High tumour-infiltrating lymphocytes correlate with distinct gene expression profile and favourable survival in single hormone receptor-positive breast cancer

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Introduction: This study aimed to evaluate the impact of tumour-infiltrating lymphocytes (TILs) on the expression of immune-related genes and prognosis in single hormone receptor-positive breast cancer. Material and methods: Tumour-infiltrating lymphocytes were analysed according to the guidelines of the International TILs Working Group in a cohort of 206 patients with single hormone receptor-positive breast cancer. Of these, 44.7% were classified as ER+/PgR–/HER2–, 18.4% as ER+/PgR–/HER2+, 26.2% as ER–/PgR+/HER2–, and 10.7% as ER–/PgR+/HER2+. Moreover, in 52 samples the analysis of gene expression profiling was performed using nCounter technology.
Results: Most cases (74.3%) showed at least 1% of stromal TILs, with a median of 4%, mean of 16.3%, and interquartile range of 0–20%. ER–/PgR+ tumours displayed significantly higher TILs density than ER+/PgR– cases (p < 0.001, Wilcoxon test), regardless of HER2 status. The abundance of TILs was positively associated with ER–/PgR+ phenotype, higher Ki-67, and higher grade, but not with age, tumour size, or regional and distant metastases at diagnosis. Additionally, in ER+/PgR– subgroup higher TILs were associated with HER2-positive status. Stromal TILs > 5% were associated with better survival in the whole group, but this effect was less prominent in ER–/PgR+ patients. We identified 50 differentially expressed genes (DEGs) between single hormone receptor-positive breast tumours with high and low TILs, including 39 up-regulated and 11 down-regulated genes in the high TILs group.
Conclusions: The up-regulated expression of immune-related genes was consistent also among separately analysed single hormone receptor-positive groups (ER+/PgR– and ER–/PgR+).

Key words: gene expression, tumour infiltrating lymphocytes, prognosis, breast cancer.

Introduction

Mutations in cancer cells generate new cancer cell-specific antigens (neoantigens), eliciting a host immune response against cancer cells [1]. In a favourable scenario, activated innate and adaptive immune cells may effectively eliminate neoplastic cells. However, tumours are capable of immune escape facilitating cancer development. The biomarker of the persistent immune response against cancer is the presence of tumour-infiltrating lymphocytes (TILs) inside the tumour (intratumoural TILs) and on its borders (stromal TILs). According to the recommendations of the International TILs Working Group TILs assessment should include the percentage of lymphocytes in the stroma within the invasive tumour borders, excluding areas of crush artifacts, necrosis, reactive stromal hyalinisation, and the site after a previous biopsy. Tumour-infiltrating lymphocytes should be evaluated as an averaged continuous parameter, without focusing on hotspots [2]. The abundance of TILs reflects the tumour mutational burden (TMB), which in breast cancer (BC) is average compared to other cancers, with only 5% of BC considered TMB high [3, 4]. The main component of TILs are T cells, B cells, monocytes, and NK cells, which usually account for about 75%, < 20%, < 10%, and < 5%, respectively [5]. The cellular composition and prevalence of TILs vary in different types of BCs. Some subpopulations promote whereas others suppress tumour progression. Inflammatory infiltration already occurs in benign ductal hyperplasia and ductal carcinoma *in situ* (DCIS) [6]. Interestingly, in DCIS dense TILs are associated with negative oestrogen receptor (ER) status and shorter recurrence-free survival (RFS) [7, 8]. Number

of TILs, cellular composition of the inflammatory infiltrate, and expression of immune-related genes have predictive and prognostic significance in patients with HER2– amplified/overexpressing (HER2+ BC) and triple-negative breast cancer (TNBC). In the group of 294 BC patients, higher number of TILs was linked to ER– and HER2+ phenotype, larger tumour size, higher grade, higher Ki-67, and metastases to lymph nodes [9]. In the neoadjuvant setting high TILs in biopsy specimen predict favourable response to chemotherapy, and the presence of TILs in residual disease correlates with better outcomes in TNBC [10]. Similar trends were observed in HER2+ BC [11]. Much less is known about the role of TILs in hormone-dependent BC, especially single hormone receptor-positive BC (i.e. ER+/PgR– and ER–/PgR+). In a large study on ER+/HER2+ and ER+/HER2– tumours, high TILs were associated with unfavourable clinicopathological features but in patients treated with adjuvant chemotherapy correlated with longer distant disease-free survival (DFS) [12]. Interestingly, in the metastatic lesions, TILs are usually less abundant [13]. In a group of 94 patients with TILs assessed in BC metastases, lower abundance of TILs was found in younger patients, while higher TILs were correlated with prolonged overall survival (OS). Moreover, the composition of TILs differed depending on the location of the metastasis [14].

In BC the abundance of TILs correlates with immune-related gene expression profiles [15]. Interestingly, specific immunological profiles of BC microenvironment correlate with steroid hormone receptor status. Among luminal BCs, 3 subgroups were distinguished based on the expression of immune-related genes, one of which was characterised by higher amount of TILs and lower expression of *ESR1/ESR2,* supporting the relationship between weaker hormone receptor expression and enhanced immune reaction in BC [16].

In the current study, we aimed to investigate the role of TILs in single hormone receptor-positive BCs. We analysed associations with clinicopathological features, gene expression profiles, and long-term outcomes.

Material and methods

Study group

Tumours from 212 female patients diagnosed with single hormone receptor-positive BC were collected. Basic clinicopathological data (ER, PgR and HER2 status, Ki-67 proliferation index, grade, histological type, pathological tumour-node-metastasis [pTNM], use of neoadjuvant systemic treatment, age at diagnosis, date of diagnosis, date of relapse, and date of death) were retrieved from local databases. All ER–/PgR+ BC cases incorporated in the study had their phenotype adequately confirmed as described previously [17]. Subsequently, available microscopic slides were collected and subjected to analysis. Six patients were excluded from the subsequent analyses due to lack of slides for the assessment of TILs, leaving 206 patients in the study.

Evaluation of tumour-infiltrating lymphocytes

Assessment of stromal TILs was performed according to the International TILs Working Group guidelines by 2 pathologists (MK and RP) [2]. In the case of discrepant opinion, the final score was established after a discussion between pathologists. In general, one selected representative haematoxylin and eosin section from each case was analysed under 200–400× magnification. The percentage of the stromal TILs was assessed only within the invasive tumour borders. Tumour-infiltrating lymphocytes were scored as continuous variables and later subcategorised as low (0–5% of stromal TILs), and high (> 5% of stromal TILs).

RNA extraction

Total RNA was extracted from 52 samples prepared as formalin-fixed paraffin-embedded primary tumour tissue cores (4 cores of 0.6 mm diameter per patient, sampled from representative tumour areas) using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, US) with an Agilent RNA 6000 Pico Kit (Agilent Technologies).

nCounter gene expression assay

nCounter gene expression analysis technology was employed to decipher transcriptomic differences between single hormone receptor-positive BCs according to TILs abundance. Extracted RNA (4 µl) was pre-amplified using nCounter Low RNA Input Kit (NanoString Technologies, USA) with a dedicated Primer Pool covering sequences of 758 genes included in nCounter Breast Cancer 360 Panel (NanoString Technologies, USA). Pre-amplified samples were analysed using NanoString nCounter Analysis System (NanoString Technologies, USA) according to the manufacturer's procedures for hybridisation, detection, and scanning, with BC 360 Panel Standard included in each run.

Background correction and normalisation were performed using nSolver 4.0 software (NanoString Technologies, USA). In brief, the background level was estimated by thresholding over the mean plus 2 standard deviations of negative control counts. Subsequently, the data were normalised according to the global mean of the counts of positive controls and the 3 most stably expressed housekeeping genes – *OAZ1*, *TFRC*, and *PUM1*. Breast Cancer 360 Panel Standard was used for batch calibration. The negative and positive control probes were included in the assay.

Following normalisation, low-expression genes ($\log_2$ median count in all samples < 6) were excluded, leaving 533 target genes for analysis. The collected RNA expression results were then analysed according to the TILs score of the corresponding primary tumour. Differences in gene expression between the TILs groups were presented as $\log_2$ fold change ($\log_2$FC). Genes with median-based $\log_2$FC ≥ 1 were considered as up-regulated and genes with $\log_2$FC ≤ -1 as down-regulated. Selected genes were associated with GO and Reactome terms using the STRING database [18].

Statistics

Statistical analyses were performed with the use of Statistica 13 (RRID:SCR_014213, Tibco, CA, USA) licensed to the Medical University of Gdańsk, and R statistical environment [19]. Categorical variables were compared by

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Fisher's exact test or χ^2 test with Yates correction. Continuous variables were analysed utilising the Wilcoxon test or Kruskal-Wallis test when applicable. Overall survival was defined as the time from the diagnosis to the date of death from any cause. Disease-free survival was defined as the time from the diagnosis to the date of relapse (either local or distant) or death and was analysed only in M0 cases (199 cases). Event-free survival (EFS) was calculated from the date of diagnosis to the earliest date of disease progression, relapse, or death from any cause. Kaplan-Meier curves were plotted to calculate the survival rates. The log rank test was used to compare survival between groups. Hazard ratios (HRs) were estimated using Cox regression analysis. A p-value < 0.05 was considered significant; in cases of multiple comparisons, p-values were adjusted with Benjamini-Hochberg correction. Boxplots and scatterplots were produced using the 'ggplot2' and 'ggstatsplot' packages [20, 21]. Kaplan-Meier curves were plotted using the 'survminer' and 'ggsci' packages [22, 23].

Data availability statement

All data generated or analysed during this study are included in this article. Further enquiries can be directed to the corresponding author.

Results

In the study group, 130 (63.1%) patients were classified as ER+/PgR– and 76 (36.9%) as ER–/PgR+ (Table 1). The frequency of HER2 overexpression/amplification did not differ between groups (p = 0.718, χ^2). The mean age of the patients was 61 years (range 28–91, interquartile range, IQR 53–69). Patients in ER+/PgR– were significantly older (p = 0.0218, Wilcoxon test), and their tumours had lower Ki-67 index (p < 0.001, Wilcoxon test). Tumours with ER–/PgR+ phenotype more commonly displayed grade 3 histology when compared to ER+/PgR– cancers that were predominantly grade 2 (p < 0.001, χ^2). Neoadjuvant treatment was administered to 34 (44.7%) patients in the ER–/PgR+ group and 9 patients (7%) in the ER+/PgR– group (p < 0.001, χ^2). ER–/PgR+ tumours were significantly larger (p < 0.001, Wilcoxon test) and had higher pT descriptors in TNM (p = 0.044, χ^2). Moreover, ER–/PgR+ tumours were more commonly associated with the presence of distant metastases (p = 0.048, χ^2). However, there was no significant difference in the frequency of nodal metastases (p = 0.187, χ^2). Median follow-up was 38.5 months (IQR 21.5–76 months). Twenty-four (18.5%) ER+/PgR– patients died and 22 (16.9%) had a relapse/progression during follow-up, compared to 12 (15.8%) and 11 (14.5%) ER–/PgR+

Table 1. Main clinicopathological features of the study groups

Parameters	TILs analysis population, N = 206		Gene expression analysis population, N = 52	
Feature	*n*	%	*n*	%
Phenotype				
ER+/PgR–/HER2–	92	44.5	14	26.9
ER+/PgR/HER2+	38	18.4	11	21.2
ER–/PgR+/HER2–	54	26.3	17	32.7
ER+/PgR/HER2+	22	10.7	10	19.2
T				
1	88	42.7	20	38.5
2–4	114	55.3	32	61.5
n/a	4	1.9	0	0
N				
0	123	59.7	34	65.4
1–3	79	38.3	18	34.6
n/a	4	1.9	0	0
M				
0	195	94.7	52	100
1	7	3.4	0	0
n/a	4	1.9	0	0
Grade				
1	20	9.7	2	3.8
2	92	44.7	15	28.8
3	94	45.6	35	67.3
Neoadjuvant treatment				
No	162	78.6	46	88.5
Yes	43	20.9	6	11.5
n/a	1	0.5	0	0

n/a – not applicable, TILs – tumour-infiltrating lymphocytes
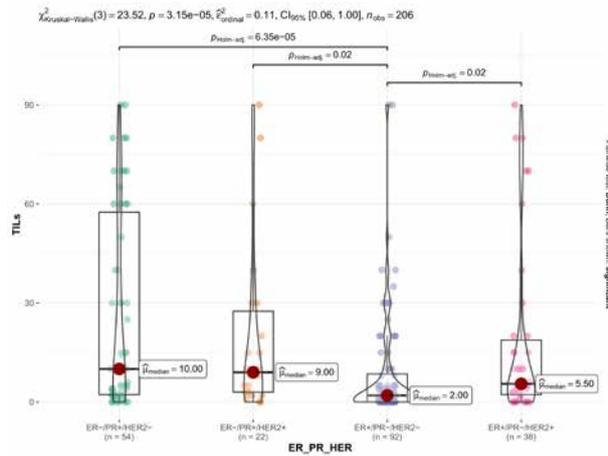** The percentages may not add up to 100% due to rounding.*

Fig. 1. Tumour-infiltrating lymphocytes density according to joint ER/PgR/HER2 phenotype
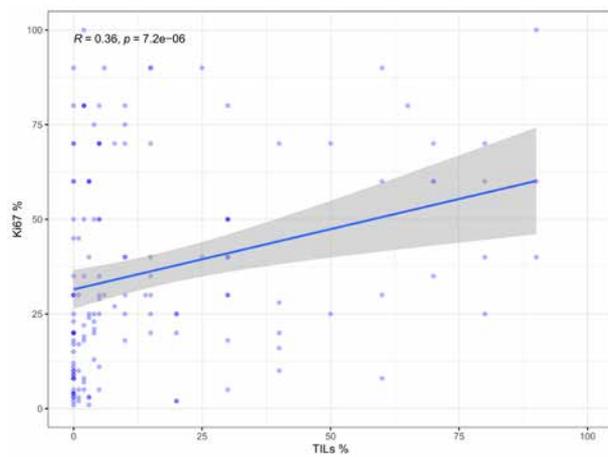

Fig. 2. Correlation between Ki-67 and tumour-infiltrating lymphocytes percentage in the whole cohort
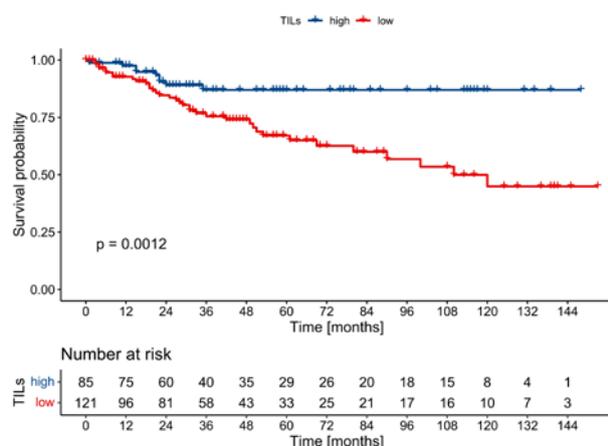

Fig. 3. Kaplan-Meier curves for event-free survival in the whole cohort according to tumour-infiltrating lymphocytes status

patients, respectively. There were no significant differences in OS, DFS, and EFS between ER+/PgR– and ER–/PgR+ patients. When subcategorised according to HER2 status, ER–/PgR+/HER2+ patients had the best OS, followed by ER+/

PgR–/HER2– patients, while ER+/PgR–/HER2+ and ER–/PgR+/HER2– patients had poorer OS ($p = 0.006$, log rank).

Tumour-infiltrating lymphocytes score

Fifty-three (25.7%) cases were scored as TILs negative. The remaining cases showed at least 1% of stromal TILs (median 4%, mean 16.3%, IQR 0–20%). ER–/PgR+ tumours displayed significantly denser TILs than ER+/PgR– cases ($p < 0.001$, Wilcoxon test), irrespectively of HER2 status (Fig. 1). HER2 overexpression was associated with higher TILs but only in the ER+/PgR– subgroup (as a dichotomised variable – $p = 0.006$, χ^2 and as a continuous variable – $p = 0.003$, Wilcoxon test). The abundance of TILs positively correlated with higher grade ($p < 0.001$, Kruskal-Wallis), higher Ki-67 ($p < 0.001$, $R = 0.36$, Spearman) (Fig. 2), and younger age ($p = 0.008$, $R = –0.19$, Spearman) but not with tumour size ($p = 0.54$, $R = –0.04$, Spearman), status of regional lymph nodes ($p = 0.983$, Wilcoxon test), and distant metastases at the time of diagnosis ($p = 0.841$, Wilcoxon test).

Association of tumour-infiltrating lymphocytes status with long-term outcomes

Tumour-infiltrating lymphocytes percentage as a continuous variable was associated with better EFS with borderline statistical significance (HR 0.98, 95% CI: 0.96–0.99, $p = 0.043$, Cox), and it had no impact on DFS (HR 0.98, 95% CI: 0.96–1.0, $p = 0.083$, Cox) and OS (HR 0.98, 0.97–1.0, $p = 0.102$, Cox). When dichotomised, high-TILs (i.e. > 5%) correlated with better EFS in the whole cohort, as well as in the ER+/PgR– and ER–/PgR+ subgroups (Figs. 3–5). The 5-year EFS rate in the whole cohort was 87% in the high TILs group, and 67% in the low TILs group ($p = 0.001$, log rank). Significant differences in OS were noted in the whole cohort and in the ER–/PgR+ subgroup, whereas significant differences in DFS were observed in the whole cohort and in the ER+/PgR– subgroup (Suppl. Figs. 1–6).

Multivariate analysis was restricted to EFS due to insufficient numbers of DFS and OS events. In the whole cohort nodal status, the presence of distant metastases, TILs, and Ki-67 index were associated with EFS in multivariate analysis (Table 2). In the analysis restricted to ER–/PgR+ patients, only TILs status and distant metastases were associated with EFS (Table 3), whereas in ER+/PgR– patients, T-descriptor, nodal status, HER2, and TILs correlated with EFS (Table 4).

Association of tumour-infiltrating lymphocyte status with gene expression profile

The analysis covered 758 genes associated with BC biology, microenvironment, and immune response. When ER+/PgR– and ER–/PgR+ cases were analysed together, 50 DEGs were identified, including 39 up- and 11 down-regulated genes in the high TILs group (Table 5). The most up-regulated genes included *CXCL13* (C-X-C Motif Chemokine Ligand 13), *BCL11A* (BCL11 Transcription Factor A), *CD27* (CD27 Molecule), *GABRP* (γ-aminobutyric acid type A receptor subunit Pi), *GNLY* (granulysin), and *KLRK1* (killer cell lectin-like receptor K1). The genes with higher expression in high TILs tumours were associated with immune response, including NK chemotaxis regulation, antigen
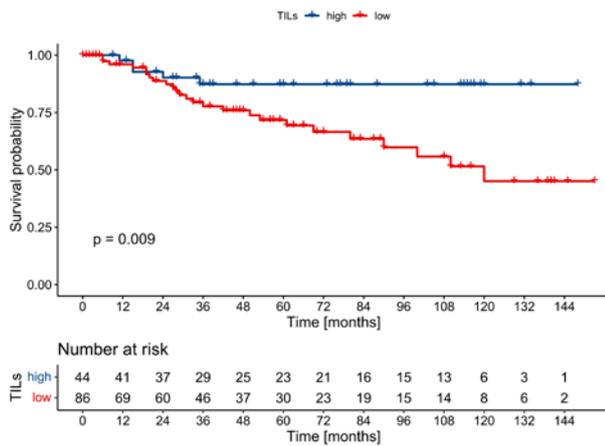
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Fig. 4. Kaplan-Meier curves for event-free survival in ER+/PgR– patients according to tumour-infiltrating lymphocytes status
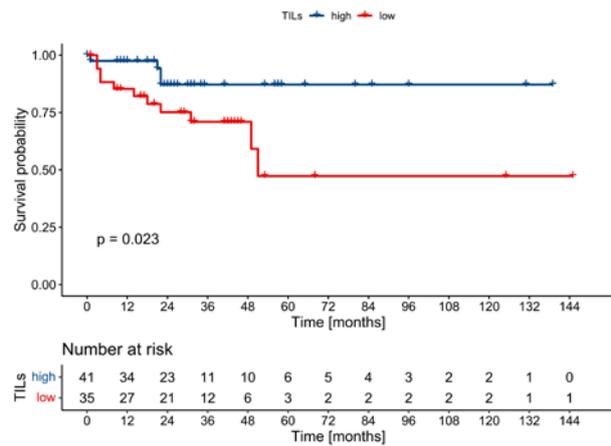

Fig. 5. Kaplan-Meier curves for event-free survival in ER–/PgR+ patients according to tumour-infiltrating lymphocytes status

Table 2. Multivariate Cox regression analysis for event-free survival in the whole cohort

Characteristic	HR	95% CI	*p*-value
T (low vs. high)	0.754	0.328–1.734	0.506
N (0–1 vs. 2–3)	0.328	0.134–0.804	0.014
M (0 vs. 1)	0.069	0.017–0.289	< 0.001
Grade (low vs. high)	0.589	0.117–0.298	0.522
HER2 (neg vs. pos)	0.910	0.409–2.028	0.819
Ki-67 (high vs. low)	2.527	1.024–6.235	0.044
TILs (low vs. high)	6.549	2.536–16.914	< 0.001

HR – hazard ratio

Table 3. Multivariate Cox regression analysis for event-free survival in ER–/PgR+ cohort

Characteristic	HR	95% CI	*p*-value
T (low vs. high)	1.836	0.506–6.661	0.355
N (low vs. high)	0.488	0.114–2.084	0.333
M (0 vs. 1)	0.085	0.011–0.654	0.018
Grade (low vs. high)	n/a	n/a	n/a
HER2 (neg vs. pos)	5.308	0.844–33.393	0.075
Ki-67 (high vs. low)	0.634	0.065–7.772	0.712
TILs (low vs. high)	5.259	1.346–20.544	0.018

HR – hazard ratio, n/a – not applicable, TILs – tumour-infiltrating lymphocytes

Table 4. Multivariate Cox regression analysis for event-free survival in ER+/PgR– cohort

Characteristic	HR	95% CI	*p*-value
T (low vs. high)	0.253	0.076–0.847	0.025
N (0–1 vs. 2–3)	0.189	0.050–0.723	0.015
M (0 vs. 1)	n/a	n/a	n/a
Grade (low vs. high)	0.548	0.097–3.09	0.994
HER2 (neg vs. pos)	0.264	0.089–0.778	0.016
Ki-67 (high vs. low)	2.45	0.804–7.462	0.115
TILs (low vs. high)	7.237	1.768–29.616	0.005

HR – hazard ratio, n/a – not applicable, TILs – tumour-infiltrating lymphocytes

presentation, chemokine receptor binding, or interleukin and interferon signalling (Suppl. Table 1). Importantly, the upregulated expression of immune-related genes was consistent also across single hormone receptor-positive groups analysed separately (ER+/PgR–, ER–/PgR+) (Fig. 6). On the other hand, TILs-high tumours showed reduced expression of genes involved in tight-junction formation (*CLDN3*, *OCLN*), most probably resulting from lower abun-

Table 5. Differently expressed genes identified for tumour-infiltrating lymphocytes high (n = 25) vs. tumour-infiltrating lymphocytes low (n = 27) comparison in single hormone-receptor positive breast cancers (ER+/PgR– and ER–/PgR+ cases analysed together)

Gene	TILs low (median)	TILs high (median)	Log$_2$FC	*p*-value
CCL5	6739	18127	1.43	0.000
CCL2	499	1250	1.32	0.000
LTB	111	325	1.54	0.000
TAP1	52	133	1.35	0.000
ENPP2	248	689	1.47	0.000
NKG7	139	420	1.59	0.000
MARCO	194	828	2.09	0.000
B3GNT3	215	1180	2.45	0.000
CD27	0	82	6.37	0.000
TGFB2	78	188	1.25	0.000
CXCL13	0	212	7.74	0.000
CXCL9	59	188	1.64	0.000
S100A7	24	204	3.06	0.000
GNLY	0	69	6.13	0.000
MFNG	43	111	1.35	0.000
CXCL10	38	119	1.61	0.000
BMP7	23	157	2.71	0.000
KRT6B	48	187	1.93	0.000
MIA	59	231	1.95	0.000
RARRES3	37	104	1.48	0.000
HLA-A	2834	6269	1.15	0.000
CCL8	16	138	3.00	0.001
GABRP	0	74	6.23	0.001
IL2RB	185	506	1.45	0.001
LAG3	38	104	1.44	0.001
HLA-B	842	1929	1.19	0.001
IDO1	46	174	1.90	0.001
IKZF3	247	791	1.68	0.002
PSMB9	50	134	1.41	0.002
PTGDS	30	88	1.54	0.002
JAK3	45	95	1.06	0.003
EGFR	3158	1549	−1.03	0.003
PSAT1	260	108	−1.26	0.003
KLRK1	0	65	6.04	0.004
CMKLR1	224	82	−1.43	0.007
STAT1	42	87	1.03	0.008
HLA-DRA	396	169	−1.22	0.008
CD8A	34	72	1.05	0.010
HLA-DPB1	202	89	−1.18	0.010
PYCARD	401	176	−1.18	0.013
BCL11A	0	92	6.54	0.016
IL7R	32	105	1.66	0.016
EDN1	97	46	−1.05	0.018
SOCS1	42	100	1.23	0.019
CD274	179	86	−1.05	0.029
RASGRP1	1143	3032	1.41	0.033
HLA-DQA1	130	57	−1.18	0.034
RUNX3	42	98	1.19	0.037
CHI3L1	116	53	−1.10	0.038
PLA2G2A	193	87	−1.14	0.049

TILs – tumour-infiltrating lymphocytes
Statistical significance was evaluated with the Mann-Whitney-Wilcoxon test.

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dance of epithelial cells in tissue cores sampled from high-TILs tumours.

The only gene that had significantly different (higher) expression among TILs-high ER–/PgR+ ($n = 14$), comparing to TILs-high ER+/PgR– tumours ($n = 11$) was *SOX10* ($\log_2$FC = 7.75, p = 0.0201, Wilcoxon test).

Discussion

Single hormone receptor-positive BC accounts for about 15–20% of BCs, and the vast majority display the ER+/PgR– phenotype, whereas ER–/PgR+ tumours are very rare [24]. The latter is a controversial group, and many authors disregard ER–/PgR+ cancers as technical artifacts. Moreover, given the infrequency of ER–/PgR+ tumours, there is a significant scarcity of studies dedicated to them, and we were able to include a relatively substantial number of such cases in our study. Nevertheless, multiple studies have confirmed their existence and demonstrated similarities to TNBC [25]. Therefore, the assessment of predictive and prognostic factors in this subpopulation is of great clinical importance. ER–/PgR+ BC are more common in younger women, they are more often poorly differentiated, larger, more frequently have high proliferation index, positive lymph node status, distant metastases, and high glucose metabolism, and carry worse prognosis compared to ER+/PgR+ tumours [26, 27]. Similarly, in the current study, ER+/PgR– BCs occurred in older patients and had lower Ki-67 index, while ER–/PgR+ BCs were larger, had higher grade, were more likely to have high TILs, and were more often associated with distant metastases. In one study survival in both groups of single hormone receptor-positive BCs was similar, although in ER–/PgR+ cancers higher expression of the PgR was associated with longer RFS and DFS [28]. On the contrary, in our cohort of ER–/PgR+/HER2– BCs, a negative prognostic significance of higher PgR expression was demonstrated [17]. In ER+ BCs a lack of PgR expression is predictive for higher incidence of pCR after neoadjuvant chemotherapy and poorer response to anti-ER treatment [26].

ER-positive BCs are less immunogenic compared to hormone receptor-negative BCs; therefore, they are less likely to have high TILs [8, 29], which may explain the lower prevalence of intermediate/high TILs in ER+/PgR– cancers in our study. Data on the prognostic and predictive value of TILs in BC are inconsistent. Moreover, there are few data on the prognostic significance of TILs in single hormone receptor-positive BCs. Most of the available data relate to triple-negative and HER2+ cancers, in which higher TILs are associated with better response to neoadjuvant chemotherapy and improved prognosis; however, in luminal BC high TILs seem to be a negative prognostic factor [30–32], including worse OS [9]. This unexpected correlation may be explained by confounding effects of age, grade, and stage on the prognostic significance of TILs [32]. In the current study high TILs as a continuous parameter correlated positively with EFS in both groups of single hormone receptor-positive BCs. Alike in TNBC, we demonstrated that also in single hormone receptor-positive BCs, higher TILs are associated with higher tumour grade [7].
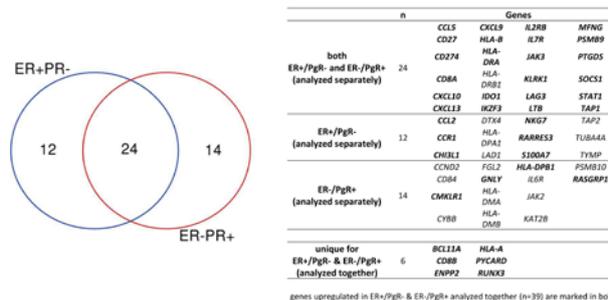

	n	Genes			
both ER+/PgR- and ER-/PgR+ (analyzed separately)	24	CCL5	CXCL9	IL2RB	MFNG
		CD27	HLA-B	IL7R	PSMB9
		CD274	HLA-DRA	JAK3	PTGDS
		CD8A	HLA-DRB1	KLRK1	SOCS1
		CXCL10	IDO1	LAG3	STAT1
		CXCL13	IKZF3	LTB	TAP1
ER+/PgR- (analyzed separately)	12	CCL2	DTX4	NKG7	TAP2
		CCR1	HLA-DPA1	RARRES3	TUBA4A
		CHI3L1	LAD1	S100A7	TYMP
ER-/PgR+ (analyzed separately)	14	CCND2	FGL2	HLA-DPB1	PSMB10
		CD84	GNLY	IL6R	RASGRP1
		CMKLR1	HLA-DMA	JAK2	
		CYBB	HLA-DMB	KAT2B	
unique for ER+/PgR- & ER-/PgR+ (analyzed together)	6	BCL11A	HLA-A		
		CD8B	PYCARD		
		ENPP2	RUNX3		

genes upregulated in ER+/PgR- & ER-/PgR+ analyzed together (n=39) are marked in bold

Fig. 6. Venn diagram presenting the overlap of upregulated genes identified for tumour-infiltrating lymphocytes high vs. tumour-infiltrating lymphocytes low comparison in single receptor-positive breast cancers analysed together and separately

There are differences in gene expression among high and low TILs BCs. In the group of 200 HER2+ BCs, high TILs were associated with up-regulation of immune genes, down-regulation of stromal and ER genes, and better response to trastuzumab [33]. Similarly, in our group of single hormone receptor-positive BCs, we identified a relationship between high TILs and higher expression of immune-related genes, and lower expression of stromal genes. One of the immune-related genes which were up-regulated in our cohort is *CXCL13*, which plays an important role in the formation of organised structures composed of B, T lymphocytes, and dendritic cells – tertiary lymphoid structures (TLSs), which are involved in the immune response against cancer cells. *CXCL13* has a prognostic significance in BC, especially in HER2+ BC, positively affecting DFS [5]. In addition, CXCL13/CXCR5 signalling is of significant prognostic importance in TNBC immunotherapy with atezolizumab [34].

Another interesting finding is the difference in *SOX10* expression between TILs-high ER–/PgR+ and ER+/PgR– tumours. SOX10 is a useful immunohistochemical marker of both primary and metastatic TNBC even if standard breast lineage markers are reduced [35]. In a study on HER2+/ER– BC *SOX10* expression was associated with features typically seen in TNBC, i.e. large acellular zone or prominent TILs [36]. On the other hand, HER2+/ER+ tumours are almost exclusively SOX10-negative [37]. Thus, higher expression of *SOX10* mRNA in TILs-high ER–/PgR+ tumours may indicate their phenotypic resemblance to other ER– BCs, and future studies should elucidate the role of SOX10 as a potential biomarker of ER–/PgR+ BC.

The optimal cut-offs for TILs in various types of BC can vary depending on the specific subtype and the assessment method used. It is important to note that the field of TILs and their prognostic or predictive value in BC is still evolving, and there are no universally accepted cut-offs for all subtypes. It ranges from 5 to 75% in different research studies or clinical trials, and the cut-offs are generally higher in HER2+ BCs and TNBC compared to luminal BCs [12, 38–41]. In our study we did not predefine the cut-off for TILs assessment, and long-term outcomes analysis was based on the median TILs percentage identified in our cohort, and this is a potential limitation of our study.

Other major limitations are the study's retrospective nature and the small size of the cohort. Moreover, while

we assessed only chemo-naïve cases, in some instances, TILs were assessed in biopsy material or surgical specimens, depending on the availability of specimens, which might have impacted the results. Thus, our findings should be analysed with caution.

Conclusions

Our study confirms that single hormone receptor-positive BCs and both their subgroups (ER+/PgR– and ER–/PgR+) are biologically distinct groups of BCs in which the assessment of TILs carries prognostic significance. Additionally, in patients with single hormone receptor-positive BC the presence of TILs is associated with distinct gene expression profiles and better outcomes. Our findings suggest that the assessment of TILs should be considered as part of routine assessment of single hormone receptor-positive BC, although the role of TILs in clinical decision-making in these BC subtypes warrants further research. Future studies should validate these findings in larger cohorts and address the potential role of TILs as a predictive factor in these tumours.

Disclosures

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