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Single-cell immunoprofiling and spatial analysis of hormone receptor subtypes in HER2+ and HER2low breast tumors using multiplexed immunofluorescence

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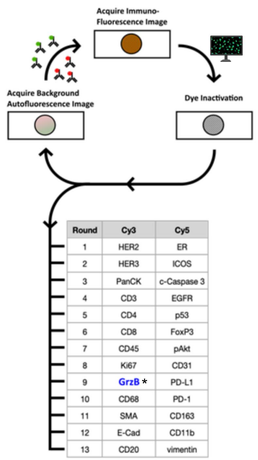
Abstract#4639

Background: Breast cancer is characterized by distinct molecular subtypes based on expression of the ER and PR hormone receptors, and epidermal growth factor receptor 2 (HER2). To investigate presence and location of multiple distinct immune cell populations on the single cell level we utilized the multiplexed immunofluorescence (mIF) platform MultiOmyx™ to investigate the tumor immune microenvironment (TME) in HER2+ breast cancer. We have built on growing data implicating distinct immunophenotypes in the breast cancer TME with breast cancer outcomes by profiling 1) prevalence; 2) location (e.g. intratumoral, stromal); 3) phenotype (e.g. activated, exhausted) of infiltrating immune cells.

Methods: We optimized a custom 26-marker MultiOmyx panel interrogating HER2 immunofluorescence (IF) expression (HER2exp), HER2 signaling, stromal, and immune markers. This mIF platform leverages serial IF image capture to allow concurrent profiling of all 26 markers on a pathologic section at single cell resolution. We applied the 26-marker panel to a tissue microarray of 208 unique patients with matched tumor/normal tissue cores (1-4 cores/patient; total 333 tumor and 307 normal cores). HER2-positive was defined via ASCO/CAP guidelines; HER2-low was defined as HER2 immunohistochemistry (IHC) 1+/2+ but HER2 in-situ hybridization (ISH) negative.



Round	Cy3	Cy5
1	HER2	ER
2	HER3	ICOS
3	PanCK	c-Caspase 3
4	CD3	EGFR
5	CD4	p53
6	CD8	FoxP3
7	CD45	pAkt
8	Ki67	CD31
9	GrzB *	PD-L1
10	CD68	PD-1
11	SMA	CD163
12	E-Cad	CD11b
13	CD20	vimentin

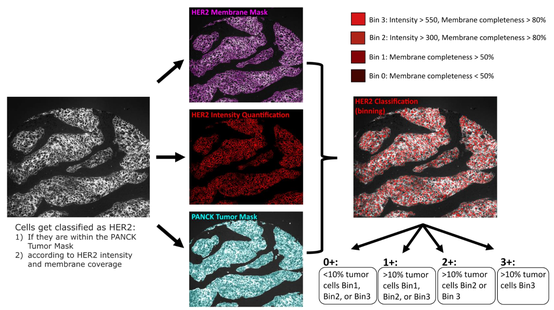
Characteristic	HER2-Positive N=151	HER2-Low* N=20
Median Age at Diagnosis (range) - Years	53 (27-88)	60 (40-79)
Self-Reported Race or Ethnicity (%)		
Caucasian	135 (89.4)	19 (95)
Black	10 (6.6)	1 (5)
Hispanic	2 (1.3)	0
Asian	1 (0.7)	0
Other	3 (2.1)	0
Primary Cancer Histology (%)		
Infiltrating Ductal Carcinoma	143 (95)	18 (90)
Infiltrating Lobular Carcinoma	3 (2.0)	1 (5)
Mixed Infiltrating Ductal and Lobular Carcinoma	1 (0.7)	1 (5)
Metaplastic Carcinoma, NOS	1 (0.7)	0
Papillary Carcinoma	1 (0.7)	0
Adenocarcinoma, NOS	2 (1.3)	0
Histologic Grade (%)		
1	4 (2.6)	3 (15)
2	44 (29.1)	11 (55)
3	102 (67.5)	6 (30)
Unknown	1 (0.7)	0
Overall Pathologic Stage (%)		
I	53 (35.1)	6 (30)
II	66 (43.7)	9 (45)
III	32 (21.2)	5 (25)
Lymphovascular Invasion (%)		
Yes	66 (43.7)	5 (25)
No	68 (45.0)	9 (45)
Unknown	17 (11.3)	6 (30)
Estrogen Receptor Status (%)		
Positive	83 (55)	15 (75)
Negative	68 (45)	5 (25)
Progesterone Receptor Status (%)		
Positive	65 (43)	12 (60)
Negative	86 (57)	8 (40)
Receptor Subtype* (%)		
HR+ HER2Low	0	15 (75)
HR+ HER2+	87 (57.6)	0
HR- HER2Low	0	5 (25)
HR- HER2+	64 (42.4)	0
HER2-Targeted Therapy Received (%)		
Yes	140 (92.7)	0
No	11 (7.3)	20 (100)
HER2-Targeted Therapy First Exposure Setting (%)		
Neoadjuvant	11 (7.3)	0
Adjuvant	123 (81.5)	0
Metastatic	6 (4.0)	0
None	11 (7.3)	20 (100)
Median Duration of HER2-Targeted Therapy (range) - Days	124 (17-5908)	0
Median Overall Survival (range) - Months	141 (7-215)	170.5 (21-219)

*HR = Hormone Receptor Status (Estrogen Receptor and/or Progesterone Receptor)

Figure 1. For MultiOmyx multiplexing two conjugated fluorescent antibodies are applied per round, followed by image acquisition of the stained slides. The dye is erased, enabling a subsequent round of staining with another pair of fluorescent antibodies. Table) Patient characteristics. *Granzyme B did not pass the image QC and was excluded from the study.

Image Analysis Workflow

We developed a novel machine-learning system to quantify single-cell HER2 IF expression



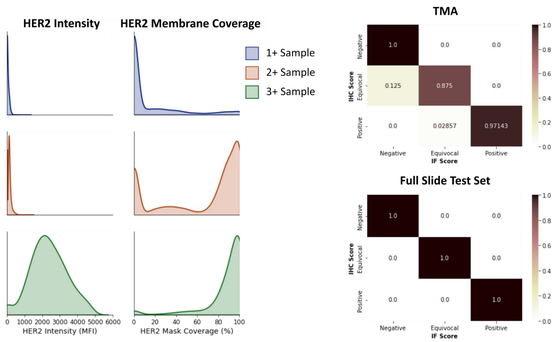


Figure 2. Our HER2 IF scoring system (**A**) fits a segmentation mask to HER2 IF membrane staining and uses this mask to extract single-cell expression metrics (**B**). Malignant cells (PanCK+, classified via the standard MultiOmyx pipeline) were scored into four bins according to HER2 intensity and membrane completeness (**A**). Approximating the ASCO/CAP HER2 guidelines, tumors were classified as 0, 1+, 2+, or 3+ based on the prevalence of cells belonging to each bin. Tumors with <10% of cells exhibiting faint/barely perceptible HER2 intensity (Bin0) were classified as HER2 0; >10% of cells exhibiting faint-strong HER2 intensity (Bin1-3) were classified as HER2 1+; >10% of cells with moderate-strong HER2 intensity (Bin2-3) were classified as HER2 2+; and >10% and strongly positive HER2 intensity (only Bin 3) were classified as HER2 3+. When comparing clinical HER2 IHC scoring with our IF based classification, the performance was excellent, achieving overall concordance of 97.9%, analytic sensitivity of 100%, and analytic specificity of 97.1% on the TMA training set and 100% concordance, 100% analytic sensitivity, and 100% analytic specificity on the full slide test set (**C**).

Sample-level Cluster Analysis

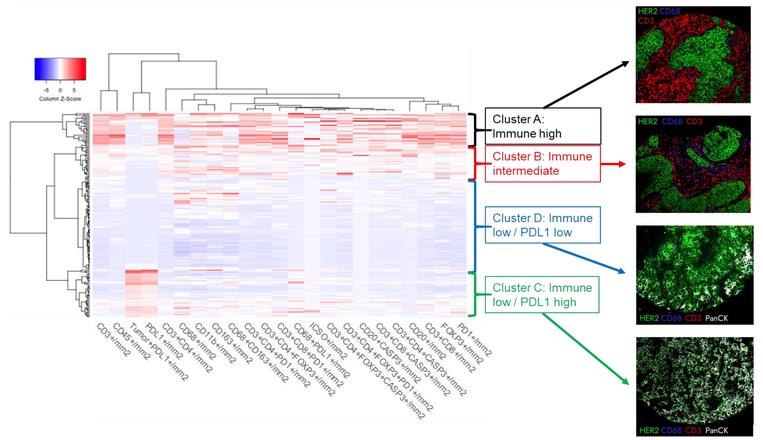


Figure 3. Immune lineage-specific and functional markers were interrogated as single markers and established immune cell phenotypes based on combinations of markers, with number of positive cells per mm² (mean per patient for multiple cores). Patterns of patient-level immunophenotypes were interrogated via unsupervised hierarchical clustering, which identified four main clusters: immune-high, immune-intermediate, immune-low/tumor PDL1 (tPDL1) high, immune-low/tPDL1 low. Example images provided.

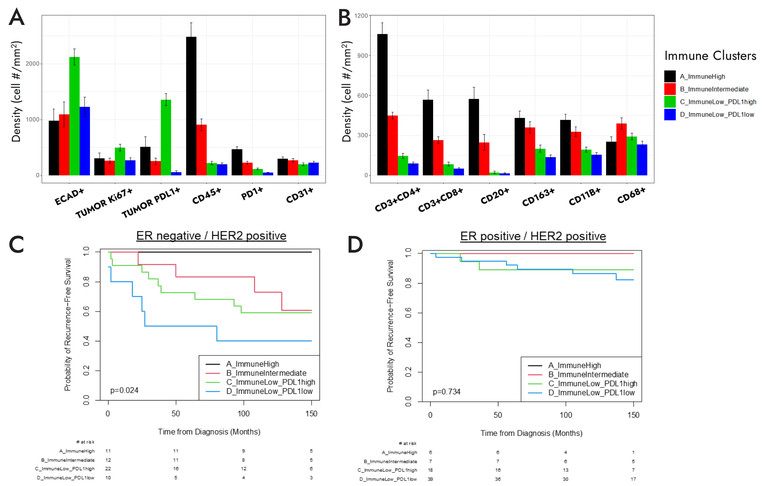


Figure 4. **(A-B)** Quantification results of the density of selected biomarkers in the MultiOmyx panel were generated by applying the proprietary deep-learning based cell classification platform NeoLYTX™ to MultiOmyx multiplexed IF images. Error bars show SEM, and significance was calculated by a two-tailed, unpaired t-test. **(C,D)** Among hormone receptor (HR)-negative, HER2+ tumors, there was significant association with immune cluster and RFS. Among HR+/HER2+ tumors, there were fewer immune-high or -intermediate cancers and fewer events, but a similar trend was seen.

Spatial Cluster Analysis by HR Subtype

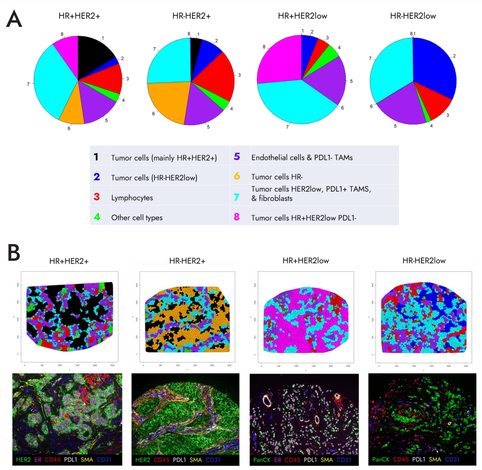


Figure 5. Spatial immune composites. The phenotypes of each cell and its 9 nearest neighbors (a "neighborhood" of 10 cells) are tallied. High-dimensional clustering is applied to the neighborhood phenotype frequencies, resulting in eight clusters or neighborhood types corresponding to different types of tumor and stromal tissue. (A) Prevalence of the eight clusters by cancer subtype. (B) Voronoi diagrams (colored by each cell's cluster) and IF overlays for representative ROIs for the different cancer types.

Key Take-Aways

- We developed a novel machine-learning system to quantify single-cell HER2 IF expression and translate this expression data into a clinically relevant HER2 scoring system.
- Patient-level immunophenotype based on 23 distinct immune cell types reveals unique subsets associated with outcome
- Among hormone receptor (HR)-negative, HER2+ tumors there was significant association with immune clusters and RFS.

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