelial mesenchymal transition (EMT). ApoStream™ is an antibody independent enrichment platform which utilizes the principle of continuous flow dielectrophoretic field-flow fractionation (DEP-FFF) to isolate and enrich for CTCs. A head to head comparison of CellSearch® to ApoStream™ for recovery of CTCs in 10 patients with metastatic prostate cancer was performed, as part of a lead-in to a larger project evaluating DEP-FFF in patients with metastatic prostate cancer receiving palliative radiotherapy with or without lapatinib.

Methods

Two 7.5 ml blood samples were collected at a single time point for each patient who was diagnosed with Stage IV prostate cancer. One sample was analyzed by CellSearch® CTC enumeration kit and one sample was analyzed by ApoStream™. CTCs recovered by both devices were immunophenotyped using antibodies against cytokeratin (CK), CD45 and DAPI. CTCs were defined as CD45⁻/CD45-DAPI⁻/CK⁺ intact cells. CTCs recovered by ApoStream™ were further analyzed by quantitative laser scanning cytometry (LSC). A paired t-test was used to compare the cell counts in the two devices.

Biophysical Basis for Separation of CTCs

A) In response to AC electrical field stimulation, cells are attracted to, or repulsed from, the source of that field. The frequency at which the cell shifts from attraction to repulsion is known as the crossover frequency. The crossover frequency of cancer cells is different from peripheral blood mononuclear cells, and allows for tumor cells to be attracted to the electrical plate while normal cells are repulsed into the center of the flow chamber.

B) Dielectrophoretic, hydrodynamic, and sedimentation forces are balanced to attract CTCs to, and repel normal cells from, the chamber flow. PHMCs are positioned in the center of the flow column, and move through the chamber quickly into a waste port. CTCs are attracted to the electrical plate, move slowly along the flow column, and are collected through a port located in the chamber floor. Cells remain viable after collection.

Table 1

<table>
<thead>
<tr>
<th>Patient</th>
<th>CellSearch® CK⁺/CD45⁻/DAPI⁺</th>
<th>ApoStream™ CK⁺/CD45⁻/DAPI⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>32.5 (range 40-174)</td>
<td>4.5 (range 0-41)</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>90</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>40</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>174</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>138</td>
</tr>
<tr>
<td>6</td>
<td>8</td>
<td>41</td>
</tr>
<tr>
<td>7</td>
<td>11</td>
<td>75</td>
</tr>
<tr>
<td>8</td>
<td>8</td>
<td>152</td>
</tr>
<tr>
<td>9</td>
<td>8</td>
<td>50</td>
</tr>
<tr>
<td>10</td>
<td>21</td>
<td>50</td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th>CTC Type</th>
<th>Pre-RT</th>
<th>Post-RT</th>
</tr>
</thead>
<tbody>
<tr>
<td>pERK</td>
<td>277967</td>
<td>506394</td>
</tr>
<tr>
<td>pERBB1</td>
<td>28266</td>
<td>26830</td>
</tr>
</tbody>
</table>

Table 3

<table>
<thead>
<tr>
<th>CTC Type</th>
<th>Pre-RT</th>
<th>Post-RT</th>
</tr>
</thead>
<tbody>
<tr>
<td>pERK</td>
<td>277967</td>
<td>506394</td>
</tr>
<tr>
<td>pERBB1</td>
<td>28266</td>
<td>26830</td>
</tr>
</tbody>
</table>

Table 4. The ApoStream™ DEP-FFF CTC enrichment platform isolated a greater number of CTCs from blood of ten patients compared to CellSearch® (p<0.01).

CTC analysis not limited to prostate cancer or other EpCAM positive tumors.

Future Directions

The Massey Cancer Center will continue to utilize the ApoStream™ device for therapeutic clinical trials in prostate cancer, breast cancer, and hepatocellular cancer. Further exploration into the epithelial mesenchymal transformation (EMT) phenotype in CTCs is also planned.

Conclusions

- The ApoStream™ DEP-FFF platform is differentiated from EpCAM dependent platforms and successfully isolates a greater number of putative CTCs than CellSearch®.
- The antibody independent nature of the ApoStream™ platform is well suited for detection and recovery of CTCs in advanced stage disease where tumor cell heterogeneity is common.
- Multiple modalities for second stage analysis are available for use.
- ApoStream™ is well suited to advance clinical research in prostate cancer patients.
**Abstract #2381**

**Background**

Biopsies of HCC can be technically difficult, given the vascular nature of the liver and underlying liver disease. Many HCC patients do not get biopsies, and molecular characterization of these tumors is not possible. Capture of CTCs from blood allows for analysis of cancer cells in metastatic dissemination. The use of EpCAM-based enrichment platforms limits the type of tumor cells that can be recovered, as it selects only for cells which express the antigen of interest. Nonselective methods of CTC analysis are needed. An ongoing study is evaluating the recovery of CTCs in HCC patients with elevated serum alpha-fetoprotein (AFP) using the novel antibody-independent ApoStream™ platform, which utilizes the principle of dielectrophoretic field-flow fractionation to position cells in a hydrodynamic flow profile for sorting.

**Methods**

Paired 7.5 ml blood samples from HCC patients were analyzed by CellSearch® and ApoStream™. Collected cells were immunophenotyped using antibodies against cytokeratin (CK), CD45, DAPI, and AFP, and enumerated by quantitative laser scanning cytometry.

**Biophysical Basis for Separation of CTCs**

<table>
<thead>
<tr>
<th>Cancer Cell and Human CTCs</th>
<th>Normal Blood Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Viable cell</td>
<td>- Viable cell</td>
</tr>
<tr>
<td>- EpCAM+</td>
<td>- EpCAM+</td>
</tr>
<tr>
<td>- EpCAM-</td>
<td>- EpCAM-</td>
</tr>
<tr>
<td>- Phenotypically identical</td>
<td>- Phenotypically identical</td>
</tr>
<tr>
<td>- ExpressesAFP</td>
<td>- Does not express AFP</td>
</tr>
<tr>
<td>- ExpressesCD45</td>
<td>- Does not express CD45</td>
</tr>
<tr>
<td>- Expresses CK</td>
<td>- Does not express CK</td>
</tr>
<tr>
<td>- Expresses ApoStream™</td>
<td>- Does not express ApoStream™</td>
</tr>
<tr>
<td>- Expresses EpCAM</td>
<td>- Does not express EpCAM</td>
</tr>
</tbody>
</table>

**Crossover Frequency**

A) In response to AC electrical field stimulation, cells are attracted to, or repulsed from, the source of that field. The frequency at which the cell shifts from attraction to repulsion is known as the crossover frequency. The crossover frequency of cancer cells is different from peripheral blood mononuclear cells, and allows for tumor cells to be attracted to the electrical plate while normal cells are repulsed into the center of the flow chamber.

B) Dielectrophoretic, hydrodynamic, and sedimentation forces are balanced to attract CTCs to, and repel normal cells from, the chamber floor. PBMCs are positioned in the center of the flow column, and move through the chamber quickly into a waste port. CTCs are attracted to the electrical plate, move more slowly along the flow column, and are collected through a port located in the chamber floor. Cells remain viable after collection.

<table>
<thead>
<tr>
<th>Patient #</th>
<th>Subject ID</th>
<th>Serum AFP level ng/mL</th>
<th>Microvascular Invasion</th>
<th>Cytodiagnostic Disease</th>
<th>Therapy at time of sample collection</th>
<th>CTC count by CellSearch®</th>
<th>CTC count by ApoStream™</th>
<th>Total</th>
<th>D45/CD45 CK+AFP (ApoStream™)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1-1-01</td>
<td>30955</td>
<td>Yes</td>
<td>Abdominal LN</td>
<td>Sorafenib-30mg/Rid</td>
<td>0</td>
<td>0</td>
<td>NA</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>1-1-02</td>
<td>728</td>
<td>No</td>
<td>No</td>
<td>Sorafenib-30mg/Rid</td>
<td>1</td>
<td>21</td>
<td>512,033</td>
<td>13 (82%)</td>
</tr>
<tr>
<td>3</td>
<td>1-1-03</td>
<td>78</td>
<td>No</td>
<td>No</td>
<td>Sorafenib-30mg/Rid</td>
<td>1</td>
<td>125</td>
<td>1,841,480</td>
<td>90 (72%)</td>
</tr>
<tr>
<td>4</td>
<td>1-1-04</td>
<td>60</td>
<td>No</td>
<td>No</td>
<td>Sorafenib-30mg/Rid</td>
<td>0</td>
<td>554</td>
<td>3,600,799</td>
<td>540 (87%)</td>
</tr>
<tr>
<td>5</td>
<td>1-1-05</td>
<td>2278</td>
<td>No</td>
<td>No</td>
<td>Sorafenib-30mg/Rid</td>
<td>1</td>
<td>1165</td>
<td>3,806,105</td>
<td>1049 (90%)</td>
</tr>
<tr>
<td>6</td>
<td>1-1-06</td>
<td>3049</td>
<td>Yes</td>
<td>Abdominal LN</td>
<td>Sorafenib-30mg/Rid</td>
<td>0</td>
<td>29</td>
<td>0</td>
<td>29 (95%)</td>
</tr>
<tr>
<td>7</td>
<td>1-1-07</td>
<td>31522</td>
<td>Yes</td>
<td>Abdominal LN</td>
<td>Sorafenib-30mg/Rid</td>
<td>0</td>
<td>380</td>
<td>506,989</td>
<td>576 (99%)</td>
</tr>
<tr>
<td>8</td>
<td>1-1-08</td>
<td>29</td>
<td>No</td>
<td>Abdominal LN</td>
<td>Sorafenib-30mg/Rid</td>
<td>0</td>
<td>198</td>
<td>0</td>
<td>198 (100%)</td>
</tr>
<tr>
<td>9</td>
<td>1-1-09</td>
<td>4083</td>
<td>No</td>
<td>No</td>
<td>Sorafenib-30mg/Rid</td>
<td>0</td>
<td>121</td>
<td>1,300,207</td>
<td>52 (43%)</td>
</tr>
<tr>
<td>10</td>
<td>1-1-10</td>
<td>19219</td>
<td>No</td>
<td>Abdominal LN</td>
<td>Sorafenib-30mg/Rid</td>
<td>0</td>
<td>805</td>
<td>2,164,083</td>
<td>746 (93%)</td>
</tr>
</tbody>
</table>

**Results**

Results from the 10 patients in this pilot project are listed in the table above. Serum AFP at time of collection, the presence of portal or hepatic vein invasion by tumor, presence of metastases, and therapy at time of CTC analysis are listed. In comparison with the CellSearch® platform, ApoStream™ isolated a higher number of Ck+/CD45-/DaPi+ CTCs in HCC cancer patients. Nine out of ten (90%) of patients had detectable CTCs using the typical definition (21-1165 cells). Within most individual patients, AFP+ and AFP- CTCs were collected, demonstrating tumor heterogeneity. ApoStream™ also isolated potential CTCs with the ApoStream™ Ck+/CD45-/DaPi+ phenotype (1.85 cells), and cells with ApoStream™ Ck+/CD45+/DaPi+ phenotype (1.3472 cells). ApoStream™ detected EpCAM+ cells and EpCAM- cells within the same patient.

**Conclusions**

- The ApoStream™ system successfully recovers CTCs from HCC patients.
- ApoStream™ captures more CTCs from HCC patients than CellSearch®.
- ApoStream™ isolates CTCs with multiple phenotypes within the same patient.
-AFP+ and AFP- CTCs
- EpCAM+ and EpCAM- CTCs
- ApoStream™ is well suited to advance clinical research in HCC patients.

**Future Directions**

ApoStream™ CTC analysis is being used for pharmacodynamic analysis in an ongoing phase I study of the combination of sorafenib and vandetanib in patients with advanced HCC. In preclinical models, the combination synergistically induces tumor cell death through activation of CD95. In this study, CTC analysis will compare the expression of CD95 prior to and after drug combination exposure. ApoStream™ is also being used for CTC analysis in other tumor types.
Antibody-independent enrichment of circulating tumor cells (CTCs) from a variety of cancer types

Vishal Gupta, Miguel Garza, Weiguo Wu, Margaret Pace, Chris Neal, Yujian Zhang, Sujita Sukumaran, Brad Redden, Geoffrey M. Copper, Yuan Wang, Jacky Woo, Vlada O. Melnikova, Dave Hasegawa, Darren W. Davis
ApoCell Inc., Houston, TX 77054

Abstract

Background: Circulating tumor cells (CTCs) are used clinically as biomarkers for monitoring metastatic disease progression. However, CTC capture has been hampered due to the limitations of EpCAM dependent capture methods, non-specific nature of filtration methods and rarity of CTCs. We developed a new CTC enrichment device, ApoStream™, that is based on dielectrophoretic field-flow fractionation (DEP-FFF) in a continuous flow microfluidic chamber enabling antibody independent isolation of viable CTCs. ApoStream™’s DEP-FFF technology leverages inherent differences in cell morphology between normal cells and cancer cells to separate CTCs from other healthy blood cells. Methods: To demonstrate performance, ovarian cancer cells were spiked into peripheral blood mononuclear cells (PBMCs) from normal donor blood and isolated using the ApoStream™ device. Cancer cell recovery was demonstrated for cells isolated from the ApoStream™ device from blood of patients with lung, prostate, breast cancer and melanoma. Lung cancer blood samples were compared in a paired sample study with CellSearch®. Cells isolated from ApoStream™ were stained for cytokeratin (CK), CD45, and DAPI, and melanoma CTCs with S100 and CD45, and imaged and enumerated using laser scanning cytometry (LSC). CTC morphology was confirmed with H&E staining. Results: ApoStream™ yielded a recovery of 80 ± 5% with more than 4000 times enrichment from samples spiked with ovarian cancer cells (SKOV3). High CTC recovery from cancer patient blood samples was achieved with counts ranging from 0 - 2104 (lung, n=33), 0 – 3490 (prostate, n=15), 176 – 968 (breast, n=3), and 4 – 3120 (melanoma, n=11) CTCs per 7.5 ml blood. There were no false-positive CTCs from normal donor blood controls demonstrating ApoStream™’s specificity. In a side-by-side sample comparison with CellSearch® system, ApoStream™ isolated a significantly higher number of CTCs from lung cancer patient blood samples (range: 3-487, mean:163, n=9 versus range: 0-8, mean:2, n=9) showing the effectiveness of ApoStream™ in isolating EpCAM-negative CTCs. In addition, FISH analysis was successfully performed on ApoStream™ enriched cells. Conclusion: ApoStream™ technology provides an antibody-independent method for CTC enrichment from various types of cancers with high recovery enabling downstream characterization including protein, RNA and DNA analysis. Isolation of CTCs enriched by ApoStream™ will have broad applications including drug screening, ultimately facilitating implementation of personalized cancer therapy.

Conclusions

- ApoStream™ is a high throughput CTC isolation system that permits antibody-independent enrichment of CTCs from various types of cancers with high recovery. ApoStream™ technology isolates higher number of CTCs from patients with NSCLC than the CellSearch® method, evidently due to capturing EpCAM-negative cancer cells.
- CTC capture with ApoStream™ allows downstream CTC characterization such as protein expression, gene expression, mutation analysis and FISH.
- ApoStream™ can serve as a new effective tool with broad applications in cancer biomarker discovery and implementation of personalized cancer therapy.

Acknowledgments

Funded in part by NCI Contract No. HHSN261200800001E