Transcriptomic and immune heterogeneity drive aggressive ILC

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Introduction

Invasive Lobular Carcinoma (ILC) is the second most common histological subtype of breast cancer (BC) accounting for 10 - 15% of all BCs. It is a unique disease entity with distinct histological appearances, molecular alterations and clinicopathologic features. It also has a unique yet poorly understood tumour immune microenvironment.

A subgroup of ILC patients have clinically aggressive disease with metastases occurring early (< 3 years) after primary diagnosis. In particular pleomorphic ILC, a distinct histological subtype has been associated with more aggressive clinical features. These patients have limited treatment options and represent a clinically unmet need. There is thus a need to better understand the molecular basis and transcriptional drivers of aggressive ILC, as well of the immune landscape to help identify potential new drug targets to improve patient outcomes.

Methods

In this project we:

1) Perform RNA sequencing in needle macro-dissected pleomorphic ILCs (n = 47) and create a prognostic gene expression risk predictor using a random forest model to validate this in independent ILC cohorts
2) Characterise the immune microenvironment histologically through the quantification of stromal TILs in non-pleomorphic (n = 100) and pleomorphic (n = 63) ILC
3) Characterise the immune subpopulations at the protein level in pleomorphic ILCs (stromal TILs ≥ 5%, n = 20) using NanoString Digital Spatial profiling with the GeoMx® Human Immuno-Oncology (IO) Panel and validate findings using immunohistochemistry (IHC) for CD68 and dual IHC (CD68/CD163)
4) Characterise transcriptomic heterogeneity and associations with the immune infiltrate in pleomorphic ILCs (stromal TILs ≥ 4%, n = 10) using NanoString GeoMx® Human Whole Transcriptome Atlas

Identification of a pleomorphic gene expression risk predictor

356 prognostic genes from KHP discovery cohort predict overall survival in TCGA, METABRIC, SCAN-B

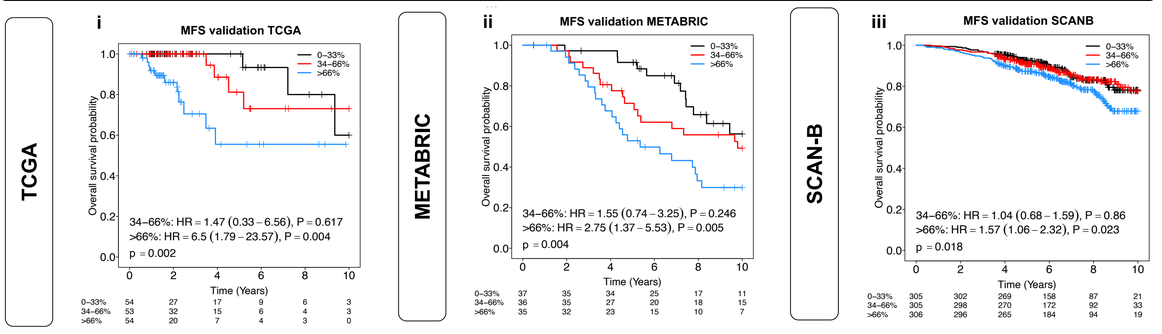


Figure 1: Validation of prognostic gene expression signature in independent ILC cohorts: 356 genes were identified as significantly associated with metastasis-free survival (MFS) in the pleomorphic KHP cohort. Using a random forest model these predict overall survival (OS) in i) TCGA (n = 161, p = 0.002), ii) METABRIC (n = 109, p = 0.004), iii) SCAN-B (n = 917, p = 0.018)

Pleomorphic ILC has higher stromal TILs

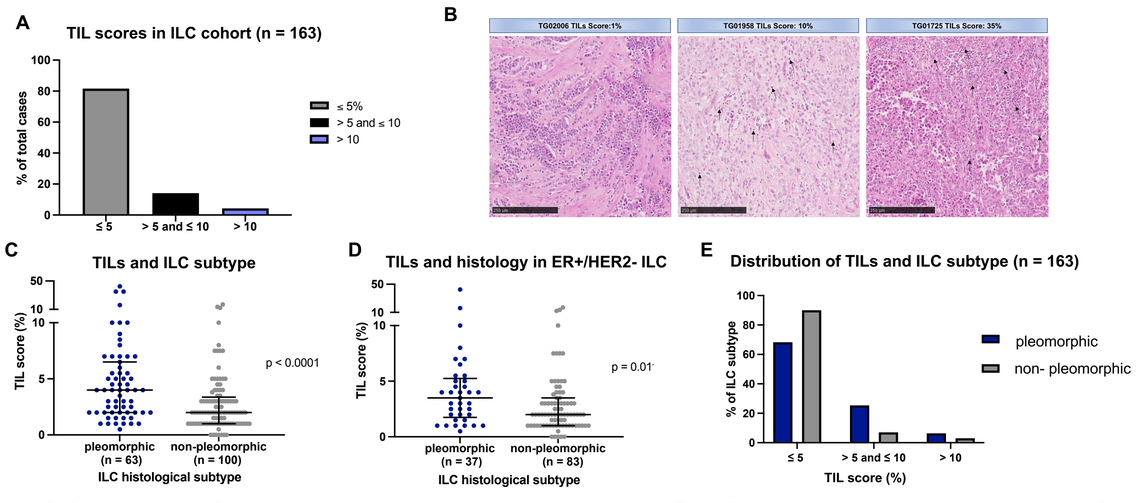


Figure 2: Pleomorphic ILC is more immunogenic than non-pleomorphic ILC: A) Bar chart showing the proportions of all ILCs within each TIL assessment category (n = 163) B) H&E sections from pleomorphic ILC cases showing low, intermediate and high TIL scores C) Scatter plot showing TIL scores for pleomorphic (n = 63) and non-pleomorphic (n = 100) ILC with higher scores in the pleomorphic group (p < 0.0001, Mann-Whitney U test) D) Scatter plot showing individual TIL scores for ER+/HER2- pleomorphic (n = 37) and non-pleomorphic (n = 83) ILC showing higher TIL scores in the pleomorphic group (p = 0.01, Mann-Whitney U test) E) Bar chart showing proportions of pleomorphic (n = 63) and non-pleomorphic (n = 100) ILCs within each TIL group.

High CD68+ cells are associated with early relapse in pleomorphic ILC

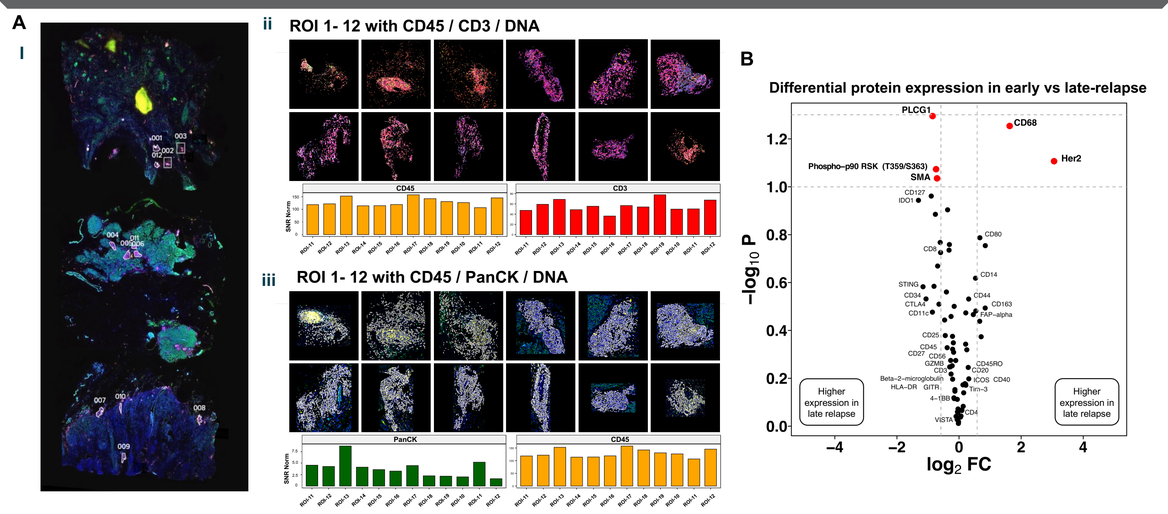


Figure 3: NanoString DSP identifies association between high CD68+ cells and early relapse: A) Representative Immunofluorescent NanoString DSP images demonstrating immune heterogeneity across selected ROIs: i) IF image showing morphology markers: PanCK/CD45/CD3/DNA and 12 selected ROIs ii) IF images showing individual ROIs with **CD45 (yellow)**, **CD3 (red)** and **DNA (blue)** channels **iii)** IF images showing individual ROIs with **PanCK (green)**, **CD45 (yellow)** and **DNA (blue)** channels B) Differential protein expression in early vs late relapsing pleomorphic ILCs (n = 9) showing higher levels of CD68+ cells (macrophages) in early relapse.

The macrophage M2/M1 ratio is prognostic in pleomorphic ILC

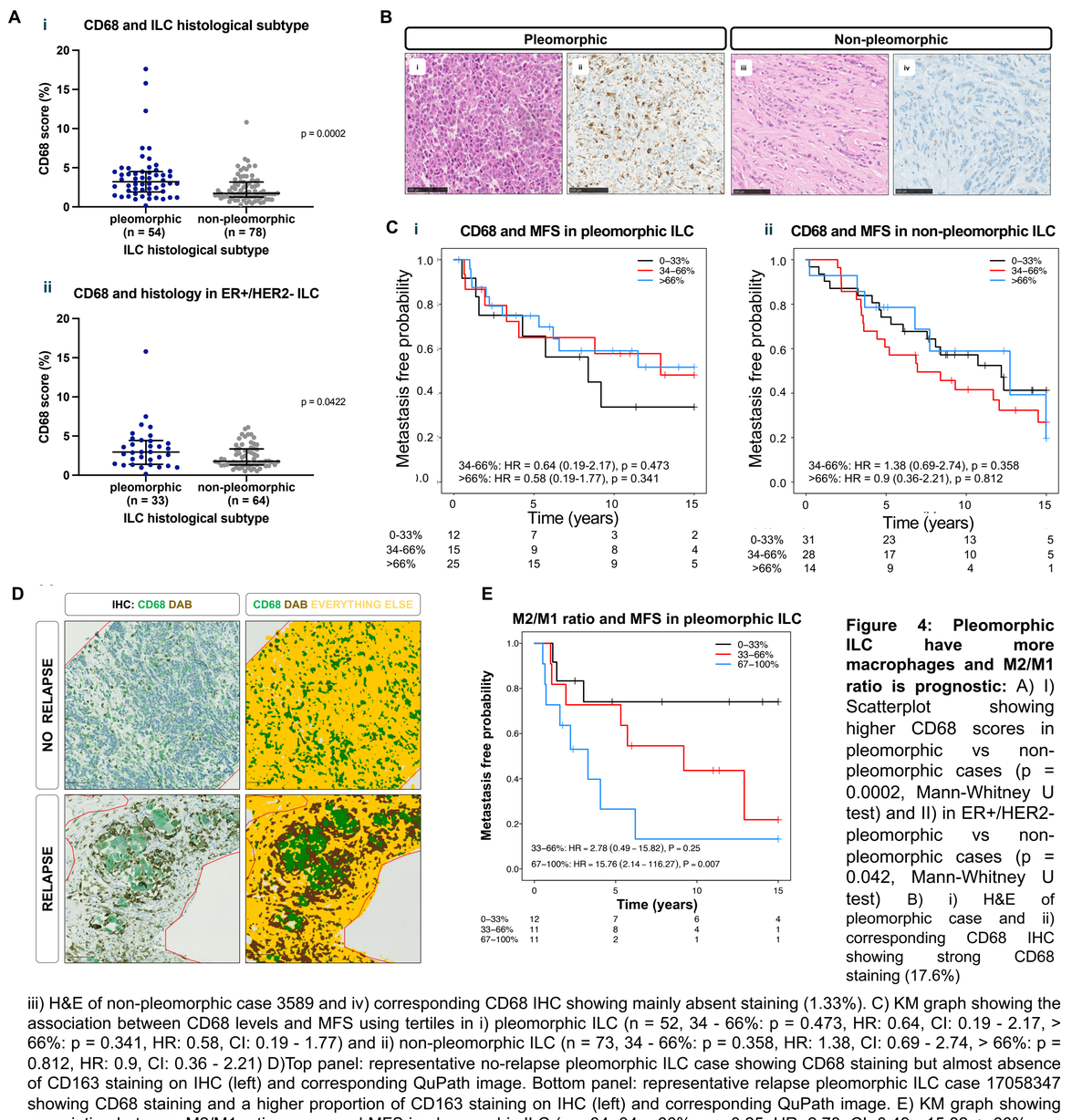
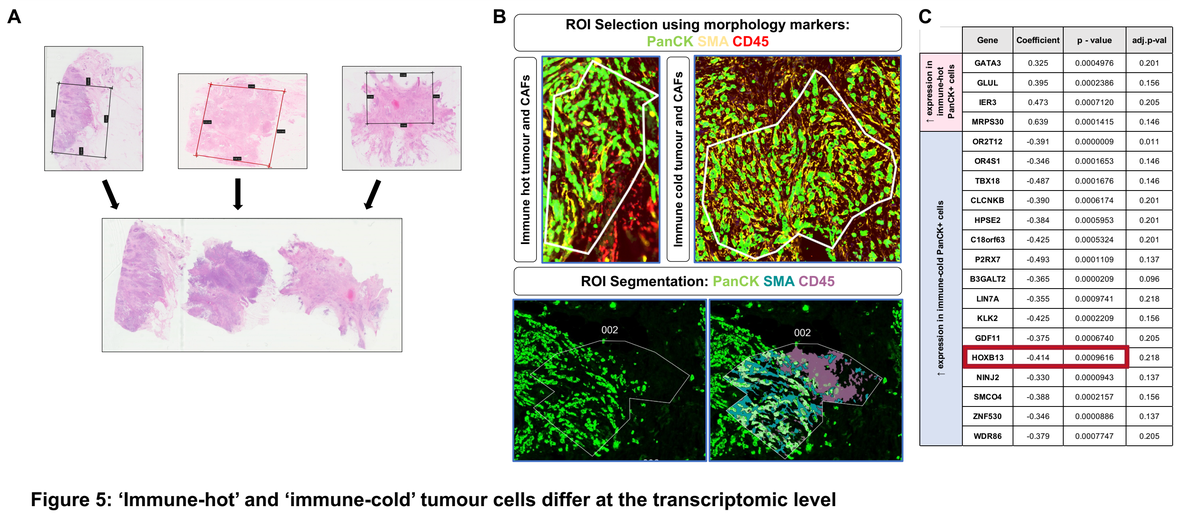


Figure 4: Pleomorphic ILC have more macrophages and M2/M1 ratio is prognostic: A) I) Scatterplot showing higher CD68 scores in pleomorphic vs non-pleomorphic cases (p = 0.0002, Mann-Whitney U test) and II) in ER+/HER2- pleomorphic vs non-pleomorphic cases (p = 0.042, Mann-Whitney U test) B) i) H&E of pleomorphic case and ii) corresponding CD68 IHC showing strong CD68 staining (17.6%) iii) H&E of non-pleomorphic case 3589 and iv) corresponding CD68 IHC showing mainly absent staining (1.33%). C) KM graph showing the association between CD68 levels and MFS using tertiles in i) pleomorphic ILC (n = 52, 34 - 66%: p = 0.473, HR: 0.64, CI: 0.19 - 2.17, > 66%: p = 0.341, HR: 0.58, CI: 0.19 - 1.77) and ii) non-pleomorphic ILC (n = 73, 34 - 66%: p = 0.358, HR: 1.38, CI: 0.69 - 2.74, > 66%: p = 0.812, HR: 0.9, CI: 0.36 - 2.21) D)Top panel: representative no-relapse pleomorphic ILC case showing CD68 staining but almost absence of CD163 staining on IHC (left) and corresponding QuPath image. Bottom panel: representative relapse pleomorphic ILC case 17058347 showing CD68 staining and a higher proportion of CD163 staining on IHC (left) and corresponding QuPath image. E) KM graph showing association between M2/M1 ratio scores and MFS in pleomorphic ILC (n = 34, 34 – 66%: p = 0.25, HR: 2.78, CI: 0.49 - 15.82, > 66%: p = 0.007, HR: 15.76, CI: 2.14 - 116.27).

'Immune-hot' and immune-cold' tumour cells differ at the transcriptomic level



	Gene	Coefficient	p - value	adj.p-val
↑ expression in immune-hot / PanCK+ cells	GATA3	0.325	0.0004976	0.201
	GLUL	0.395	0.0002386	0.156
	IER3	0.473	0.0007120	0.205
	MRPS30	0.639	0.0001415	0.146
↑ expression in immune-cold PanCK+ cells	OR2T12	-0.391	0.0000009	0.011
	OR4S1	-0.346	0.0001653	0.146
	TBX18	-0.487	0.0001676	0.146
	CLCNKB	-0.390	0.0006174	0.201
	HPSE2	-0.384	0.0005953	0.201
	C18orf63	-0.425	0.0005324	0.201
	P2RX7	-0.493	0.0001109	0.137
	B3GALT2	-0.365	0.0000209	0.096
	LIN7A	-0.355	0.0009741	0.218
	KLK2	-0.425	0.0002209	0.156
	GDF11	-0.375	0.0006740	0.205
	HOXB13	-0.414	0.0009616	0.218
	NINJ2	-0.330	0.0000943	0.137
	SMCO4	-0.388	0.0002157	0.156
	ZNF530	-0.346	0.0000886	0.137
	WDR86	-0.379	0.0007747	0.205

Figure 5: 'Immune-hot' and 'immune-cold' tumour cells differ at the transcriptomic level

A) Selection of representative 10 x 13mm tumour regions from 3 pleomorphic ILCs orientated on the NanoString slides C) Selection and segmentation of 'immune-hot' and 'immune-cold' tumour regions of interest (ROIs): Top panel shows two distinct ROIs; left is an 'immune-hot' ROI characterised by the presence of CD45+ immune cells, PanCK+ tumour cells and *α-SMA* CAFs and right showing an 'immune-cold' ROI characterised by the presence of PanCK+ tumour cells (green) and *α-SMA* CAFs. Bottom panel shows the segmentation of an individual 'immune-hot' ROI into distinct PanCK+ tumour, *α*-SMA+ CAF and CD45+ immune cell compartments known as 'areas of interest' (AOIs). C) Twenty differentially expressed genes between 'immune-hot' and 'immune-cold' ROIs (P < 0.001). *HOXB13* is associated with disease relapse and early relapse (< 3 years) in SCAN-B (n = 386).

Conclusions

- **We generated a prognostic gene expression signature associated with metastases-free survival that validates in independent ILC cohorts.**
- **The majority of ILCs have low immune infiltrates, yet a minority of cases have higher infiltrates.**
- **Pleomorphic ILC has higher stromal TILs compared to non-pleomorphic ILC but the gross quantification of stromal TILs is not associated with clinical outcome in pleomorphic or non-pleomorphic ILC.**
- **Pleomorphic ILC has higher levels of macrophages compared to non-pleomorphic ILC. Whilst total CD68+ cells (macrophages) are not prognostic in pleomorphic and non-pleomorphic ILC, a high M2/M1 ratio is associated with worse metastasis-free survival in pleomorphic ILC.**
- **NanoString GeoMx® Human Whole Transcriptome identifies differences between 'immune-hot' and 'immune-cold' tumour cells at the gene expression level in pleomorphic ILC and identifies genes associated with a poor prognosis, highlighting that the immune landscape may shape the aggressive nature of the tumour cells.**

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Conflict of Interest: The authors declare no conflict of interest.