

Predictive factors of the tumor immunological microenvironment for long-term follow-up in early stage breast cancer

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Key words

Early stage breast cancer, phosphatase and tensin homolog, programmed death 1, programmed death ligand 1, tumor infiltrating lymphocytes

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The aim of this research was to investigate the correlation of immunologic factors in the tumor environment of breast cancer, using immunohistological staining to evaluate the expression of programmed death 1/programmed death ligand 1 (PD-1/PD-L1), phosphatase and tensin homolog (PTEN), tumor infiltrating lymphocytes (TILs), and macrophages, and to analyze the association between the immunologic factors and clinical outcome for patients with early stage breast cancer (EBC). A total of 97 EBC patients who underwent standard surgery were investigated. Expression of PD-1/PD-L1 and PTEN and the density of CD3⁺ TILs, CD8⁺ TILs, and CD163⁺ macrophages were evaluated by immunohistochemical analysis. The association between the immunologic factors and clinical outcome was statistically analyzed. The density of CD3⁺ TILs, CD8⁺ TILs, and CD163⁺ macrophages and non-expression of PTEN was significantly higher in cases of triple negative breast cancer. CD8⁺ TIL density and CD8⁺/PD-L1⁺ expression were predictive factors for disease-free survival and overall survival (OS). Human epidermal growth factor 2 (HER2)-positive patients with PTEN expression and luminal/HER2-negative patients without PD-L1 expression had significantly longer OS compared to patients without PTEN expression ($P = 0.049$) and with PD-L1 expression ($P = 0.036$), respectively. Furthermore, patients with PD-L1⁺/CD8⁺ expression had worse median progression-free survival ($P = 0.022$) and median OS ($P = 0.037$) compared with patients without PD-L1⁺/CD8⁺ expression. The CD3⁺ TILs, CD8⁺ TILs, and CD163⁺ macrophages were shown to infiltrate the tumor area of EBC. In particular, triple negative breast cancer had a higher rate of TIL infiltration within the tumor environment. Expression of PTEN and lack of PD-L1 expression were associated with favorable survival in HER2-positive and luminal/HER2-negative EBC patients, respectively. The PD-L1 expression combined with CD8⁺ density was significantly associated with an aggressive clinical outcome.

Recent advances in diagnosis, neo-adjuvant or adjuvant chemotherapies, hormonal therapies, and anti-HER2 therapies have significantly improved the prognosis of patients with EBC. Breast cancer is traditionally thought of as a kind of low immunogenic carcinoma, but the influence of local immunologic factors on clinical outcome of BC is still under evaluation. It has been indicated that FOXP3 expression on lymphocytes and tumor cells of EBC is significantly associated with clinical outcome and that the peptide vaccines of tumor-associated antigens could induce and boost tumor-specific immune response in patients with metastatic BC.^(1–3) Novel investigations have shown that immunologic factors, such as TILs, and immune relevant factors, including PD-1, PD-L1, and PTEN, are significantly associated with responses to treatment and clinical outcome.^(4–9) Furthermore, PD-L1 and PTEN expression were suggested as predictive markers for clinical survival time of metastatic BC.^(9,10) Accumulating evidence has confirmed that the immunologic relevance of TILs and

immune checkpoint markers, including PD-1/PD-L1, are associated with response to treatment and clinical outcome in patients with BC. Despite these recent advances in immunological research, investigations into these immune markers on the molecular level remain insufficient and more immunopathologic evaluations involving the interaction of TILs and immunologic markers like PD-1, PD-L1, and PTEN in the local tumor area may provide novel clinical prognostic information for EBC.

We retrospectively investigated the density of TILs and macrophages in 97 EBC tumor samples, according to the intrinsic subtype, by immunohistochemistry staining the expression of CD3, CD8, and CD163 molecules. The intratumoral and stromal (peritumoral) expression of PD-1, PD-L1, and PTEN were simultaneously evaluated. The aim of this study was to analyze the correlation between these immunologic factors and clinical outcome, as well as to identify the novel predictive markers for long-term follow-up of EBC.

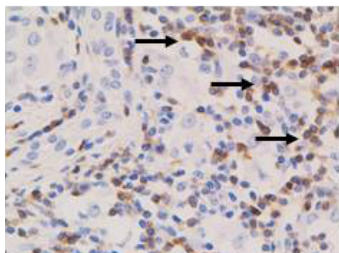
Materials and Methods

Patients. A total of 97 patients with clinical stage I–III operable BC who underwent breast surgery from February 1995 to November 2005 in the Kurume University Hospital (Kurume, Japan) were analyzed for inclusion in the present study. The

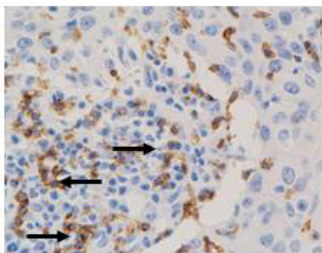
postoperative standard adjuvant chemotherapies were routinely carried out; anti-HER2 therapies with trastuzumab were not available in Japan for neo-adjuvant or adjuvant treatment within this period. Patients were divided into three different intrinsic subtypes as follows: luminal type (ER-positive/HER2-

Intratumoral area (×400)

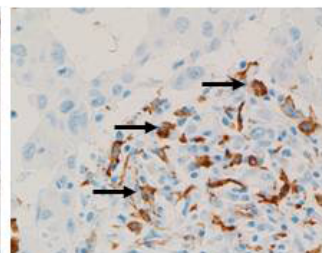
CD3+ TILs



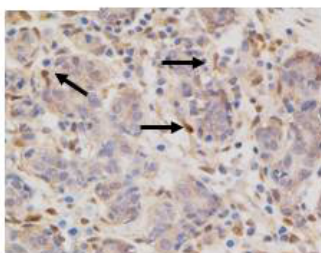
CD8+ TILs



CD163+ Macrophages

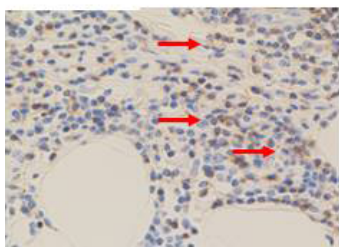


PD-1 +

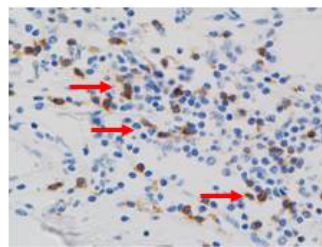


Stromal area (×400)

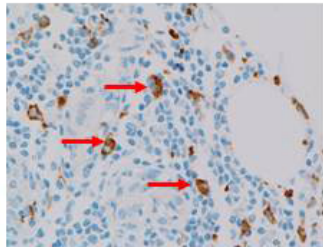
CD3+ TILs



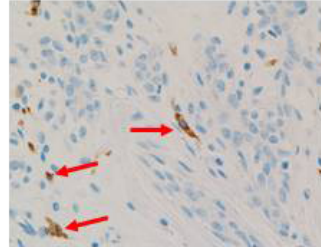
CD8+ TILs



CD163+ Macrophages

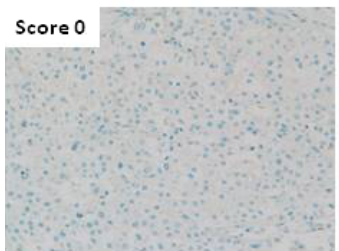


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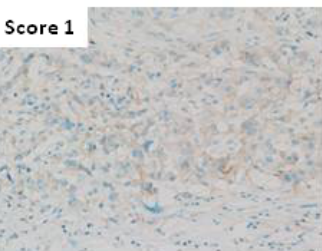


PD-L1 (×400)

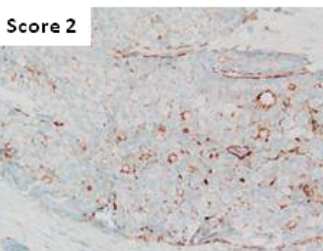
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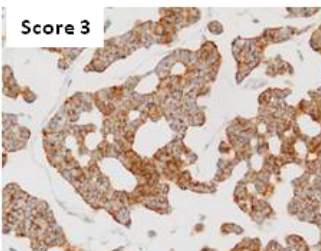
Score 1



Score 2

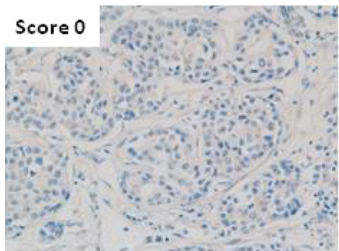


Score 3

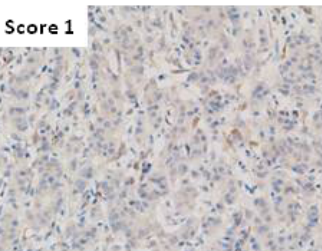


PTEN (×400)

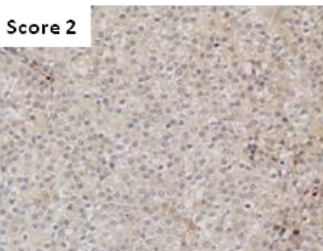
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Score 2



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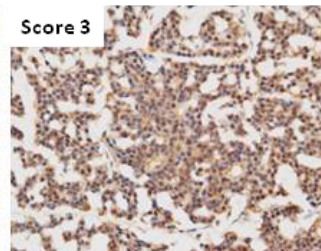


Fig. 1. 1-1. Immunohistochemical staining for the infiltration of CD3⁺ tumor-infiltrating lymphocytes (TILs), CD8⁺ TILs, and CD163⁺ macrophages, and the expression of programmed death 1 (PD-1) in early stage breast cancer (EBC). Images of TILs, macrophages, and PD-1-positive cells in invasive ductal carcinoma (IDC) (immunostaining ×400). Upper panel, CD3⁺ TILs, CD8⁺ TILs, CD163⁺ macrophages, and PD-1 were stained in the intratumoral (black arrows) areas of IDC. Lower panel, CD3⁺ TILs, CD8⁺ TILs, CD163⁺ macrophages, and PD-1 were stained in the stromal (red arrows) areas of IDC. 1-2. Expression of programmed death ligand 1 (PD-L1) and phosphatase and tensin homolog (PTEN) in EBC. Expression of PD-L1 and PTEN were evaluated in the local tumor area by immunohistochemistry obtained from two EBC cases. Upper panel, PD-L1 (×400) was defined as positive with a score of 2+ or 3+. Lower panel, PTEN (×400) was defined as positive with a score of 1+, 2+, or 3+.

negative), HER2-positive type (IHC score 3+ or *HER2* gene/chromosome 17 ratio >2.2 in FISH), and TNBC (hormone receptor-negative and HER2-negative). The study protocol was approved by the Kurume University Ethical Committee. All patients were given a full explanation of the protocol and provided their informed consent before starting the analysis.

Immunohistochemistry staining protocol. This was a retrospective study to evaluate the association between tumor immunological microenvironment factors and clinical outcome in EBC patients for over 10 years of follow-up. A histopathological assessment was carried out using conventional IHC. Each primary tumor tissue was sliced at 4- μ m intervals for fixation and paraffin embedding and examined by routine H&E staining for biological parameters and histological grading, according to the Nottingham combined histological grade (Scarff–Bloom–Richardson grading system).⁽¹¹⁾ The immunohistological staining was undertaken with mAbs for PD-1/PD-L1, PTEN, CD3, CD8, and CD163 using a conventional peroxidase–antiperoxidase staining method. The paraffin-embedded tissue samples were cut at 4 μ m and examined on a coated slide glass, and labeled with the following antibodies using the BenchMark ULTRA (Ventana Automated Systems, Tucson, AZ, USA) and Bond-Max autostainer (Leica Microsystems, Newcastle, UK). Primary antibodies (with dilutions) were as follows: anti-PD-L1 mAb (\times 200, EPR1161(2); Abcam, Cambridge, MA, USA); anti-PD-1 mAb (\times 200, NAT105; Abcam); anti-CD3 mAb (\times 300, LN10; Leica Microsystems); anti-CD8 mAb (\times 200, 1A5; Leica Microsystems); anti-CD163 mAb (\times 100, 10D6; Leica Microsystems); and anti-PTEN mAb (\times 100, 6H2.1; DakoCytomation, Glostrup, Denmark). Briefly, each slide was heat-treated using Ventana's CC1 retrieval solution for 32 min, and incubated with the antibody for 30 min. This automated system used the streptavidin–biotin complex method with DAB as the chromogen (UltraVIEW DAB detection kit; Ventana Automated Systems). Immunostaining with PD-1, CD3, CD8, and PTEN were carried out on the same fully automated Bond-Max system using onboard heat-induced antigen retrieval with Epitope Retrieval Solution 2 for 30 min and a Refine polymer detection system (Leica Microsystems). We used DAB as the chromogen in all these immunostainings.

Pathological analysis of all cases was carried out to measure the total expression area of each antibody, using images scanned by a charge coupled device digital (CCD) camera (DXM 1200; Nikon, Tokyo, Japan), and the digitized data of the expression area (μm^2) were measured and sequentially analyzed by WinROOF (version 5.7; Mitani Corp., Osaka, Japan) computer software, as previously reported.⁽¹²⁾ Images of expression in the cytoplasm/membrane were selected for clarity in each of 10 high-power fields at \times 400 from each IHC specimen. CD3⁺ or CD8⁺ TILs, CD163⁺ macrophages, and the expression of PD-1⁺ were measured in two locations in each tumor: the intratumoral compartment (direct contact with tumor cells and the edge of a tumor) (Fig. 1-1, upper panel), and the peritumoral (stromal area: between tumor cells but within the tumor stroma) (Fig. 1-1, lower panel).^(13,14) The expression of PTEN and PD-L1 was only measured in the intratumoral compartment (Fig. 1-2). All IHC images were evaluated by two experienced observers (A.K. and M.A.) who were unaware of the conditions of enrolled patients in the study. We determined \geq 50% expression area of CD3⁺ TIL as the cut-off point for high CD3⁺ TIL density; high CD8⁺ TIL density and the TAMs were similarly counted on the expression area of the CD163 population. The staining intensity was evaluated according to the following scale: 0, no staining; 1,

weak staining; 2, moderate staining; and 3, strong staining (Fig. 1-2). The proportion of all tumor cells found to express PD-L1 was determined and then multiplied by the staining intensity score to obtain a final semiquantitative *H* score (maximum value of 300, corresponding to 100% of tumor cells positive for PD-L1 with an overall staining intensity score of 3), as previously reported.^(9,15)

Statistical analyses. Correlations between the expression of PD-L1, PD-1, and PTEN or the infiltration of CD3⁺ TIL, CD8⁺ TIL, and CD163⁺ TAM and patient characteristics were analyzed. The Mann–Whitney *U*-test and Fisher–Freeman–Halton exact test were used to examine statistical differences for continuous values and categorical values, respectively. *P*-values <0.05 were considered to be statistically significant. Multivariate regression was carried out using the Cox proportional hazards model. Disease-free survival time and OS time were calculated from the date of the first surgical treatment until the date of disease progression or death, respectively, or

Table 1. Clinicopathological characteristics of breast cancer patients included in this study

Characteristic	<i>n</i> (%)
Total no. of patients	97 (100)
Age, years	
Range, 27–84	
Median, 58	
<50	31 (32.0)
\geq 50	66 (68.0)
Operation procedure	
Mastectomy	81 (83.5)
Conservation surgery	16 (16.5)
Histopathological stage	
Tumor staging (<i>n</i> = 97)	
pT1 (0.6–20 mm)	56 (57.7)
pT2 (21–50 mm)	38 (39.2)
pT3	3 (3.1)
Axillary nodal status	
pN0	52 (53.6)
pN1 (1,2)	40 (41.2)
pN1 (3)	5 (5.2)
pN2/3	0 (0.0)
Nuclear grade	
G1	47 (48.5)
G2	28 (28.9)
G3	22 (22.7)
Subtype†	
Luminal	55 (56.7)
HER2	21 (21.6)
TN	21 (21.6)
Pathology	
Invasive ductal carcinoma	83 (85.6)
Invasive lobular carcinoma	5 (5.2)
Special type	9 (9.3)
(mucinous/apocrine/micropapillary/mixed matrix-producing)	(3/2/1/3)
Adjuvant therapy	
Chemotherapy	69 (71.1)
Endocrine therapy	53 (54.6)
Radiation	23 (23.7)

†HER2, human epidermal growth factor receptor 2-positive; Luminal, estrogen receptor-positive; TN, triple negative (hormone receptor-negative and HER2-negative).

the last date when the patient was known to be alive. The survival analysis was undertaken using the Kaplan–Meier method, and a comparison of the survival curves with the log–rank test. Statistical tests were carried out using JMP Pro version 11 (SAS Institute, Cary, NC, USA).

Results

Patient characteristics. Between February 1995 and November 2005, 97 operable EBC patients were investigated in this study. The patient characteristics are shown in Table 1 for the overall patient group. The median age of patients at diagnosis

was 58 years (range, 27–84 years). A total of 31 (32%) patients were under 50 years old and 66 (68%) patients were older. Among the 97 patients, 55 patients (56.7%) were determined to have the luminal/Her2-negative intrinsic subtype, while 21 patients (21.6%) and the remaining 21 patients (21.6%) were determined to have the HER2-positive intrinsic and triple negative intrinsic subtypes, respectively. Of the 97 patients who underwent standard mastectomy or conservation surgery, 56 patients (57.7%) were identified with a preoperative tumor stage of T1 (<2 cm) and the remaining 42 patients (42.3%) were diagnosed with T2 or T3 (≥2 cm). After the surgery, 83 patients were histopathologically diagnosed with

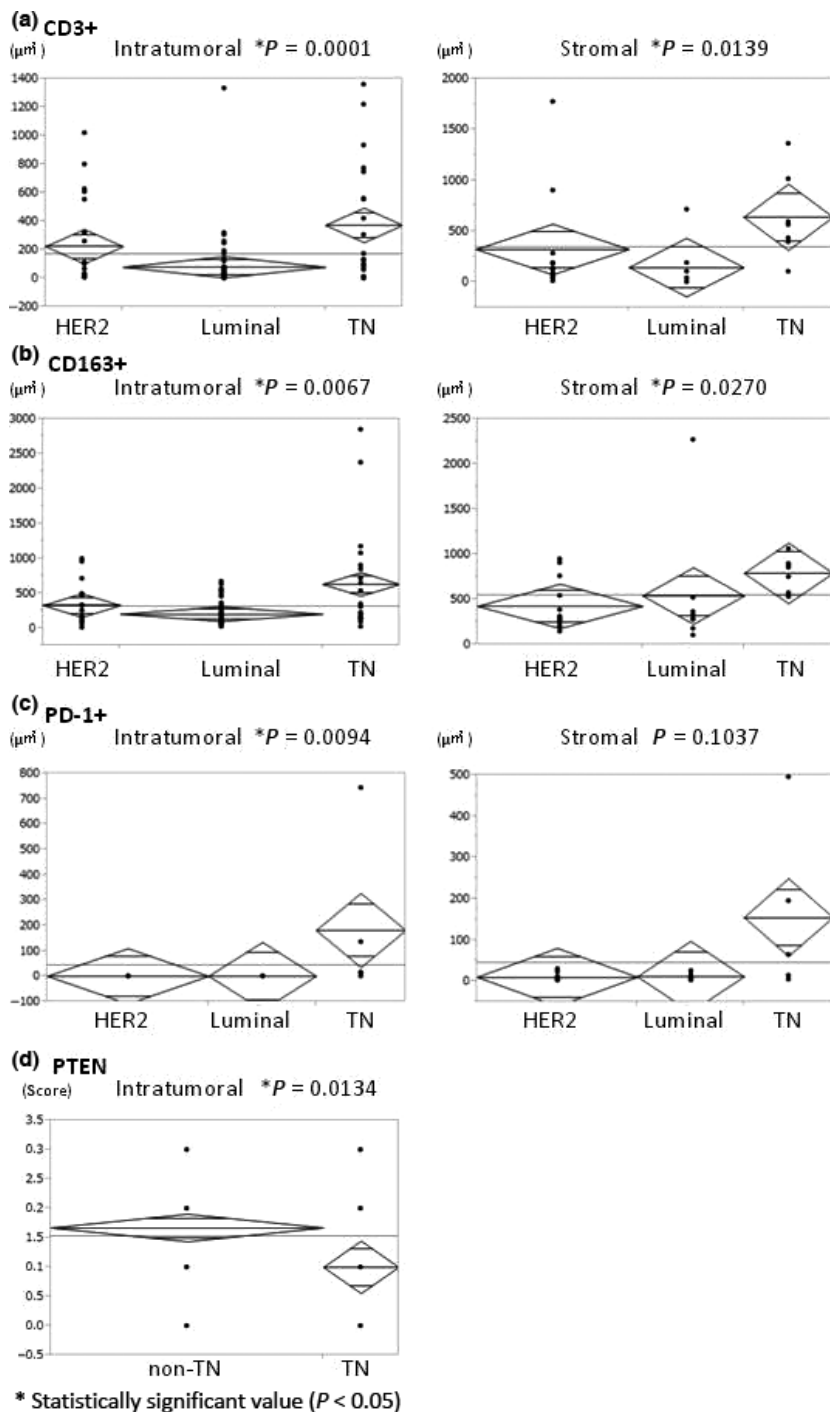


Fig. 2. Infiltration of tumor-infiltrating lymphocytes (TILs) and macrophages and expression of programmed death 1 (PD-1) and phosphatase and tensin homolog (PTEN) between three intrinsic subtypes of early stage breast cancer (EBC). The infiltration of CD3⁺ TILs and CD163⁺ macrophages and expression of PD-1 and PTEN between the three intrinsic subtypes were evaluated for all 97 patients by immunochemical staining. (a) The density of CD3⁺ TILs was significantly higher in triple negative breast cancer (TNBC) samples compared to human epidermal growth factor 2 (HER2)-positive and luminal/HER2 EBC, both in the intratumoral ($P = 0.0001$) and stromal ($P = 0.0139$) areas. (b) The density of CD163⁺ macrophages was significantly higher in TNBC compared to HER2-positive and luminal/HER2 EBC, both in the intratumoral ($P = 0.0067$) and the stromal ($P = 0.027$) areas. (c) Intratumoral PD-1 was expressed significantly higher in TNBC than the other breast cancer subtypes ($P = 0.0094$), whereas PD-1 showed moderate expression between each intrinsic subtype ($P = 0.1037$). (d) Expression of intratumoral PTEN was significantly lower in the TNBC group than in the non-TNBC group ($P = 0.0134$).

invasive ductal BC, including 39 (40.2%) with papillitubular carcinoma, 15 (15.5%) with solid tubular carcinoma, 29 (29.9%) with scirrhous carcinoma, 5 (5.2%) with lobular carcinoma, and 9 (9.3%) with more specific types of carcinoma that included 3 mucinous, 3 mixed matrix-producing carcinomas, 2 apocrine, and 1 micropapillary. None of the patients diagnosed with ductal carcinoma in situ were included in the present study (Table 1).

Density of CD3⁺ and CD8⁺ TILs and CD163⁺ macrophages and expression of PD-L1 or PD-1, according to intrinsic subtype of EBC. Figure 2 shows that the density of CD3⁺ TILs and CD163⁺ macrophages was significantly higher in TNBC compared to the HER2-positive and luminal/HER2-negative EBCs, both in the intratumoral ($P = 0.0001$ and 0.0067 , respectively) and stromal areas ($P = 0.0139$ and 0.027 , respectively) (Fig. 2a,b). Intratumoral PD-1 showed significantly higher expression in TNBC ($n = 5$) than in the other subtypes of BC ($n = 15$) ($P = 0.0094$) (Fig. 2c). In contrast, intratumoral PTEN had significantly lower expression in the TNBC group than in the non-TNBC group ($P = 0.0134$) (Fig. 2d). However, there was no significant difference among each subtype of EBC in terms of CD8⁺ TIL infiltration (intratumoral area, $P = 0.7518$; stromal area, $P = 0.9793$) or intratumoral PD-L1 expression ($P = 0.8389$).

In this study, the expression of PD-L1 was observed in 7 of 21 cases (33.3%) with the TNBC subtype, 18 of 55 cases (32.7%) with the luminal/HER2-negative subtype, and 7 of 21 cases (33.3%) with the HER2-positive subtype. There was no significant difference between TNBC and other subtypes ($P = 0.998$).

Correlation between expression of PTEN and PD-L1 and clinicopathologic factors. The PTEN protein was represented in the intratumoral area in a total of 75 cases (77.3%), with a

staining score range from 1 to 3 (Fig. 1-2). In contrast, the remaining 22 (22.7%) cases were PTEN-negative, significantly fewer than 10 of 21 TNBC cases (47.6%, $P = 0.002$). Table 2 shows the correlation between PTEN expression and various clinicopathologic factors. Multivariate analysis showed that PTEN expression was significantly correlated with tumor size (≥ 2.0 cm diameter) ($P = 0.0286$), tumor nuclear grade 3 ($P < 0.0001$), ER negativity ($P = 0.0023$), and triple negative status ($P = 0.0020$). There was no statistically significant difference between PTEN expression and age, lymph node status, and HER2 status, or between PD-L1 expression and clinicopathologic factors. Only patient age showed a significant association with PD-L1 expression ($P = 0.0155$) (Table 2).

Correlation between TIL density and intratumoral PD-L1 or PTEN expression. Figure 3 shows that CD3⁺ TIL infiltrated density was significantly associated with intratumoral PD-L1⁺ expression (Fig. 3a; $P = 0.0235$), whereas no significant correlation was found between PTEN expression and the density of CD3⁺ TILs ($P = 0.5016$) in EBC patients (Fig. 3b). In addition, CD8⁺ TIL density was not associated with either intratumoral PD-L1 ($P = 0.5702$) or PTEN expression ($P = 0.491$). No significant correlation was observed between the expression of PTEN and PD-L1 ($P = 0.8377$) (Fig. 3c).

Survival analyses by density of TILs and expression of PD-L1 or PTEN. At the time of analysis, the duration of follow-up was 127.3 months. The median DFS time was 88.9 months, when the median OS time of these EBC patients was still not complete. Survival analyses along with analyses of the density of CD3⁺ TILs and CD8⁺ TILs were carried out. There was no significant difference between the density of CD8⁺ TILs and OS, regardless of the EBC intrinsic subtype. Figure 4-1 shows the survival curves for patients with and without increased CD3⁺ TIL and CD8⁺ TIL density for all patients. High CD8⁺

Table 2. Correlation between clinicopathological features of patients with breast cancer and the expression of phosphatase and tensin homolog (PTEN) or programmed death ligand 1 (PD-L1)

Clinicopathologic feature	n	PTEN expression on tumor cells			PD-L1 expression on tumor cells		
		Negative (%)	Positive (%)	P-value	Negative (%)	Positive (%)	P-value
Age, years							
<50	31	10 (32.3)	21 (67.7)	0.1226	26 (83.9)	5 (16.1)	0.0155*
≥50	66	12 (18.2)	54 (81.8)		39 (59.1)	27 (40.9)	
Tumor size, cm							
<2.0	55	8 (14.5)	47 (85.5)	0.0286*	36 (65.5)	19 (34.5)	0.7092
≥2.0	42	14 (33.3)	28 (66.7)		29 (69.1)	13 (30.9)	
No. of positive lymph nodes							
0	52	8 (15.4)	44 (84.6)	0.1728	35 (67.3)	17 (32.7)	0.7453
1-2	40	12 (30.0)	28 (70.0)		26 (65.0)	14 (33.3)	
≥3	5	2 (40.0)	3 (60.0)		4 (80.0)	1 (20.0)	
Tumor grade							
I-II	75	10 (13.3)	65 (86.7)	<0.0001*	52 (69.3)	23 (30.7)	0.3689
III	22	12 (54.5)	10 (45.5)		13 (59.1)	9 (40.9)	
HER2 type							
Positive	21	6 (28.6)	15 (71.4)	0.4664	14 (66.7)	7 (33.3)	0.9698
Negative	76	16 (21.1)	60 (78.9)		51 (67.1)	25 (32.9)	
ER							
Positive	54	6 (11.1)	48 (88.9)	0.0023*	36 (66.7)	18 (33.3)	0.9357
Negative	43	16 (37.2)	27 (62.8)		29 (67.4)	14 (32.6)	
TN	21	10 (47.6)	11 (52.4)	0.0020*	14 (66.7)	7 (33.3)	0.9698
Non-TN	76	12 (15.8)	64 (84.2)		51 (67.1)	25 (32.9)	

*Statistically significant ($P < 0.05$). ER, estrogen receptor; HER2, human epidermal growth factor 2; n, number of a total cases; TN, triple negative.

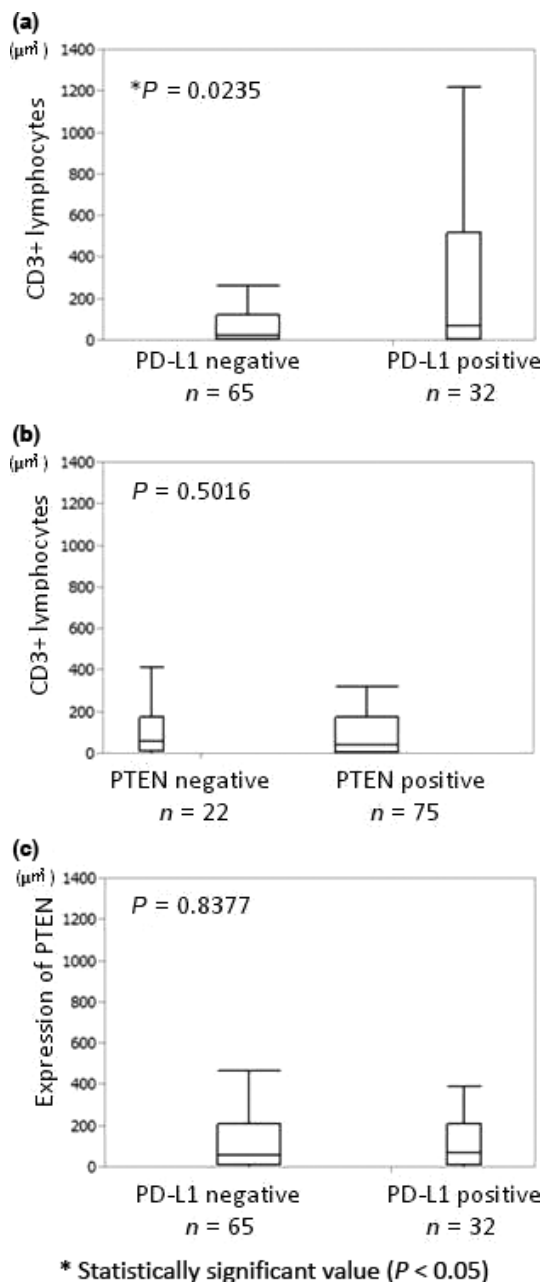


Fig. 3. Correlation between tumor-infiltrating lymphocytes (TILs) and expression of programmed death ligand 1 (PD-L1) and phosphatase and tensin homolog (PTEN) in early stage breast cancer. The correlation between CD3⁺ TILs and expression of PD-L1 and PTEN was analyzed with the Mann-Whitney *U*-test for all 97 patients. (a) The CD3⁺ TIL infiltrated density was significantly associated with intratumoral PD-L1⁺ expression ($P = 0.0235$). (b) There was no significant correlation between PTEN expression and the density of CD3⁺ TILs ($P = 0.5016$). (c) There was also no significant correlation between the expression of PD-L1 and PTEN ($P = 0.8377$).

TIL density was suggested to be a potential prognostic factor for DFS ($P = 0.0447$; Fig. 4-1a), but not for OS ($P = 0.4768$; Fig. 4-1b), whereas there was no significant difference between high CD3⁺ TIL density and these prognoses for DFS ($P = 0.4875$) and OS ($P = 0.895$).

Table 3 shows the correlation for immunologic factors and clinicopathologic factors in all patients. Multivariate analysis indicated that the density of CD8⁺ TILs was an independent

and significant factor for OS ($P = 0.041$) and DFS ($P = 0.054$), as well as the clinical factor of patient age (>50 years, $P = 0.015$) for favorable OS. In contrast, the number of metastatic axillary lymph nodes (>3 nodes) was significantly associated with both decreased OS ($P < 0.0001$) and DFS ($P < 0.0001$). However, neither the immunologic factors of CD3⁺ TIL density, CD163⁺ macrophage density, PD-L1 expression, or PTEN expression, nor clinicopathologic factors such as tumor size, <3 metastatic axillary lymph nodes, or nuclear grade were significantly associated with OS or DFS (Table 3).

Figure 4-2 shows the survival curves for patients with and without PTEN expression in each intrinsic subtype. For each intrinsic subtype, the survival curves were compared for patients with PTEN expression and non-expression (PTEN loss). Compared with patients with non-expression of PTEN, HER2-positive patients with PTEN expression showed significantly better OS ($P = 0.0489$; Fig. 4-2b), whereas no significant differences were seen in other patients with intrinsic subtypes ($P = 0.1913$; Fig. 4-2a), with each luminal/HER2-negative subtype ($P = 0.3953$; Fig. 4-2c), or the TNBC subtype ($P = 0.546$; Fig. 4-2d). In contrast, non-expression of PD-L1 (PD-L1 negativity) was a significant prognostic factor for DFS in luminal/HER2-negative patients ($P = 0.0355$; Fig. 4-3), whereas there was no significant difference between PD-L1 expression and these prognoses in TNBC and HER2-positive patients, or in all patients together (HER2-positive type, $P = 0.5773$, Fig. 4-3b; TNBC, $P = 0.4094$, Fig. 4-3d; and all patients without PD-L1 expression, $P = 0.5508$, Fig. 4-3a).

In addition, further analysis of PD-L1 expression combined with CD3⁺ or CD8⁺ TIL density was subsequently carried out to examine the relationship between these factors and EBC prognosis. Patients were divided into various groups by their positive or negative PD-L1 expression and high or low density of CD3⁺ and CD8⁺ TILs, in order to identify the impact on clinical outcome of patients with EBC. Figure 5 shows the results of Kaplan-Meier survival curves for patients in the PD-L1⁺/CD8⁺ high group and non-PD-L1⁺/CD8⁺ high group, including the PD-L1⁻/CD8⁺ high or low groups and the PD-L1⁺/CD8⁻ low group. However, no significant difference was observed in DFS ($P = 0.6102$) or OS ($P = 0.6702$) by the combination of PD-L1 expression and CD3⁺ density, and the median DFS was not reached in the non-PD-L1⁺/CD8⁺ high group. However, the duration was significantly shorter in the PD-L1⁺/CD8⁺ high group (DFS = 38.1 months, log-rank = 0.0224) (Fig. 5a). Furthermore, the median OS was also not reached, but was significantly shorter in the PD-L1⁺/CD8⁺ high group compared to the non-PD-L1⁺/CD8⁺ high group (log-rank = 0.0367) (Fig. 5b).

Discussion

Despite the fact that recent research has outlined immunologic molecules, such as high PD-1/PD-L1 expression in BC, and novel treatment strategies like immunotherapy,⁽¹⁶⁻²⁰⁾ there are few clinical studies focused on the immune microenvironment. The research investigating clinical biomarkers related to immunologic factors or TILs involving EBC is still insufficient. Our previous studies showed that the expression of FOXP3 on lymphocytes or tumor cells of EBC was significantly associated with clinical outcome and tumor-specific immune responses, as well as the observation that clinical benefits in patients with metastatic BC could be augmented by

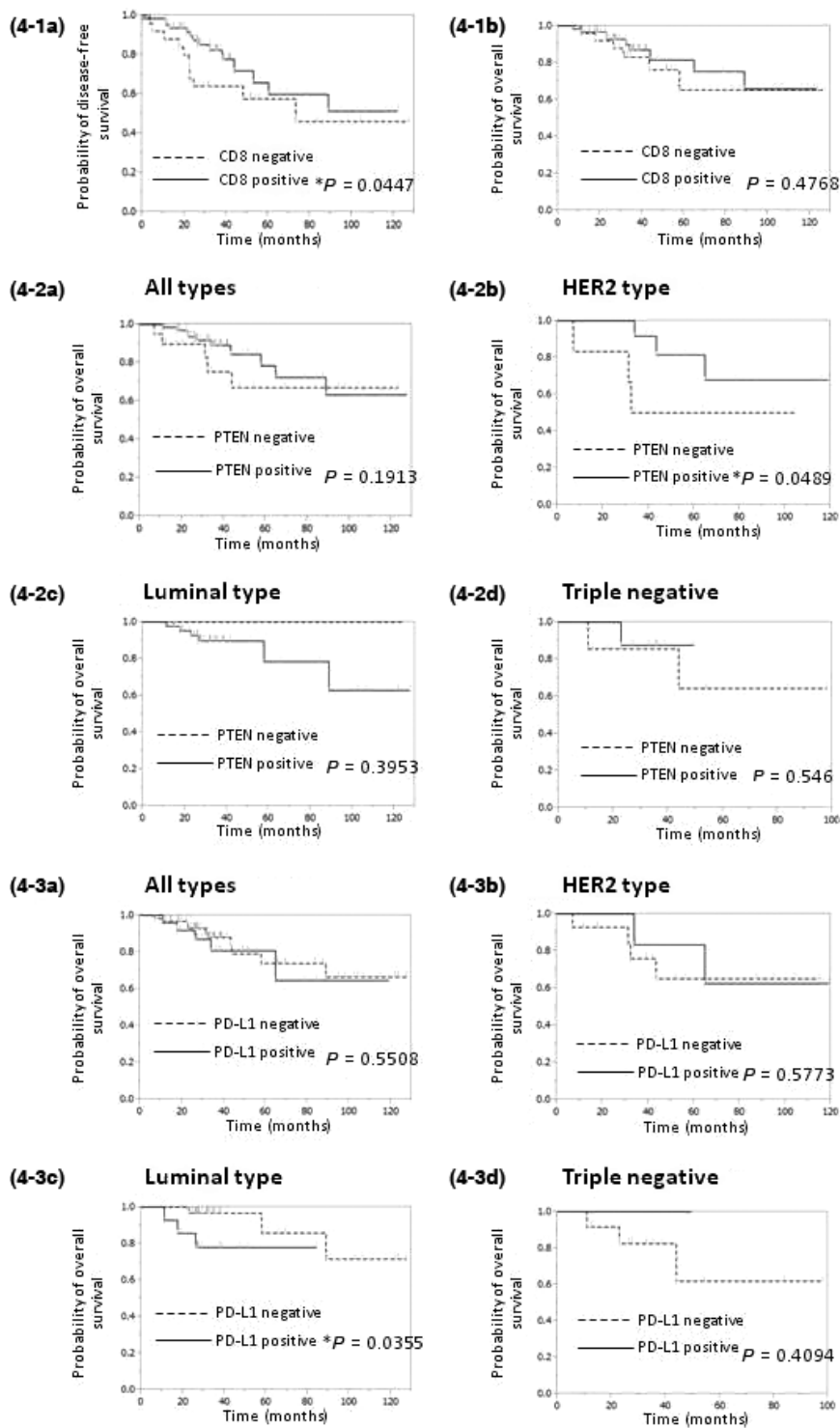


Fig. 4. Survival curves for early stage breast cancer (EBC) patients with and without CD8⁺ tumor-infiltrating lymphocyte (TIL) infiltration, expression of phosphatase and tensin homolog (PTEN), and expression of programmed death ligand 1 (PD-L1). 4-1. There was a significant survival advantage for disease-free survival (DFS) (a), but not for overall survival (OS) (b), in patients who showed high CD8⁺ TIL infiltration, compared to those without CD8⁺ TIL infiltration ($P = 0.0447$ and 0.4765 , respectively). 4-2. Positive expression of PTEN was a significant prognostic indicator for OS, but not for DFS, in human epidermal growth factor 2 (HER2)-positive patients, whereas there was no significant difference between positive PTEN expression and prognosis in the patients overall, luminal/HER2-negative, or triple negative breast cancer (TNBC) patients. (a) All patients, $P = 0.1913$. (b) HER2-positive, $P = 0.0489$. (c) Luminal/HER2-negative, $P = 0.3953$. (d) TNBC, $P = 0.546$; log-rank test. 4-3. Negative expression of PD-L1 was a significant prognostic factor for OS, but not for DFS. Luminal/HER2-negative, in contrast, showed no significant difference between negative PD-L1 expression and prognosis in patients overall, TNBC, or HER2-positive patients. (a) All patients, $P = 0.5508$. (b) HER2-positive, $P = 0.5773$. (c) Luminal/HER2-negative, $P = 0.0355$. (d) TNBC, $P = 0.4094$; log-rank test. 4-4. Summary of correlation between survival time and expression of PD-L1 or PTEN for figures 4-2 and 4-3.

(4-4)	EBC subtype	PTEN	PD-L1
	All cases	0.1913	0.5508
	HER2	0.0489*	0.5773
	Luminal	0.3953	0.0355*
	Triple negative	0.5460	0.4094

* Statistically significant value ($P < 0.05$)

Figure 4-4 shows the summary of correlation between survival time and expression of PD-L1 or PTEN for figures of 4-2 and 4-3

Table 3. Multivariate Cox regression analysis for all subtypes of early stage breast cancer in 97 patients

Multivariate analysis Variable	OS			DFS		
	HR	95%CI	P-value	HR	95%CI	P-value
Age (>50 years)	0.13	0.02–0.65	0.0130*	0.94	0.33–2.71	0.9010
Tumor size (>2 cm)	0.29	0.05–1.41	0.1260	1.37	0.47–4.07	0.5660
No. of metastatic lymph nodes	1.50	0.34–6.33	0.5850	1.29	0.48–3.77	0.6180
No. of metastatic lymph nodes (>3)	3.43	1.42–9.65	<0.0001*	3.77	1.83–7.31	<0.0001*
Nuclear grade 3	1.50	0.33–6.04	0.5830	1.02	0.34–2.89	0.9710
PD-L1 positive	2.65	0.73–9.04	0.1320	2.05	0.81–4.93	0.1250
PTEN negative	1.52	0.26–3.90	0.6560	0.81	0.23–2.45	0.7250
Density of CD3+ TILs	0.40	0.08–2.06	0.2630	0.65	0.21–2.50	0.5000
Density of CD8+ TILs	0.43	0.05–0.95	0.0410*	0.39	0.14–0.22	0.0540

*Statistically significant value ($P < 0.05$). CI, confidence interval; DFS, disease-free survival; HR, hazard ratio; OS, overall survival; PD-L1, programmed death ligand 1; TIL, tumor-infiltrating lymphocyte.

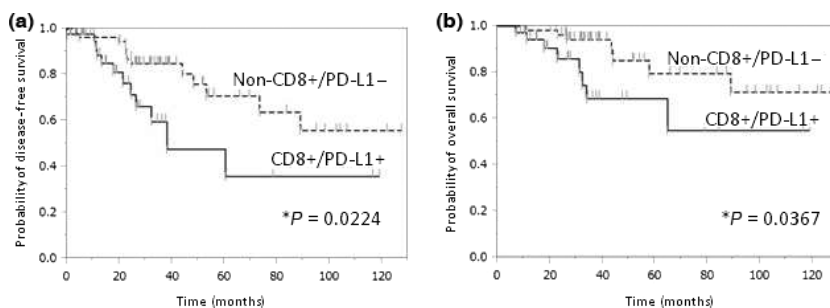


Fig. 5. Survival curves for early stage breast cancer patients with and without programmed death ligand 1 (PD-L1)⁺/CD8⁺ expression. Expression of PD-L1⁺/CD8⁺ was a significant prognostic factor for overall survival (OS) and disease-free survival (DFS) in all patients. (a) Duration of DFS was significantly shorter in the PD-L1⁺/CD8⁺ group (DFS = 38.1 months; log-rank test, $P = 0.0224$) compared to the non-PD-L1⁺/CD8⁺ group (DFS, not reached). (b) Duration of OS was also significantly shorter in the PD-L1⁺/CD8⁺ group compared to the non-PD-L1⁺/CD8⁺ group (median OS, not reached; log-rank test, $P = 0.0367$).

* Statistically significant value ($P < 0.05$).

peptide vaccines derived from various tumor-associated antigens.^(1–3) In this study, we described the tumor environmental interaction between the density of TILs or macrophages and various immunologic molecules, including PD-L1, PD-1, and PTEN, by the intrinsic biological subtypes in EBC. We analyzed the relationship, if any, of these immunologic molecules and the clinical outcome of OS and PFS to identify novel biomarkers for predicting the clinical outcome of EBC.

Previous studies had shown that TILs frequently infiltrate into highly proliferative tumors, such as TNBC and HER2-positive BC, and were a predictive factor for responses to trastuzumab in HER2-positive BC. Infiltration of CD8⁺ TILs was reported to be an independent favorable prognostic factor for TNBC.^(7,14,21–23) The immunologic factors, including PD-1/PD-L1 expression and PTEN expression and their interaction with CD3⁺ and CD8⁺ TILs, or CD163⁺ macrophages in the tumor area, have still been insufficiently investigated for EBC. In the present study, we have indicated the relationship between expression of immunologic factors and density of infiltrated TILs and macrophages by the biologic subtypes of EBC, in which the density of CD3⁺ TILs and CD163⁺ macrophages in the intratumoral or stromal (peritumoral) area and intratumoral PD-1 expression are significantly higher in TNBC compared to HER2-positive or luminal/HER2-negative EBC (Fig. 2). There was, however, no significant difference in intratumoral or stromal PD-L1 expression between TNBC and other BC subtypes. In contrast, intratumoral PTEN expression of TNBC was significantly lower compared to the non-TNBC group. The multivariate analysis shows PD-L1 expression was significantly associated with patient age at EBC diagnosis. In addition, PTEN negativity (PTEN loss) was significantly associated with the subtype of TNBC, tumor size, nuclear grade,

and hormonal receptor status. Previous studies have shown the remarkable immunological relevance of increased infiltration of TILs and TAMs in metastatic TNBC compared with the tumors of other intrinsic subtypes,^(24–27) whereas PD-1 expression was correlated with unfavorable outcomes for EBC patients.^(9,28) Our data suggest that, even in early stage TNBC, there was a stronger immunologic association of TILs and TAMs within the tumor area, as well as that observed in metastatic recurrent breast cancer. Additionally, the expression of intratumoral PD-1, an immune checkpoint molecule, was higher in TNBCs, whereas PD-L1 expression was only significantly associated with patient age, regardless of the intrinsic subtype of EBC.

Several studies have reported that the deletion or silencing of the *PTEN* gene, a phosphatase and tensin homolog, altered PD-L1 expression in glioblastoma tumors,⁽²⁹⁾ was correlated with ER/progesterone receptor-negative tumors,⁽³⁰⁾ and enhanced PD-L1 expression on the TNBC cell surface, leading to decreased T cell proliferation and resulting in increased apoptosis.⁽¹⁷⁾ To our knowledge, our study showed the first clinical description of the relationship between CD3⁺ and CD8⁺ TILs and the expression of PTEN or PD-L1 in EBC. Although there was no significant correlation observed between expression of PTEN and PD-L1, or with the density of CD3⁺ or CD8⁺ TILs, a high expression of intratumoral PD-L1 was significantly correlated with infiltrated density of CD3⁺ TILs (Fig. 3). These data indicated that the infiltration of TILs was significantly associated with the expression of PD-L1 within the EBC tumor area.

In previous studies, Mahmoud *et al.* and Rathore *et al.* showed that both high intratumoral and stromal infiltrations of CD3⁺ or CD8⁺ TILs were associated with better survival of

EBC patients, and that PTEN expression was a predictive and prognostic biomarker for trastuzumab treatment in HER2-positive metastatic BC, but the clinical effect of PD-L1 remains controversial.^(26,27) The present study shows that, although there were no significant benefits associated with the density of CD3⁺ or CD8⁺ TILs in relation to OS, the median DFS of the patients with a high density of CD8⁺ TILs was significantly longer than of those with a low density of CD8⁺ TILs (Fig. 4). The density of CD8⁺ TILs and other clinical factors including patient age and the number of metastatic axillary lymph nodes were also independent predictive factors for OS and/or DFS in patients with EBC (Table 3), consistent with previous clinical evidence.⁽³¹⁾ In addition, we found that PTEN expression (PTEN loss) was significantly associated with tumor size, tumor nuclear grade, and ER negativity (Table 2); similar results have been previously reported elsewhere.^(32,33) Notably in this study, PTEN expression was a significant prognostic factor for favorable OS in HER2-positive patients who were without adjuvant anti-HER2 therapy (trastuzumab), which was not true for other intrinsic EBC subtypes. In this study, the multivariate analysis also showed PD-L1 expression was significantly correlated with younger age at EBC diagnosis (Table 2), in accordance with previously reported findings.^(9,34) Furthermore, PD-L1 expression was a significant prognostic factor for OS in patients with luminal/HER2-negative EBC.

Because the infiltration of TILs producing γ -interferon, particularly CD8⁺ TILs, can augment PD-L1 expression in tumor cells,⁽³⁵⁾ the prognostic relevance of PD-L1 expression, CD8⁺ TILs, and CD3⁺ TILs, was subsequently analyzed. Our data shows that the PD-L1⁺/CD8⁺ group had significant unfavorable DFS and OS compared to the non-PD-L1⁺/CD8⁺ group, whereas there was no significant difference between the PD-L1⁺/CD3⁺ group and non-PD-L1⁺/CD3⁺ group. It suggests that in EBCs, despite high levels of infiltration, the CD8⁺ TILs may contribute a favorable immunologic effect for preventing recurrence or metastasis from the primary BC by promoting and enhancing antitumor cytokine production and cytolytic activity.^(26,36–38) Simultaneously, CD8⁺ TIL infiltration may subsequently promote PD-L1 expression in tumor cells to develop the immune escape mechanism for some BCs, particularly TNBC, that express significantly high PD-1 and PD-L1.^(17,39) Therefore, the combination of CD8⁺ TILs with PD-L1 expression may be more important than either CD8⁺ TIL infiltration or PD-L1 expression alone for clinical analysis of predictive or prognostic effects in EBC. Further large cohort studies are warranted to validate the association of CD8⁺ TILs and PD-L1 expression, and to identify whether this combination has clinical meaning as a prognostic biomarker for EBC.

As this study was evaluated with a retrospective dataset, there were several limitations, including the small, non-

uniform sample size, to investigate the association with each immunologic factor. Expression of PD-L1 was counted regionally, not only in the tumor cells, but also in the lymphocytes or macrophages, and the threshold for positivity for each immunologic factor was not clearly defined. However, this study indicated the association between various immunologic factors among patients with EBC for a long-term follow-up over 10 years.

In conclusion, this study showed that the immunologic relevance of the infiltration of TILs and macrophages and the expression of PD-1/PD-L1 and PTEN were significantly associated with biologic intrinsic subtypes of EBC. Triple negative BC showed significant infiltration of TILs and macrophages, expression of PD-1, and non-expression of PTEN compared with other BC subtypes. Although the high density of CD8⁺ TILs was a significant prognostic factor for favorable PFS and OS in EBC patients, PTEN expression and PD-L1 expression were significant prognostic factors for favorable OS in HER2-positive patients and in patients with luminal/HER2-negative BC, respectively. The combination of CD8⁺ TILs with PD-L1 expression may also be important for predicting clinical outcome in EBC.

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Disclosure Statement

The authors have no conflict of interest.

Abbreviations

BC	breast cancer
DAB	3,3'-diaminobenzidine
DFS	disease-free survival
EBC	early stage breast cancer
ER	estrogen receptor
FOXP3	Forkhead box P3
HER2	human epidermal growth factor 2
IHC	immunohistochemistry
OS	overall survival
PD-1	programmed death 1
PD-L1	programmed death ligand 1
PTEN	phosphatase and tensin homolog
TAM	tumor-associated macrophage
TIL	tumor-infiltrating lymphocyte
TNBC	triple negative breast cancer

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