Prognostic Significance of Tumor-Infiltrating Lymphocytes and the Tertiary Lymphoid Structures in HER2-Positive Breast Cancer Treated With Adjuvant Trastuzumab

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ABSTRACT

Objectives: *Tumor-infiltrating lymphocytes (TILs) have prognostic significance in breast cancer. The tertiary lymphoid structure (TLS) is related to the influx of TILs, and expression of major histocompatibility complex (MHC) I in tumor cells is necessary for the effective action of TILs.*

Methods: *We retrospectively evaluated the relationship of TILs and TLS and the expression of MHC I in 447 HER2-positive breast cancers treated with chemotherapy and 1 year of trastuzumab.*

Results: *TILs were more abundant in hormone receptor (HR)–/HER2+ tumors than in HR+/HER2+ tumors. HR–/HER2+ breast cancers with abundant TILs showed a higher histologic grade, the absence of lymphovascular invasion, the presence of peritumoral lymphocytic infiltration, moderate to abundant TLSs in adjacent tissue, and stronger HLA-ABC and HLA-A expression. Abundant TILs and the absence of lymphovascular invasion were found to be good, independent prognostic factors for disease-free survival in patients with HR–/HER2+ breast cancer. The level of TILs was not associated with the patients' prognosis in HR+ tumors.*

Conclusions: *Abundant TILs are an independent prognostic factor in HR–/HER2+ breast cancers. Evaluation of TILs in HR–/HER2+ breast cancers may provide valuable information regarding the prognosis of patients treated using adjuvant chemotherapy and trastuzumab.*

HER2 is overexpressed in 15% to 20% of breast cancers and is associated with aggressive disease progression and poor outcomes. We previously analyzed surgically resected, HER2-positive breast cancers from patients treated with adjuvant chemotherapy and trastuzumab and found that hormone receptor–negative/HER2-positive (HR−/HER2+) tumors had more diffuse and intense lymphocytic infiltration, a higher nuclear grade, less tubule formation, a higher histologic grade, more frequent apocrine features, stronger HER2 immunohistochemical staining, a higher average *HER2* copy number and *HER2*/CEP17 ratio, the absence of *HER2* genetic heterogeneity, and more p53 expression than hormone receptor–positive/HER2-positive (HR+/HER2+) tumors.[1] HR–/HER2+ tumors also have a higher pathologically complete response rate to neoadjuvant systemic therapy than HR+/HER2+ tumors, and the patterns of relapse, metastatic spread, and patient survival also differ between these subtypes.[2-4]

The prognostic and predictive values of tumor-infiltrating lymphocytes (TILs) have been determined for breast cancer. In general, increased lymphocytic infiltration in tumors is inversely correlated with estrogen receptor (ER) and/or progesterone receptor (PR) expression and is positively correlated with HER2 expression, the pathologic complete response rate, and increased patient survival.[2,5-10] Trastuzumab is a monoclonal antibody (mAb) targeting overexpressed HER2 and with great improvement of the clinical outcome in HER2+ breast cancers.[11] Recent studies have shown that the therapeutic effect of trastuzumab depends on innate and adaptive, immune-mediated mechanisms.[12-19] For example, CD8+ T-cell–mediated

immune response enhances the therapeutic effects of the anti-HER2 antibody.[17] In HER2+ breast cancer, increased TIL was associated with decreased distant recurrence in patients who also received trastuzumab in addition to chemotherapy.[20] In one study, patients with a high level of TIL, but not those with a low level of TIL, showed a significant benefit from the addition of trastuzumab to their chemotherapy. In addition, immunostimulatory approaches with antibodies against programmed death 1 or 4-1BB improved the therapeutic activity of anti–ErbB-2 mAb in an immunocompetent mouse model.[19] Therefore, a better understanding of TILs and their related features and the mechanism of TIL influx could facilitate the development of combined immunotherapeutic approaches using anti-HER2 mAb.

Tertiary lymphoid structures (TLSs) are ectopic lymph node–like structures characterized by lymphoid aggregation with high endothelial venules (HEVs), and they have been identified in breast cancer.[21,22] HEVs are specialized blood vessels with plump, cuboidal endothelial cells that express peripheral node addressin (PNAd), and the PNAd ligand CD62L is expressed in lymphocytes. Therefore, the expression of PNAd in HEVs facilitates the extravasation of lymphocytes from the blood vessel to the tissue.[23] High densities of tumor HEVs were correlated with increased naïve, central memory, and activated effector memory T-cell infiltration and T-cell cytotoxicity in breast cancer and were associated with a significantly longer disease-free survival period.[21] However, to date, the TLS location and the relationship between TILs and TLSs in HER2+ breast cancer have not been clearly elucidated.

Cytotoxic T cells recognize antigens that are presented by major histocompatibility complex I (MHC I) on the surface of tumor cells. MHC I proteins are encoded by human leukocyte antigen (*HLA*)–*A*, –*B*, and –*C*, and the *HLA-A* alleles of MHC I produce the most accurate peptide-binding affinity predictions.[24] HLA-A expression has been reported to be related to improved survival in patients with breast cancer.[25] Brown et al[24] analyzed the RNA sequencing data of six tumor sites, including breast cancer, from The Cancer Genome Atlas data and confirmed the relationship between a high *HLA-A* expression level and better overall patient survival. However, the association of TIL and MHC I protein expression has not been analyzed in HER2+ breast cancer.

We analyzed the location of TLSs and the relationship between TLSs and TILs in a large number of HER2+ breast cancers treated with adjuvant chemotherapy and trastuzumab. HLA-ABC and HLA-A protein expression in invasive carcinoma were assessed by immunohistochemistry. We also analyzed their prognostic significance in HR+/HER2+ and HR–/HER2+ breast cancer.

Materials and Methods

Patients and Tissue Specimens

A total of 447 patients with HER2+ breast cancer who underwent surgery for primary breast cancer between 2006 and 2011 at Asan Medical Center and who had available formalin-fixed, paraffin-embedded breast tissue samples for analysis were included in this study, as previously described.[1] All of these patients were preoperatively chemo- and radiotherapy naïve, and all underwent adjuvant treatment. Of the 447 patients, 161 with node-negative breast cancer were treated with four cycles of adjuvant anthracycline and cyclophosphamide (AC) as well as 1 year of trastuzumab as standard therapies. The remaining 286 patients had node-positive breast cancer and were treated with either four cycles of AC followed by four cycles of paclitaxel or four cycles of AC followed by four cycles of docetaxel and 1 year of trastuzumab. Trastuzumab was given every 3 weeks. Clinicopathologic information was obtained from the patients' medical records and surgical pathology reports.

Expressions of standard biomarkers, including ER, PR, and HER2, were reviewed in full sections that were immunohistochemically stained at the time of diagnosis. The ER and PR levels were regarded as positive if there was at least 1% positive tumor nuclei staining.[26] The HR+ group was defined as ER- or PR-positive tumors. The Allred score was also calculated for ER and PR.[27] HER2-overexpressing tumors were defined as those with scores of 3+ by immunohistochemistry (IHC) or gene amplification by fluorescence in situ hybridization or silver in situ hybridization.[28] Exemption from informed consent after de-identification of patient information was approved by the Institutional Review Board of Asan Medical Center.

Histologic Evaluation

H&E-stained sections were histopathologically analyzed for the number of invasive tumors, histologic subtype and grade, ductal carcinoma in situ (DCIS) percentage, the presence or absence of extensive intraductal component and Paget disease, tumor size, pT stage, pN stage, ratio of metastatic to the total number of examined lymph nodes, and lymphovascular invasion, as previously described.[1] TILs (defined as the mean percentage of stroma of invasive carcinoma infiltrated by lymphocytes and plasma cells in 10% increments; if less than 10% of stroma was infiltrated by TILs, 1% or 5% criteria were used; all available full sections were evaluated), peritumoral inflammatory cell infiltrate according to the Klintrup criteria (score 0, no inflammatory cells at the tumor's invasive margin; score 1, mild and patchy inflammatory cells; score 2, prominent band-like inflammatory reaction; and score 3, florid cup-like

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inflammatory infiltrate), lymphoid aggregation in terminal duct lobular units within 5 mm from the invasive or in situ carcinoma, necrosis in the invasive area, the amount of TLSs in adjacent tissue including the in situ component (none, minimal, moderate, or abundant), and the presence or absence of a germinal center in TLSs were also evaluated.[5,9,29,30] TILs were also divided into three groups: minimal (≤10%), moderate (10%-60%), and abundant (>60%).

Tissue Microarray Construction

Formalin-fixed, paraffin-embedded tissue samples were arrayed by a tissue-arraying instrument. After the review of HER2 expression in immunostained whole tumor section slides, three areas were selected and arrayed in 1-mm diameter cores. If the tumor showed heterogeneous staining for HER2, representative areas of different staining were chosen.

Immunohistochemical Staining

Formalin-fixed, paraffin-embedded tissue sections were stained with an automatic immunohistochemical staining device (BenchMark XT; Ventana Medical Systems, Tucson, AZ). Antibodies to HLA-ABC (1:1,600 dilution; EMR8-5, ab70328; Abcam, Cambridge, England) and HLA-A (1:1,600 dilution; EP1395Y, ab52922; Abcam) were used. The percentage of membranous and cytoplasmic expressions of HLA-A and HLA-ABC was evaluated. Then we categorized the expression levels according to the three criteria, as previously described (strong positive [2+], expression in more than 75% of the tumor cells; weak positive [1+], expression between 25% and 75%; and negative [0], loss of expression in more than 75% of the tumor cells).[31]

Statistical Analysis

All statistical analyses were performed using SPSS statistical software (version 18; SPSS, Chicago, IL). An unpaired Student *t* test, Fisher exact test, linear-by-linear association, Spearman correlation coefficients, the log-rank test, and the Cox proportional hazards regression model were used, as appropriate. Disease-free survival was defined as the time from trastuzumab treatment to the recurrence of breast cancer at any site. All tests were two-sided, and the statistical significance was set at 5%.

Results

TILs and Associated Features

TILs were more abundant in HR–/HER2+ tumors than in HR+/HER2+ tumors ($P < .001$) **Figure 1**. Of the 447 tumors, 205 (45.9%) had a minimal amount of TIL, 180 (40.3%) had a moderate amount of TIL, and 62 (13.9%) had abundant TIL. Peritumoral lymphocytic infiltration was seen in 401 (89.7%) tumors: score 1 in 225 cases, score 2 in 136 cases, and score 3 in 40 cases. TLSs were present in 402 (89.9%) tumors and were mainly associated with an adjacent, terminal-duct lobular unit or with in situ carcinoma **Image 1**. Most tumors (98.7%) showed lymphoid aggregation in the terminal-duct lobular unit within 5 mm from the carcinoma. Strong HLA-ABC expression was seen in 233 (52.1%) cases, weak expression was in 85 (19.0%) cases, and no expression in 119 (26.6%) cases **Image 2**. Strong HLA-A expression was also seen in 221 (49.4%) cases, weak expression in 96 (21.5%) cases, and no expression in 124 (27.7%) cases.

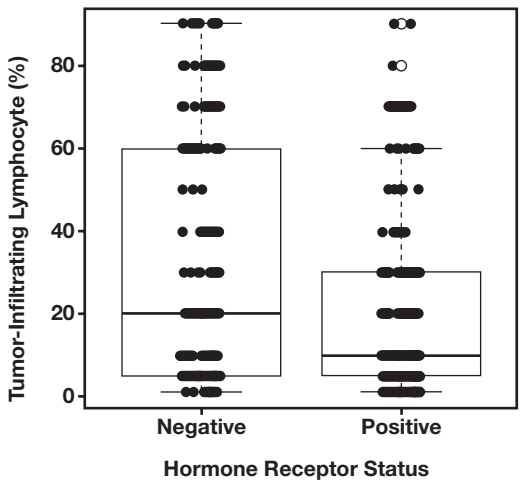

Figure 1 Tumor-infiltrating lymphocytes in HER2-positive breast cancer. Lymphocytic infiltration is more abundant in hormone receptor–negative tumors than in hormone receptor–positive tumors ($P < .001$).

Correlation Between TIL and the Histopathologic Characteristics

The degree of TIL was significantly associated with the histologic grade, lymphovascular invasion, peritumoral lymphocytic infiltration, the degree of adjacent TLS, the presence of a germinal center in TLSs, the *HER2* gene amplification level, HER2 immunohistochemical score, and HLA-ABC, HLA-A, and HR expression **Table 1**. Patient age, histologic type, pT, lymph node metastasis, pTNM stage, percentage of in situ component, the presence of an extensive intraductal component, Paget disease, and necrosis in the invasive area were not associated with the degree of TIL.

Since HER2+ breast cancer showed significantly different histopathologic characteristics, we analyzed the association of TILs with other clinicopathologic factors in the subgroups according to the HR expression. Compared with

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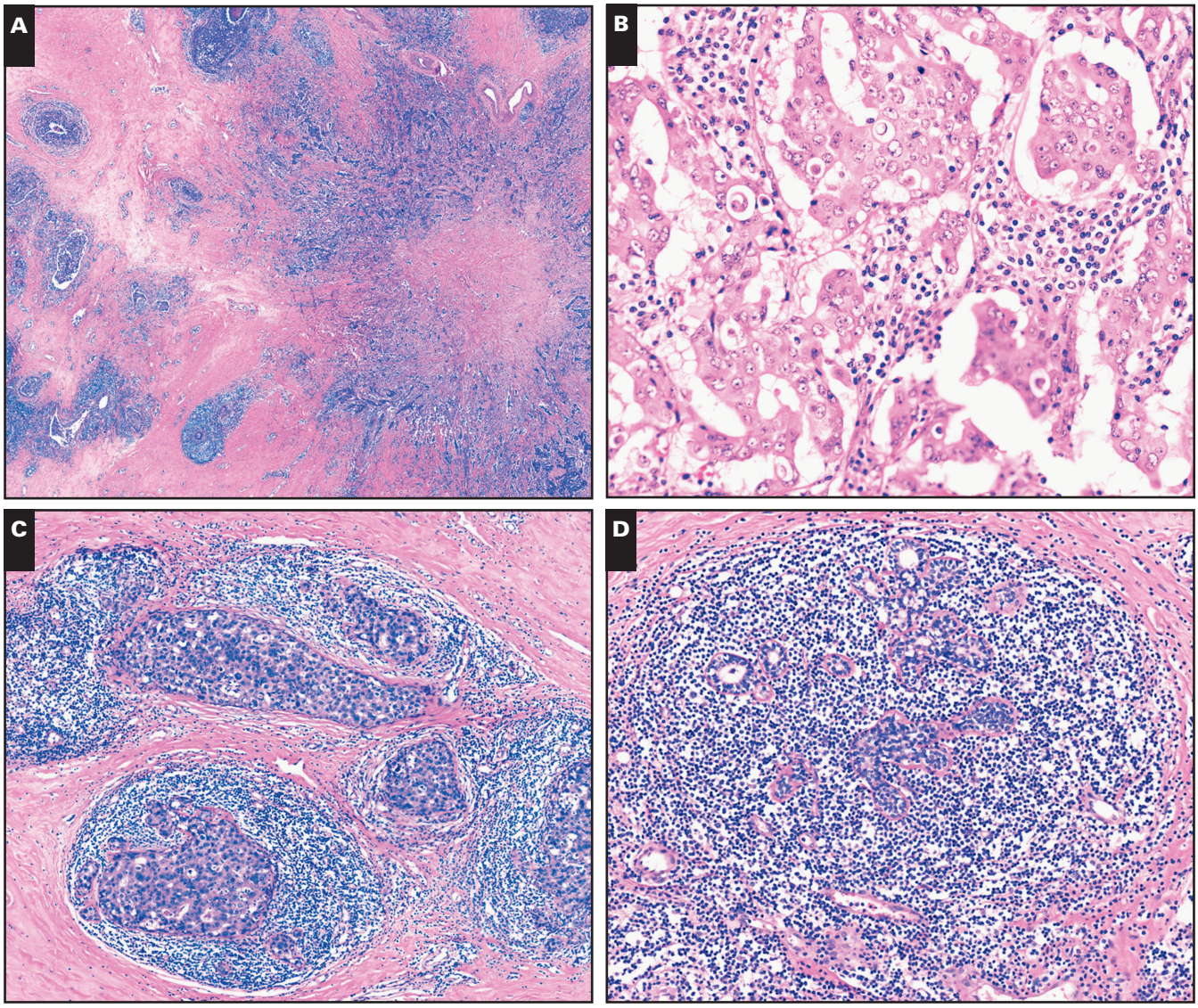

Image 1 HER2-positive breast cancers with abundant tertiary lymphoid structures. **A**, The tumor has an invasive tumor area (right side) with a large amount of tumor-infiltrating lymphocytes (**B**) and abundant tertiary lymphoid structures in ductal carcinoma in situ (**C**) and adjacent, terminal-duct, lobular units (**D**).

HR+/HER2+ tumors, HR–/HER2+ tumors had less frequent lymphovascular invasion when they had more TILs. Conversely, HR+/HER2+ tumors with a high level of TILs were associated with a 3+ HER2 immunohistochemical score. In both groups, a higher level of TILs was correlated with a higher histologic grade, higher peritumoral lymphocytic infiltration score, abundant TLS in adjacent tissue, the presence of a germinal center in TLSs, and stronger expression of HLA-ABC and HLA-A proteins.

Correlation of Continuous Variables

The TIL level was significantly correlated with the peritumoral lymphocytic infiltration score, the degree of adjacent TLS, HLA-ABC and HLA-A expression, the HER2 immunohistochemical score, and the *HER2* gene copy number **Table 2**. Peritumoral lymphocytic infiltration was also significantly associated with the adjacent TLS score, HLA-ABC and HLA-A expression, and the *HER2* gene copy number. The adjacent TLS score showed a significant positive correlation with the DCIS percentage in addition to HLA-ABC and HLA-A expression, the HER2 immunohistochemical score, and the *HER* gene copy number. However, the ER Allred score was inversely correlated with TIL, peritumoral lymphocytic infiltration, and the adjacent TLS score.

In the subgroup analysis according to the HR status, strong correlations among the level of TIL, the peritumoral lymphocytic infiltration score, the degree of adjacent TLS, and HLA-ABC and HLA-A expression were present in both HR–/HER2+ and HR+/HER2+ tumors. However, the

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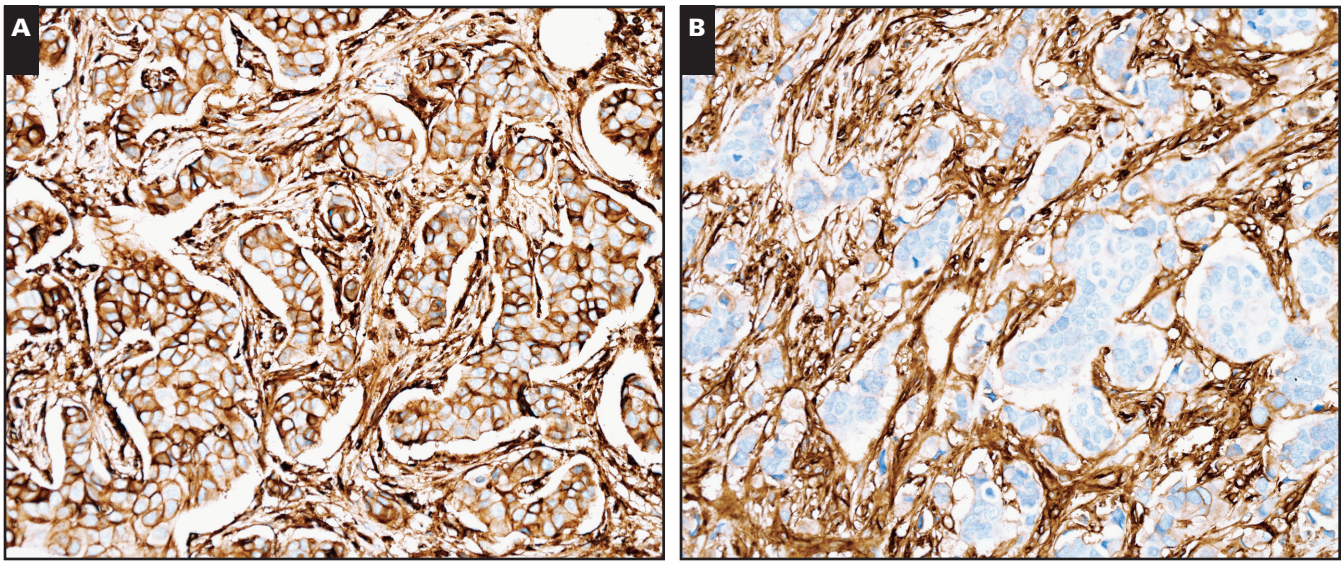

Image 2 Human leukocyte antigen (HLA)–ABC expression in HER2-positive breast cancer. **A**, The tumor cells are strongly positive for HLA-ABC. **B**, The tumor cells are negative for HLA-ABC.

correlation between HLA and HER2 expression differed. In HR–/HER2+ tumors, HLA-ABC and HLA-A expression was inversely correlated with the HER2 immunohistochemical score. Conversely, there were positive correlations in HR+/HER2+ tumors.

Prognostic Significance of the Clinicopathologic Factors

The median patient follow-up was 49 months (range, 18-104 months). As shown in **Table 3**, pT, pTNM stage, lymphovascular invasion, TIL, and peritumoral lymphocytic infiltration were prognostic factors for disease-free survival in patients with HR–/HER2+ tumors. According to Kaplan-Meier analysis, patients with a minimal amount of TIL showed a significantly worse disease-free survival rate than those with moderate to abundant TIL (Image 2). The amount of TIL (hazard ratio, 0.979; 95% confidence interval [CI], 0.960-0.998; $P = .03$) was an independent prognostic factor in multivariate analysis, including the pT stage (hazard ratio, 1.737; 95% CI, 0.976-3.093; $P = .061$) and lymphovascular invasion (hazard ratio, 2.256; 95% CI, 1.072-4.747; $P = .032$). In HR+ tumors, only the presence of lymphovascular invasion was a worse prognostic factor for disease-free survival, and the TIL level did not have prognostic significance in this patient group **Figure 2**.

Discussion

Immunotherapy is a promising therapeutic modality for personalized cancer therapy. To facilitate the development of effective immunotherapeutic tools, a better understanding of TILs and the related features as well as the mechanism of TIL influx is crucial. In this study, we identified the presence of TLSs, the relationship between TILs and TLSs, and the prognostic significance of TILs in HER2+ breast cancer treated with adjuvant chemotherapy and trastuzumab.

Lymphocytic infiltration in DCIS lesions has been described as being present immediately adjacent to the involved duct spaces and associated with nodular aggregates of lymphocytes and a lymphoid follicle or complete, targetoid, periductal aggregates.[32] The presence of TLSs in breast cancer has also been described, and the more frequent association of TLSs with DCIS than with invasive carcinoma has also been reported.[33,34] HER2+ DCIS is characterized by high-grade nuclei and extensive comedonecrosis.[35] Abundant lymphocytic aggregation in the stroma surrounding DCIS is also a characteristic feature of HER2+ DCIS. In our study, we found significant correlations between the degree of TLSs and the HER2 IHC score or *HER2* gene copy number. There was also a strong correlation between the DCIS percentage and the degree of TLSs. Therefore, we can speculate that increased HER2 protein expression or associated mutations may function as an immunogenic factor with attraction of lymphocytes to the tissue and being associated with the formation of TLSs. Another possible explanation is that the frequent comedonecrosis in HER2+ DCIS might be associated with increased macrophage infiltration, which has an important role as antigen-presenting cells in an immune reaction.

DiCaro et al[36] reported that the amount of TLSs was linearly correlated with the density of TILs and suggested that TLSs might allow recruitment of TILs and cooperate

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Table 1
Comparison of the Clinicopathologic Variables According to the TIL Level in HER2-Positive Breast Cancer[a]

Variable	All Patients				Hormone Receptor Negative				Hormone Receptor Positive			
	Minimal TIL	Moderate TIL	Abundant TIL	*P* Value	Minimal TIL	Moderate TIL	Abundant TIL	*P* Value	Minimal TIL	Moderate TIL	Abundant TIL	*P* Value
Age, mean ± SD, y	48.5 ± 9.9	48.8 ± 9.1	51.5 ± 7.3	.072	51.0 ± 10.6	49.6 ± 9.2	52.1 ± 7.3	.267	46.6 ± 9.0	47.7 ± 8.9	49.7 ± 7.4	.374
Histology												
Invasive carcinoma of no special type	189 (92.2)	167 (92.8)	61 (98.4)	.519	86 (98.9)	100 (94.3)	46 (97.9)	.297	103 (87.3)	67 (90.5)	15 (100.0)	.610
Carcinoma with micropapillary differentiation	9 (4.4)	10 (5.6)	1 (1.6)		0	5 (4.7)	1 (2.1)		9 (7.6)	5 (6.8)	0	
Carcinoma with mucinous differentiation	6 (2.9)	2 (1.1)	0		0	0	0		6 (5.1)	2 (2.7)	0	
Metaplastic carcinoma	1 (0.5)	1 (0.6)	0		1 (1.1)	1 (0.9)	0		0	0	0	
Histologic grade												
2	101 (49.3)	57 (31.7)	9 (14.5)	<.001	31 (35.6)	26 (24.5)	6 (12.8)	.004	70 (59.3)	31 (41.9)	3 (20.0)	.001
3	104 (50.7)	123 (68.3)	53 (85.5)		56 (64.4)	80 (75.5)	41 (87.2)		48 (40.7)	43 (58.1)	12 (80.0)	
pT												
1	95 (46.3)	78 (43.3)	34 (54.8)	.247	42 (48.3)	53 (50.0)	26 (55.3)	.254	53 (44.9)	25 (33.8)	8 (53.3)	.810
2	100 (48.8)	97 (53.9)	28 (45.2)		41 (47.1)	51 (48.1)	21 (44.7)		59 (50.0)	46 (62.2)	7 (46.7)	
3	10 (4.9)	5 (2.8)	0		4 (4.6)	2 (1.9)	0		6 (5.1)	3 (4.1)	0	
Lymphovascular invasion												
Negative	108 (52.7)	105 (58.3)	46 (76.7)	.003	45 (51.7)	62 (58.5)	35 (77.8)	.008	63 (53.4)	43 (58.1)	11 (73.3)	.181
Positive	97 (47.3)	75 (41.7)	14 (23.3)		42 (48.3)	44 (41.5)	10 (22.2)		55 (46.6)	31 (41.9)	4 (26.7)	
Lymph node metastasis												
Negative	74 (36.1)	51 (28.3)	36 (58.1)	.107	30 (34.5)	32 (30.2)	26 (55.3)	.075	44 (37.3)	19 (25.7)	10 (66.7)	.758
Positive	131 (63.9)	129 (71.7)	26 (41.9)		57 (65.5)	74 (69.8)	21 (44.7)		74 (62.7)	55 (74.3)	5 (33.3)	
pTNM stage												
I	52 (25.4)	33 (18.3)	20 (32.3)	.279	21 (24.1)	22 (20.8)	15 (31.9)	.130	31 (26.3)	11 (14.9)	5 (33.3)	.954
II	97 (47.3)	97 (53.9)	34 (54.8)		39 (44.8)	54 (50.9)	25 (53.2)		58 (49.2)	43 (58.1)	9 (60.0)	
III	56 (27.3)	50 (27.8)	8 (12.9)		27 (31.0)	30 (28.3)	7 (14.9)		29 (24.6)	20 (27.0)	1 (6.7)	
Percentage of in situ component, mean ± SD	26.7 ± 28.0	24.2 ± 27.0	24.8 ± 32.4	.671	31.0 ± 30.8	25.8 ± 28.3	27.5 ± 34.7	.502	23.5 ± 25.4	21.8 ± 25.0	16.3 ± 22.7	.562
Extensive intraductal component												
Negative	127 (62.0)	111 (61.7)	41 (66.1)	.687	48 (55.2)	63 (59.4)	30 (63.8)	.328	79 (66.9)	48 (64.9)	11 (73.3)	.924
Positive	78 (38.0)	69 (38.3)	21 (33.9)		39 (44.8)	43 (40.6)	17 (36.2)		39 (33.1)	26 (3.1)	4 (26.7)	
Paget disease												
Negative	194 (94.6)	173 (96.1)	62 (100.0)	.146	79 (90.8)	100 (94.3)	47 (100.0)	.069	115 (97.5)	73 (98.6)	15 (100.0)	1
Positive	11 (5.4)	7 (3.9)	0		8 (9.2)	6 (5.7)	0		3 (2.5)	1 (1.4)	0	
Adjuvant systemic therapy												
AC	74 (36.1)	51 (28.3)	36 (58.1)	<.001	30 (34.5)	32 (30.2)	26 (55.3)	.012	44 (37.3)	19 (25.7)	10 (66.7)	.010
ACT	131 (63.9)	129 (71.7)	26 (41.9)		57 (65.5)	74 (69.8)	21 (44.7)		74 (62.7)	55 (74.3)	5 (33.3)	
Peritumoral lymphocytic infiltration												
Score 0	44 (21.5)	2 (1.1)	0	<.001	15 (17.2)	1 (0.9)	0	<.001	29 (24.6)	1 (1.4)	0	<.001
Score 1	149 (72.7)	75 (41.7)	1 (1.6)		66 (75.9)	42 (39.6)	1 (2.1)		83 (70.3)	33 (44.6)	0	
Score 2	12 (5.9)	95 (52.8)	29 (46.8)		6 (6.9)	57 (53.8)	20 (42.6)		6 (5.1)	38 (51.4)	9 (60.0)	
Score 3	0	8 (4.4)	32 (51.6)		0	6 (5.7)	26 (55.3)		0	2 (2.7)	6 (40.0)	
Tertiary lymphoid structure in adjacent tissue												
None	43 (21.0)	2 (1.1)	0	<.001	12 (13.8)	0	0	<.001	31 (26.3)	2 (2.7)	0	<.001
Minimal	76 (37.1)	22 (12.2)	4 (6.5)		31 (35.6)	10 (9.4)	3 (6.4)		45 (38.1)	12 (16.2)	1 (6.7)	
Moderate	65 (31.7)	77 (42.8)	11 (17.7)		30 (34.5)	41 (38.7)	8 (17.0)		35 (29.7)	36 (48.6)	3 (20.0)	
Abundant	21 (10.2)	79 (43.9)	47 (75.8)		14 (16.1)	55 (51.9)	36 (76.6)		7 (5.9)	24 (32.4)	11 (73.3)	

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Table 1 (cont)
Comparison of the Clinicopathologic Variables According to the TIL Level in HER2-Positive Breast Cancer[a]

	All Patients				Hormone Receptor Negative				Hormone Receptor Positive			
Variable	Minimal TIL	Moderate TIL	Abundant TIL	*P* Value	Minimal TIL	Moderate TIL	Abundant TIL	*P* Value	Minimal TIL	Moderate TIL	Abundant TIL	*P* Value
Germinal center in the tertiary lymphoid structure												
Negative	180 (87.8)	116 (64.4)	31 (50.0)	<.001	75 (86.2)	61 (57.5)	22 (46.8)	<.001	105 (89.0)	55 (74.3)	9 (60.0)	.001
Positive	25 (12.2)	64 (35.6)	31 (50.0)		12 (13.8)	45 (42.5)	25 (53.2)		13 (11.0)	19 (25.7)	6 (40.0)	
Necrosis in the invasive area												
Negative	123 (60.0)	89 (49.4)	34 (55.7)	.161	47 (54.0)	42 (39.6)	24 (52.2)	.438	76 (64.4)	47 (63.5)	10 (66.7)	.981
Positive	82 (40.0)	91 (50.6)	27 (44.3)		40 (46.0)	64 (60.4)	22 (47.8)		42 (35.6)	27 (365)	5 (33.3)	
HER2 gene amplification												
Low level	56 (27.3)	32 (17.8)	10 (16.1)	.028	14 (16.1)	13 (12.3)	6 (12.8)	.573	42 (35.6)	19 (25.7)	4 (26.7)	.426
High level	146 (71.2)	148 (82.2)	51 (82.3)		72 (82.8)	93 (87.7)	40 (85.1)		74 (62.7)	55 (74.3)	11 (73.3)	
HER2 immunohistochemistry												
2+	40 (19.5)	17 (9.4)	6 (9.7)	.006	9 (10.3)	5 (4.7)	5 (10.6)	.715	31 (26.3)	12 (16.2)	1 (6.7)	.031
3+	165 (80.5)	163 (90.6)	56 (90.3)		78 (89.7)	101 (95.3)	42 (89.4)		87 (73.7)	62 (83.8)	14 (93.9)	
HLA-ABC immunohistochemistry												
0	78 (39.0)	34 (19.4)	7 (11.3)	<.001	38 (44.7)	28 (26.4)	5 (10.6)	<.001	40 (34.8)	6 (8.7)	2 (13.3)	<.001
1+	37 (18.5)	38 (21.7)	10 (16.1)		16 (18.8)	22 (20.8)	7 (14.9)		21 (18.3)	16 (23.2)	3 (20.0)	
2+	85 (42.5)	103 (58.9)	45 (72.6)		31 (36.5)	56 (52.8)	35 (74.5)		54 (47.0)	47 (68.1)	10 (66.7)	
HLA-A immunohistochemistry												
0	80 (40.0)	38 (21.2)	6 (9.7)	<.001	39 (45.9)	29 (27.4)	3 (6.4)	<.001	41 (35.7)	9 (12.3)	3 (20.0)	.001
1+	41 (20.5)	42 (23.5)	13 (21.0)		13 (15.3)	24 (22.6)	10 (21.3)		28 (24.3)	18 (24.7)	3 (20.0)	
2+	79 (39.5)	99 (55.3)	43 (69.4)		33 (38.8)	53 (50.0)	34 (72.3)		46 (40.0)	46 (63.0)	9 (60.0)	
Hormone receptor												
Negative	87 (42.4)	106 (58.9)	47 (75.8)	<.001								
Positive	118 (57.6)	74 (41.1)	15 (24.2)									

AC, anthracycline and cyclophosphamide; ACT, anthracycline, cyclophosphamide, and taxane; HLA, human leukocyte antigen; TIL, tumor-infiltrating lymphocyte.
[a] Values are presented as number (%) unless otherwise indicated.

with TILs in the antitumor immune response in colorectal cancers. We also noted that the degree of TILs was significantly associated with the degree of adjacent TLSs in HER2+ breast cancers. Further evaluation of the antitumor immunologic effect of TILs and TLSs is, therefore, required.

Recent studies have shown that the therapeutic effect of trastuzumab depends on innate and adaptive, immune-mediated mechanisms.[12-18] For example, a combination therapy inhibiting the PI3K/AKT pathway for trastuzumab resistance increases intratumoral T-cell infiltration, thus enhancing the antitumor effect, and the CD8+ T-cell–mediated immune response enhances the therapeutic effects of the anti-HER2 antibody.[12,17] In HER2+ breast cancer, increased TIL is also associated with decreased distant recurrence in patients who also received trastuzumab in addition to undergoing chemotherapy.[17,19,20,37] In this study, we validated the TIL effect on the clinical outcome in a large cohort of patients with HR–/HER2+ breast cancer treated with trastuzumab. These results suggest that trastuzumab is more effective in treating tumors with increased TIL. Therefore, assessment of TILs at the initial diagnosis might be necessary to subdivide patients with HER2+ breast cancer who would benefit more from the additional use of trastuzumab. The development of strategies that can facilitate effective immune response is also warranted.

MHC I expression on the surface of tumor cells is essential for their annihilation by cytotoxic T cells. We demonstrated that there is a strong positive correlation between the level of TIL and HLA expression. These results suggest that MHC I expression on the surface of tumor cells might be a possible mechanism of attracting cytotoxic T lymphocytes to tumors. MHC I expression has been reported to have an inverse correlation with HER2 expression in breast cancer.[38] However, we could find such a correlation only between HLA expression and the HER2 immunohistochemical score in HR–/HER2+ tumors. In HR+ tumors, HLA and HER2 expression showed significantly positive correlations. Therefore, the mechanism of HLA expression on the surface of tumor cells might differ in HR–/HER2+ and HR+/HER2+ tumors.

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Table 2
Correlation Coefficients Between Each Continuous Variable

Variable (*P* Value)	Peritumoral Lymphocytic Infiltration	Adjacent TLS	HLA-ABC Percentage	HLA-A Percentage	ER Allred Score	HER2 IHC Score	*HER2* Copy Number	DCIS Percentage
All								
Tumor-infiltrating lymphocyte	0.762 (<.001)	0.558 (<.001)	0.246 (<.001)	0.249 (<.001)	–0.242 (<.001)	0.136 (.004)	0.138 (.004)	–0.018 (.703)
Peritumoral lymphocytic infiltration		0.583 (<.001)	0.274 (<.001)	0.267 (<.001)	–0.231 (<.001)	0.073 (.123)	0.097 (.041)	–0.081 (.089)
Adjacent TLS			0.201 (<.001)	0.213 (<.001)	–0.305 (<.001)	0.202 (<.001)	0.181 (<.001)	0.231 (<.001)
HLA-ABC percentage				0.770 (<.001)	0.033 (.489)	0.029 (.550)	0.019 (.697)	–0.72 (.131)
HLA-A percentage					0.019 (.685)	0.017 (.716)	0.076 (.112)	–0.064 (.183)
ER Allred score						–0.258 (<.001)	–0.285 (<.001)	–0.108 (.022)
HER2 IHC score							0.521 (<.001)	0.127 (.007)
HER2 copy number								0.112 (.018)
Hormone receptor negative								
Tumor-infiltrating lymphocyte	0.784 (<.001)	0.558 (<.001)	0.275 (<.001)	0.279 (<.001)	–0.094 (.148)	0.030 (.644)	0.098 (.131)	–0.116 (.074)
Peritumoral lymphocytic infiltration		0.545 (<.001)	0.241 (<.001)	0.302 (<.001)	–0.068 (.292)	–0.051 (.435)	0.014 (.826)	–0.132 (.041)
Adjacent TLS			0.202 (.002)	0.166 (.010)	–0.046 (.480)	0.067 (.304)	0.025 (.698)	0.282 (<.001)
HLA-ABC percentage				0.812 (<.001)	–0.026 (.694)	–0.140 (.031)	–0.012 (.857)	–0.060 (.357)
HLA-A percentage					–0.076 (.241)	–0.130 (.046)	–0.061 (.350)	–0.087 (.179)
ER Allred score						0.002 (.979)	–0.069 (.289)	0.070 (.278)
HER2 IHC score							0.296 (<.001)	0.118 (.067)
HER2 copy number								0.049 (.455)
Hormone receptor positive								
Tumor-infiltrating lymphocyte	0.724 (<.001)	0.650 (<.001)	0.284 (<.001)	0.284 (<.001)	–0.104 (.134)	0.218 (.002)	0.169 (.015)	–0.097 (.163)
Peritumoral lymphocytic infiltration		0.582 (<.001)	0.318 (<.001)	0.288 (<.001)	–0.096 (.168)	0.116 (.097)	0.076 (.277)	–0.219 (.002)
Adjacent TLS			0.236 (.001)	0.247 (<.001)	–0.172 (.013)	0.226 (.001)	0.195 (.005)	0.104 (.137)
HLA-ABC percentage				0.779 (<.001)	–0.019 (.789)	0.167 (.017)	0.154 (.028)	–0.127 (.070)
HLA-A percentage					–0.080 (.262)	0.148 (.037)	0.086 (.227)	–0.090 (.205)
ER Allred score						–0.327 (<.001)	–0.279 (<.001)	–0.159 (.022)
HER2 IHC score							0.631 (<.001)	0.130 (.062)
HER2 copy number								0.135 (.053)

DCIS, ductal carcinoma in situ; ER, estrogen receptor; HLA, human leukocyte antigen; IHC, immunohistochemistry; TIL, tumor-infiltrating lymphocyte; TLS, tertiary lymphoid structure.

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Table 3
Univariate Analyses of the Clinicopathologic Variables Affecting the Clinical Outcomes

	Hormone Receptor Negative		Hormone Receptor Positive	
Variable	**HR (95% CI)**	***P* Value**	**HR (95% CI)**	***P* Value**
Age: <50 vs ≥50 y	0.573 (0.253-1.300)	.183	0.181 (0.023-1.411)	.103
Histologic grade: 3 vs 2	1.341 (0.498-3.614)	.561	1.249 (0.381-4.092)	.714
pT	2.667 (1.308-5.440)	.007	1.804 (0.651-4.999)	.256
Lymphovascular invasion: positive vs negative	2.378 (1.029-5.495)	.043	5.969 (1.289-27.641)	.022
Lymph node metastasis: positive vs negative	2.002 (0.741-5.408)	.171	5.162 (0.656-40.635)	.119
pTNM stage	2.173 (1.158-4.075)	.016	2.118 (0.839-5.347)	.112
Percentage of in situ component	1.003 (0.991-1.016)	.601	0.998 (0.973-1.023)	.849
Chemotherapy: AC vs ACT	0.707 (0.430-1.162)	.171	0.44 (0.157-1.235)	.119
TIL (per 10%)	0.971 (0.950-0.992)	.008	0.975 (0.937-1.015)	.213
Peritumoral lymphocytic infiltration	0.454 (0.259-0.793)	.006	0.666 (0.282-1.571)	.353
Tertiary lymphoid structure in adjacent tissue	0.777 (0.505-1.195)	.25	0.695 (0.380-1.269)	.236
Germinal center in adjacent tissue: positive vs negative	0.848 (0.349-2.062)	.716	0.435 (0.056-3.402)	.428
HER2 immunohistochemistry: 3+ vs 2+	0.589 (0.175-1.982)	.392	0.734 (0.195-2.766)	.647
HER2 SISH status: high-level vs low-level	1.015 (0.300-3.432)	.98	0.536 (0.163-1.759)	.304
HLA-ABC percentage	1 (0.990-1.010)	.972	0.992 (0.990-1.010)	.236
HLA-A percentage	1.054 (0.831-1.336)	.666	0.899 (0.651-1.242)	.52

AC, anthracycline and cyclophosphamide; ACT, anthracycline, cyclophosphamide, and taxane; CI, confidence interval; HLA, human leukocyte antigen; HR, hazard ratio; SISH, silver in situ hybridization; TIL, tumor-infiltrating lymphocyte.

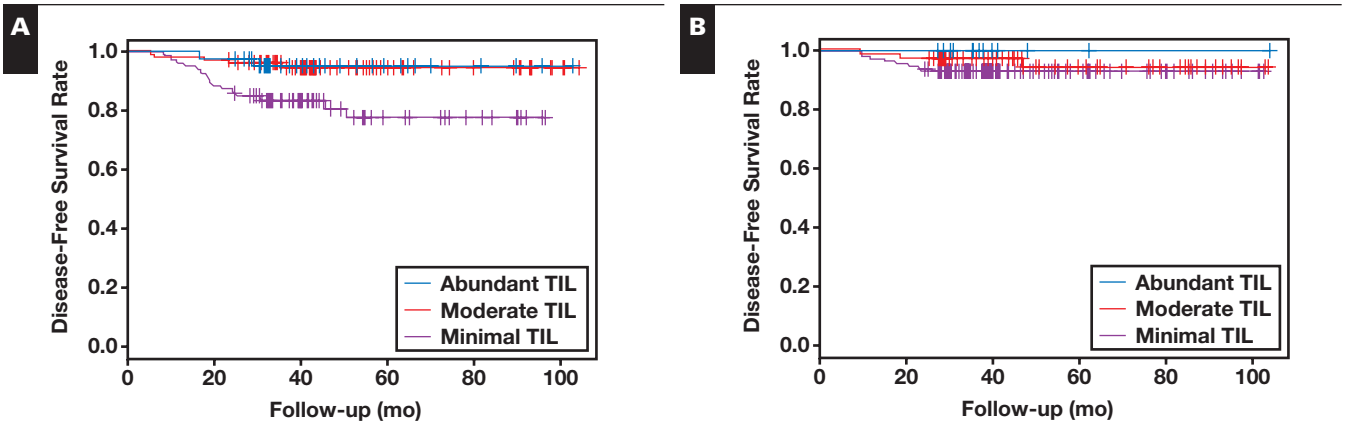

Figure 2 Kaplan-Meier survival curves for patients with hormone receptor (HR)–negative and HR-positive HER2-positive tumors. **A**, Moderate to abundant, tumor-infiltrating lymphocytes (TILs) are associated with significantly longer disease-free survival in patients with HR-negative tumors. **B**, The TIL level is not associated with the survival outcome in patients with HR-positive tumors.

In conclusion, TLSs were identified in 89.9% of HER2+ breast cancers and were primarily associated with the terminal-duct lobular unit and DCIS. TILs were more abundant in HR–/HER2+ tumors than in HR+/HER2+ tumors. HR–/HER2+ breast cancers with abundant TILs showed a higher histologic grade, absence of lymphovascular invasion, the presence of peritumoral lymphocytic infiltration, moderate to abundant TLSs in adjacent tissue, and stronger expression of HLA-ABC and HLA-A. Abundant TILs and the absence of lymphovascular invasion were independent prognostic factors in HR–/HER2+ breast cancers.

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