**Ethics Approval**

The study was approved by Zucker School of Medicine at Hofstra/Northwell at Staten Island University Hospital’s IRB #: 19-0922

**REFERENCE**


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**QUANTIFYING PHARMACODYNAMIC BIOMARKER CHANGES IN IMMUNO-ONCOLOGY BY MASS SPECTROMETRY**

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**Background**

Quantifying pharmacodynamic biomarker changes enables decision making and clinical trials in drug development. Pharmacodynamic biomarkers are used to determine the effects of treatment on disease. Mass spectrometry offers a quantitative, selective, and multiplex platform for pharmacodynamic protein biomarker analysis in clinical samples (e.g. blood and tumor) that is feasible across multiple sample conditions (e.g. fresh, frozen and formalin-fixed paraffin-embedded (FFPE)). To date, however, methodologies for targeted protein analysis by mass spectrometry (i.e. quantitative proteomics) are underdeveloped for application in immunoncology.

**Methods**

To address this, we sought to extract the immunoncology-associated T cell membrane proteins CD3, CD4 and CD8 from peripheral blood mononucleate cells (PBMC) and develop a multiplexed mass spectrometry method to quantify their expression. PBMC were isolated from whole blood and using detergent-based lysis buffers fractionated into a cytosolic and membrane protein lysate (figure 1). Analytical methods were then developed to detect proteotypic peptides of all three proteins (table 1 and figure 6) from the lysates by mass spectrometry.

**Results**

CD3, CD4 and CD8 were detected in the membrane protein fraction but not in the cytosolic protein fraction after whole-proteome tryptic digestion using a filter-aided sample preparation (or FASP) technique but with a signal-to-noise ratio of ≤ 2.0 (figure 2). Applying an additional immunoaffinity (IA) enrichment step with antibody-conjugated magnetic beads, prior to digestion, dramatically improved the analyte signal-to-noise ratios to > 100 (figure 3). Reverse-phase nanoflow liquid chromatography (LC) was used to separate all three analytes in multiplex over a 12-minute run prior to tandem mass analysis (MS/MS) (figure 4). Together, this IA-LC-MS/MS method resulted in detection of endogenous CD3, CD4 and CD8 proteins from small volumes of whole blood (< 0.1 mL) and the analyte responses were linear over at least two orders of magnitude (figure 5).

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**Abstract Table 1**

Multivariable Cox regression analysis for association of baseline peripheral blood biomarkers and overall survival.

<table>
<thead>
<tr>
<th>Protein</th>
<th>CD3</th>
<th>CD8</th>
<th>CD4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptic peptide</td>
<td>GSKPEDAFNLGYLR</td>
<td>AAEGLDTQRR</td>
<td>ILNGQPSFLTK</td>
</tr>
<tr>
<td>MRM transition (m/z)</td>
<td>558.2 &gt; 793.3</td>
<td>480.7 &gt; 818.4</td>
<td>580.3 &gt; 961.4</td>
</tr>
</tbody>
</table>

**Abstract Figure 1**

Detergent-based protein extraction and fractionation of PBMC

**Abstract Figure 2**

Filter-aided sample preparation (FASP) for whole-proteome analysis

**Abstract Figure 3**

Immunoaffinity enrichment of proteins from PBMC lysates

**Abstract Figure 4**

Representative multiplex analysis from 1mL of whole blood
Abstract 84 Figure 5 Multiplex analysis of endogenous CD3, CD4, and CD8

Abstract 84 Figure 6 Optimization of tryptic digestion conditions

Conclusions This method was developed specifically to quantify pharmacodynamic changes in CD4 and CD8 T cell membrane expressions from clinically feasible samples (i.e. PBMC). This work, however, provides a foundation for developing methodologies to conduct quantitative proteomics applicable to immuno-oncology, which may be used to interrogate additional pharmacodynamic biomarkers.

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Abstract 85

Spatial Heterogeneity of TAMs in the Tumor Immune Microenvironment in cCRCC

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Background Tumor associated macrophages (TAM) stimulate tumor proliferation and facilitate immune escape via production of immunosuppressive cytokines. We hypothesize that non-random spatial clustering of TAMs within the tumor are associated with poor survival in cCRCC patients.

Methods Tumor specimens were obtained from 41 patients with metastatic cCRCC who received immunotherapy (IT). Sections from the tumor core underwent multiplex immunofluorescence staining for CD68, CD163, and CD206. Digital pathologic analysis was used to convert the digital images to spatial point pattern plots (PPP). Ripley’s K function, the current standard metric for spatial heterogeneity, was utilized. Novel metrics were developed using a probability density function (PDF) for distances between cells, assuming that cells can be located anywhere with equal probability. Empirical histograms were generated from the PDFs. Deviation from the PDF demonstrates a non-random distribution. Deviations were quantified with the Kolmogorov-Smirnov (KS) test and Cramér-von Mises (CVM) criterion. Overall survival (OS) was assessed between groups stratified by the median value for each metric using Kaplan-Meier and log-rank analysis. Figure 1A.

Results 75 slides were analyzed from the 41 patients. The three metrics for measuring spatial heterogeneity had moderate and statistically significant correlation with each other (Spearman’s R: Ripley/KS=0.68, p<0.01; Ripley/CVM=0.54, p<0.01; KS/CVM=0.47, p<0.01; figure 1B). Using CVM, increasingly non-random distribution of the Tumor-CD68+ cell relationship was associated with worse OS (p<0.01, figure 1C), and increasingly non-random distribution of CD163+ cells suggested an association with worse OS without reaching statistical significance (p=0.06, figure 1C). No statistically significant associations were identified using the KS or Ripley’s K metrics.

Conclusions We describe CVM and KS as novel metrics for measuring spatial heterogeneity of immune cells. Increased spatial heterogeneity of CD68+ TAMs and tumor cells was associated with worse OS in patients with metastatic cCRCC who received IT. These findings corroborate prior reports of TAMs eliciting an immunosuppressive effect on the tumor-immune microenvironment, and demonstrate the novel finding of a clinically significant effect of TAM spatial clustering on OS.

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Abstract 86

Co-Detection of RNA and Protein in FFPE Tumor Samples by Combining RNAscope In Situ Hybridization and Immunohistochemistry Assays

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Background Spatially resolved gene expression has emerged as a crucial technique to understand complex multicellular