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Original Article

Prognostic significance of tumor-infiltrating lymphocytes in premenopausal, luminal breast cancer treated with adjuvant endocrine therapy

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Abstract: Purpose: Tumor-infiltrating lymphocytes (TILs) have strong prognostic value in triple-negative and HER2-enriched breast cancer, but their prognostic role in luminal breast cancer (LBC) is less clear. Here, we assessed the overall TIL levels and CD8+ T-cells in relation to the prognosis of LBC patients from China. Methods and results: A total of 596 patients with LBC who were premenopausal and treated with adjuvant endocrine therapy were included. Among them, 160 cases were evaluated for CD8 by immunohistochemical (IHC) staining. Whole-section hematoxylin and eosin and IHC staining were visually assessed for stromal TILs (sTILs), stromal CD8+ T-cells (sCD8), and intratumoral CD8+ T-cells (iCD8). Multivariable analyses were used to test the associations between TILs and disease-free survival (DFS) and overall survival (OS) with the adjustment for clinicopathologic characteristics and treatment. High sTILs (≥10%) were associated with high histologic grade (P<0.001), luminal B/HER2- (P<0.001), luminal B/HER2+ subtype (P=0.002), and high Ki67 expression (≥25%; P=0.014). Similar associations were observed for sCD8 but not for iCD8. While sTILs and sCD8 were not associated with either DFS or OS, the presence of iCD8 (≥1%) was associated with better DFS in both univariate (HR=0.51, 95% CI 0.26-0.96, P=0.042) and multivariate (HR=0.48, 95% CI 0.25-0.92, P=0.027) analyses. Similar but less significant associations were found for iCD8 and OS (adjusted HR=0.35, 95% CI 0.11-1.10, P=0.073). Conclusions: Among Chinese premenopausal patients with LBC, iCD8 demonstrated suggestive associations with favorable outcome. In contrast, although sTILs and sCD8 were associated with more aggressive tumor features, they did not appear to be associated with clinical outcome.

Keywords: Luminal breast cancer, stromal tumor-infiltrating lymphocytes, tumor-infiltrating CD8+ T-cell, prognostic significance

Introduction

The prognostic and predictive value of tumor-infiltrating lymphocytes (TILs) in breast cancer has been extensively studied in recent years, especially in triple negative and human epidermal growth factor receptor 2-enriched (HER2+) subtypes [1-3]. High TIL levels, which seem to reflect favorable host antitumor immune response, have been associated with improved clinical outcomes, increased rates of pathological complete response (pCR) to neoadjuvant therapy, and greater benefit from adjuvant therapy in triple negative breast cancers (TNBC) and HER2+ breast cancers [4-6]. Higher TILs at baseline also resulted in better response to trastuzumab treatment in HER2+ disease [4]. In addition to overall TIL levels, specific immune cells have also been associated with survival and therapeutic response. In particular, extensive tumor-infiltrating cytotoxic CD8 positive (CD8+) T-cells were strongly associated with patients' favorable survival and increased rates of pCR in response to neoadjuvant therapy [3, 7-9].

Unlike TNBC and HER2+ breast cancers, which are highly proliferative breast cancer subtypes

with increased level of tumor mutational burden and genomic instability, luminal breast cancer (LBC) is in general considered as low immunogenic with a low rate of lymphocyte-predominant breast cancer (LPBC, TILs ≥50%) [10, 11]. However, given LBC makes up over 70% of all breast cancer patients and a subset of LBC demonstrate a high-TIL phenotype [12], capturing this subgroup of high-TIL tumors and identifying possible immune-related prognostic markers could have great clinical significance [13]. However, studies on the potential clinical relevance of TILs in LBC have generated mixed results. Some studies reported that TILs or CD8+ T-cells had no prognostic value in LBC [1, 7], whereas two studies reported that high TILs conferred a significantly higher likelihood of pCR in LBC patients, although they did not significantly improve long-term outcome [14, 15]. Similarly, another study showed that a high CD8+ T-cell exhaustion signature score, indicating a low CD8+ T-cell level, was associated with worse disease-free survival (DFS) in LBC patients regardless of HER2 status [16]. In contrast, high TILs have also been associated with a worse prognosis in LBC [17].

Most previous studies focused on post-menopausal LBC patients receiving adjuvant chemotherapy from Europe and America [5, 18]. Research investigating the association between TILs and response to endocrine therapy among premenopausal women from Asian women is lacking. It is possible that LBC might rely on different mechanisms to modulate the tumor immune microenvironment through interactions between endocrine factors and immune cells. We therefore investigated TILs in relation to patient outcome in 596 premenopausal Chinese LBC patients treated with curative surgery and adjuvant endocrine therapy.

Materials and methods

Patient enrollment and sample collection

We retrospectively investigated a total of 8416 breast lesions in patients receiving surgery from January 2008 to December 2012 at the Cancer Hospital, Chinese Academy of Medical Science (CHCAMS), Beijing, China. Patients were eligible for this study if they were: (1) 18 to 55 years old, (2) premenopausal, (3) pathologically diagnosed with invasive lobular carcinoma (ILC) or invasive ductal carcinoma of no special type (IDC, NST) and ER or PR-positive (ER/PR ≥1%), (4) clinical stage I to III based on the AJCC 8th TNM staging system, and (5) had received endocrine therapy for at least five years after surgery with or without systematic adjuvant chemotherapy or radiotherapy. The exclusion criteria included: (1) diagnosis of microinvasive carcinoma, (2) receiving neoadjuvant chemotherapy or radiotherapy, (3) follow-up data unavailable. After the inclusion and exclusion criteria were applied, a total of 596 LBC patients were included in this study (**Figure 1**). We retrospectively reviewed the medical records to collect information on clinicopathologic features (age at diagnosis, tumor size, lymph node status, histologic grade, ER, PR and HER-2 status, Ki-67 labeling index, occurrence of peritumoral vascular invasion), type of surgery, and postoperative treatment plan. The follow-up information was obtained through hospital visits or telephone contact with patients or relatives. Up to June 11, 2019, 114 patients relapsed and 29 patients died. The median follow-up time was 88.41 months (ranging from 3.27 months to 11.83 years). The study was approved by the CHCAM internal review board for ethical issues.

Breast cancer subtypes

Data on immunohistochemical (IHC) staining of key tumor markers, including ER, PR, HER2, and Ki-67 were collected from patients' pathologic reports. IHC was performed for ER (CONFIRMTM, anti-Estrogen Receptor Rabbit Monoclonal Primary Antibody), PR (CONFIRMTM, anti-Progesterone Receptor Rabbit Monoclonal Primary Antibody), Ki-67 (MAIXIN, Ki-67 Rabbit Monoclonal Primary Antibody) and HER2 (VENTANA, anti-HER2/neu Rabbit Monoclonal Primary Antibody) according to the manufacturer's instructions. All patients with equivocal HER2 status (IHC 2+) were recommended to have fluorescence in situ hybridization (FISH). FISH staining was performed using the PathVysion HER2 DNA Probe Kit (PathVysion, Abbott Molecular, Des Plaines, Illinois, USA) according to the manufacturer's instructions. All markers were visually assessed by pathologists. For ER and PR, a 1% cut-point was used to define positive staining, consistent with recommendations of international guidelines. For HER2, a score of 3+ on IHC, or amplification of

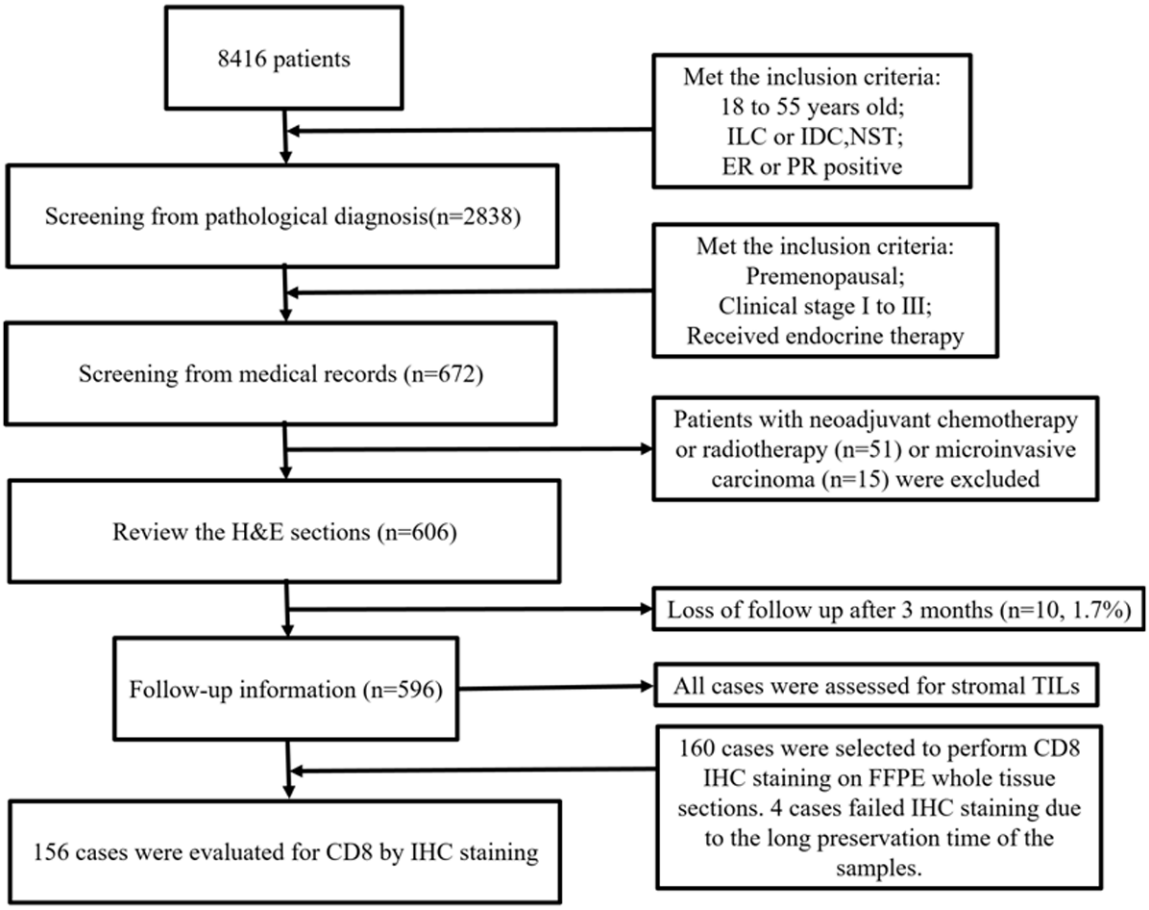


Figure 1. Flow diagram of cases included in the study.

FISH, was considered as positive according to the American Society for Clinical Oncology (ASCO)/College of American Pathologists (CAP) guidelines. Patients with HER2 IHC scores 2+ but for whom information on FISH was not available were classified as HER2-negative. Because a Ki-67 threshold of at least 25% of immunostained cells has been shown to provide the most powerful outcome prognostication, cases with Ki-67 scores above this threshold were considered positive [19]. Breast cancer molecular subtypes were classified according to the following rules: luminal A (ER positive and/or PR positive, HER2 negative, Ki-67 <25%), luminal B/HER2+ (ER positive and/or PR positive, and HER2 positive), and luminal B/HER2- (ER positive and/or PR positive, Ki-67 ≥25%, and HER2 negative). If the Ki-67 information was missing, tumor grade was used for defining luminal A (grade 1 or 2) and luminal B/HER2- (grade 3) subtypes.

Stromal TIL assessment

Whole sections of hematoxylin and eosin (H&E) stained slides were used to evaluate stromal TILs (sTILs), strictly following the criteria proposed by the International TIL Working Group [20]. Briefly, the percentage of all mononuclear cells (including lymphocytes and plasma cells) in the stromal compartment within the border of the invasive tumor was visually evaluated. We also developed a supervised machine-learning algorithm for the unbiased detection of TILs based on cell texture, size, and shape within well-defined regions of the stroma [12].

Immunohistochemistry (IHC) for CD8+ T-cells

Of these 596 patients, 160 cases were selected to perform CD8 immunohistochemical (IHC) staining on formalin-fixed paraffin-embedded (FFPE) whole tissue sections. These cases were selected based on TIL quantity measured by

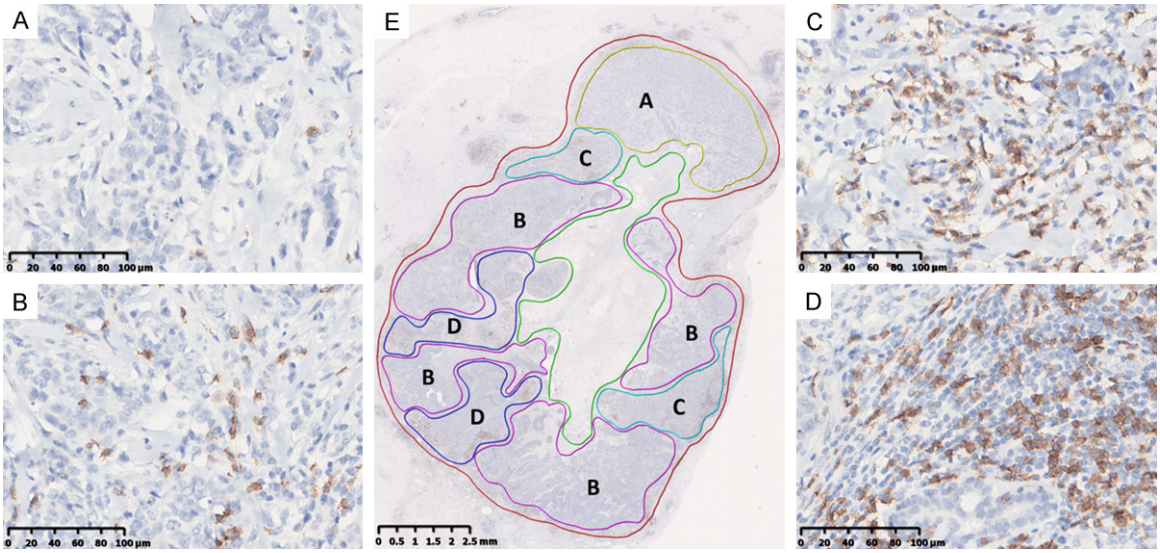


Figure 2. Evaluation method for cases with significant heterogeneity in lymphocyte distribution. Representative images showing the infiltration of CD8+ cells in (A) yellow coils scored 1% accounting for 20% of the tumor; (B) purple coils scored 10% accounting for 45% of the tumor; (C) azure coils scored 30% accounting for 15% of the tumor; (D) blue coils scored 40% accounting for 20% of the tumor. (E) The assessment area of this case is included between the red coil and the green coil. The average sCD8 for this case was 1%×20%+10%×45%+30%×15%+40%×20%= 17%. sCD8 = stromal CD8+ T-cell.

the algorithm, with 60 and 100 cases from the lowest and highest quartile, respectively. (We initially thought that using the computer-based algorithm might provide more quantitative and accurate TIL assessment. However, after a comprehensive comparison of data, we concluded that the algorithm had a better capture of stromal cell density than TILs, as we detailed in a submitted manuscript (Abubakar et al., submitted). Our initial idea was to select a subset of patients with the most contrasting TIL levels (high vs. low) for the purpose of validating the TIL assessment of H&E sections; however, because of the unexpected results based on the algorithm, we ended up having cases with a wide range of TIL levels, which could be used for the evaluation of prognosis associations. For this reason, we did not use the algorithm-based TIL levels for subsequent analyses. Instead, we focused on the pathologists' visual assessment of sTILs, which showed higher agreement (r=0.69) with CD8+ IHC results compared to the computer algorithm). The tumor-infiltrating CD8+ T-cells were evaluated by IHC using a rabbit monoclonal primary antibody against CD8 (ZA-0508, ZSGB-BIO). Automated IHC was performed on 4-µm-thick sections using the Ventana Benchmark ULTRA automated slide processing system according to the manufacturer's instructions.

All cells stained positive in the stromal compartment and tumor nests within the borders of the invasive tumor were evaluated and reported as a percentage value. CD8+ T-cells were classified as 'intratumoral CD8+ T-cell' (iCD8+, the percentage of the area of CD8+ T-cell infiltration within the tumor area if they were in direct contact with tumor cells) and 'stromal CD8+ T-cell' (sCD8+, the percentage of the area of CD8+ T-cell infiltration within the tumor stroma if they were in the stromal compartment but not in direct contact with tumor cells).

Stromal TILs and CD8+ T-cells outside of the tumor border, around DCIS and normal breast tissue, or in areas of necrosis were not included in the scoring. The visual evaluation of stromal TILs and CD8+ T-cells was performed by an expert pathologist (JZ) three times to increase the reproducibility without knowledge of the clinical information. For cases with significant region variations in lymphocyte distribution, we evaluated each region separately and calculated a weighted average of TIL values across different regions according to the size of the region (**Figure 2**). We used the third quartile as the cutoff point to define high and low TIL levels, specifically, 10% for sTILs, 5% for sCD8 and 1% for iCD8, which were consistent with previous studies [7, 21]. We also defined the lympho-

cyte-predominant breast cancer (LPBC) based on sTILs ≥50%, similar to what was previously reported [18].

Statistical analysis

Two clinical outcomes were analyzed in this study. Disease-free survival (DFS) was defined as the interval between the first operation time and the date of first relapse (local, regional, contralateral, or metastatic), and overall survival (OS) was defined as the time from surgery to the date of death from any reason. Patients who were alive (for OS) and disease free (for DFS) were censored at the date of last contact.

Statistical analyses were conducted using SPSS version 25.0 and R version 3.6.1. The differences between categorical variables were evaluated using Chi-square or Fisher's exact test. The differences between two continuous variables were evaluated using Mann-Whitney Test. Univariable and multivariable Cox proportional hazards regression models were used to assess differences in DFS and OS across different groups defined by TILs. The multivariable model contained variables that remained significant in the univariable Cox-regression model after backward elimination (significance level of 0.1). For visualization purposes, Kaplan-Meier plots were used to produce DFS and OS curves stratified by sTILs, sCD8, and iCD8 as binary variables. The log-rank test was used to compare the two groups. All reported *p* values were two-tailed, and for all analyses, P<0.05 was considered significant.

Results

Association of TILs and clinicopathologic characteristics in luminal breast cancer

TIL levels were obtained from 596 H&E sections and 156 IHC CD8+ T-cell stained sections (4 cases failed IHC staining due to the long preservation time of the samples). Pathologists' visual assessment for sTILs was used in the subsequent analyses because they showed higher agreement with CD8+ IHC results compared to the computer algorithm. There was a high correlation between TILs and sCD8, with r value of 0.694. The median percentage of sTILs, sCD8, and iCD8 was 5% (range from 0% to 90%), 1% (range from 0 to 40%), and 1% (range from 0 to 30%), respectively. Compared to luminal A patients, luminal B/HER2+ and luminal B/HER2- patients were more likely to have higher levels of sTILs (P< 0.001, **Figure 3A**). Luminal B/HER2+ patients were also more likely to have higher levels of sCD8+ (P=0.045) and iCD8+ (P=0.052) compared to luminal A patients, while the differences of sCD8+ and iCD8+ levels between luminal B/HER2- and luminal A patients were not significant (**Figure 3B**, **3C**).

When looking at TILs as dichotomized variables, we found that high sTILs (≥10%) were associated with high histologic grade (P< 0.001), luminal B/HER2- (P<0.001), luminal B/HER2+ subtype (P=0.002), and high Ki67 expression (≥25%; P=0.014) (**Table 1**). Similar associations were observed for sCD8+ T-cell levels (**Table 1**). sTILs and sCD8+ T-cell levels did not significantly vary by age at diagnosis, histologic type, vascular invasion, lymph node status, tumor size, or pTNM stage. There was also no significant difference in the number of relapses and deaths between the two groups. iCD8+ T-cells did not show significant association with any examined clinicopathologic factors (**Table 1**). The LPBC phenotype occurred in 3% of overall patients, with frequency of 2.1% in luminal A, 4.2% in luminal B/HER2-, and 5.0% in luminal B/HER2+ subtypes. LPBC was more common in histologic grade 3 (P=0.046) breast cancer but did not vary by other clinicopathologic factors (Supplementary Table 1).

Association of TILs and clinical outcome

In the overall analysis of 596 cases, sTIL levels were not significantly associated with either DFS or OS (**Table 2**). As summarized in **Table 2** and **Figure 4**, in the analysis of 156 cases with CD8 IHC staining data, the presence of iCD8 (≥1%) was associated with improved DFS in both univariable (HR=0.51, 95% CI 0.26-0.96, P=0.042) and multivariable (HR=0.48, 95% CI 0.25-0.92, P=0.027) Cox models. The presence of iCD8 also showed borderline significant associations with OS in both univariable (HR=0.33, 95% CI 0.10-1.03, P=0.057) and multivariable (HR=0.35, 95% CI 0.11-1.10, P= 0.073) analyses, while the presence of sCD8 was not associated with either DFS or OS (**Table 2**). Interestingly, when analyzing luminal A and luminal B subtypes separately, we ob-

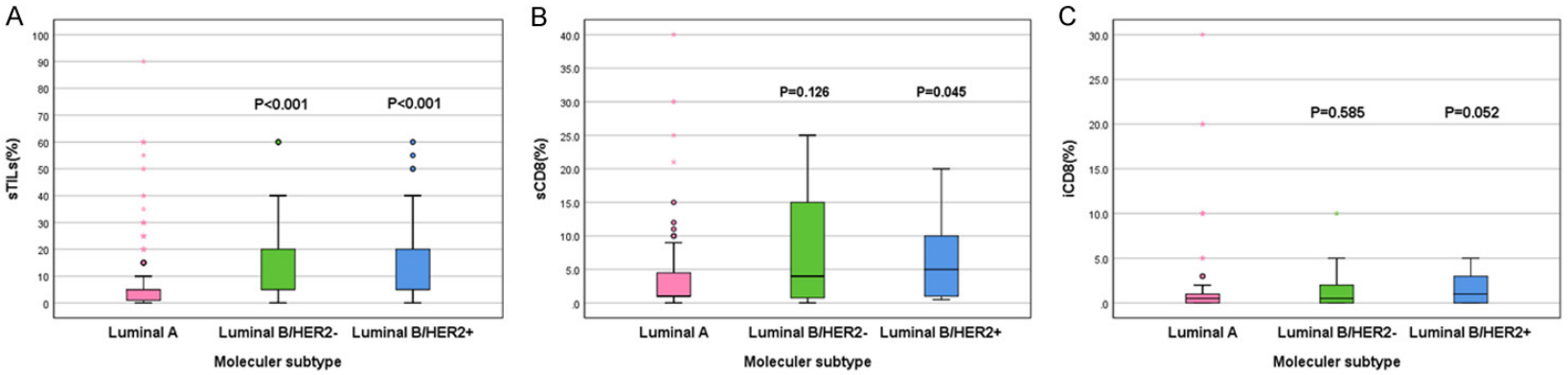


Figure 3. Stromal TILs (sTILs, A), stromal CD8+ (sCD8+, B), and intratumoral CD8+ (iCD8+, C) T-cells in different subgroups of luminal BC. *P* values were obtained from Mann-Whitney test.

Table 1. Association of sTILs and tumor-infiltrating CD8+ T-cells with clinicopathologic characteristics

	N (%)	sTILs (%)		*P**	N (%)	sCD8+ T-cell (%)		*P**	iCD8+ T-cell (%)		*P**
		<10%	≥10%			<5%	≥5%		<1%	≥1%	
Total	596	423 (71.0)	173 (29.0)		156	104 (66.7)	52 (33.3)		75 (48.1)	81 (51.9)	
Age (44, range 25-55 years)				0.179				0.261			0.788
<35 years	60 (10.0)	38 (63.3)	22 (36.7)		15 (9.6)	8 (53.3)	7 (46.7)		8 (53.3)	7 (46.7)	
≥35 years	536 (89.9)	385 (71.8)	151 (28.2)		141 (90.4)	96 (68.1)	45 (31.9)		67 (47.5)	74 (52.5)	
Histological type				1.000				0.665			0.672
ILC	12 (2.0)	9 (75.0)	3 (25.0)		151 (96.8)	100 (66.2)	51 (33.8)		2 (40.0)	3 (60.0)	
IDC, NST	584 (98.0)	414 (70.9)	170 (29.1)		5 (3.2)	4 (80.0)	1 (20.0)		79 (52.3)	72 (47.7)	
Histological grade				<0.001				0.011			0.209
1	71 (11.9)	58 (81.7)	13 (18.3)	0.037	18 (11.5)	15 (83.3)	3 (16.7)	0.182	12 (66.7)	6 (33.3)	0.216
2	385 (64.6)	287 (74.5)	98 (25.5)		102 (65.4)	72 (70.6)	30 (29.4)		54 (52.9)	48 (47.1)	
3	140 (23.5)	78 (55.7)	62 (44.3)	<0.001	36 (23.1)	17 (47.2)	19 (52.8)	0.008	15 (41.7)	21 (58.3)	0.186
Vascular invasion				0.753				1.000			0.164
Present	54 (9.1)	37 (68.5)	17 (31.5)		14 (9.0)	9 (64.3)	5 (33.1)		10 (71.4)	4 (28.6)	
Absent	542 (90.9)	386 (71.2)	156 (28.8)		142 (91.0)	95 (66.9)	47 (33.1)		71 (50.0)	71 (50.0)	
Molecular subtype				<0.001				0.003			0.173
Luminal A	379 (63.6)	293 (77.3)	86 (22.7)	<0.001	107 (68.6)	80 (74.8)	27 (25.2)	0.002	58 (54.2)	49 (45.8)	0.490
Luminal B/HER2-	96 (16.1)	58 (60.4)	38 (39.6)		24 (15.4)	12 (50.0)	12 (50.0)		14 (58.3)	10 (41.7)	
Luminal B/HER2+	121 (20.3)	72 (59.5)	49 (40.5)	0.002	25 (16.0)	12 (48.0)	13 (52.0)	0.038	9 (36.0)	16 (64.0)	0.125

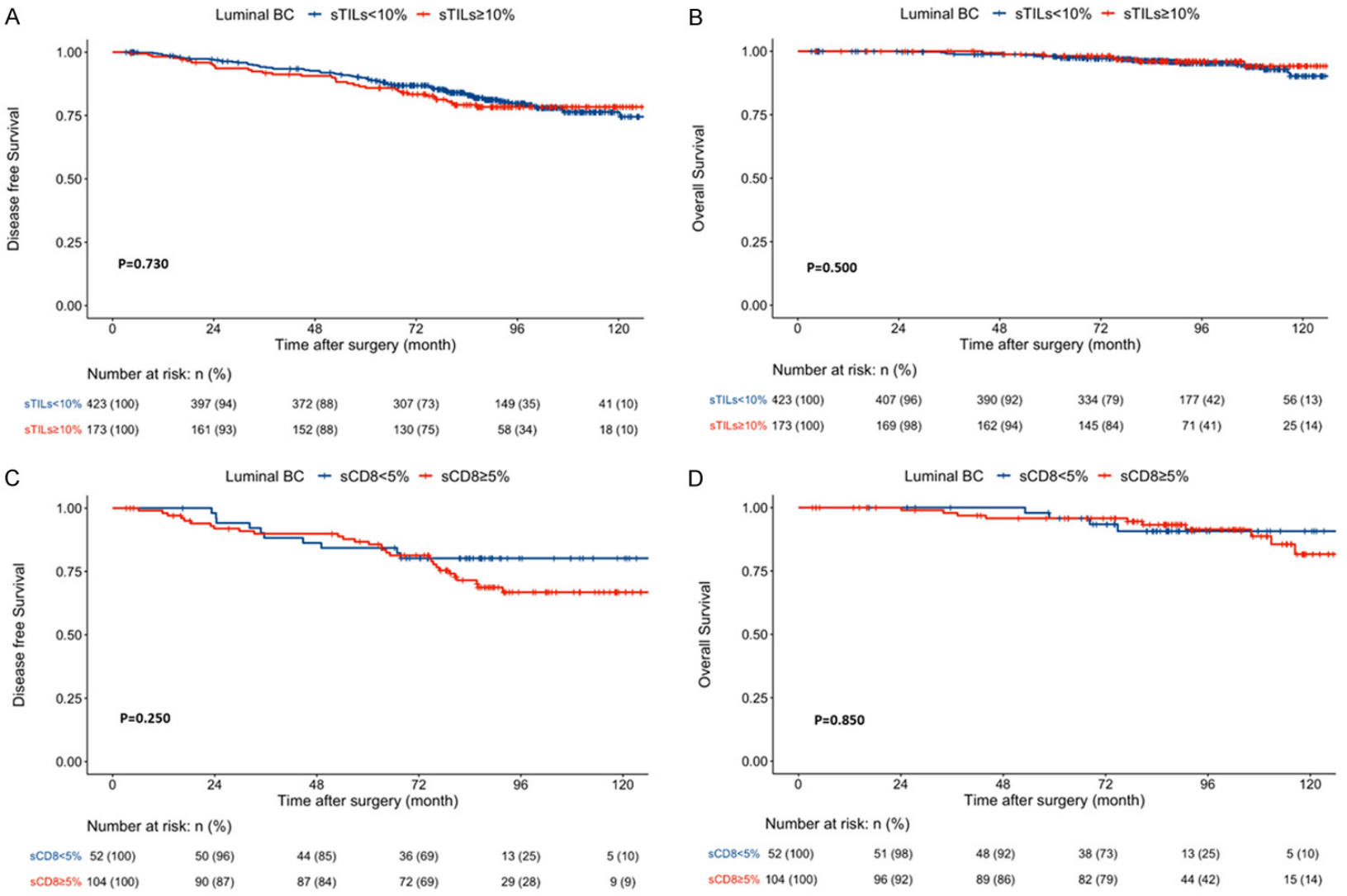
Ki67				0.014				0.036			0.356
<25%	296 (49.7)	221 (74.7)	75 (25.3)		56 (35.9)	42 (75.0)	14 (25.0)		32 (57.1)	24 (42.9)	
≥25%	162 (27.2)	103 (63.6)	59 (36.4)		68 (43.6)	34 (50.0)	34 (50.0)		33 (48.5)	35 (51.5)	
Unknown	138 (23.1)	99 (71.7)	39 (28.3)		32 (20.5)	28 (87.5)	4 (12.5)		10 (31.2)	22 (68.8)	
Tumor size (cm)				0.346				0.858			0.318
≤2	384 (64.4)	267 (69.5)	117 (30.5)		102 (65.4)	67 (65.7)	35 (34.3)		56 (54.9)	46 (45.1)	
>2	212 (35.6)	156 (73.6)	56 (26.4)		54 (34.6)	37 (68.5)	17 (31.5)		25 (46.3)	29 (53.7)	
LN status				0.521				1.000			1.000
Negative	351 (58.9)	253 (72.1)	98 (27.9)		100 (64.1)	67 (67.0)	33 (33.0)		52 (52.0)	48 (48.0)	
Positive	245 (41.1)	170 (69.4)	75 (30.6)		56 (35.9)	37 (66.1)	19 (33.9)		29 (51.8)	27 (48.2)	
pTNM stage				0.789				0.712			0.897
I	265 (44.5)	187 (70.6)	78 (29.4)		77 (49.4)	51 (66.2)	26 (33.8)		41 (53.2)	36 (46.8)	
II	252 (42.3)	182 (72.2)	70 (27.8)		62 (39.7)	43 (69.4)	19 (30.6)		32 (51.6)	30 (48.4)	
III	79 (13.3)	54 (68.4)	25 (31.6)		17 (10.9)	10 (58.8)	7 (41.2)		8 (47.1)	9 (52.9)	
Relapse				0.648				0.327			0.097
Yes	114 (19.1)	79 (69.3)	35 (30.7)		39 (25.0)	29 (74.4)	10 (25.6)		25 (64.1)	14 (35.9)	
No	482 (80.9)	344 (71.4)	138 (28.6)		117 (75.0)	75 (64.1)	42 (35.9)		56 (47.9)	61 (52.1)	
Death				0.677				0.775			0.105
Yes	29 (4.9)	22 (75.9)	7 (24.1)		15 (9.6)	11 (73.3)	4 (26.7)		11 (73.3)	4 (26.7)	
No	567 (95.1)	401 (70.7)	166 (29.3)		141 (90.4)	93 (66.0)	48 (34.0)		70 (49.6)	71 (50.4)	

Abbreviations: ILC, invasive lobular carcinoma; IDC, NST, invasive ductal carcinoma of no special type; LN, lymph node. **P* values were obtained from Chi-square or Fisher's exact test.

Table 2. Associations between tumor-infiltrating lymphocytes and patient outcome in Chinese luminal breast cancer patients

Measure	DFS						OS					
	Univariate			Multivariate*			Univariate			Multivariate*		
	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P
sTILs (<10%/≥10%)	1.07	0.72, 1.60	0.735	0.83	0.51, 1.36	0.464	0.75	0.32, 1.75	0.503	0.60	0.22, 1.63	0.314
Luminal A	1.56	0.95, 2.56	0.080	1.15	0.61, 2.17	0.658	0.64	0.18, 2.23	0.480	0.61	0.13, 2.82	0.529
Luminal B/HER2-	0.48	0.19, 1.22	0.124	0.37	0.11, 1.25	0.108	0.28	0.03, 2.33	0.238	0.50	0.03, 7.60	0.618
Luminal B/HER2+	0.79	0.30, 2.11	0.639	0.65	0.20, 2.12	0.478	0.43	0.07, 2.58	0.357	0.94	0.13, 6.97	0.955
sCD8 (<5%/≥5%)	0.66	0.32, 1.35	0.254	0.75	0.35, 1.61	0.463	0.89	0.28, 2.85	0.850	1.35	0.39, 4.67	0.638
iCD8 (<1%/≥1%)	0.51	0.26, 0.96	0.042	0.48	0.25, 0.92	0.027	0.33	0.10, 1.03	0.057	0.35	0.11, 1.10	0.073

Abbreviations: DFS, disease-free survival; OS, overall survival; HR, hazard ratio; sTILs, stromal tumor-infiltrating lymphocytes; BC, breast cancer; sCD8, stromal CD8+ T-cells; iCD8, intratumoral CD8+ T-cells. *, The multivariate model contains the prognostic factors that remained significant in the univariate Cox-regression model after backward elimination including Age, Vascular invasion, Ki-67 labeling index, Tumor size, LN status, pTNM stage, Type of surgery, Herceptin therapy for sTILs with DFS and OS; Tumor size, LN status, pTNM stage for sCD8 and iCD8 with DFS; Tumor size, pTNM stage for sCD8 and iCD8 with OS; significance level of 0.1; An additional table shows this in more detail [see Supplementary Table 2].


A
Luminal BC
sTILs<10%
sTILs≥10%
Disease free Survival
1.00
0.75
0.50
0.25
0.00
P=0.730
0
24
48
72
96
120
Time after surgery (month)
Number at risk: n (%)
sTILs<10% 423 (100) 397 (94) 372 (88) 307 (73) 149 (35) 41 (10)
sTILs≥10% 173 (100) 161 (93) 152 (88) 130 (75) 58 (34) 18 (10)
B
Luminal BC
sTILs<10%
sTILs≥10%
Overall Survival
1.00
0.75
0.50
0.25
0.00
P=0.500
0
24
48
72
96
120
Time after surgery (month)
Number at risk: n (%)
sTILs<10% 423 (100) 407 (96) 390 (92) 334 (79) 177 (42) 56 (13)
sTILs≥10% 173 (100) 169 (98) 162 (94) 145 (84) 71 (41) 25 (14)
C
Luminal BC
sCD8<5%
sCD8≥5%
Disease free Survival
1.00
0.75
0.50
0.25
0.00
P=0.250
0
24
48
72
96
120
Time after surgery (month)
Number at risk: n (%)
sCD8<5% 52 (100) 50 (96) 44 (85) 36 (69) 13 (25) 5 (10)
sCD8≥5% 104 (100) 90 (87) 87 (84) 72 (69) 29 (28) 9 (9)
D
Luminal BC
sCD8<5%
sCD8≥5%
Overall Survival
1.00
0.75
0.50
0.25
0.00
P=0.850
0
24
48
72
96
120
Time after surgery (month)
Number at risk: n (%)
sCD8<5% 52 (100) 51 (98) 48 (92) 38 (73) 13 (25) 5 (10)
sCD8≥5% 104 (100) 96 (92) 89 (86) 82 (79) 44 (42) 15 (14)

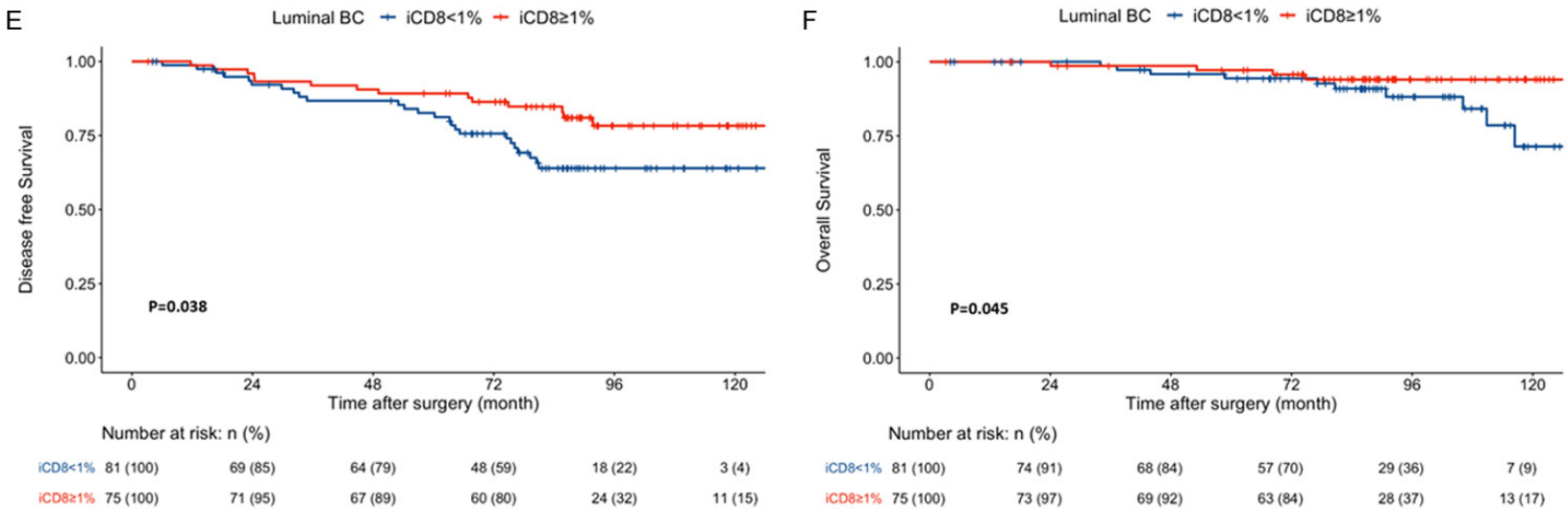


Figure 4. Kaplan-Meier analysis for disease-free survival (DFS, A, C, E) and overall survival (OS, B, D, F) of patients by stromal TILs (A and B), sCD8 (C and D) and iCD8 (E and F) in luminal breast cancer patients. *P* values were derived from a log-rank test. BC = breast cancer. TILs = tumor-infiltrating lymphocytes. Kaplan-Meier analysis for disease-free survival (DFS, A, C and E) and overall survival (OS, B, D and F) of luminal A BC patients (A and B), luminal B/HER2- BC patients (C and D) and luminal B/HER2+ BC patients (E and F) by stromal TILs. *P* values were derived from a log-rank test. BC = breast cancer. TILs = tumor-infiltrating lymphocytes.

served that higher sTILs were associated with worse DFS (HR=1.56, 95% CI 0.95-2.56, P= 0.080) among luminal A patients but improved DFS among luminal B/HER2- (HR=0.48, 95% CI 0.19-1.22, P=0.124) and luminal B/HER2+ (HR=0.79, 95% CI 0.30-2.11, P= 0.639), although none of these associations reached statistical significance (**Table 2** and Supplementary Figure 1). However, this pattern was not seen for OS.

Discussion

In this analysis of premenopausal patients with luminal breast cancer (LBC) treated with curative surgery and adjacent endocrine therapy from China, high stromal TILs (≥10%) and high stromal CD8+ T-cells were associated with high histologic grade, luminal B/HER2-, luminal B/HER2+ subtype, and high Ki-67 expression. Although the overall stromal TILs were not associated with clinical outcome, patients with intratumoral CD8+ T-cells (≥1%) were likely to have a better prognosis. Interestingly, the association between stromal TIL levels and clinical outcome might show different directions for luminal A and luminal B breast cancers.

These results are in general consistent with previous studies that reported no statistically significant association between TILs and survival in LBC [1, 18]. In particular, our findings that high TILs levels tended to have a worse DFS for luminal A breast cancer were in line with previous reports, indicating that increased TILs were an adverse prognostic factor in luminal HER2-, luminal-low Ki67 and luminal-low grade breast cancer [21-24]. Similar to basal-like tumors, this association may be due to the higher number of FoxP3+ T-cells with an unfavorable ratio to cytotoxic CD8+ T-cells [25]. We hypothesized that in different subtypes of LBC, the composition of tumor infiltrating lymphocytes might be different, as shown by Zhu et al. [12]. Therefore, further accounting for subtype differences is needed within LBC in future prognostic associations for TILs.

Previous studies evaluating the prognostic value of CD8+ T cells in LBC had reported conflicting findings. A study reported that the presence of intratumoral CD8+ lymphocytes was not associated with breast cancer-specific survival in ER+ breast tumors [7]. Another study found that higher levels of CD8+ T cells were associated with favorable response to neoadjuvant chemotherapy, but not with improved outcome [15]. In contrast, two studies revealed that LBC regardless of the HER2 status with high CD8+ T-cell infiltration was associated with unfavorable outcome [16, 26], which is contrary to our results. However, consistent with our findings, Bense et al. showed that a high CD8+ T-cell exhaustion signature score, which is equivalent to a low CD8+ T-cell level, was associated with shortened DFS in patients with luminal tumors regardless of HER2 status [16].

The inconsistent results might be due to different study populations, tissue resources, and methodologies used in these studies. For example, some studies used tissue microarrays (TMAs) to stain CD8+ T-cells, and results were based on small tissue cores, which might not accurately capture overall TILs. In addition, most studies focused on LBC patients receiving neoadjuvant chemotherapy or postmenopausal LBC patients receiving adjuvant chemotherapy who might have different immunomodulatory mechanisms compared to premenopausal patients included in this study. Further, the prognostic associations might also vary by the special context of TILs. Intertumoral CD8+ T cells directly contacting tumor cells are cytotoxic cells that play a key role in anti-tumor immunity. In this study, only iCD8, but not overall stromal TILs or sCD8, was associated with DFS and OS. The association was independent of other clinicopathologic factors and did not vary by subtype, suggesting its likely utility as a prognostic marker in all luminal cases. This finding should be further validated in studies using larger patient populations.

It has been suggested that cyclical changes in endogenous hormones may influence the immune microenvironment of the breast [27]. However, we were unable to evaluate the impact of endogenous hormones on our findings since we did not measure circulating hormone levels of the study participants. Nonetheless, we did not find any statistically significant association between TILs and age, as a proxy for endogenous hormone levels, in this population. In addition, we adjusted for age in all our models which should limit the impact of age-related differences in endogenous hormones among the study participants.

The strengths of the study include access to a large sample of Chinese premenopausal patients with LBC, a comprehensive collection of clinicopathologic factors, treatment, and clinical outcome data, a TIL assessment based on both H&E and IHC staining on whole-tissue sections, and an evaluation method to account for regional heterogeneity in lymphocyte distribution. Furthermore, the high correlation between H&E and IHC staining results suggested that our TIL assessment was reasonably accurate. One major limitation of our study is that we used IHC staining only in a subset of patients rather than all patients and we only measured a single IHC marker. Future studies exploring other TIL markers using quantitative measurements are warranted to understand the immune microenvironment of different subtypes of LBC.

In summary, we found that intratumoral CD8+ T-cells showed suggestive prognostic value for premenopausal LBC treated with curative surgery and adjuvant endocrine therapy. Specifically, the presence of intratumoral CD8+ T-cells was associated with more favorable outcomes. Although the overall stromal TILs and stromal CD8+ T-cells were not associated with clinical outcome, the associations indicated different directions in luminal A and luminal B subtypes, suggesting that there may be a different biology of the immunological infiltrate in different luminal subtypes. Additional large studies of tumor immune microenvironment accounting for specific luminal types are needed to disentangle the role of TILs in the prognosis of luminal breast cancer.

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Disclosure of conflict of interest

None.

Abbreviations

sTILs, stromal tumor-infiltrating lymphocytes; HER2, human epidermal growth factor receptor 2; TNBC, triple negative breast cancers; LBC, luminal breast cancer; IHC, immunohistochemical; iCD8+, intratumoral CD8+ T-cell; sCD8+, stromal CD8+ T-cell.

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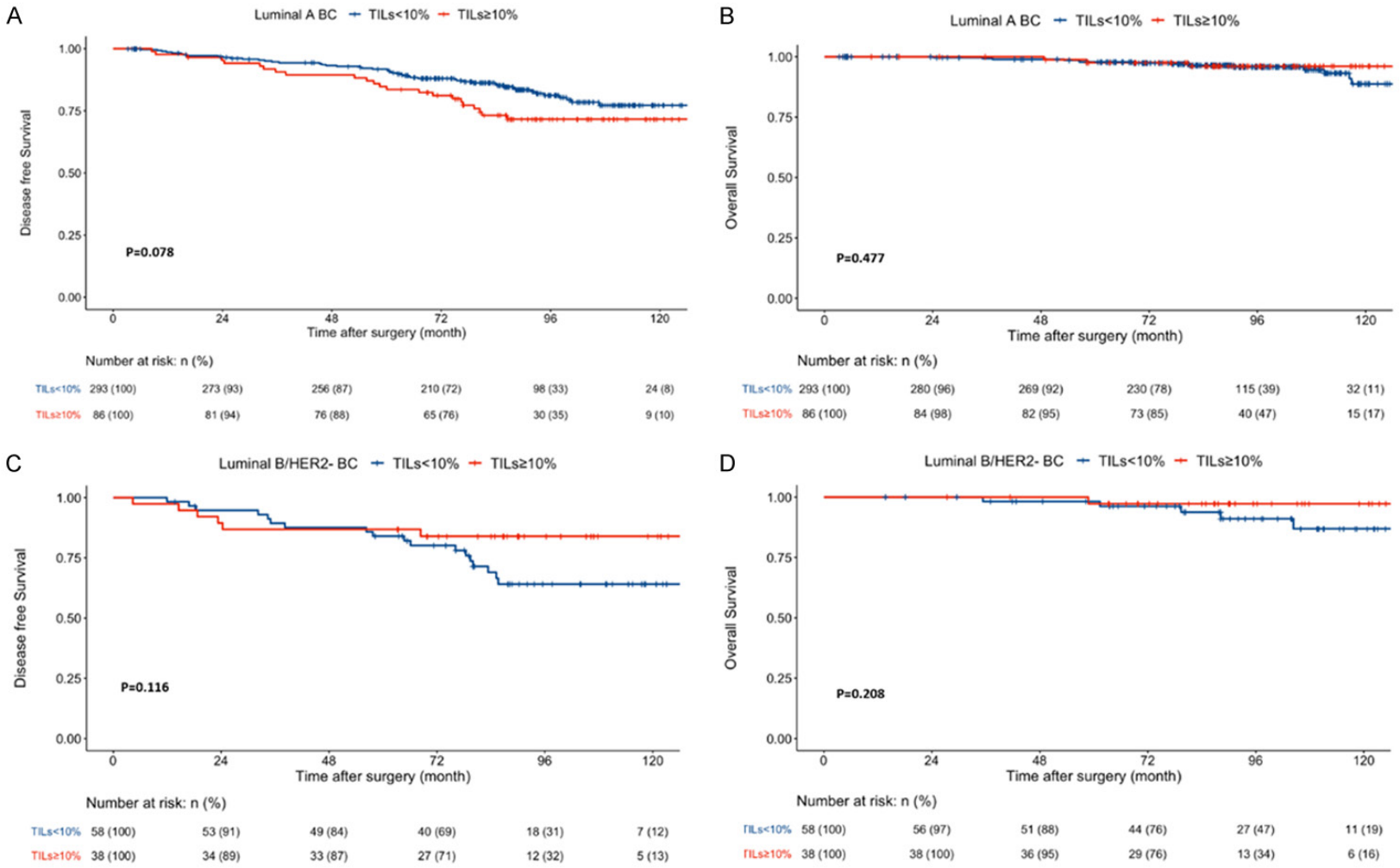
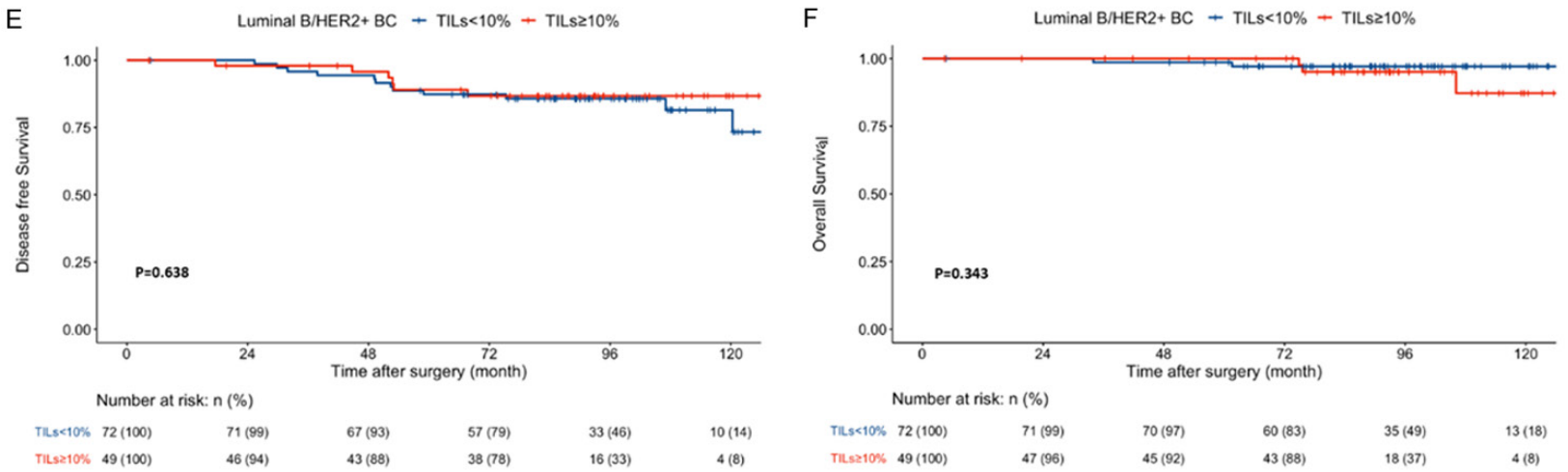
Supplementary Table 1. Association of LPBC with clinicopathologic characteristics

	N (%)	LPBC (%)		P*
		TILs <50%	TILs ≥50%	
Total	596	578 (97.0)	18 (3.0)	
Age (44, range 25-55 years)				0.412
<35	60 (10.0)	57 (95.0)	3 (5.0)	
≥35	536 (89.9)	521 (97.2)	15 (2.8)	
Histologic type				1.000
ILC	12 (2)	12 (100)	0 (0)	
IDC, NST	584 (98.0)	566 (96.9)	18 (3.1)	
Histologic grade				0.052
I	71 (11.9)	71 (100)	0 (0)	0.150
II	385 (64.6)	375 (97.4)	10 (2.6)	
III	140 (23.5)	132 (94.3)	8 (5.7)	0.046
Vascular invasion				1.000
Present	54 (9.1)	53 (98.1)	1 (1.9)	
Absent	542 (90.9)	525 (96.9)	17 (3.1)	
Molecular subtype				0.148
Luminal A	379 (63.6)	371 (97.9)	8 (2.1)	0.133
Luminal B/HER2-	96 (16.1)	92 (95.8)	4 (4.2)	
Luminal B/HER2+	121 (20.3)	115 (95.0)	6 (5.0)	0.228
Ki67				0.286
<25%	296 (49.7)	288 (97.3)	8 (2.7)	
≥25%	162 (27.2)	154 (95.1)	8 (4.9)	
unknown	138 (23.1)	136 (98.6)	2 (1.4)	
Tumor size (cm)				0.319
≤2	384 (64.4)	370 (96.4)	14 (3.6)	
>2	212 (35.6)	208 (98.1)	4 (1.9)	
LN status				0.092
Negative	351 (58.9)	344 (98.0)	7 (2.0)	
Positive	245 (41.1)	234 (95.5)	11 (4.5)	
pTNM				0.089
I	265 (44.5)	256 (96.6)	9 (3.4)	
II	252 (42.3)	248 (98.4)	4 (1.6)	
III	79 (13.3)	74 (93.7)	5 (6.3)	
Relapse				0.548
Yes	114 (19.1)	112 (98.2)	2 (1.8)	
No	482 (80.9)	466 (96.7)	16 (3.3)	
Death				1.000
Yes	29 (4.9)	29 (100)	0 (0)	
No	567 (95.1)	549 (96.8)	18 (3.2)	

Abbreviations: ILC, invasive lobular carcinoma; IDC, NST, invasive ductal carcinoma of no special type; LN, lymph node. **P* values were obtained from Chi-square or Fisher's exact test.


A
Luminal A BC
TILs<10%
TILs≥10%
Disease free Survival
1.00
0.75
0.50
0.25
0.00
P=0.078
0
24
48
72
96
120
Time after surgery (month)
Number at risk: n (%)
TILs<10% 293 (100) 273 (93) 256 (87) 210 (72) 98 (33) 24 (8)
TILs≥10% 86 (100) 81 (94) 76 (88) 65 (76) 30 (35) 9 (10)
B
Luminal A BC
TILs<10%
TILs≥10%
Overall Survival
1.00
0.75
0.50
0.25
0.00
P=0.477
0
24
48
72
96
120
Time after surgery (month)
Number at risk: n (%)
TILs<10% 293 (100) 280 (96) 269 (92) 230 (78) 115 (39) 32 (11)
TILs≥10% 86 (100) 84 (98) 82 (95) 73 (85) 40 (47) 15 (17)
C
Luminal B/HER2- BC
TILs<10%
TILs≥10%
Disease free Survival
1.00
0.75
0.50
0.25
0.00
P=0.116
0
24
48
72
96
120
Time after surgery (month)
Number at risk: n (%)
TILs<10% 58 (100) 53 (91) 49 (84) 40 (69) 18 (31) 7 (12)
TILs≥10% 38 (100) 34 (89) 33 (87) 27 (71) 12 (32) 5 (13)
D
Luminal B/HER2- BC
TILs<10%
TILs≥10%
Overall Survival
1.00
0.75
0.50
0.25
0.00
P=0.208
0
24
48
72
96
120
Time after surgery (month)
Number at risk: n (%)
TILs<10% 58 (100) 56 (97) 51 (88) 44 (76) 27 (47) 11 (19)
TILs≥10% 38 (100) 38 (100) 36 (95) 29 (76) 13 (34) 6 (16)



Supplementary Figure 1. Kaplan-Meier analysis for disease-free survival (DFS, A, C and E) and overall survival (OS, B, D and F) of luminal A BC patients (A and B), luminal B/HER2- BC patients (C and D) and luminal B/HER2+ BC patients (E and F) by stromal TILs. *P* values were derived from a log-rank test. BC = breast cancer. TILs = tumor-infiltrating lymphocytes.

Supplementary Table 2. Univariate analysis of pathologic features and TIL variables for DFS and OS in luminal BC

Data	N (596) (%)	DFS Univariate			OS Univariate		
		HR	95% CI	P	HR	95% CI	P
Age (years) (<35/≥35)	60 (10.1)/536 (89.9)	2.09	1.28, 3.42	0.003	0.29	0.12, 0.68	0.005
Histological type (IDC, NST/ILC)	584 (98.0)/12 (2.0)	2.05	0.84, 5.03	0.117	0.05	0.00, 540.32	0.522
Histologic grade				0.207			0.319
I/II and III	71 (11.9)/525 (88.1)	0.51	0.25, 1.08	0.078	0.21	0.03, 1.65	0.138
I and II/III	456 (76.5)/140 (23.5)	0.83	0.55, 1.26	0.389	0.76	0.35, 1.69	0.504
Vascular invasive (present/absent)	54 (9.10)/542 (90.9)	1.87	1.10, 3.18	0.020	1.83	0.64, 5.25	0.264
Molecular subtype (luminal HER2-/luminal HER2+)	475 (79.7)/121 (20.3)	0.69	0.42, 1.15	0.155	0.81	0.31, 2.12	0.667
Ki67 labeling index (<25%/≥25%)	227 (38.1)/231 (38.8)	1.71	1.11, 2.65	0.016	1.94	0.84, 4.47	0.121
Tumor size (cm) (≤2/>2)	384 (64.4)/212 (35.6)	1.61	1.11, 2.33	0.011	2.39	1.15, 4.98	0.019
LN status (negative/positive)	351 (58.9)/245 (41.1)	2.51	1.72, 3.66	<0.001	2.79	1.29, 6.00	0.009
pTNM				<0.001			<0.001
I/II and III	265 (44.5)/331 (55.5)	0.28	0.17, 0.47	<0.001	0.19	0.08, 0.47	<0.001
I and II/III	517 (86.7)/79 (13.3)	0.50	0.32, 0.78	0.002	0.25	0.11, 0.59	0.002
Type of surgery (RM/BC)	434 (72.8)/162 (27.2)	0.56	0.35, 0.90	0.015	0.46	0.18, 1.22	0.118
Postoperative treatment without/with AC or AR	97 (16.3)/499 (83.7)	1.44	0.82, 2.52	0.201	1.17	0.41, 3.36	0.773
Herceptin therapy (with/without)	29 (4.9)/567 (95.1)	2.12	1.14, 4.00	0.018	0.61	0.08, 4.51	0.631
sTILs (<10%/≥10%)	423 (71.0)/173 (29.0)	1.07	0.72, 1.60	0.735	0.75	0.32, 1.75	0.503
LPBC (TILs <50%/TILs ≥50%)	578 (97.0)/18 (3.0)	1.80	0.44, 7.28	0.412	21.0	0.01, 682295.12	0.566

DFS, disease-free survival; OS, overall survival; HR, hazard ratio; IDC, NST, invasive ductal carcinoma of no special type; ILC, invasive lobular carcinoma; ER+, Estrogen receptor-positive; HER2, human epidermal growth factor receptor 2; LN, lymph node; RM, Radical mastectomy; BCS, Breast conserving surgery; AC, Adjuvant chemotherapy; AR, Adjuvant radiotherapy.