#2637. Gene Expression Profiling, Digital Spatial Profiling and Multiplex Immunohistochemistry of the Tumor-immune Microenvironment in HNSCC Tumors

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Introduction

The complex and dynamic nature of the tumor-immune microenvironment (TME) presents challenges for identification of robust and predictive biomarkers in immuno-oncology (IO). A combination of different immune cells characterized by specific immuno-activating or inhibiting expression patterns, in addition to cytokines in the environment, present several unique niche environments not only across but within tumor samples. Gene expression profiling allows for sensitive and high-throughput analysis of genes and signatures associated with the tumor, the immune response, and the TME, allowing examination of tumor-immune cell interactions at a whole tissue high level view. Multiplex immunohistochemistry (mIHC) facilitates the ability to detect, phenotype, and quantify spatial relationships of individual cells within the tumor microenvironment. We used these approaches to generate multiple data sets from a cohort of HNSCC tumor samples.

Methods

Formalin-fixed paraffin-embedded (FFPE) specimens from HNSCC patients were cut into 5µm sections for all technologies.

nCounter PanCancer IO360™ Panel
RNA was extracted from the tissues using Roche High Pure™ FFPET RNA Isolation kit and analyzed for 770 genes utilizing the NanoString PanCancer IO 360 Gene Expression Panel. Transcripts were quantitated using a NanoString nCounter® and target gene counts were normalized to internal housekeeping genes. The IO 360 data set was further investigated using the Tumor Inflammation Signature (TIS)[1] algorithm comprised of a weighted sum of 18 functional genes known to be associated with the PD-1/PD-L1 blockade pathway.

Digital Spatial Profiling
Epitope retrieval and dewaxing of the tissues was performed using a Leica BOND™ Rx and slides were manually stained with core modules provided by NanoString containing probes (antibody or RNA probes) with photocleavable oligonucleotides followed by morphology reagents with conjugated fluorophores. Slides were scanned on the NanoString DSP, Regions of Interest (ROIs) were selected based on morphological markers, and UV-cleaved oligonucleotides were hybridized with GeoMx™ Hyb Code reagents and counted with the NanoString nCounter platform.

Multiplex Fluorescent IHC
FFPE tissues were prepared and stained on a Leica Bond Rx with two Ultivue UltiMapper® I/O Kits: PD-L1 Kit (CD8, CD68, PD-L1, panCK/SOX10, and nuclear counterstain) and T-act Kit (CD3, Granzyme B, Ki67, panCK/SOX10 and nuclear counterstain). Scanning and data analyses were performed with the Akoya Vectra® Polaris™ and HALO® software (Indica Labs), respectively.

Experimental Design and Workflow

Gene Expression IO360 and TIS
- 770-gene CodeSet for tumor profiling containing I/O-relevant gene signatures

Digital Spatial Profiling (DSP)
- Spatially resolved and quantifiable data sets of 24 proteins and 78 genes

Multiplex Immuno-fluorescence (mIF)
- Spatial identification of I/O relevant biomarkers and co-expression in cells for phenotyping

Reference

1. Ayers, Mark, et al. "IFN-γ-related mRNA profile predicts clinical response to PD-1 blockade." The Journal of Clinical Investigation 127.8 (2017).

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Results

18- Gene Tumor Inflammation Signature			
IFN Activity	**T Cell Exhaustion**	**T Cell/ NK Cell Abundance**	**Antigen Presenting Cell Abundance**
CCL5	CD8A	HLA- E	PSMB10
CXCL9	TIGIT		
CD27	LAG3		HLA-DQA1
CXCR6	PD-L2	NKG7	HLA-DRB1
IDO1	PD-L1/CD274		
STAT1	CD276		CMKLR1

The tumor inflammation signature (TIS) calculates a weighted average of 18 functional genes known to be associated with the PD-1/PD-L1 blockade pathway. A total of 46 tumor samples, across 4 indications were tested. The HNSCC samples showed the largest dynamic range and were selected for further analysis. The cases indicated as “Extreme Hot” and “Extreme Cold” were chosen and will herein be referred as “Hot Tumor” and “Cold Tumor” respectively.

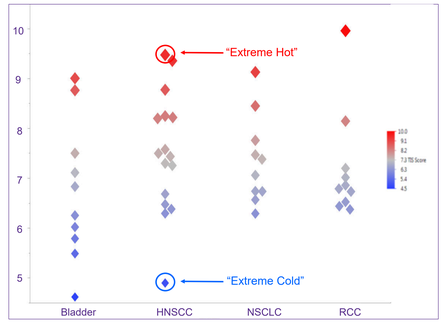

Figure 1. Scatter plot of 46 tumor samples, stratified by indication, showing the dynamic range of tumor inflammation in bladder cancer, HNSCC, NSCLC and RCC. The TIS scale ranges from 4 to 10 with a score of 10 indicating a high abundance of inflammation within the tumor.

We used NanoString's GeoMx Digital Spatial Profiler (DSP) to examine the “Hot” and “Cold” tumors identified by the IO360 panel. The DSP allowed us to also obtain spatially resolved analyte abundance. We showed that both protein and RNA expression from a region of interest selected on each tumor showed similar expression patterns to the nCounter data set.

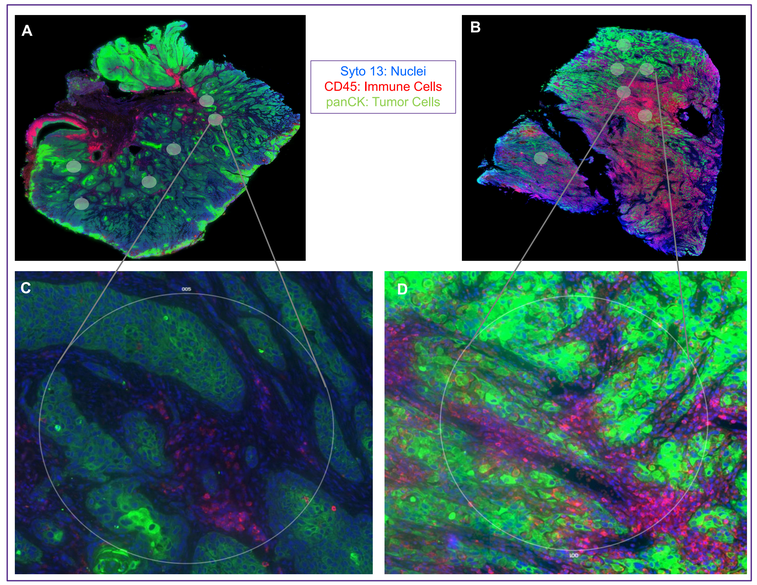

Figure 2. Images obtained from the NanoString GeoMx instrument. (A) Full slide scan and (C) high magnification image region of interest (ROI) of “Cold Tumor” HNSCC tissue in the left. (B) Full slide scan and (D) high magnification image ROI of “Hot Tumor” HNSCC on the right. The DSP allows for the selection of ROIs across any part of the tumor resulting in quantifiable protein or RNA expression. This degree of spatial profiling helps provide high parameter analysis across tumors that are known to have a high degree of cellular complexity and heterogeneity.

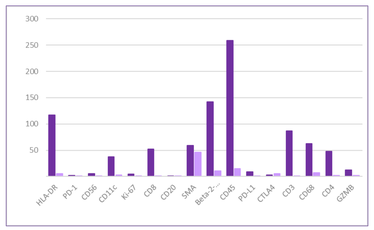

Figure 3. Protein counts for the above tumors from the NanoString DSP. The counts were corrected for background signal (IgG Isotype). The hot tumor shows a higher infiltration of all I/O proteins compared to the cold tumor.

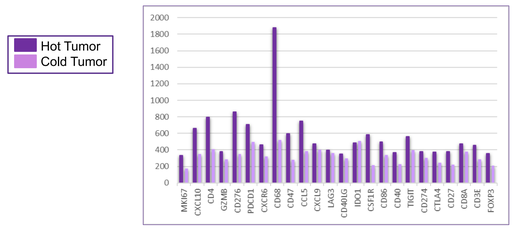

Figure 4. RNA counts for the above tumors from the NanoString DSP. The counts were corrected for background from the average of 4 negative control probes. A subset of the RNA data, abbreviated for display clarity, of the most well known I/O targets. RNA data shows higher expression of all targets in the hot versus cold tumor.

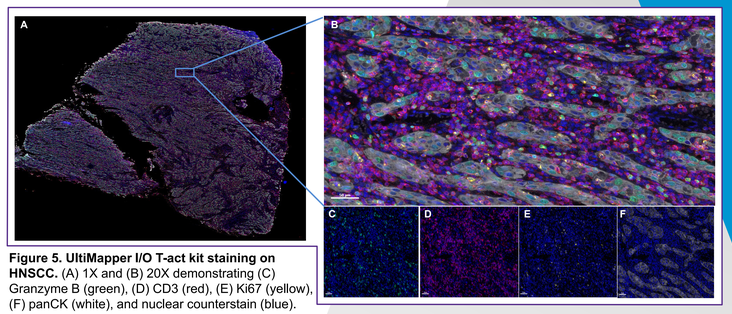

Figure 5. UltiMapper I/O T-act kit staining on HNSCC. (A) 1X and (B) 20X demonstrating (C) Granzyme B (green), (D) CD3 (red), (E) Ki67 (yellow), (F) panCK (white), and nuclear counterstain (blue).

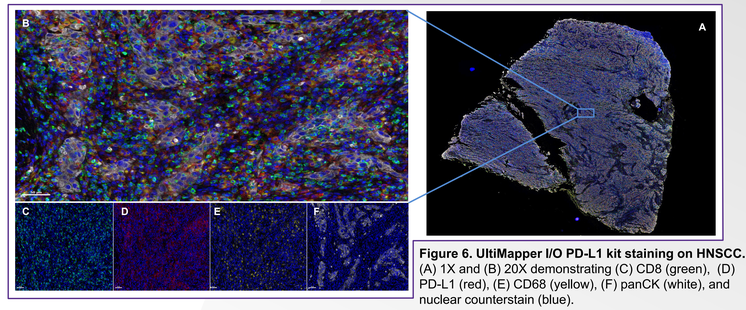

Figure 6. UltiMapper I/O PD-L1 kit staining on HNSCC. (A) 1X and (B) 20X demonstrating (C) CD8 (green), (D) PD-L1 (red), (E) CD68 (yellow), (F) panCK (white), and nuclear counterstain (blue).

Multiplex immunofluorescence (mIF) allows the staining of multiple proteins with a large dynamic range giving the power to characterize cells with spatial context. The HNSCC samples seen above, the” Hot Tumor” case identified by the Nanostring IO360 panel, showing the high amount of I/O relevant cell types in the tumor microenvironment. Additionally, we utilized automated quantitative digital pathology software to quantify and phenotype cells across the whole tissue.

Table 1. Partial List of Cell Phenotypes Identified by Staining with Ultivue’s PD-L1 Staining Kit and Using Indica Lab’s HALO Quantitative Digital Pathology

Cell Quantifying and Phenotyping		
Phenotype	**Cold Tumor**	**Hot Tumor**
T Cells (CD3+)	10,778	133,954
Cytotoxic T Cell (CD8+)	408	10,533
Macrophages (CD68+)	501	12,079
Immunosuppresive Macrophages (CD68+/PD-L1+)	390	6002
Immune Evading Tumor Cells (panCK+/PDL1+)	222	9722

Conclusions

The technologies described enable the investigation of the TME for use in biomarker discovery, drug discovery, and IO pathway interrogation. We used gene expression panels to screen a broad range of targets, then further investigated HNSCC specimens using the spatial analysis capabilities of DSP and mIHC. These technologies provided greater insight into the immune infiltrate population by quantification of cell types and co-expressing phenotypes with spatial resolution at the cell level. These complementary technologies provide useful tools in the IO biomarker toolkit.

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