Spatial biomarkers to improve the predictive power of response to immunotherapy in operable Lung Cancer

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Immunotherapy has reshaped the field of lung cancer therapeutics but, despite positive results, only a minority (<20%) of the patients derived a very long-term benefit from Immune Checkpoint Inhibitor[1] (ICI) therapy warranting a companion diagnostic[2]. In this work, total expression of relevant transcripts and spatially resolved proteins was quantitated using multiplexed methods (NanoString nCounter and GeoMx platforms). This analysis identified numerous differentially expressed transcripts associated with the response to ICI. In addition, significant alterations in the expression and/or spatial distribution of immunologically relevant proteins in different regions (tumor cell rich versus immune cell rich) of the tumor microenvironment provide additional insight into the predictive immunological effects of clinically relevant adjuvant ICI therapy for resectable lung cancer. Taken together, these results identify gene expression profiles within the tumor microenvironment and provide preliminary evidence on biomarkers that may influence the suitability of ICIs

OBJECTIVES

Non-small cell lung cancer (NSCLC) accounts for about 85% of all lung cancers[3]. There is a strong rationale for incorporating immunotherapy into the treatment of early-stage NSCLC, given the breakthrough results with PD-1 checkpoint inhibitors in advanced-stage NSCLC[4]. How immunotherapy should be implemented in patients who are operable is still unclear. 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)[5]. In order to overcome these limitations, we used spatial biomarkers assays that preserve this critical information about which cells are influencing treatment response and how they are spatially distributed relative to each other.

MATHERIALS AND METHODS

Frozen sections from retrospectively collected surgically resected NSCLC (adenocarcinoma and squamous cell carcinoma) tumors treated with adjuvant pembrolizumab therapy were used. Patients were classified in two groups according to their Objective Response Rate (ORR): Complete Response (CR) and Progression Disease (PD) for spatial transcriptomic and proteomics assays.

Transcript levels of 780 predefined immunologically relevant and signaling tumor pathways genes (*Tumor Signaling 360 Panel*) were assessed using the NanoString nCounter platform in bulk RNA extracted from tissue sections. Unsupervised hierarchical clustering of genes and samples was carried out by uncentered Pearson correlation.

Figure 1 Gene expression analysis workflow.

Tissue sections of lung tumors were stained with fluorescently labeled antibody anti-Pan-Cytokeratin and anti-CD45 to "map" the tissue. NanoString GeoMx™ Digital Spatial Profiling (DSP) technology was used to determine differential expression of immune markers (*Human immune cell profiling and immune activation status protein modules*) in Regions of Interest (ROIs). Wilcoxon and Mann-Whitney U statistic tests were used to evaluate differences between ORR groups.

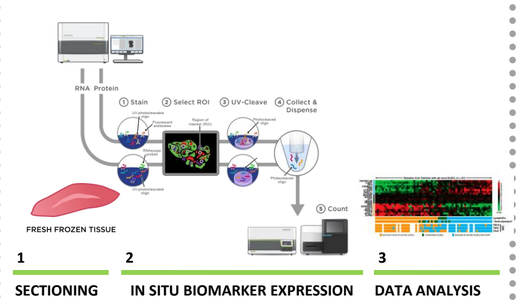


Figure 2 Protein expression analysis workflow.

T-CELL EXHAUSTION IN PD PATIENT

Unsupervised hierarchical clustering (Figure 3-A) revealed that the gene expression data clustered into objective response rate (ORR) with pathways of immunogenicity overexpressed in the complete response (CR) patient. T cell exhaustion phenotype was highly represented in the progression disease (PD) patient with the statistically significant downregulation of the marker CD44 (Figure 3-B).

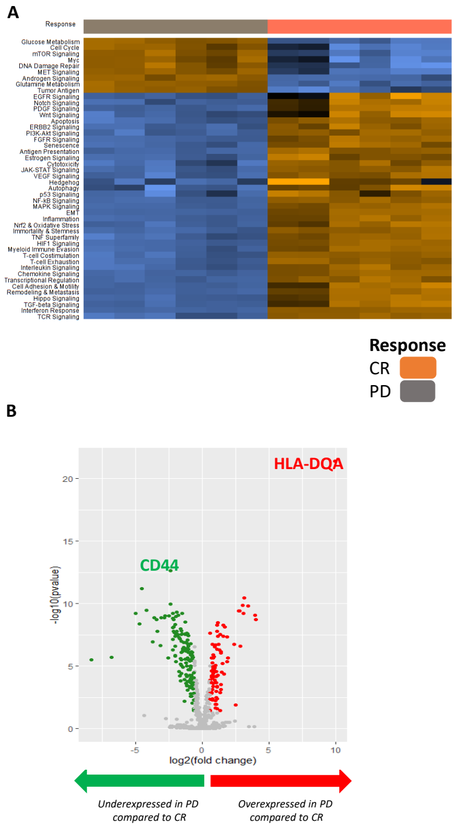
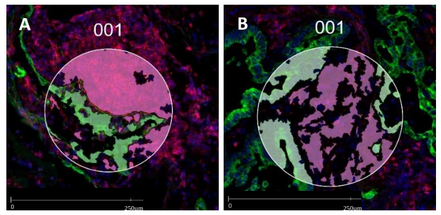


Figure 3 Response to ICI is associated with substantial alterations in immunologically relevant expression A-Heatmap clustering of gene expression in NSCLC samples resected from patients who received adjuvant ICI therapy (n = 1 patient/ORR, 6 replicates per patient). Each column represents one functional pathway, and each row represents 1 patient replicate. Unsupervised hierarchical clustering of genes and samples was carried out by uncentered Pearson correlation. Color indicates normalized counts of each gene, with yellow/orange representing higher expression and blue relatively lower expression. B- Volcano plot depicting differentially expressed gene P value as a function of fold change between the PD response compared to CR response.

TUMOR IMMUNE INFILTRATE

4 regions of interest (ROIs) per sample were selected based on morphological staining of immune cell (CD45) and tumor cells (PanCK) (Figure 4-A&B). Based on these fluorescent labels, "immune cell– rich" regions were selected that were enriched for CD45 staining, and "tumor rich" regions enriched with PanCK were detected.

Within the immune cell-rich regions, 21 proteins differed significantly across the ORR (Figure 4-C). Response to ICI therapy was associated with high immune infiltration within the vicinity of the tumor. Indeed, CD8 and CD45 markers as well as the target of the drug PD-1 were highly expressed in the CR patient. In contrast, in the PD patient, Fibronectin was highly expressed and no immunosuppression marker was identified in this patient.



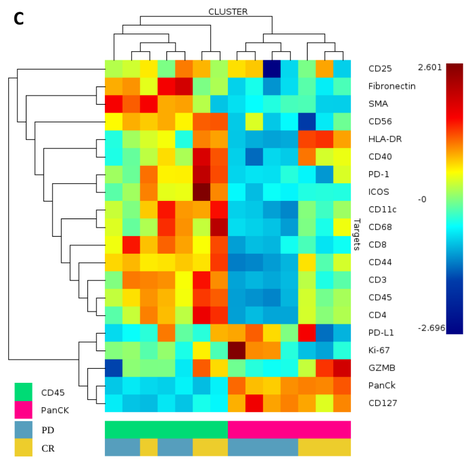


Figure 4 A,B-Selection of tumor-rich and immune rich regions within NSCLC tumors. As part of the GeoMx workflow, frozen slides of lung tumors from patients who received ICI therapy were stained with fluorescently labeled anti–pan-cytokeratin (green) and anti-CD45 (magenta). A representative region of interest (ROI) 001 is shown. C-Expression levels of immunologically relevant proteins in immune and tumor cells-rich regions. Heatmap clustering of expression of the indicated proteins. Individual regions of interest were derived from each patient tumor and stroma from a total of n = 2 patients.

Among the statistically significant target that were differentially expressed across ORR in the immune cell-rich regions; **CD4**, **HLA-DR**, **Granzyme B** and **CD40** were upregulated in the patient that responded to ICI (Figure 5). This emphasized the importance of activation of T cell and the antigen presenting cells in ICI response.

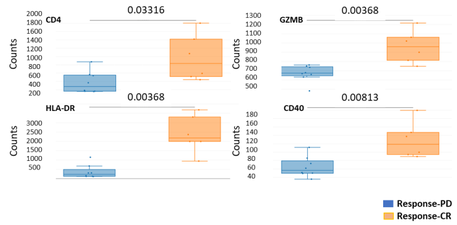


Figure 5 Response to ICI is associated with elevated expression of T cell activation and antigen presenting cell activation markers. Expression levels of the indicated proteins, CD4, HLA-DR, GZMB and CD40 are represented.

CONCLUSION

Using the spatial analysis, we identified 4 protein markers independently associated with benefit from single-agent PD-1 checkpoint blockade in spatial context. High expression of CD4, CD40, Granzyme B and HLA-DR were significantly associated with all favorable clinical outcome, whereas upregulation of extracellular matrix (ECM) proteins related to pro-tumoral reprogramming of the stroma were associated with immunotherapy resistance. These observations argue that ICI therapy is associated with differential modulation of immune related genes.

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