Spatial transcriptomics identifies unique pharmacodynamic effects of checkpoint inhibitor treatment on the tumor microenvironment in NSCLC

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Introduction

- Immune checkpoint inhibitor (ICI) therapy has improved outcomes in non-small cell lung cancer (NSCLC). However, the long-term benefit of ICI therapy is only observed in a subset of patients and often lacks duration.
- Identifying biomarkers associated with the initial response to ICI treatment and subsequently developed resistance in the tumor microenvironment (TME) can inform therapeutic development for sustained NSCLC remission.
- We sought to identify pharmacodynamic changes in the TME from chemotherapy experienced patients before and after ICI therapy using a spatial transcriptomics platform, the GeoMX Digital Spatial Profiler (DSP) from NanoString.
- The technology uses a photocleavable DNA barcode strategy to multiplex antibodies or in-situ RNA hybridization probes.
- A fluorescent marker such as anti-Pan-Cytokeratin (PanCK) can be used to select regions of interest (ROIs) and separately characterize adjacent tumor and stromal cell microenvironments.
- In this study we profiled 18 NSCLC patients before and after ICI treatment for 1,800 genes, allowing investigation of genes associated with response, pharmacodynamic effects, and potential resistant mechanisms to ICI treatment.
- Pharmacodynamic effects of ICI treatment have been well studied using bulk RNA sequencing; this technology allows characterization of molecular changes with high resolution in the TME.

Objectives

- Leverage the GeoMx DSP platform to characterize the tumor microenvironment (TME) for enriched genes, pathways, and immune cell composition changes after ICI treatment
- Identify genes and pathways at baseline that are associated with partial response to ICI therapy
- Identify changes in genes and pathways that may constitute resistance mechanisms in patients who initially responded to ICI treatment but lacked durable response.

Methods

Patients

- FFPE non-small lung resections from 18 patients were sourced commercially (Capital Biosciences, MD) (Figure 1).
- Patient history is as follows: patients had surgical resection of tumors then adjuvant chemotherapy. Upon progression, patients received monotherapy ICI (nivolumab or pembrolizumab). Once progressed on ICI, resections were performed.
- All patients were then treated with docetaxel, either alone or in combination with platinum, gemcitabine, or vinorelbine; patients were followed until progression and/or death.
- Best overall response (BOR) and progression free survival (PFS) from all patients was available for ICI therapy.

Spatial Genomics with NanoString Digital Spatial Profiler (GeoMx DSP)

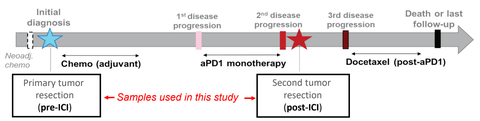
- FFPE tumors before and after ICI treatments were sectioned and hybridized with GeoMx DSP Cancer Transcriptome Atlas (CTA) panel probes followed by staining with anti-PanCK, anti-CD3 (clone UM500048, Origene), and anti-PD-L1 (clone E1L3N, Cell Signaling Technology) using standard immunofluorescence techniques with fluorophore conjugated antibodies.
- Six circular regions of ~600µm in diameter containing tumor (PanCK+) and stromal (PanCK-) areas were selected per patient (Figure 2) and profiled for gene expression using the CTA panel (1,800 genes).
- Sections were analyzed for CD3 and PDL1 positive cells using IHC and digital pathology scoring.

Bioinformatics

- Differential gene expression analysis was performed using linear models in R.
- Pathway gene sets were obtained from NanoString and immune cell signatures were extracted from the literature [1,2] and the Molecular Signatures Database (mSigDB) [3]. Gene set enrichment was computed using the gene set variation analysis (GSVA) R package [4].

Validation of Spatial Transcriptomic Platform in FFPE NSCLC Tumor Resections

- Samples from 18 patients who experienced chemo- and checkpoint inhibitor therapy (Figure 1) were analyzed by DSP to characterize the tumor and stromal microenvironment (Figure 2A, B).
- Tumor and stromal segments clustered separately (Fig 2C). Differential expression analysis showed Keratins and EpCAM were highly expressed in tumor, while collagens, CD45 (PTPRC) and immune associated cadherins (PECAM1) were highly expressed in the stromal microenvironment (Figure 2D).
- RNA expression levels of CD3 and PDL1 were highly correlated IHC scores (Figure 2E, F).



Gender	Female 4 (22%)	**Best Overall Response to ICI**	PR 9 (50%)
	Male 14 (78%)		PD 9 (50%)
Age	69 years (63-74)	**Progression Free Survival during ICI**	Median 13.5 months
Race	Caucasian (100%)		Range 8-25
Stage	IIIA/B (AJCC7)	**Histology**	Adenocarcinoma 6 (33%)
EGFR	Negative (100%)		Adenosquamous Cell Carcinoma 2 (11%)
			Squamous cell carcinoma 10 (56%)

Figure 1: Patient Treatment History and Demographics.

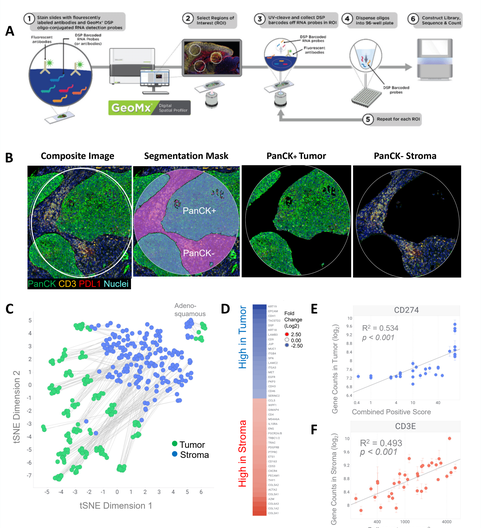


Figure 2: Nanostring DSP Workflow and Tumor Segmentation Strategy. (A) Workflow for DSP RNA profiling. (B) PanCK immunofluorescence was used to generate an image mask (AOI) to direct the UV illumination photocleavage of oligo probes. Sequential microcapillary collection allowed separate profiling of PanCK positive (tumor) and negative (stromal) regions. (C) Visualization of tumor and stromal using t-distributed stochastic neighbor embedding (tSNE); lines connect adjacent microenvironments. (E,F) correlation of gene expression to IHC scores for PD-L1 (CD274) and CD3.

Changes in the Tumor and Stromal Microenvironment after Checkpoint Inhibitor Therapy

- Differential expression analysis was used to identify genes upregulated after ICI therapy in the tumor and stromal compartments between samples collected 8-25 months apart. Magnitude of differential expression was moderate but significant after multiple test correction (Figure 3A,B).
- Pathway enrichment was performed using mSigDB Hallmark Pathways [3] to summarize broad molecular changes. Pathways upregulated post-ICI suggested a strong immune response consistent with T-cell activation including IL2, TNFα signaling, IFNγ and Complement (Figure 3C).
- Ayers et. al performed a multi-tumor study to identify a prognostic signature for pembrolizumab response [2]. We assessed the pharmacodynamic change on the 28 gene signature (26 on the DSP CTA panel), the majority showed higher expression post ICI; 8 were significant (Figure 3D).

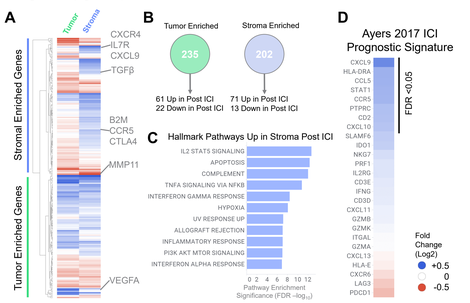


Figure 3: Differential Expression Analysis. (A) Heatmap of fold changes (log_2) between pre-ICI and post-ICI samples. (B) Summary of significantly differentially expressed genes by microenvironment compartment. (C) Most significant pathways enriched post-ICI in stroma. (D) Fold change heatmap of genes prognostic for ICI response (log_2 of 0.5 is 40% change).

- We interrogated the stromal microenvironment using expression of immune cell signatures as proxies for cell levels with a single-sample gene set enrichment approach [1,4]. CD4 T-cell subtypes and Macrophages showed the most heterogeneity (Figure 4A).
- The post-ICI stromal microenvironment was enriched for CD8 cells and depleted for DCs and Macrophages (Figure 4B,C).

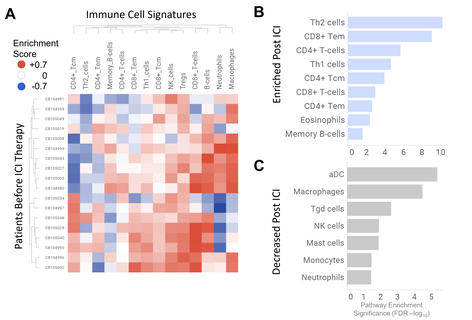


Figure 4: Immune Cell Signature Analysis. (A) Patient level immune cell enrichment scores at baseline, scale is from -1 to 1. (B, C) Immune cell signatures significantly changed during ICI therapy.

Analysis of Partial Response Predictors Uncovers Attenuation of Immune Activation after Long Term Checkpoint Inhibitor Therapy

- Tumor samples were collected by resection after progression on ICI, thus the second tumor sample can be interrogated for both effects of ICI and subsequent resistance mechanisms.
- We sought to identify genes, immune cell populations and pathways that were associated with partial response and showed changes over time. The statistical technique used was an interaction model between timepoints, microenvironment and best overall response or progression free survival on ICI.
- Signatures for CD4+ T-cell subtypes including T central memory (T_{cm}), T helper typ1 (T_h1), and T-regulatory (T_{regs}) were higher in patients who experienced progressive disease (Figure 5). These subtypes were also increased at the conclusion of ICI therapy. T helper type 2 (T_h2) was significantly higher post ICI.
- CD8+ T cells increased over time, consistent with known mechanism of αPD1 blockade. CD8+ T effector memory (T_{em}) signature was higher pre-ICI in the PD group, in the PR group the level rose to match by the 2nd progression. This suggests that countervailing factors, potentially T_{regs}, limit the effectiveness of cytotoxic T cells in anti-tumor response.

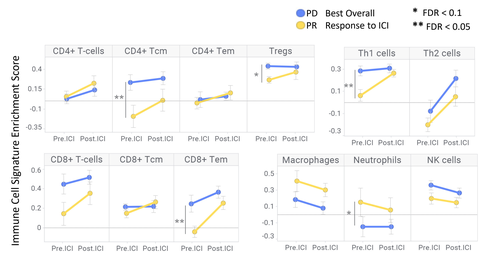


Figure 5: Immune Cell Signatures in Stroma Associated with Partial Response. Line plots illustrate averages of gene signature score among patients with partial response or progressive disease on ICI. A score of 1 indicates enrichment and -1 indicates depletion relative to other samples, error bars indicate standard error of the mean.

- Pathway analysis revealed T_{reg} Differentiation and Type II Interferon (IFNγ) signaling was higher in the PD group at baseline (Figure 6A). These pathway scores increased in the PR group by the 2nd timepoint, suggesting that patients experiencing PR had increased inflammation during ICI treatment (Figure 6B).
- Arginine metabolism was higher in the PR group, consistent with its role in T-cell survival [5].

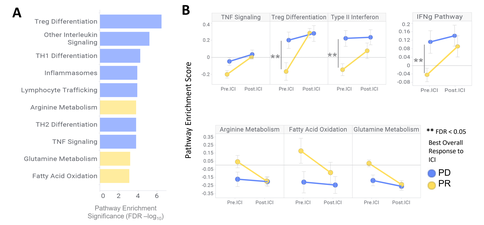


Figure 6: Pathways in Stroma Associated with Partial Response. (A) Top pathways using the NanoString CTA panel annotation, ranked by significance. Yellow indicates higher in the PR group. (B) Line plots of average pathway enrichment score by response group. IFNγ pathway was extracted from a study on αCTLA4 resistance [6].

Spatial Transcriptomics Enables Mapping of Costimulatory Interactions in the Tumor Microenvironment

- The spatial resolution of DSP allows profiling of ligand-receptor pairs expressed by tumor or stromal cells within 600µm of each other. Understanding this complex interplay is enables patient stratification and combination hypothesis generation (Figure 7A).
- *CTLA4* and *TIGIT* inhibition has shown efficacy in NSCLC (ipilimumab and tiragolumab), in this cohort they were higher in PD but showed an increase by progression in the PR group (Figure 7B).
- *CXCR4* binds *CXCL12* and *MIF*, it has roles in cytotoxicity and macrophage recruitment, also has been associated with poor survival in NSCLC [7].
- *IL7R* was highly significant for association with response and increase post-ICI. A recent single cell analysis of NSCLC TILs associated *IL7R* expression as a prognostic factor and expressed on tissue resident CD8s [8].

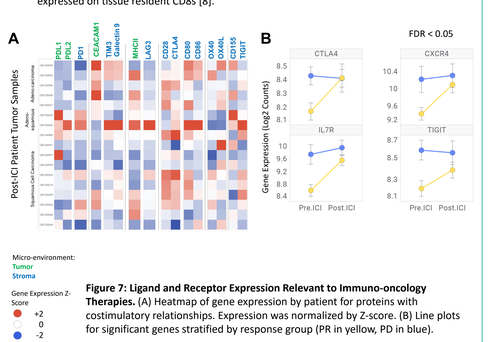


Figure 7: Ligand and Receptor Expression Relevant to Immuno-oncology Therapies. (A) Heatmap of gene expression by patient for proteins with costimulatory relationships. Expression was normalized by Z-score. (B) Line plots for significant genes stratified by response group (PR in yellow, PD in blue).

Conclusions

- The DSP spatial transcriptomics platform enables focused interrogation of the tumor microenvironment for 1,800 genes.
- Gene expression can be used to estimate T-cell cell abundances and activation states in the heterogenous stromal microenvironment.
- We observed an increase in T-cell activation after long term checkpoint inhibitor treatment, evidenced by increased cytotoxic T populations and pro-inflammatory pathways (IFNγ and TNFα).
- Comparison of patients who experienced partial response to αPD1 revealed a less inflamed tumor microenvironment before treatment.
- Patients who experienced progressive disease had higher CD8 activity before treatment, but also had higher Treg activity. Post-ICI, the PR group showed similar activation levels to the PD group.
- We hypothesize that pretreatment with neoadjuvant chemo before resection may drive an initial immune response and a compensatory Treg expansion.
- In our cohort, patients who may derive additional benefit from αPD1 could have a larger window for increasing inflammation in the tumor microenvironment due to less Treg or tumor directed inhibition.

References

1. Charoentong P, Finotello F, Angelova M, et al. *Cell Rep.* 2017;18(1):248-262.
2. Ayers M, Lunceford J, Nebozhyn M, et al. J Clin Invest. 2017;127(8):2930-2940.
3. Liberzon A, Birger C, Thorvaldsdóttir H, Ghandi M, et al. *Cell Syst.* 2015;1(6):417-425.
4. Hänzelmann S, Castelo R, Guinney J. *BMC Bioinform.* 2013;14:7.
5. Geiger R, Rieckmann JC, Wolf T, et al. *Cell.* 2016;167(3):829-842.e13.
6. Gao J, Shi LZ, Zhao H, et al. *Cell.* 2016;167(2):397-404
7. Rivas-Fuentes S, Salgado-Aguayo A, Pertuz-Belloso S, et al. *J Cancer.* 2015; 6, 938–952
8. Caushi JX, Zhang J, Ji Z, et al. *Nature.* 2021;596(7870):126-132.

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