Spatially-Resolved, High-Plex Digital Profiling Enables Characterization of Complex Immune Biology of the Colorectal Cancer Tumor Microenvironment

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Abstract

Background:
Spatial characterization of the tumor microenvironment (TME) interface between cancer cells, stroma and immune cells is essential for understanding tumor progression and discovering prognostic and predictive biomarkers. However, it has proven difficult to perform such studies in a highly multiplexed manner using limited sample quantity. GeoMx™ Digital Spatial Profiling (DSP) has been developed as a research use instrument, software and chemistry for hi-plex profiling of mRNA or protein using an optical-barcode read-out. In this study, microsatellite stable (MSS) or instable (MSI) colorectal tumors were characterized using DSP with 40 proteins or 80 RNA probes to evaluate active and suppressive immune mechanisms in both immune dense and tumor dense regions.

Methods:
Eighteen FFPE colorectal tumors were previously characterized for microsatellite stability status. They were also characterized for Tumor Inflammation Signature (TIS) status using NanoString’s PanCancer IO360™ gene expression panel. These samples were profiled using GeoMx DSP with 40 proteins and 80 mRNAs.

Results:
We show that deep profiling of CD45-enriched regions from the invasive margin and tumor center of MSS and MSI tumors have different immunosuppressive and activated immune phenotypes. Comparing colorectal tumors characterized as MSS, DSP was able to differentiate immune hot and cold tumors despite MSS status. Further evaluation using tumor and stroma-enriched profiling also identified specific immune pathways that were distinctly related to tumor and stroma-enriched compartments that were different between MSI and MSS tumors.

Conclusions:
Our results suggests DSP has the potential to be used in the evaluation of patients' response to PD-1 immune checkpoint blockade with greater sensitivity than standard MSS/MSI profiling, and furthermore DSP may allow identification of unique localized immune characteristics that could guide combination therapeutic approaches.
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The Tumor Inflammation Signature (TIS) contains 18 genes that measure a peripherally suppressed immune response and distinguishes tumors as immune hot and cold. (Ayers, et. al. JCI 2017)

**Figure 1**

<table>
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<th>IFN(\gamma) Biology</th>
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GeoMx™ DSP Workflow

Workflow

Tissue sections were stained with a cocktail of pan-cytokeratin (pan-CK), CD45, CD3 and DNA fluorescent morphology markers and 80 RNA probes or a 40 protein cocktail of antibodies conjugated to UV-photocleavable DNA barcodes. Regions of interest (ROI) were delineated using the immunofluorescence followed by UV excitation of the defined ROIs, which releases the DNA barcodes for downstream quantitation on the NanoString nCounter® platform.

Two strategies were used for selecting ROIs,
1. Geometric profiling of 12 400µm diameter circle CD45-enriched hotspots in the tumor center, invasive margin or pan-CK-enriched tumor regions using 40 protein markers.
2. Geometric profiling of 12 300 µm diameter circular ROIs from tumor (pan-CK-enriched) or stromal (non-pan-CK-enriched) areas using a 80-plex RNA panel.
Results for Geometric Profiling of MSI-MSS Colorectal Tumors

GeoMx Profiling of Eighteen MSI and TIS Characterized Colon Tumors

Figure 3
Representative whole slide and ROI images for the eighteen colon samples, 6 each MSI-TIS-H, MSS-TIS-H and MSS-TIS-L were stained for morphology markers pan-CK, CD45 and CD3 and a 40-plex oligo-conjugated antibody mix. Each sample was profiled with 400 µm ROI circles in CD45-enriched areas: 5 in the invasive margin (IM) and 4 in the tumor center (CT) as well as 3 in pan-cytokeratin enriched areas.

CD45-enriched Hotspots

Invasive Margin | Tumor Center | Cytokeratin-enriched
Differential Expression of Immune Markers by Location Within the TME

Figure 4
Comparison of samples and ROI location described in Figure 3. Left: Unsupervised hierarchal clustering of ROIs for eighteen samples. Right: Differential Expression averaged for each ROI type within a sample (CT, IM, Tumor) per marker. A Kruskal-Wallis test was used to test for differences in expression between either location (CT, IM, Tumor) or phenotype (MSI:TIS-H, MSS:TIS-H, MSS:TIS-L). P-values were adjusted for false discovery using the Benjamini-Yekutieli method and plotted against each other.

Figure 5
CD20, CD163, PD-L1, and PMS2 expression by location (CT, IM or tumor from cohort described in Figure 3.)
Immune Hot MSI and MSS Tumors Have Distinct Immune Biomarkers

Figure 6.
Left: Markers differentially expressed (DE) in MSI and MSS TIS-H tumors. Center and Right: Box plots comparing MSI status and ROI location for Ki67 and CD163.
Results for Geometric Profiling Using RNA

Figure 7
In situ hybridization of 80 RNA probes on a colorectal tumor using pan-CK and CD45 morphology immunofluorescence to select 12 300 µm diameter circular geometric ROIs. Heatmap of unsupervised hierarchal clustering of 80 RNA probes based on tumor (pan-CK-enriched), stromal (non-pan-CK) and stromal CD3-enriched regions.
Conclusions

Results: We show that deep profiling of CD45-enriched regions from the invasive margin and tumor center of MSS and MSI tumors have different immunosuppressive and activated immune phenotypes. Comparing colorectal tumors characterized as TIS high (immune hot), DSP was able to differentiate unique immune biology despite MSS/MSI TIS status. Evaluation of tumor-enriched versus stromal-enriched regions also identified RNA pathways that were distinctly related to each compartment.

Conclusion: Our results suggests DSP has the potential to be used in the evaluation of patients’ response to PD-1 immune checkpoint blockade with greater sensitivity than standard MSS/MSI profiling, and furthermore DSP may allow identification of unique localized immune characteristics that would guide combination therapeutic approaches.