

Comparison of tumor-infiltrating lymphocytes between primary and metastatic tumors in breast cancer patients

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Key words

Immune microenvironment, immunohistochemistry, metastatic breast tumor, primary breast tumor, tumor-infiltrating lymphocytes

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Funding Information

Ministry of Education, Culture, Sports, Science and Technology, Japan.

Received July 25, 2016; Revised September 29, 2016;
Accepted October 2, 2016

Cancer Sci 107 (2016) 1730–1735

doi: 10.1111/cas.13101

The presence of tumor-infiltrating lymphocytes (TILs) is associated with favorable long-term outcome in breast cancer. However, little is known about changes in TILs during metastatic progression. To confirm our hypothesis that malignant tumors escape from the host immune system during metastasis, we evaluated the percentage of TILs in paired samples of primary and metastatic breast tumors. We retrospectively identified 25 patients with human epidermal growth factor receptor-2 (HER2⁺, *n* = 14) and triple negative (TN, *n* = 11) early breast cancer diagnosed between 1990 and 2009 at Tokai University Hospital (Isehara, Japan) and who subsequently experienced regional or distant recurrence confirmed by tumor biopsy/resection. Hematoxylin–eosin-stained slides of these paired samples were evaluated for stromal TILs. Immunohistochemical staining was carried out using primary antibodies against CD4, CD8, Foxp3, programmed cell death ligand 1 (PD-L1), PD-L2, and HLA class I for characterizing the TILs and breast tumors. The percentage of TILs in the primary tumors was significantly higher (average 34.6%) than that in metastatic tumors (average 15.7%) (paired *t*-test, *P* = 0.004) and that of CD8⁺ and CD4⁺ T cells significantly decreased from primary to metastatic tumors (paired *t*-test, *P* = 0.008 and *P* = 0.026, respectively). The PD-L1, PD-L2, and HLA class I antibody expression changed from positive to negative and vice versa from the primary to the metastatic tumors. Tumors at first metastatic recurrence in HER2⁺ and TN breast cancers have a lower percentage of TILs and CD8⁺ and CD4⁺ T cells compared to primary tumors, which indicates that immune escape plays a role in tumor progression.

The presence of tumor-infiltrating lymphocytes (TILs) is associated with favorable long-term outcome in breast cancer.^(1,2) Previous studies have reported that immune activation at the baseline, as assessed by pathology or gene expression arrays, is associated with a higher likelihood of pathological complete response after neoadjuvant chemotherapy (NAC), particularly in human epidermal growth factor receptor-2 (HER2)-positive and triple negative (TN) breast cancers.^(3–10) Furthermore, trastuzumab has been predicted to have beneficial effects.⁽¹¹⁾ Increased expression of a subset of immune function genes may provide a means of predicting the benefits of adjuvant trastuzumab treatment.⁽¹²⁾ Tumor-infiltrating lymphocytes in breast tumors mainly comprise cytotoxic (CD8⁺) T cells, followed by helper (CD4⁺) T cells and natural killer cells.⁽¹³⁾ A high CD8⁺/Foxp3⁺ ratio in the TILs of biopsy specimens was found to be a strong predictor of pathological complete response after NAC in TN breast cancers.⁽¹⁴⁾ In addition, the presence of TILs in residual disease after NAC is associated with better prognosis in TN breast

cancers patients. This suggests that chemotherapy could convert low-TIL tumors into high-TIL tumors. This finding supports the concept that chemotherapy could partly exert its antitumor effect through the immune system.⁽¹⁵⁾ Preclinical studies have also suggested that cytotoxic agents may partly exert their antitumor activity by inducing immune responses against tumor cells.⁽¹⁶⁾

However, little is known about the change in TILs during metastatic progression and the prognostic impact of TILs in metastatic sites.^(17,18) The current concept of cancer immunoeediting leading from immune surveillance to immune escape is proposed to comprise three essential phases: (i) elimination; (ii) equilibrium; and (iii) escape.⁽¹⁹⁾ In the elimination phase, tumor cells undergo angiogenesis and stromal remodeling, resulting in tumor cell variants with low immunogenicity and resistance to immune attack. These tumor cell variants then proceed to the equilibrium phase but the elimination phase continues through immune selection pressure. Tumor progression then leads to the release of tumor-derived soluble factors

that are involved in several mechanisms of immune evasion in the escape phase.⁽²⁰⁾

We hypothesized that malignant tumors escape from the immune system of the host during the process of metastasis. We therefore aimed to study the immune escape by evaluating TILs in paired samples from primary and metastatic breast tumors. We also evaluated the prognostic impact of TILs in the metastatic sites.

Methods

Patients. This study was reviewed and approved by the Institutional Review Board for Clinical Research, Tokai University (Isehara, Japan). We retrospectively identified 25 patients with TN or HER2⁺ early breast cancer diagnosed between 1990 and 2009 at Tokai University Hospital and who subsequently experienced a regional or distant recurrence confirmed by tumor biopsy/resection. Patients who had only local events were excluded because it is difficult to determine whether the tumor has recurred or is a new primary tumor.⁽²¹⁾ The clinical characteristics of all the patients were obtained from their medical records.

Pathological assessment. All the tumor specimens were fixed in 10% formalin and embedded in paraffin, and 4- μ m-thick sections were prepared for H&E staining and immunohistochemistry (IHC) and were reviewed by a pathologist. Immunohistochemistry was carried out using the following primary antibodies: anti-estrogen receptor (ER) (–2009, clone 1D5; Dako, Carpinteria, CA, USA; 2010–, clone SP1; Roche Diagnostics, Basel, Switzerland), anti-HER2 (–2009, polyclonal, HercepTest II, Dako; 2010–, clone 4B5, Roche Diagnostics), anti-CD4 (clone SP35; Spring Bioscience, Pleasanton, CA, USA), anti-CD8 (clone C8/144B; Nichirei, Tokyo, Japan), anti-Foxp3 (clone 236A/E7; Abcam, Cambridge, MA, USA), anti-programmed cell death ligand 1 (PD-L1) (polyclonal, ab58810; Abcam), anti-Pdcd-IL2 (PD-L2, clone XX19; Santa Cruz Biotechnology, Dallas, TX, USA), and anti-HLA class I A, B, C (clone EMR8-5; Hokudo, Sapporo, Japan). The specimens were considered positive for hormone receptor if $\geq 1\%$ of the cancer cells expressed ER. For the patients determined as HER2⁺ by IHC, FISH (PathVysion kit; Abbott, Des Plaines, IL, USA) was used to confirm HER2⁺ disease. The breast cancer subtypes were classified using IHC as previously described:⁽²²⁾ HER2-positive or HER2-overexpressing (HER2⁺) and TN (ER[–] and HER2[–]). Hematoxylin–eosin-stained slides for the paired match cases were evaluated for stromal TILs using full sections in 10% increments (<10%, 10%–100%) by a pathologist (N.K.) blinded to the clinicopathological characteristics of the patients, as recommended.⁽²³⁾ The specimens were classified into three groups: low TIL (<10%), intermediate TIL (10%–60%), and lymphocyte-predominant breast cancer (LPBC) ($\geq 60\%$).

To quantify the TILs in each antibody-stained slide, we used a NanoZoomer 2.0 HT (Hamamatsu Photonics, Hamamatsu, Japan) at $\times 40$ magnification. Three non-overlapping fields with high numbers of TILs on the H&E-stained slides were selected.⁽¹⁴⁾ CD4, CD8, and PD-L1 positivity was determined by membranous lymphocyte staining, and Foxp3 and PD-L2 positivity was determined by nuclear lymphocyte staining. The expression of CD4, CD8, Foxp3, PD-L1, and PD-L2 by TILs was recorded in 10% increments, and the score of three fields was averaged. The expression of PD-L1, PD-L2, and HLA class I in the tumors was scored as 0 (negative), 1 (weak), or 2 (strong).

Statistical analyses. Associations of the percentage of TILs with the positivity for each antibody between the primary and metastatic tumors were evaluated using Fisher's exact test for categorical variables and using the two-sided *t*-tests for continuous variables. Overall survival (OS) was defined as the time from date of the first biopsy of metastatic tissue to the date of death resulting from any cause. Patients who were alive and disease-free were censored at the date of last contact. Post-progression OS curves of the patients were drawn using the Kaplan–Meier method, and the statistical difference between two survival curves was calculated using the log–rank test. The correlation between the percentage of TILs and the expression of each of the antibodies was calculated using Spearman's rank correlation coefficient test. In all the analyses, the differences were considered significant at $P < 0.05$. Statistical analyses were carried out using SPSS, version 23 (Armonk, New York, USA).

Results

Comparison of TILs between primary and metastatic tumors. The characteristics of the 25 breast cancer patients (HER2⁺, $n = 14$; TN, $n = 11$) at the time of diagnosis of the primary breast cancer are presented in Table 1. Six primary tumors and one metastatic tumor were core needle biopsy specimens, and the rest were surgical specimens. We evaluated the core needle biopsy specimens before chemotherapy in the patients who received neoadjuvant therapy for excluding the possibility of alterations in the immune microenvironments of the tumors caused by the neoadjuvant therapy. The first biopsy sites of the metastatic tumors were the skin ($n = 7$), brain ($n = 6$), lymph node ($n = 4$), lung ($n = 3$), bone ($n = 2$), and bone marrow/liver/muscle ($n = 1$). The median follow-up time after the first biopsy of recurrent tumors was 54 months (range, 2–176 months). Ten (40%) patients had died of metastatic disease at the last follow-up.

The TILs of the primary and metastatic tumors are shown in Table 2. Of the primary tumors, 28% were LPBC, 52% were intermediate TIL tumors, and 20% were low TIL tumors. Among the corresponding first metastatic tumors, 44% were intermediate TIL tumors and 56% were low TIL tumors (Table 3). Overall, the percentage of TILs in the primary tumors was significantly higher (average, 34.6%) than that in the metastatic tumors (average, 15.7%) (paired *t*-test, $P = 0.004$). This difference was similar in the HER2⁺ ($P = 0.036$) and TN ($P = 0.06$) breast cancer groups. The percentage of TILs decreased in 13 of the 25 cases (66%) and increased in 3 of the 25 cases (12%) from the primary tumors to the metastatic tumors (difference $> 10\%$). We next undertook an exploratory analysis of the post-progression OS according to the percentage of TILs at a distant site of recurrence ($n = 17$). The group with low TILs had a significantly lower OS than that with intermediate TILs (hazard ratio = 3.77; 95% confidence interval, 0.99–14.9; log–rank test, $P = 0.038$) (Fig. S1).

Characteristics of TILs. Immunohistochemical evaluations could not be carried out in five primary tumors and two metastatic tumors because of the small quantity of the specimens. The results of the comparison of the expression of antibodies between the primary and metastatic tumors are shown in Table 4 and Figures S2–S4. Representative photographs of each antibody in the primary and metastatic tumors from the same patient are shown in Figure 1. The median percentage of CD8⁺ T cells was 15.8% (range, <10–37%) and 10.0% (range,

Table 1. Clinicopathological characteristics of primary surgical breast tumor specimens

Characteristics	Total patients (n = 25)	HER2 ⁺ (n = 14)	TN (n = 11)
Age, years			
Median (range)	48 (28–64)	46 (28–61)	49 (40–63)
T			
1	7 (28)	4 (29)	3 (27)
2	12 (48)	8 (57)	3 (27)
3	2 (8)	1 (7)	1 (9)
4	2 (8)	0 (0)	2 (18)
Unknown	2 (8)	1 (7)	2 (18)
N			
0	14 (56)	8 (57)	6 (55)
1	6 (24)	5 (36)	1 (9)
2	0 (0)	0 (0)	0 (0)
3	3 (12)	1 (7)	2 (18)
Unknown	2 (8)	0 (0)	2 (18)
Stage			
1	5 (20)	2 (14)	3 (27)
2	13 (52)	10 (71)	3 (27)
3	4 (16)	1 (7)	3 (27)
4	0 (0)	0 (0)	0 (0)
Unknown	3 (12)	1 (7)	2 (18)
Histological grade			
1	5 (20)	2 (14)	3 (27)
2	13 (52)	8 (57)	5 (45)
3	7 (28)	4 (29)	3 (27)
ER			
Positive	11 (44)	11 (79)	0 (0)
Negative	14 (56)	3 (21)	11 (100)
Chemotherapy			
No	5 (20)	4 (29)	1 (9)
Neoadjuvant	6 (24)	3 (21)	3 (27)
Adjuvant	13 (52)	7 (50)	6 (55)
Unknown	1 (4)	0 (0)	1 (9)
Trastuzumab			
No	21 (84)	12 (86)	9 (82)
Yes	3 (12)	2 (14)	1 (9)
Unknown	1 (4)	0 (0)	1 (9)
Endocrine therapy			
No	11 (44)	4 (29)	7 (64)
Yes	13 (52)	10 (71)	3 (27)
Unknown	1 (4)	0 (0)	1 (9)
Radiotherapy			
No	16 (64)	10 (71)	6 (55)
Adjuvant	8 (32)	4 (29)	4 (36)
Unknown	1 (4)	0 (0)	1 (9)
Alive at last follow-up			
No	10 (40)	6 (43)	4 (36)
Yes	15 (60)	8 (57)	7 (64)

Data are shown as n (%) unless otherwise indicated. ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; TN, triple negative.

<10–37%) and that of CD4⁺ T cells was 40.0% (range, <10–77%) and 25% (range, <10–83%) in the primary and metastatic tumors, respectively. The percentages of CD8⁺ and CD4⁺ T cells in the primary tumors were significantly higher than those in the metastatic tumors (paired *t*-test, *P* = 0.008 and *P* = 0.026, respectively). Moreover, there was a strong correlation between the percentage of CD4⁺ T cells and CD8⁺

Table 2. Comparison of tumor-infiltrating lymphocytes (TILs) between primary and metastatic breast cancer tumors for each subtype

Subtype	First site of biopsy	Primary tumor TILs, %	Metastatic tumor TILs, %	Comparison
HER2 ⁺	Bone marrow	10	<10	→
	Brain	20	<10	▼
	Brain	<10	<10	→
	Brain	80	10	▼
	Brain	<10	10	→
	Brain	30	50	△
	Liver	60	10	▼
	LN (Ax)	50	20	▼
	LN (rotter)	70	20	▼
	LN (SCLN)	70	10	▼
	Lung	10	<10	→
	Muscle (pectoralis)	<10	30	△
	Skin (thoracic wall)	30	<10	▼
TN	Skin (thoracic wall)	<10	<10	→
	Bone	10	20	→
	Bone	20	20	→
	Brain	50	<10	▼
	LN (rotter)	70	<10	▼
	Lung	10	50	△
	Lung	30	<10	▼
	Skin (abdominal wall)	80	30	▼
	Skin (head)	10	<10	→
	Skin (thoracic wall)	<10	<10	→
Skin (thoracic wall)	50	20	▼	
Skin (thoracic wall)	80	10	▼	

The percentage of TILs in the primary tumors was significantly higher (average, 34.6%) than in the metastatic tumors (average, 15.7%) (paired *t*-test, *P* = 0.004). This difference was similar in the human epidermal growth factor receptor 2 (HER2)⁺ (*P* = 0.036) and triple negative (TN) (*P* = 0.06) breast cancer groups. Ax, axillary lymph node; SCLN, supraclavicular fossa lymph node.

Table 3. Comparison of tumor-infiltrating lymphocytes (TILs) between primary and metastatic breast cancer tumors

	Primary	Rate, %	Metastatic	Rate, %
Low TIL	5	20	11	44
Intermediate TIL	13	52	14	56
LPBC	7	28	0	0

LPBC, lymphocyte-predominant breast cancer.

T cells in both the primary and the metastatic tumors (*r* = 0.607 and 0.656, *P* = 0.005 and 0.001, respectively). The percentage of Foxp3⁺ T cells was very low (approximately <10%) and stable in the primary and metastatic tumors.

With regard to the TILs, the median percentage of PD-L1⁺ TILs was <10% (range, <10–90%) and <10% (range, <10–15%) and that of PD-L2⁺ TILs was 42% (range, <10–80%) and 30% (range, <10–80%) in the primary and metastatic

Table 4. Comparison of positivity rate between primary and metastatic breast cancer tumors for each antibody

	Primary tumor	Metastatic tumor	<i>P</i> -value
Total breast tumors, <i>n</i> (%)	20 (100)	23 (100)	
CNB specimens	5 (25)	1 (4)	
Surgical specimens	15 (75)	22 (96)	
TIL positivity rate, median % (range)			
CD4	40 (<10–77)	25 (<10–83)	0.03
CD8	16 (<10–37)	10 (<10–37)	0.01
Foxp3	<10 (<10–10)	<10 (<10)	0.16
PD-L1	<10 (<10–90)	<10 (<10–15)	0.21
PD-L2	42 (<10–80)	30 (<10–80)	0.09
Expression in tumor cells, <i>n</i> (%)			
PD-L1			
Strong: 2	8 (40)	5 (25)	0.46
Weak: 1	10 (50)	15 (75)	
Negative: 0	2 (10)	3 (15)	
PD-L2			
Strong: 2	6 (30)	9 (45)	0.78
Weak: 1	10 (50)	11 (55)	
Negative: 0	4 (20)	3 (15)	
HLA			
Strong: 2	4 (20)	6 (30)	0.89
Weak: 1	14 (70)	15 (75)	
Negative: 0	2 (10)	2 (10)	

CNB, core needle biopsy; PD-L1/2, programmed cell death ligand 1/2; TIL, tumor-infiltrating lymphocyte.

tumors, respectively. There was no significant difference in the percentage of PD-L1⁺ and PD-L2⁺ TILs between the primary and metastatic tumors.

The expression of PD-L1, PD-L2, and HLA class I antibodies changed from strong or weakly positive to negative and vice versa from the primary to the metastatic tumor cells. There was a strong correlation between the expression of PD-L1 and PD-L2 ($r = 0.602$, $P = 0.005$) and between the percentage of PD-L2⁺ TILs and the expression of PD-L2 in the primary tumor cells ($r = 0.788$, $P < 0.001$). There was no strong correlation between the primary and metastatic tumors; the results of the correlation test for the antibody expression between the primary and metastatic tumors were: PD-L1, $r = 0.28$; PD-L2, $r = -0.13$; and HLA class I, $r = 0.43$. We next undertook an exploratory analysis of the post-progression OS according to the expression score for PD-L1, PD-L2, and HLA class I of the tumors at a distant site of recurrence ($n = 17$), but no significance was observed (log-rank test: PD-L1, $P = 0.13$; PD-L2, $P = 0.00012$; and HLA class I, $P = 0.35$). The P -value for PD-L2 was <0.05 , but the order of the survival curve was not theoretical (upper, score 1; middle, score 2; and lower, score 0).

Discussion

Previous studies have shown that a loss of concordance in the status of biomarkers such as ER, Progesterone receptor (PR), and HER2 can occur between primary and metastatic breast tumors.^(24–27) According to the concept of cancer immunoediting, the possibility of discordance of immune microenvironments between primary and metastatic breast tumors should also be considered. In this study, we found that tumors at the first metastatic recurrence in HER2⁺ and TN breast cancers

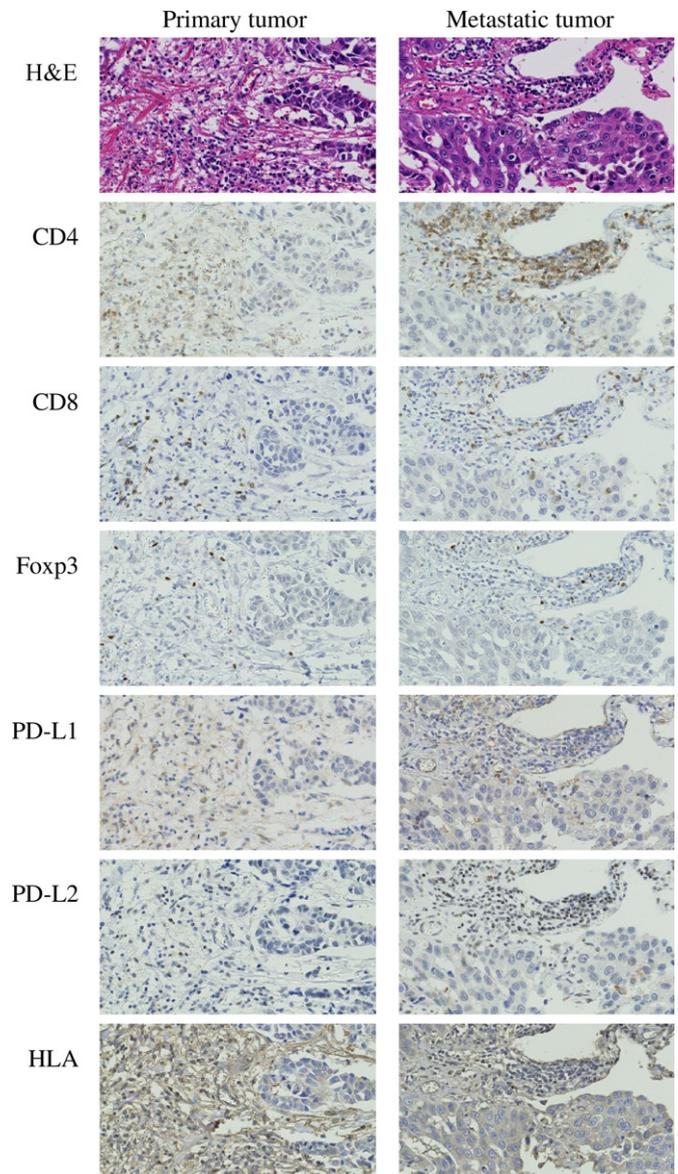


Fig. 1. Immunohistochemical staining for primary antibodies against CD4, CD8, Foxp3, programmed cell death ligand 1 (PD-L1), PD-L2, and HLA class I to characterize tumor-infiltrating lymphocytes. Representative photographs are shown from the same patient who had human epidermal growth factor receptor-2-positive primary breast tumor (left column) and lung metastasis (right column). Original magnification, $\times 400$.

have a lower percentage of TILs and CD8⁺ and CD4⁺ T cells compared to primary tumors, suggesting that immune escape plays a role in tumor progression. To the best of our knowledge, this is one of the first studies to evaluate changes in the tumor microenvironment during the process of metastasis using pair-matched specimens. Our study was similar to previous articles in reporting that TILs and the subset percentages of metastatic sites was lower than that of primary sites,^(17,18) however, we focused on HER2⁺ and TN breast cancers, because TILs are a reliable predictive and prognostic biomarker in these subtypes. Furthermore, we found that the expression of PD-L1, PD-L2, and HLA class I were changeable between primary and metastatic breast cancer tumors.

The clinical utility of TILs in most patients is limited because the determination of the potential of TILs as a specific immune

marker or their ability to facilitate the prediction of the use of T-cell checkpoint inhibitors is a major challenge and because no methods to successfully modulate immunity to reduce mortality have been established so far.⁽²⁸⁾ Antibodies targeting cytotoxic T-lymphocyte-associated antigen 4/programmed death 1/PD-L1 have resulted in clinical responses in multiple tumor types including advanced melanomas, advanced non-small-cell lung cancers, and advanced renal cell carcinomas.^(29–31) These treatment methods are expected to slow cancer progression and significantly prolong the survival of patients with advanced cancer. Given that immune checkpoint therapy only benefits a fraction of patients, there are ongoing efforts being made to identify predictive biomarkers that could be used to select patients that will respond well to such treatment.⁽³²⁾

There are many candidates for biomarkers, such as PD-L1 expression in tumors, the percentage of TILs, the percentage of CD8⁺ T cells in the TILs, the level of cytokines and chemokines produced by lymphocytes in the peripheral blood, and myeloid-derived suppressor cells in tumor lesions.^(9,33) However, sometimes these biomarkers are also detected in primary tumors. These biomarkers could also be influenced by metastatic processes and cytotoxic chemotherapy.⁽³⁴⁾ In our study, the expression of PD-L1, PD-L2, and HLA class I antigen was also found to change from primary to metastatic tumors. Therefore, the evaluation of targeted lesions just before the start of immunotherapy might be needed in future clinical trials.

Although many adjuvant and neoadjuvant studies have assessed infiltrating lymphocytes and stromal lymphocytic infiltration has been found to constitute a robust prognostic factor in primary HER2⁺ tumors or TN breast cancers,^(8,9,35) whether lymphocytic infiltration in metastatic tumors could be a prognostic factor has not yet been evaluated. In our study, the group with low TILs in metastatic tumors had a significantly lower OS than the group with intermediate TILs. Thus, our results indicate that a higher percentage of TILs could have a prognostic impact, even in metastatic tumors.

Previous studies showed that the results of evaluation of the stromal compartment were more reproducible than those of the evaluation of intratumoral TILs.⁽²³⁾ We evaluated TILs within the borders of the invasive tumor and found that it was quite difficult to distinguish the invasive margin TILs clearly from stromal TILs. Although there are few studies involving the evaluation of the invasive edge, there is currently no evidence indicating that TILs at the invasive edge are functionally different from stromal TILs. We therefore evaluated stromal TILs of the breast tissue and other organs. Recommendations for TIL evaluation have been published previously,⁽²³⁾ and guidelines for the same will be standardized in the years ahead. However, we encountered some difficulties in the evaluation of

TILs from other tissues. In some cases, there was very little stromal area in the biopsy specimens, which was not the case in the surgical specimens. It was also difficult to precisely detect TILs among the background lymphocytes in the recurrent tumors in the lymph nodes or the bone marrow on the H&E-stained slides. The TILs were differentiated from the background lymphocytes based on the structural patterns of infiltration in the case of bone marrow tumors, and in case of the tumors in the lymph nodes, the lymph node structure had been totally replaced by the tumor in our study.

One limitation of this study was the small number of patients; in particular, patients with LPBCs were few, which limited our ability to determine the prognostic value of lymphocyte predominance in breast cancer. The reason for the small number of cases is that metastatic biopsy samples were very rare. Previous articles that compared primary and metastatic breast tumors consisted of ER⁺/HER2⁻ cases and the number of HER2⁺ and TN cases was approximately 30–40 in their cohorts.^(17,18) Tumor-infiltrating lymphocytes are associated with a better neoadjuvant chemotherapy response and prognosis in HER2⁺ and TN breast cancers. Therefore, we focused only on HER2⁺ and TN breast cancers, which resulted in a small number of cases.

In summary, we found that tumors at the first metastatic recurrence in HER2⁺ and TN breast cancer patients have a lower percentage of TILs and CD8⁺ and CD4⁺ T cells compared to primary tumors, suggesting a role for immune escape in tumor progression. These differences could occur in a time-, site-, and therapy- (chemotherapy, radiotherapy, and surgery) dependent manner; therefore, the evaluation of targeted lesions just before the start of immunotherapy might be needed in future clinical trials. Furthermore, a low percentage of TILs at the recurrence sites seemed to be associated with poor OS, suggesting a more aggressive phenotype. These findings warrant independent confirmation in future studies.

Acknowledgments

We would like to thank Toshiya Tanaka, Division of Diagnostic Pathology and Support Center for Medical Research and Education for technical assistance with staining. This research was supported in part by the Japanese Ministry of Education, Culture, Sports, Science and Technology's Supported Program for the Strategic Research Foundation at Private Universities, 2012–2016. The authors thank Editage for providing editorial assistance.

Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Overall survival after first recurrence. The group with low tumor-infiltrating lymphocytes (TILs; <10%) had a significantly lower overall survival than the intermediate TIL group (≥10% TILs) (hazard ratio = 3.77; 95% confidence interval, 0.99–14.9; log-rank, $P = 0.038$).

Fig. S2. Comparison of positivity rate between primary and metastatic tumor-infiltrating lymphocytes for each antibody. CD4, CD8, and programmed cell death ligand 1 (PD-L1) positivity was defined by membranous lymphocyte staining, and FoxP3 and PD-L2 positivity was defined by nuclear lymphocyte staining. CD4, CD8, Foxp3, PD-L1, and PD-L2 expression by the tumor-infiltrating lymphocytes was recorded in 10% increments and the score of three fields was averaged.

Fig. S3. Comparison of expression score between primary and metastatic tumor cells for each antibody. The expression of programmed cell death ligand 1 (PD-L1), PD-L2, HLA class I A, B, and C in the tumor cells was scored as 0 (negative), 1 (weak), or 2 (strong). The number of cases is noted above the bars and each bar without annotation represents only one case.

Fig. S4. Representative photographs. The expression of programmed cell death ligand 1 (PD-L1), PD-L2, and HLA class I in the tumors was scored as 0 (negative), 1 (weak), or 2 (strong).