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ORIGINAL ARTICLE

Novel tumor-infiltrating lymphocytes ultrasonography score based on ultrasonic tissue findings predicts tumor-infiltrating lymphocytes in breast cancer

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Abstract

Background The presence of tumor-infiltrating lymphocytes (TILs) is a prognostic factor for breast cancer. However, because of tumor tissue heterogeneity, an accurate and simple evaluation method is needed. We determined if preoperative characteristic ultrasonography (US) image findings are predictive of lymphocyte-predominant breast cancer (LPBC).

Methods We evaluated 191 patients with invasive breast cancer treated by curative surgery between January 2014 and December 2017. Stromal lymphocytes in surgical pathological specimens were evaluated. Fifty-two patients with ≥ 50% stromal TILs were defined as having LPBC. Preoperative US images were examined for indicators of TILs. The US images with characteristic TILs were scored for prediction of LPBC.

Results Shape (more lobulated), internal echo level (weaker), and posterior echoes (stronger) were predictors of LPBC and used to assign the TILs-US scores (0–7 points); the score cutoff for predicting LPBC was 4 points (sensitivity, 0.73; specificity, 0.87; accuracy, 0.83) based on the receiver operating characteristics (ROC) curves (AUC 0.88). Multivariate logistic regression analysis identified nuclear grade (NG), OR 3.4; estrogen receptor (ER), OR 5.7; human epidermal growth factor receptor type-2 (HER2), OR 4.1; and TILs-US score, OR 14.9 as LPBC predictors (all, $p < 0.05$). The sensitivity, specificity, and accuracy for predicting LPBC were 0.75, 0.69, and 0.71 for NG and 0.33, 0.96, and 0.79 for ER and HER2, respectively. ROC analysis showed that the diagnostic abilities of NG, ER, and HER2 were lower than that of the TILs-US score.

Conclusions LPBC showed characteristic US imaging findings. The TILs-US score was an accurate preoperative predictor of LPBC.

Keywords Breast cancer · Tumor-infiltrating lymphocytes · Lymphocyte-predominant breast cancer · Ultrasonography

Introduction

Immunological parameters, including tumor-infiltrating lymphocytes (TILs), have been identified as prognostic factors for breast cancer [1–7]. TILs are predictive of response to neoadjuvant chemotherapy in luminal human epidermal growth factor receptor type 2 (HER2)-negative type breast cancer, HER2-positive breast cancer, and triple-negative breast cancer [3, 5, 8–13]. Thus, the presence of TILs is an important biological marker predictive of prognosis and drug treatment effect. Pathological evaluation of TILs is recommended by the International Immuno-Oncology Biomarker Working Group guidelines [14]. The use of biopsy specimens for preoperative evaluation of TILs is possible, but the expression and distribution of TILs in tissues are heterogeneous. Therefore, TILs should only be evaluated after determining the distribution and expression of TILs in all specimens [14]. Although there are reports [3] on the usefulness of the evaluation of TILs in residual tumors after neoadjuvant chemotherapy, it is difficult to define the evaluation range because tumor cells remain diffuse. Additionally,

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evaluation of TILs by tissue microarray is not recommended at this stage because of heterogeneity problem [14]. With these considerations, the presence of TILs is a useful predictor of prognosis and treatment effect in breast cancer. However, an accurate and convenient preoperative evaluation method is urgently needed.

Ultrasonography (US) has been reported to be useful for breast cancer detection [15–21]. It has also been reported that US tissue characterization is useful for discrimination between benign and malignant tumors by evaluating the morphology and internal characteristics of the tumor [22–24]. Thus, US is a useful modality in breast cancer diagnosis. However, there has been no report on imaging methods, including US, for evaluation of TILs.

In this study, we determined if preoperative characteristic US image findings are predictive of lymphocyte-predominant breast cancer (LPBC).

Patients and methods

Participants and study design

We included 191 consecutive patients with clinical stage I–III breast cancer who underwent preoperative US between January 2014 and December 2017. Patients who received neoadjuvant chemotherapy were excluded, and all patients were treated by either mastectomy or breast-conserving surgery.

All procedures involving human participants were performed in accordance with the ethical standards of our institutional research committee (IRB no. 1166) and the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. For this retrospective cohort study, the need for formal consent was waived.

Ultrasonography

Conventional US images were acquired by using a HIVISION Ascendus (Hitachi–Aloka Medical, Mitaka, Tokyo, Japan) with a 5–13 MHz linear-array transducer, in longitudinal and transverse planes, from supine patients with raised arms. Lesions were measured, and representative images of the index cancer (lesion with the largest diameter) were acquired. Three surgeons who acquired the US images and two other sonographers who assessed the images confirmed correspondence among the findings. Three specialist breast surgeons and two breast US technicians made decision without knowing the TIL status. The final decision in the evaluation in each case was taken by three specialist breast surgeons and two breast US technicians via discussions and consensus.

Scoring of US imaging characteristics for predicting LPBC

Tumor US images were examined according to the ultrasonic tissue characterization method defined by the Japan Association of Breast and Thyroid Sonology (JABTS) [20]. Regarding the characteristic of shape, a tumor with ≥ 2 lobulated areas, < 5 mm in size, was defined as small lobulated. In ultrasonic tissue characterization, we analyzed the most suitable setting to predict LPBC. As a standard method, characteristic factors of LPBC in the US tissue characterization were respectively scored, and various receiver operating characteristics (ROC) analyses in LPBC prediction were conducted for all patients. We identified the best AUC values for TILs-US scores from ROC analyses.

Pathological diagnosis

Pathological diagnoses were established from specimens obtained during surgery. Primary tumors were evaluated for nuclear grade (NG), estrogen receptor (ER) status, HER2 status, and Ki67 proliferation index. The NG was assigned according to the General Rules for Clinical and Pathological Recording of Breast Cancer, 17th edition (Japanese Breast Cancer Society 2012). ER positivity was assessed by immunohistochemical analysis and scored according to the Allred system. HER2 positivity was defined by the following scores: 3+ by immunohistochemistry alone; 2+ by immunohistochemistry and HER2/CEP17 ≥ 2.0 using fluorescent in situ hybridization; and 2+ by immunohistochemistry, HER2/CEP17 < 2.0, and average HER2 copy number ≥ 6.0 signals/cell by fluorescent in situ hybridization. The Ki67 proliferation index was determined by using a quantitative visual approach. Tumor zones with the most positive nuclei within the invasive component were scored, and the Ki67 proliferation index was expressed as the ratio (%) of Ki67-positive malignant cells within that area (≥ 1000). Tumors were classified as having low or high proliferative activity on the basis of Ki-67 nuclear staining values of $< 20\%$ and $\geq 20\%$, respectively. Molecular subtypes of breast cancer were classified as luminal type (ER positive, HER2 negative), luminal-HER2 (ER positive, HER2 positive), HER2 positive (ER negative and HER2 positive), or triple negative (negative for progesterone receptor, ER, and HER2) on the basis of immunohistochemistry and fluorescent in situ hybridization. TIL evaluation was performed on hematoxylin and eosin (H&E)-stained sections in a routine diagnostic setting. Stromal lymphocytes were evaluated on H&E-stained sections according to the current

recommendations (International TILs Working Group 2014) by two experienced pathologists. Breast cancer samples with ≥ 50% stromal TILs (s-TILs) were defined as LPBC as a predefined categorical parameter, as described in previous reports [1, 6, 12, 25].

Statistical analysis

The clinicopathological characteristics of the patients are shown as means ± standard deviations for continuous variables and as number (%) for categorical variables. The patient population was subdivided as those having breast cancers with LPBC (LPBC) and without LPBC (non-LPBC). The association between LPBC or non-LPBC and clinicopathological factors was analyzed by using the χ^2 test. The association between LPBC or non-LPBC and US tissue characterization was also analyzed by using the χ^2 test. The predictive performance regarding the identification of LPBC in the TILs-US score was evaluated by using ROC analysis. An ROC analysis was performed by incrementally increasing the cutoff values of the TILs-US score and recalculating the corresponding true-positive and false-negative rates. Univariate and multivariate logistic regression analyses were employed for each potential predictor variable of LPBC. The sensitivity, specificity, accuracy, positive-predictive value, and negative-predictive value were compared among the TILs-US score, NG, ER, and HER2 potential predictors and calculated according to standard formulas. Odds ratios and 95% confidence intervals (CIs) are reported. All analyses were performed by using JMP version 13.0 (SAS Institute Cary, NC, USA), and a p value of < 0.05 was considered as indicative of statistical significance in all comparisons.

Results

Associations between clinicopathological factors and LPBC and non-LPBC

We included 191 patients with invasive breast cancer who received preoperative US. The clinicopathological characteristics of the study cohort according to TILs predominance are summarized in Table 1. There was a significant association of TILS with higher tumor size ($p = 0.01$), higher NG ($p < 0.001$), higher Ki67 proliferation index ($p < 0.001$), ER negativity ($p < 0.001$), and HER-2 positivity ($p < 0.001$). We classified tumors as luminal type, luminal-HER2, HER2 positive, and triple negative in 160, 13, 10, and 8 patients, respectively. There was a significant association of TILs with subtype ($p < 0.001$).

Table 1 Clinicopathological characteristics of patients with LPBC or non-LPBC

Characteristic	LPBC $n = 52$; n (%)	Non-LPBC $n = 139$; n (%)	p
Age (years), mean ± SD	54.5 ± 13.2	59.1 ± 13.5	
Clinical T			
T1	23 (44.2)	90 (64.7)	0.01
T2	29 (55.8)	46 (33.1)	
T3	0 (0)	3 (2.2)	
Clinical N			
Negative	39 (75.0)	107 (77.0)	0.78
Positive	13 (25.0)	32 (23.0)	
Nuclear grade			
1	4 (7.7)	21 (15.1)	< 0.001
2	9 (17.3)	75 (54.0)	
3	39 (75.0)	43 (30.9)	
Ki67			
< 20	3 (5.8)	59 (42.4)	< 0.001
≥ 20	49 (94.2)	80 (57.6)	
ER			
Positive	35 (67.3)	133 (95.7)	< 0.001
Negative	17 (32.7)	6 (4.3)	
HER2			
Positive	17 (32.7)	6 (4.3)	< 0.001
Negative	35 (67.3)	133 (95.7)	
Subtype			
Luminal type	29 (55.8)	131 (94.2)	< 0.001
Luminal-HER2	8 (15.4)	5 (3.6)	
HER2 positive	9 (17.3)	1 (0.7)	
Triple negative	6 (11.5)	2 (1.4)	

T1, tumor ≤ 2 cm; T2, tumor > 2–5 cm; T3, tumor > 5 cm; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; Ki67, Ki67 proliferation index; luminal type, ER positive and HER2 negative; Luminal-HER2, ER positive and HER2 positive; HER2 positive, ER negative and HER2 positive; Triple negative, negative for ER, progesterone receptor, and HER2

Associations between US tissue characterization and LPBC and non-LPBC

There was a significant association in shape ($p < 0.001$), margin ($p < 0.001$), internal echo level ($p < 0.001$), posterior echoes ($p < 0.001$), and vascularity ($p < 0.02$) between LPBC and non-LPBC cases. In contrast, internal echo homogeneity ($p = 0.22$) and echogenic foci ($p = 0.18$) were not significantly associated with LPBC or non-LPBC. The shapes of the LPBC cases were characterized by a large number of lobulated areas (lobulated, 23.1%; small lobulated, 40.4%), whereas the non-LPBC cases tended to have few lobulated areas (lobulated, 7.2%; small lobulated, 3.6%). In this way, especially, small lobulated was a characteristic finding of LPBC. The LPBC cases were characterized by low internal

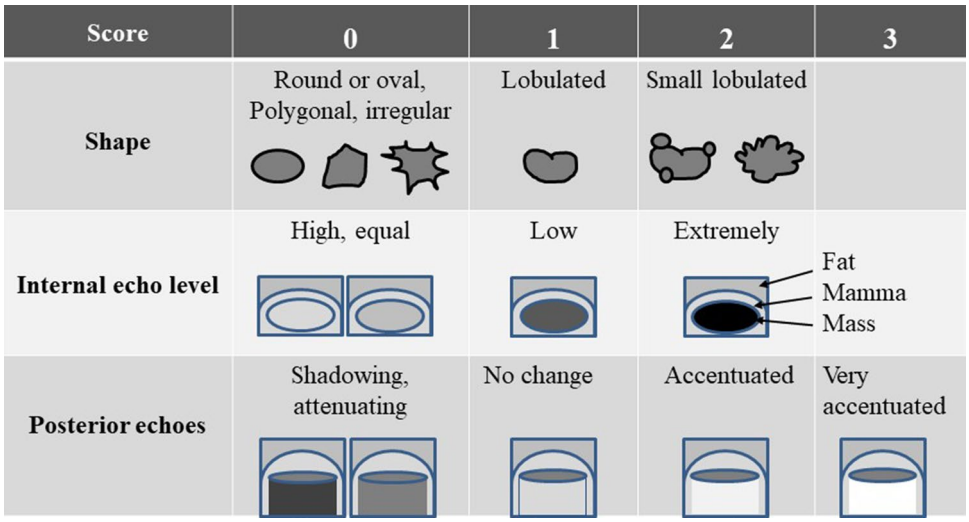

Fig. 1 Examples of the TILs-US score based on US tissue characterization. **a** Shape is irregular, 0 points; internal echo level is low, 1 point; posterior echoes show shadowing, 0 points. The TILs-US score is 1 point. **b** Shape is irregular, 0 points; internal echo level is low, 1 point; posterior echoes show no change, 1 point. The TILs-US score is 2 points. **c** Shape is lobulated, 1 point; internal echo level is low, 1 point; posterior echoes are accentuated, 2 points. The TILs-US score is 4 points. **d** Shape is small lobulated, 2 points; internal echo level is extremely low, 2 points; posterior echoes are very accentuated, 3 points. The TILs-US score is 7 points

echo level (low, LPBC vs. non-LPBC; 59.6% vs. 84.9%, respectively). Extremely low internal echo level was especially a characteristic finding in the LPBC cases (extremely low, LPBC vs. non-LPBC, 40.4% vs. 10.1%, respectively). Posterior echoes were one of the most characteristic US findings. LPBC cases were found more frequently in tumors in which posterior echoes were accentuated (not changed, 15.4%; accentuated, 50.0%; very accentuated, 32.7%), whereas there were fewer non-LPBC cases in which the posterior echoes were accentuated (not changed, 42.4%; accentuated, 21.6%; very accentuated, 4.3%).

Optimal TILs-US score for predicting LPBC by ultrasonography for predicting LPBC

We constructed various ROC curves to predict LPBC. The best AUC values for the TILs-US scores were 0.88 (95% CI 0.82–0.94; $p < 0.001$) (Fig. 2). Factors and scores in this

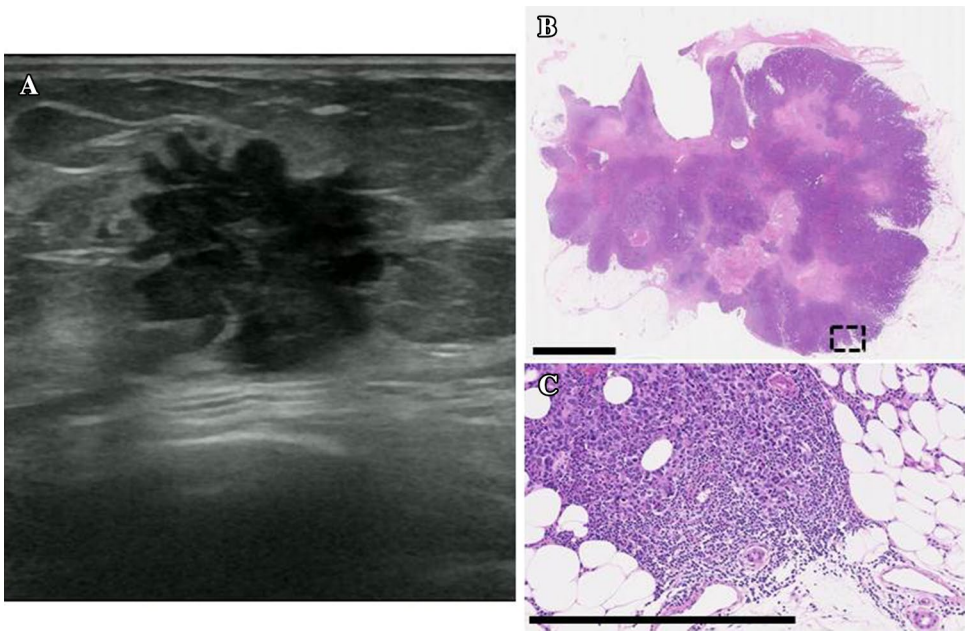

Fig. 2 Representative US images (**a**) and hematoxylin and eosin staining (**b, c**) on LPBC. Ultrasonic tissue characterization shows findings of small lobulated areas, extremely low internal echo levels, and highly accentuated posterior echoes (**a**). Hematoxylin and eosin (H&E) staining shows invasive ductal carcinoma with LPBC; scale bars = 5 mm (**b**), 500 µm (**c**). Dotted line in **b** indicates the high-power field site in **c**

US tissue characterization are mentioned below. TILs-US characteristic US image findings predicting LPBC were scored on the basis of three ultrasonic tissue characteristics: shape (more lobulated), internal echo level (weaker), and posterior echoes (stronger). For these three ultrasonic tissue characterizations, more characteristic findings were assigned higher scores, and the TILs-US score was the total score, which ranged from 0 to 7 total points comprising the subscores for shape (round, oval, polygonal or irregular, 0 points; lobulated, 1 point; small lobulated, 2 points), internal echo level (high or equal, 0 points; low, 1 point; extremely low, 2 points), and posterior echoes (shadowing or attenuating, 0 points; not changing, 1 point; accentuated, 2 points; very accentuated, 3 points) (Fig. 1). The TILs-US scores were classified as low (0–1 points), intermediate (2–3 points), high (4–5 points), and very high (6–7 points). The reviewers assigned TILs-US scores according to US tissue characterization findings. Figure 1 shows examples of TILs-US scores according to ultrasonic tissue characterization.

Distribution of TILs-US scores of LPBC and non-LPBC

Table 2 summarizes the distribution of TILs-US scores, which were classified as low (0–1 points), intermediate (2–3 points), high (4–5 points), and very high (6–7 points). The following distribution was obtained: Low score, 1 (2.5%) case in LPBC and 39 (97.5%) cases in non-LPBC; intermediate score, 13 (13.7%) cases in LPBC and 82 (86.3%) cases in non-LPBC; very high score, 18 (100%) cases in LPBC and no (0%) cases in non-LPBC. Figure 1 shows a case of LPBC. The TILs-US score was very high (7 points) for the ultrasonic tissue findings of small lobulated areas, extremely low internal echo level, and very accentuated posterior echoes (Fig. 2a). Pathological analysis of the tissue showed that the occupied area of the mononuclear cells accounted for 90% of the whole tumor interstitial region (Fig. 2b, c). Figure 3 shows a case of non-LPBC. The TILs-US score was low (1 point) for the US tissue findings of irregular shape, low internal echo level, and attenuated posterior echoes (Fig. 3a). Pathological analysis of the tissue showed that the

Table 2 Distribution of the TILs-US score for predicting LPBC using ultrasonography

	Low (0–1 point) *n* (%)	Intermediate (2–3 points) *n* (%)	High (4–5 points) *n* (%)	Very high (6–7 points) *n* (%)
LPBC *n* = 52	1 (2.5)	13 (13.7)	20 (52.6)	18 (100)
Non-LPBC *n* = 139	39 (97.5)	82 (86.3)	18 (47.4)	0

LPBC, lymphocyte-predominant breast cancer

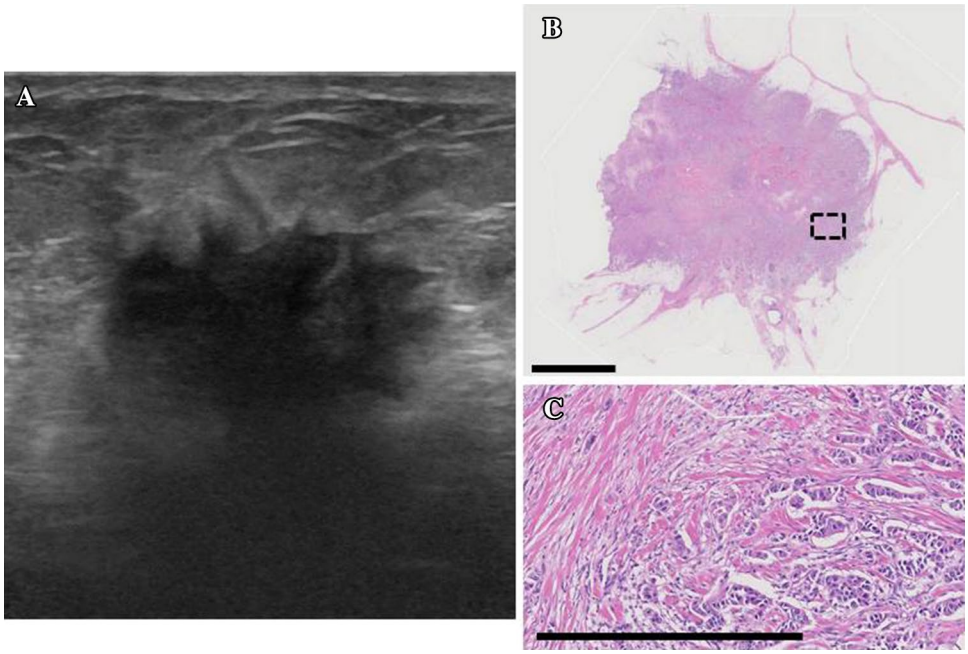

Fig. 3 Representative US images (**a**) and hematoxylin and eosin staining (**b, c**) of non-LPBC tissue. US tissue characterization shows findings of irregular shape, low internal echo levels, and accentuated posterior echoes (**a**). Hematoxylin and eosin staining shows invasive ductal carcinoma with non-LPBC; scale bars = 5 mm (**b**), 500 µm (**c**). Dotted line in **b** indicates the high-power field site in **c**

occupied area of the mononuclear cells accounted for 10% of the whole tumor interstitial region (Fig. 3b, c).

Univariate and multivariate logistic regression analyses of significant predictive clinicopathological factors for LPBC

We set cutoffs for LPBC on the basis of data derived from the TILs-US score using ROC curves (Fig. 2). We set the TILs-US score cutoff for predicting LPBC at 4 points on the basis of the ROC curves. The significant predictive clinicopathological factors for LPBC determined by univariate and multivariate logistic analyses are shown in Table 3. In the univariate logistic analysis, clinical T, NG, Ki67, ER status, HER-2 status, and TILs-US score were significant independent predictors for LPBC. In the multivariate logistic analysis, NG, ER status, HER-2 status, and TILs-US score were significantly associated with good prognosis in LPBC.

Sensitivity, specificity, positive-predictive value, and negative-predictive value in using TILs-US score for predicting LPBC in US

The sensitivity, specificity, accuracy, positive-predictive value, and negative-predictive value of the TILs-US score for predicting LPBC were 0.73 (0.63–0.81), 0.87 (0.83–0.90), 0.83 (0.78–0.88), 0.68 (0.58–0.76), and 0.90 (0.86–0.93), respectively; for predicting NG: 0.75 (0.64–0.8), 0.69 (0.65–0.72), 0.71 (0.65–0.38), 0.48 (0.41–0.53), and 0.88 (0.83–0.92), respectively; and for predicting ER and HER2: 0.33 (0.24–0.39), 0.96 (0.93–0.98), 0.79 (0.74–0.82), 0.74 (0.55–0.87), and 0.79 (0.77–0.81), respectively. The diagnostic abilities of NG, ER, and HER2 were all lower than that of the TILs-US score (Fig. 4).

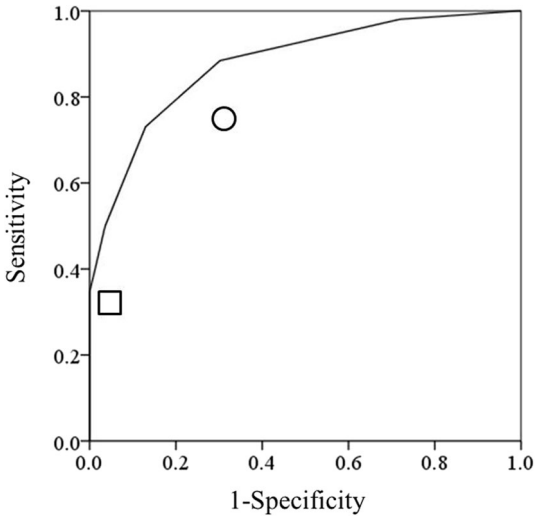

Fig. 4 Receiver operating characteristics curve of TILs-US score to predict LPBC in all patients. The AUC value of TILs-US for LPBC was 0.88 (95% CI, 0.82–0.94; $p < 0.001$). The sensitivity, specificity, and accuracy of NG (white circle) were 0.75 (0.64–0.8), 0.69 (0.65–0.72), 0.71 (0.65–0.38), and those of ER and HER2 (white square) were 0.33 (0.24–0.39), 0.96 (0.93–0.98), and 0.79 (0.74–0.82) for both, respectively

Discussion

Recent imaging studies have reported that contrast-enhanced sonography [26, 27], whole-body positron emission tomography (PET) [28–30], and dedicated breast PET [31] predicted malignancy grade and prognosis of breast cancer. However, there have been no reports on imaging findings predictive of LPBC before surgery. The TILs-US scoring method described in the present report is noninvasive and convenient to perform because it uses US findings. The TILs-US score showed high diagnostic ability for LPBC before surgery and was found be an accurate and convenient evaluation method of TILs.

Ultrasonic tissue characterization in LPBC is characterized by many lobulated areas and small lobulated areas,

Table 3 Univariate and multivariate logistic analysis of significant predictive clinicopathological factors in LPBC cases

Characteristic	Univariate logistic analysis			Multivariate logistic analysis		
	OR	95% CI	*p*	OR	95% CI	*p*
Clinical T, T2–T4 vs. T1	2.3	1.2–4.5	0.01	1.3	0.48–3.3	0.63
Clinical N, positive vs. negative	1.1	0.5–2.3	0.78	1.5	0.46–4.7	0.52
Nuclear grade, 3 vs. 1–2	6.7	3.3–14.2	< 0.001	3.4	1.2–9.8	0.02
Ki67, ≥ 20 vs. < 20	12.1	4.2–51.2	< 0.001	2.5	0.60–10.4	0.21
ER, negative vs. positive	10.8	4.1–31.7	< 0.001	5.7	1.6–20.3	0.007
HER2, positive vs. negative	10.8	4.1–31.7	< 0.001	4.1	1.1–15.2	0.04
TILs-US score, ≥ 4 vs. < 3	26.8	10.2–85.2	< 0.001	14.9	4.6–48.7	< 0.001

Ki67, Ki67 proliferation index; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2

a high frequency of low and extremely low internal echo levels, and a high frequency of accentuated and highly accentuated posterior echoes. Among the ultrasonic tissue characteristics of LPBC characterization, attenuation and back scattering were strongly associated with organizational construction. Attenuation increases in tissues with many collagen fibers, so posterior echoes are attenuated. On the other hand, tissues rich in water-soluble components have less attenuation, so posterior echoes are accentuated. Therefore, posterior echoes are attenuated as fibrosis increases in stromal tissue [32, 33], and posterior echoes of tumors with many water-soluble components are accentuated [32, 34]. Also, the internal echo level decreases in invasive carcinoma with increased cellular components because of back scattering [32]. The s-TILs have a high fraction of total stromal nuclei that represent mononuclear inflammatory cell nuclei, whereas there is a tendency for the increase in collagenous tissue to decrease. We applied the pathological features of these s-TILs and the US characteristics of tissue to the diagnostic prediction of LPBC. The tumors in LPBC contain more mononuclear inflammatory cell nuclei than those in tumor cells. Therefore, in comparisons between H&E-stained specimens and US images, an advantage of US is that LPBC shows posterior echo enhancement and low internal echo level. Additionally, LPBC with more lymphocytes may show small lobulated areas because of the distribution of mononuclear inflammatory cell nuclei that surround the cancer alveolar surrounding the tumor margin. We have demonstrated that TILs can be predicted conveniently by ultrasonic tissue characterization factors. The TILs-US score can accurately predict LPBC and be applied to future breast cancer treatment strategies.

The limitations of our study need to be acknowledged. First, it was a retrospective study involving a single institute. Second, we have not reported whether TILs-US scores based on preoperative US could accurately predict LPBC with the use of surgical pathological specimens compared with the use of preoperative biopsy tissues. To evaluate this, specimens of preoperative biopsy tissues as well as surgical pathological specimens were needed. This should also have included an analysis of whether the diagnostic ability of the TILs-US score in predicting LPBC was useful and greater than that of preoperative biopsy tissues. Further, this should also have included an analysis of whether the TILs-US score based on US images obtained before neoadjuvant chemotherapy was useful as an alternative method to LPBC evaluation of preoperative biopsy tissues for the prediction of complete pathological response. Our data showed excellent performance of LPBC prediction, but the underlying mechanism for this remains unclear. Future studies are needed to comparatively analyze US and pathological images to clarify the mechanism underlying these features. This could help demonstrate that US images are applicable in predicting clinical outcomes.

Conclusion

The presence of TILs is predictive of prognosis and therapeutic effect for breast cancer. Our method for determining the TILs-US score based on conventional US findings was found to be an accurate and convenient diagnostic method for LPBC and can be easily performed preoperatively. The clinical application of the TILs-US score in LPBC is expected to lead to a new treatment strategy for breast cancer.

Compliance with ethical standards

Conflict of interest None of the authors has a conflict of interest related to this study and manuscript.

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