An Integrated Multiplexing Approach Identifies IDO1 as a Biomarker for Recurrence of Rare Ovarian Granulosa Cell Tumors

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Background and Results

Background: Granulosa cell tumors (GCTs) are rare tumor in ovaries accounting for 2-5% of all ovarian cancers. The main current treatment for GCT is surgery, however a subset require chemotherapy for residual and recurrent disease. GCT malignancies are often low-grade with a five-year survival rate up to 90%, however a clinical characteristic of these tumors is a tendency for late recurrence and a high recurrent rate is the most critical factor for GCT death.

Methods: As GCTs are rare tumors and tissue availability is very limited, we used a dual multiplexing approach in order to maximize the data output from a total of 14 FFPE tumor samples (6 primary tumors, and 8 recurrent tumors). With this approach we used a single 4 µm section to detect 15 markers in an IF multiplexing assay, and an adjacent 10 µm section to analyze expression of 770 immuno-related cancer genes with the aim to spatially profile immune cell subsets, angiogenic vessels, as well as markers differentially expressed between primary and recurrent tumors.

Results: Of the 770 gene targets in the NanoString PanCancer Immune Profiling panel, we detected a differential gene expression for 66 genes in recurrent tumors compared to primary tumors. Of these 66 genes IDO1 showed the highest differential with a significant 14-fold increase in recurrent tumors. In addition to promoting tumor angiogenesis, IDO1 is a key regulator of immune tolerance as an activator of immuno-suppressive cell types such as Tregs and MDSCs, and thereby represents a therapeutic target within immuno-oncology beyond checkpoint blockade.

When analyzing the presence of macrophages on protein level in the tumor microenvironment, we found a 113% increase in TAM density in recurrent tumors compared to primary tumors, corresponding to increase in the macrophage score on mRNA level. In a nearest neighbor analysis for TAMs and angiogenic vessels, we found M2-type TAMs to be in closer proximity to vessels in recurrent versus primary tumors. TAMs are key cells in controlling tumor angiogenesis and can be recruited by tumor cells and reprogrammed to secrete factors mediating the angiogenic switch. One such recruitment factor is VEGFA, the gene for which was found to be up-regulated more than 10-fold in the recurrent tumor samples.

Multiplexing Setup – MultiOmyx™ (protein) & NanoString nCounter® (mRNA)

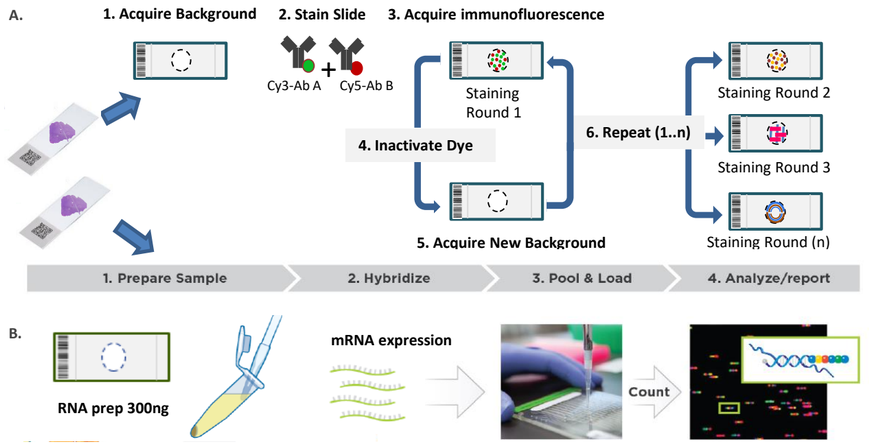


Figure 1. Multiplexing Assay Workflow. Two adjacent sections were cut from each FFPE tumor sample. **A. MultiOmyx multiplexing IF staining protocol**. For each round of staining, conjugated fluorescent antibodies were applied to a 4 µm section, followed by image acquisition of stained slides. The dye was erased, enabling a subsequent round of staining with another pair of fluorescent antibodies. Proprietary cell segmentation algorithms generate unique IDs for every cell allowing them to be tracked through multiple rounds of staining. **B. Nanostring nCounter assay**. RNA was extracted from the adjacent 10 µm section and then proceeded with hybridization, purification and immobilization and count based on manufacturer's protocol.

Panel Specifications

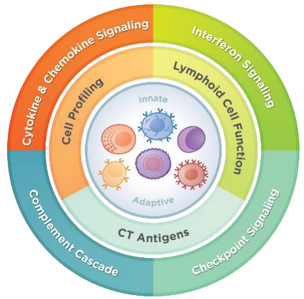
16-Marker Panel		
#	Cy3	Cy5
1	CTLA-4	CD34
2	PanCK	CD163
3	CD4	PD-1
4	CD3	PD-L1
5	CD8	FoxP3
6	CD20	HLA-DR
7	Ki67	CD68
8	Vimentin	S100

Table 1. Antibody Staining Sequence for MultiOmyx multiplexing staining. Table 2. Phenotyping of human tumor-associated lymphocytes and myeloid cells. Cell surface markers associated with cell subsets analyzed in the tumor samples. TAM: tumor-associated macrophage. PanCK: pan cytokeratin.

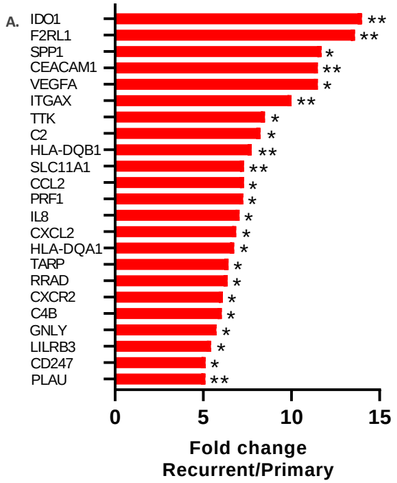
Co-expression	Phenotypes
CD3+CD4+	T helper
CD3+CD4+FoxP3+	T regulatory
CD3+CD4+PD1+	T helper PD-1
CD3+CD4+FoxP3+PD1+	T regulatory PD-1
CD3+CD4+CTLA4+	T helper CTLA-4
CD3+CD4+FoxP3+CTLA4+	Treg CTLA-4
CD3+CD8+	T cytotoxic (CTL)
CD3+CD8+PD1+	CTL PD-1
CD3+CD8+CTLA-4+	CTL CTLA-4
CD3-CD20+	B cells
CD3-CD68+	TAM
CD3-CD68+Ki67+	Proliferating TAM
CD3-CD68+HLADR+CD163-	M1 TAM
CD3-CD68+HLADR-CD163+	M2 TAM
PanCK+Ki67+	Tumor proliferation

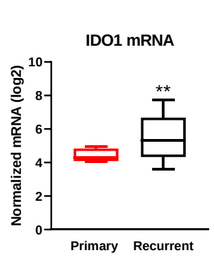
PanCancer Immune Profiling Panel :

- 770 genes from 24 different immune cell types, common checkpoint inhibitors, and CT antigens.
- Assesses mechanistic pathway activity.
- Identifies TILs in the tumor microenvironment.
- 40 reference genes.



NanoString nCounter data – Differential Gene Expression





IDO1 is a key regulator of immune tolerance, expressed by both tumor cells and immune cells such as tumor-associated macrophages. High IDO1 expression in tumors is generally correlated with poor survival and prognosis.

Figure 2. Nanostring Analysis for Differential Gene Expression. A, Bargraph displaying the 23 genes with the highest fold change in recurrent GCTs compared to primary tumors ranging from 5-fold (PLAU) to 14-fold (IDO1) increases. **B, Box plot with min/max whiskers** displaying normalized mRNA levels (log2 fold change) for IDO1.

Macrophage Increase in Recurrent Tumors – mRNA & Protein Correlation

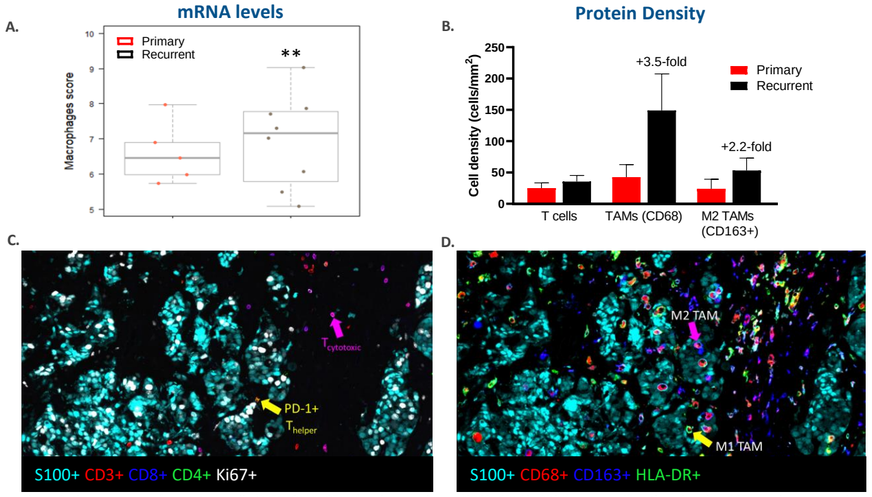
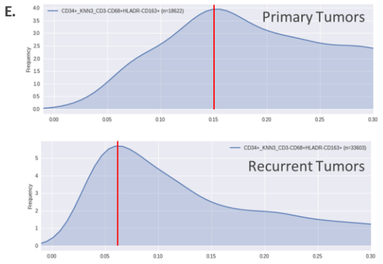
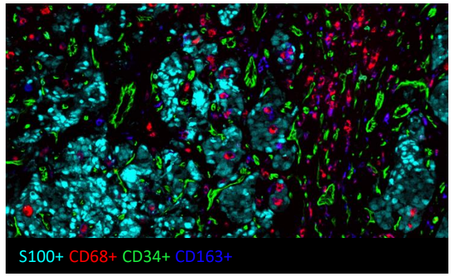


Figure 3. the gene scores for cytotoxic cells (PRF1, GNLY, GZMA) and macrophages (CD68, CD163, CD84) were significantly reduced in GCT samples compared **B**. Quantitation of protein density (cell #/mm²) of T cells (CD3+), tumor-associated macrophages/TAMs (CD68+), and M2-type TAMs (CD3-CD68+HLADR-CD163+). **C+D+F, multiplexed overlay images** of S100, CD34, CD3, CD68, Ki67, CD68, HLA-DR, and CD163. **G. Nearest Neighbor Spatial Analysis.** The average of the distance to the 5 nearest neighbors from vessels (CD34+ cells) to M2 TAMs is calculated. M2 TAMs were found to be in closer proximity to angiogenic vessels in primary tumors compared to recurrent tumor samples.

MultiOmyx Spatial Analytics – Nearest Neighbor Analysis



M2 TAMs are closer to vessels in recurrent compared to primary tumors.



Key Findings

- We have used a dual multiplexing approach to immunoprofile the tumor microenvironment of rare ovarian Granulosa Cell Tumor (GCT) FFPE samples on both mRNA level (Nanostring nCounter assay), and protein level (MultiOmyx analysis).
- IDO1 is one of several genes found to be significantly upregulated in recurrent GCTs compared to primary tumors.
- Concordance between the two assays was observed for tumor-associated macrophages (TAMs), as both the gene signatures and protein levels were increased in recurrent tumors compared to primary tumors.
- In a spatial analysis of angiogenic vessels and TAMs, M2 type TAMS were found to be in closer proximity to vessels in recurrent tumors compared to primary tumors.

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