**Introduction**

Invasive lobular breast cancer (ILC) is an understudied subtype of breast cancer with late recurrence, metastasis to visceral surfaces, such as the peritoneum, and dismal long-term survival. The dynamic interaction between the tumor and its microenvironment (TME) leads to phenotypic changes in stromal cells and the extracellular matrix to promote growth or invasion of malignant cells. ILC is histologically distinct from invasive ductal carcinoma, characterized by discohesive tumor cells that grow as a “single file” due to lack of the cell adhesion molecule E-cadherin. We therefore expect the TME to be quite unique in ILC. We hypothesized that offering levels of expression of nuclear receptors by tumor cells would impact cells residing within the stroma, presumably through paracrine mechanisms.

The nanotilting GenoMa Digital Spatial Profiling (DSP) platform is a powerful tool to spatially resolve and quantify RNA and protein expression in archived or fresh tissue samples. Tissues can be optimally segmented to profile tumor cells and tumor-associated microenvironment (TME) separately. We performed DSP using segmentation into Pan-cytokeratin-positive (tumor) and negative sectors (TME) to interrogate different gene and protein expression between GR-positive and GR-negative tumors in malignant cells and associated stromal regions in the tumor microenvironment (TME) of A+ in 12 invasive lobular breast cancer (ILC). We also utilized the spatial deconvolution tool to assess the contribution of tumor and immune cells in the tumor and TME in specimens with disparate expression of the glucocorticoid receptor (GR). A comparison of GR high and GR low ILC tumors will lend insight into the profibrotic and adhesive gene expression changes elicited by GR activation.

**Methods**

The ILC FFPE tissue sections were stained with four morphology markers: Fluorescent antibody markers for DNA (SYTO13), Pan-cytokeratin (Cy3), CD45 (Texas Red; pseudo-colored yellow). For the RNA assays, a RNAscope probe for GR (G1) tagged with Cy5. For the protein assays, a Cy5-labeled antibody was used. Selection of regions of interest (ROIs), segmentation for Pan-cyt and statistical analyses were conducted with the GeoMa DSP Analyser Suite Version 2.4.0.421. Spatial deconvolution to obtain cell type abundances was performed using a plugin tool from nanotilting and the tumourimmune cell matrix (https://plosone.org/article?id=10.1371/journal.pone.0158445). Pathway analyses were performed using Gene Set Enrichment Analysis (GSEA).

**Results**

Figure 1. (A) IHC for GR showing 2 cases positive for GR; 2 negative and 2 mixed (top). Bottom: Fluorescent images showing fluorescent antibody staining for morphology markers: PanCK (green), PanCK (yellow) and GR (red). (B) Segmentation for PanCK, ROIs are indicated by rectangles and numbers left (left). Shows the tissue for the stroma. From each ROI, RNA tags were collected for PanCK and PanCK- C. (C) Unsupervised clustering for all cases. Each row is a gene upregulated in GR+ cells. Color bar lower right may show PanCK and PanCK and GR and GR-ROIs.

Figure 2. Linear mixed model (LMM) analyses were performed to test different gene or protein expression between the tumors of 5 GR+ (ILC) and 5 GR– (ILC). Gene expression GSEA pathway enrichments were used to identify cell-type specific pathways. (A) Spatial deconvolution to measure the abundance of cells subsets based on RNA expression. Each bar represents one ROI. Color bars beneath graph is colored by patient and GR+ or GR–. (B) Violin plots from LMM analysis of proteins differentially expressed between regions of invasive (ILC) vs in situ (LCIS) cancer. Analyses were performed separately for tumor or TME.

**Results cont.**

Figure 3. Linear mixed model (LMM) analyses were performed to test different gene or protein expression between the tumors of 5 GR+ (ILC) and 5 GR– (ILC). Gene expression GSEA pathway enrichments were used to identify cell-type specific pathways. (A) Spatial deconvolution to measure the abundance of cells subsets based on RNA expression. Each bar represents one ROI. Color bars beneath graph is colored by patient and GR+ or GR–. (B) Violin plots from LMM analysis of proteins differentially expressed between regions of invasive (ILC) vs in situ (LCIS) cancer. Analyses were performed separately for tumor or TME.

Figure 4. (A) Immunofluorescent images from protein DSP showing increase ILC (left) and in situ LCIS (right) regions of the same tumor from case 36733 (ILC) - (B) Fluorescent image from protein DSP showing ILC and LCIS regions of the same tumor from case 30507-3 (GR variable expression). (C-F) Violin plots from LMM analysis of proteins differentially expressed between regions of invasive (ILC) vs in situ (LCIS) cancer. Analyses were performed separately for tumor or TME.

**Conclusions**

- GR promotes an immune suppressive environment in ILC
- Patients with ILC tumor microenvironment is heterogeneous regardless of GR expression in the tumor cells
- GR+ tumors may exclude cytotoxic T-cells
- Cancer checkpoint proteins may be disregulated in GR expressing ILC
- GeoMa DSP is a powerful tool to resolve gene expression in a spatial context
- Tumors heterogeneity between and within tumors
- Gross insight into the localized variations in the complex milieu of the stroma
- Can analyze RNA and protein within the same ROIs