A multiparameter assay for HER2 protein detection on circulating tumor cells in non-small cell lung cancer

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Abstract
Lung cancer biopsy can be difficult to perform successfully; it has a 30% adverse event risk and often provides insufficient material to test. Therefore, a liquid biopsy method to analyze the tumor is advantageous. Analysis of circulating tumor cells (CTCs) by multiparameter immunofluorescence (IF) microscopy allows non-invasive characterization of cancer cell biomarker expression. HER2 is a well-known therapeutic target in breast cancer and studies have shown that HER2 status on CTCs may provide prognostic information about response to anti-HER2 therapies. Less is understood about its frequency and clinical importance in non-small cell lung cancer (NSCLC). The RareCyte platform is uniquely suited for CTC identification and phenotypic characterization with a sensitive, accurate, simple and repeatable workflow from blood collection to CTC characterization and single-cell isolation.

Methods
We developed an assay for detecting HER2 expression on lung CTCs using the RareCyte platform and validated the assay on model CTC (mCTC) spike-in samples using cell lines characterized for HER2 expression by NanoString analysis to measure mRNA, and meso scale discovery and Quantibrite assays to measure protein levels in the cell lines. The final cell line panel demonstrated a range of HER2 expression (high (BT-474), medium (H1650 and OVCAR-3) and absent (MDA-MB-468)) HER2 protein levels. Blood was drawn into AccuCyte® Blood Collection Tubes and processed to slides using the AccuCyte Sample Preparation System and stained with an anti-HER2 antibody together with the RarePlex® CTC Panel Kit for CTC identification (nuclear dye, anti-CD45 to exclude WBC, and anti-cytokeratin/EpCAM for CTCs). Scanning of slides was performed on a CyteFinder® Instrument and images were analyzed using CyteMapper software and analysis tools. CTCs were analyzed by a trained reviewer, and CTC HER2 status was determined with a fluorescence intensity threshold.

Results
Spike-in samples showed the expected trend in per-cell HER2 mean fluorescence intensity (MFI) values. The percentage of HER2-positive cells was >90% for the HER2-high, medium and low cell lines, and <5% for the HER2-negative cell line. The assay was applied to 10 advanced stage (III or IV) post-treatment NSCLC patient samples. At least 1 CTC was found in 6 samples (range 0-2043) and HER2 expression was confirmed in 5 out of the 6 samples.

Conclusion
We have developed and analytically validated an immunofluorescence assay to measure HER2 protein expression on CTCs. The assay was used to characterize HER2 protein expression in NSCLC patient samples. A larger clinical sample set is needed to determine optimal clinical MFI thresholds.

Sample preparation and analysis workflow

Figure 1. Verification of HER2 expression using cell line controls.

Figure 2. The RareCyte CTC assay workflow.

Figure 3. HER2 expression on mCTC cell lines measured using the RarePlex HER2 CTC assay.

Figure 4. HER2 sensitivity, specificity and accuracy.

Table 1. Sensitivity, Specificity and Accuracy determined from HER2-negative (MDA-MB-468) and each HER2+ mCTC line.

<table>
<thead>
<tr>
<th>HER2 Status</th>
<th>Cell line</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Positive Predictive Value</th>
<th>Negative Predictive Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>MDA-MB-468</td>
<td>0.0000</td>
<td>1.0000</td>
<td>1.0000</td>
<td>1.0000</td>
</tr>
<tr>
<td>Low</td>
<td>H1650</td>
<td>0.9254</td>
<td>0.6934</td>
<td>0.7115</td>
<td>0.7880</td>
</tr>
<tr>
<td>Medium</td>
<td>OVCAR-3</td>
<td>0.9038</td>
<td>0.6893</td>
<td>0.7129</td>
<td>0.7880</td>
</tr>
<tr>
<td>High</td>
<td>BT-474</td>
<td>0.9888</td>
<td>0.9998</td>
<td>0.9999</td>
<td>0.9999</td>
</tr>
</tbody>
</table>

Figure 5. Representative images of mCTCs.

Each row shows the indicated representative images for a single mCTC. HER2 images were scaled in two different ways to accommodate the high dynamic range of HER2 expression across the cell lines. Autoscale was used to optimally visualize the HER2 signal on all mCTCs regardless of expression level and is achieved by using linear display scaling with the dimmest pixel displayed as black and the brightest pixel displayed as white. To perceive relative difference in HER2 intensity across the cell lines, we also used constant scaling with display saturation set to 2,000 intensity counts. All positive mCTCs show displayed the expected HER2 membrane localization.

Figure 6. Testing HER2 assay on NSCLC samples.

Blood from ten NSCLC patients was collected and processed with the RarePlex HER2 CTC assay. A) CTC count and HER2 status (determined using an MFI threshold of 100) for each patient is indicated in the table. The increased threshold was used due to higher non-specific background staining found on clinical slides. **Patient 3 HER2 expression data was extrapolated from 1 slide. HER2 expression in patient 3 had mean MFI of 141 (interquartile range 75-153). CTC numbers in patient 3 are reflective of the clinical status of the patient (stage IIIb with disease progression). B) Four representative CTC images from patient 3 are shown, including a cluster of 2 CTCs in middle row.