The Use of an Antibody Independent Method, ApoStream®, to Isolate Circulating Tumor Cells (CTCs) Isolated from Non-Small Cell Lung Cancer Patients and Identification of EGFR Mutations

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Abstract # C16

Background: A variety of methods for capture of rare CTCs of epithelial origin are available, most rely on antibodies to cell surface markers such as epithelial cell adhesion molecule (EpCAM) and cytokeratin (CK). In contrast, CTCs may elude capture as they originate from primary tumor cells that have undergone epithelial-to-mesenchymal transition (EMT) and therefore lack EpCAM. We previously reported the use of EpCAM-negative CTCs in a novel deoxycytidine kinase (dCK) assay to isolate CTCs from blood. Methods: Blood was collected from consenting NSCLC patients and processed using ApoStream®. For CTC enumeration comparison, the CellSearch® FTA approved kit was used. Isolated cells were evaluated with a multiparameter immunofluorescent assay and laser scanning cytometry was employed to identify multiple combinations of positive and negative staining for EpCAM and CD45. Cells with EpCAM+/CD45− expression were isolated and subjected to Sanger sequencing. Results: Blood samples from 40 NSCLC patients and healthy volunteers were processed in the normal, healthy volunteers, ApoStream® isolated 0–1 CTCs per 33 CR-COCA cells. From the 36 of 40 NSCLC patients, ApoStream® identified 1–7 CTCs of EpCAM− and CK− and 0–2 CTCs of EpCAM− and CF−. PCR amplification and sequencing was performed from EpCAM− CTCs in 7 patient samples tested. From our whole immunofluorescent analysis, 25% of CTCs were EpCAM− CK+ cells. From the 38 of 40 NSCLC patients, ApoStream® of standard ICE COLD PCR analysis was performed using 15% of CTC DNA specimen. (A) Modified pre-amplification PCR combined with a semi-nested PCR followed by sequencing. (B) Modified pre-amplification PCR combined with standard ICE COLD PCR.

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Results: From the 38 of 40 NSCLC patients, ApoStream® of standard ICE COLD PCR analysis was performed using 15% of CTC DNA specimen. (A) Modified pre-amplification PCR combined with a semi-nested PCR followed by sequencing. (B) Modified pre-amplification PCR combined with standard ICE COLD PCR.

Figure 2: Overview of ICE COLD-PCR Process and Theory (A)

Step 1: Add DNA to double stranded "Amplicon"
Step 2: The "R-Target" binds to one of the wild-type and mutant sequences; mismatched "Amplicon" forms a "Mismatch"
Step 3: The reaction is initiated at the "R" to the mismatch sequence; mismatched "Amplicon" forms a "Mismatch"
Step 4: Around the PCR primer, the forward and reverse PCR primers will bind to both strands of the mismatch DNA and only one strand of the wild-type.
Step 5: Sequence of the PCR primers along the mutant and wild-type DNA sequences. The mismatch sequence will undergo exponential amplification while the amplification of the wild-type sequence will be suppressed.

Step 6: cycle of Assisted Sanger Sequencing reactions.

Step 7: Analysis using an ICE COLD PCR Sequencing

Figure 3: CellSearch® and ApoStream® CTC Enrichment and Downstream Analysis Workflows

Figure 4: Representative Immunofluorescent Images of Cells Isolated by ApoStream®

Figure 5: Summary of CTC Enumeration and Mutation Analysis Results

Table 1: ApoStream® CTC Counts and EGFR Mutation Status in Tissue Pathology Report and CTCs

<table>
<thead>
<tr>
<th>Sample</th>
<th>CTC Count</th>
<th>EGFR Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method 1</td>
<td>Method 2</td>
<td>Method 3</td>
</tr>
<tr>
<td>Healthy Donor Samples</td>
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<td>0</td>
</tr>
<tr>
<td>Chronic Obstructive Pulmonary Disease Lung Cancer Samples</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
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Table 2: Summary of CTC Enumeration and Mutation Analysis Results

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Summary & Clinical Significance

- A novel, antibody independent platform ApoStream® successfully isolated CTCs from the blood of patients with advanced NSCLC and compared. ApoStream® isolated more CN/CD40/ NSCLC CTCs compared to the CellSearch® platform in 4 out of 7 NSCLC patient samples with detectable CN/CD40− cells; neither system detected CTCs in 1 patient sample.

- Phenotypic immunofluorescent analysis of cells isolated by ApoStream® revealed the presence of CR-COCA CTCs as well as CK-COCA CTCs. Levels of CR-COCA CTCs was detected in NSCLC samples as compared to 0 in healthy donor blood.

- The higher levels of sensitivity of detection is approximately one mutated cell per five total cells in sample (20%).

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

- Overall, from the isolated CTCs using ApoStream® ICE COLD-PCR identified mutation status in 15 cases (EGFR exon 19 deletions, 5; exon 19 −/L858R (2) and wild type in 7 cases) in concordance to tumor tissue analysis by Sanger sequencing.

- The mutation detection of other samples (n=20) did not match tumor mutation status by Sanger sequencing.

- EGFR exon 21 results were concordant with the corresponding tumor tissue by Sanger sequencing.

- To date, no mutations were observed in the tumor tissue from this set of patients. Using standard ICE COLD-PCR followed by Sanger sequencing on the template DNA extracted from the CTCs isolated by ApoStream®, the mutations were found, thus the results from the ICE COLD-PCR analysis were 100% concordant with the tumor samples with no false positive observed. Development of a modified ICE COLD-PCR approach (Method 2) for EGFR exon 21 CTC mutation analysis is ongoing.

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