



Original Article

Defining lymphocyte-predominant breast cancer by the proportion of lymphocyte-rich stroma and its significance in routine histopathological diagnosis

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Lymphocyte-predominant breast cancer (LPBC) defined by the density of stromal lymphocytes shows favorable behavior. However, considerable distribution heterogeneity of lymphocytes is a major problem. The present study defined LPBC by the proportion of lymphocyte-rich stroma with the cut-off values of 30, 50, and 75%, and clinicopathologically analyzed mainly LPBC (area > 30%) defined by the cut-off value of 30%. LPBCs (area > 30%), 39 cases in total, were composed mainly of triple-negative and HER2⁺/ER⁻ subtypes, without any luminal A-like subtype. LPBCs were composed predominantly of histological grade 3 tumors, without any grade 1 lesions. Multivariate analyses on 477 consecutive tumors revealed that ER-negativity and grade 3 status associated significantly with LPBC. LPBC (area > 30%) showed better disease-free survival than grade-matched controls, and it was a good indicator of complete pathological remission after pre-operative chemotherapy. Patients with LPBC with the cut-off value of 50% and that of 75% showed 100% disease-free survival. These results demonstrated the validity of our definition of LPBC. Our data also suggest that de-differentiated cancers without TILs could be regarded as high-grade cancer without lymphocyte-mediated responses. In conclusion, the definition of LPBC by the proportion of lymphoid stroma is useful for prognostication of high grade breast cancer in routine diagnosis.

Key words: anti-tumor immunity, invasive breast cancer, lymphocyte-predominant breast cancer, tumor-infiltrating lymphocytes

Tumor-infiltrating lymphocytes (TILs) are associated with the improved survival in various kinds of cancers, suggesting that TILs are involved in anti-tumor immunity.^{1–4} In breast cancer, recent studies revealed that TILs are a good predictor of complete remission after preoperative chemotherapy,^{5–9} while patients with breast cancer associated with abundant TILs showed improved rates of survival in the triple negative subtype.^{10–12} Breast cancers are classified into intrinsic subtypes: luminal A-like (estrogen receptor-positive [ER⁺], and/or progesterone receptor-positive [PgR⁺], human epidermal growth factor receptor 2-negative [HER2⁻], Ki-67 less than 15%), luminal B-like, HER2-overexpressing (HER2⁺)/ER⁻, and triple-negative (TN) subtypes.^{13,14} This subclassification of breast cancer is important for the selection of therapeutic modalities for breast cancer patients. Breast cancer with prominent TILs is designated as lymphocyte-predominant breast cancer (LPBC); currently the density of lymphocytes or plasma cells must be greater than 50–60% to be called LPBC.^{5,9,15} Recently, the International TILs Working Group 2014 recommended reporting the percentage of stromal lymphocytes by selecting areas with the average density of stromal lymphocytes.¹⁵ However, significant heterogeneity of lymphocyte distribution appears to be a major obstacle for this definition.

The authors previously analyzed TILs in gastrointestinal cancers.^{16–19} Initially, the focus was on lymphocytes that infiltrated cancer cell nests (intraepithelial lymphocytes), and then stromal TILs that form lymphoid stroma were analyzed.^{16,17} Therefore we sought to define LPBC by the proportion of lymphoid stroma and characterize LPBC in Japanese patients. This approach to stromal TILs is practical

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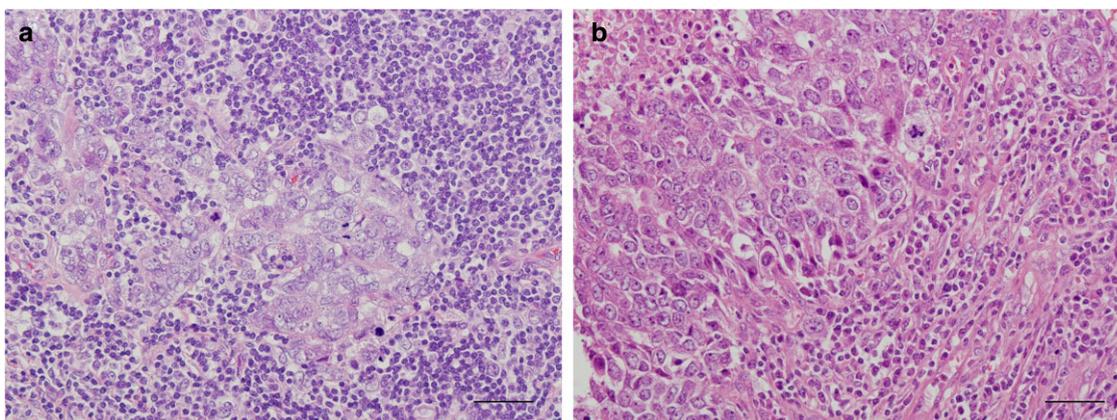


Figure 1 Hematoxylin-eosin staining of representative cases of lymphocyte-predominant breast cancer (LPBC), which have typical lymphocyte-rich stroma (**a**) and lymphoplasmacyte-rich stroma (**b**). Note undifferentiated features of cancer cells in both **a** and **b**; *i.e.*, larger nuclei and slight luminal formation. Scale bars, 50 μ m.

for diagnostic purposes. We describe here that LPBCs by our definition did not contain any well-differentiated, luminal A-like subtype or histological grade 1 tumors and that LPBCs were closely associated with poorly differentiated phenotypes. Despite these features, LPBCs showed improved survival outcomes as compared with grade-matched breast cancers. These data suggest that our method of definition of LPBC is of importance from a practical viewpoint.

MATERIALS AND METHODS

Patients

The present study is a retrospective study on surgically resected LPBCs in Mito Medical Center, Ibaraki, Japan. All specimens were fixed in formalin and embedded in paraffin. From July 2010 until September 2014, screening for TILs and subtype analysis¹³ had been performed as routine diagnosis in 477 lesions of surgically resected, consecutive invasive breast cancer (hereinafter called 'consecutive series'). Histopathological diagnosis and histological grading were done according to the World Health Organization (WHO) classification.^{20,21} Staging was performed as described in the Tumor–Node–Metastasis (TNM) classification (7th edition).²² In consecutive series, lymphocytic responses in cancer tissue were scored as follows: 1+, minimal; 2+, positive along the invasive margin, but sparse inside the cancer; and 3+, positive not only along the invasive margin but also inside the cancer tissue.

Definition and selection of LPBC

We selected LPBCs by re-observation of 3+ scored cancers. As the distribution of TILs is usually heterogeneous, histo-

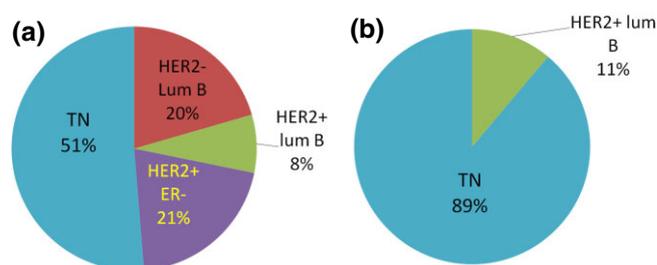


Figure 2 Subtype analyses of (**a**) lymphocyte-predominant breast cancer (LPBC) (area > 30%) and (**b**) medullary carcinoma (MC). Note that the TN subtype is the most frequent, and no luminal A-like subtype is found in LPBC.

pathological specimens of the whole cancer were observed to assess the overall lymphocytic responses in cancer tissues. The assessment of LPBCs was performed by semi-quantification of the proportion of area of lymphocyte-rich stroma to the whole cancer stroma. The lymphocyte-rich stroma was defined as stroma that contains predominantly round cell infiltrates with poorly visible collagenous tissue (Figs 1,2 and Supplementary data S1). The lymphocyte-rich stroma in this paper approximately corresponded to TIL density of greater than 80%.¹⁵ We adopted the cut-off values of 30% and 50%; *i.e.*, when lymphocyte- or lymphoplasmacyte-rich stroma exceeded 30% or 50% of all cancer stromal areas, such invasive breast cancer was designated LPBC (area > 30%) or LPBC (area > 50%), respectively (Supplementary data S1). Stroma sporadically infiltrated by lymphoplasmacytes was not included as lymphocyte-rich stroma. We also analyzed medullary carcinoma (MC), which fulfilled all the following criteria: (i) prominence of lymphocyte-rich stroma with the cut-off value of 75%; (ii) syncytial appearance of cancer cells; (iii) higher nuclear atypia; (iv) near absence of luminal formation; and (v) pushing margin. All MCs in the present study corresponded

to LPBC (area > 75%), and vice versa. LPBC (area > 30%) approximately corresponded to 'invasive carcinoma with medullary features' defined in the WHO classification.²⁰ This was the main reason we adopted the cut-off value of 30%. All LPBC patients were Japanese women, except for one non-Japanese Asian woman.

Survival analysis

Analyses of the patients' survivals were performed in 39 cases of LPBC (area > 30%), which included 11 MCs. All these patients underwent resection of cancer in Mito Medical Center during 2003–2013. 18 of these 39 cases underwent surgery during 2003–2009 (before the consecutive series). The exclusion criteria were: (i) follow-up periods less than 12 months; and (ii) patients who received pre-operative chemotherapy. For comparison, grade-matched or stage-matched controls was used and these control cases underwent resection during 2007–2009. The Kaplan–Meier method was adopted for the estimation of survival rate, and the log-rank test was used to check the difference. For multivariate analysis, Cox proportional hazards regression was adopted.

Effects on the preoperative chemotherapy

Fifty-nine patients were enrolled for the analyses on preoperative chemotherapy (all cases included in the 'consecutive series'). In this group, 10 cases of LPBCs (area > 30%) showed no residual invasive cancer in the surgically resected specimens. In those cases, core needle biopsy specimens were used to assess TILs. All patients were Japanese women with a mean age of 51.8 years (range 27–74). The regime of chemotherapy was as follows;^{23,24} the patients were first administered an anthracycline-containing regimen (FEC, 5-fluorouracil + epirubicin + cyclophosphamide) and then the drug was replaced by taxane (docetaxel or paclitaxel; 31 cases). In patients with HER2-overexpression, trastuzumab was used in combination with taxane after anthracycline (since November 2011; 29 cases). Eight cases received anthracycline only (five cases) or taxane only (three cases).

Immunohistochemical and statistical methods

For immunohistochemistry, the following primary antibodies were used: mouse monoclonal anti-ER (clone 1D5, DAKO, Glostrup, Denmark), mouse monoclonal anti-PgR (clone PgR 636, DAKO), rabbit polyclonal anti-HER2 (DAKO), and mouse monoclonal anti-Ki-67 (clone MIB1, DAKO). Statistical analysis was performed by IBM SPSS Statistics software, version 21 (IBM Inc., Armonk, NY, USA). For multivariate

analyses (logistic regression and Cox proportional hazards regression), we adopted the backward stepwise elimination method (Wald). That is, all the co-variables analyzed by univariate analysis were included in the first step. During the statistical process, co-factor with the least significance was eliminated stepwise, and finally significant co-factors were selected. The results were judged to be significant when the *P*-value was below 0.05. The present study was approved by the Ethics Committee of Mito Medical Center (approved Oct 2, 2013).

RESULTS

LPBC and breast cancer subtypes

We analyzed 'consecutive series' including 477 surgically resected, invasive breast cancers in 471 patients (age 58.1 ± 13.7 , range 27–96), in which all lesions had been classified into subtypes.¹³ The numbers of LPBC (area > 30%) and MC in this group were 39 (8.2%) (age 58.9 ± 14.1 , range 31–86) and 9 (1.9%), respectively. By the definition, all MCs were included in LPBCs (area > 30%). The occurrence rate of LPBC (area > 30%) in each subtype was the highest in the TN subtype (29% [20/70]). The second most numerous subtype was the HER2⁺/ER⁻ subtype, while HER2⁺ or HER2⁻ luminal B-like subtype showed lower ratios (Figs 2a,3a). These analyses confirmed that carcinoma cells in all LPBC (area > 30%) showed Ki-67 labelling greater than 15%. The data so far was based on the St Gallen consensus meeting 2011.¹³ The same meeting in 2013¹⁴ proposed that both ER⁺ and PgR⁺ are required for luminal A-like subtype. However, the results according to the latter definition did not show any changes to the LPBC distribution in each subtype (Supplementary data S2A). The analyses of the primary tumor (pT) and regional node status (pN) demonstrated that most cases of LPBCs were either pT1 or pT2. However, the distribution pattern of pT of LPBC (area > 30%) did not differ from that of non-LPBC cases (Supplementary data S2B). LPBC (area > 30%) had significantly more-advanced lymph node metastasis than non-LPBC cases (Supplementary data S2B). MCs were mainly composed of TN subtype (Figs 2b,3a)

LPBCs and histological grade

Grade analyses in LPBCs showed that the occurrence rate of LPBC (area > 30%) among each grade were 0% in grade 1, 8.3% (13/156) in grade 2, and 29% (26/89) in grade 3 (Fig. 3b). Nearly all MC were of grade 3. This indicated that grade 3 is the most frequent in LPBCs, and no cases of grade 1 score were found in LPBCs. In other words, our results so

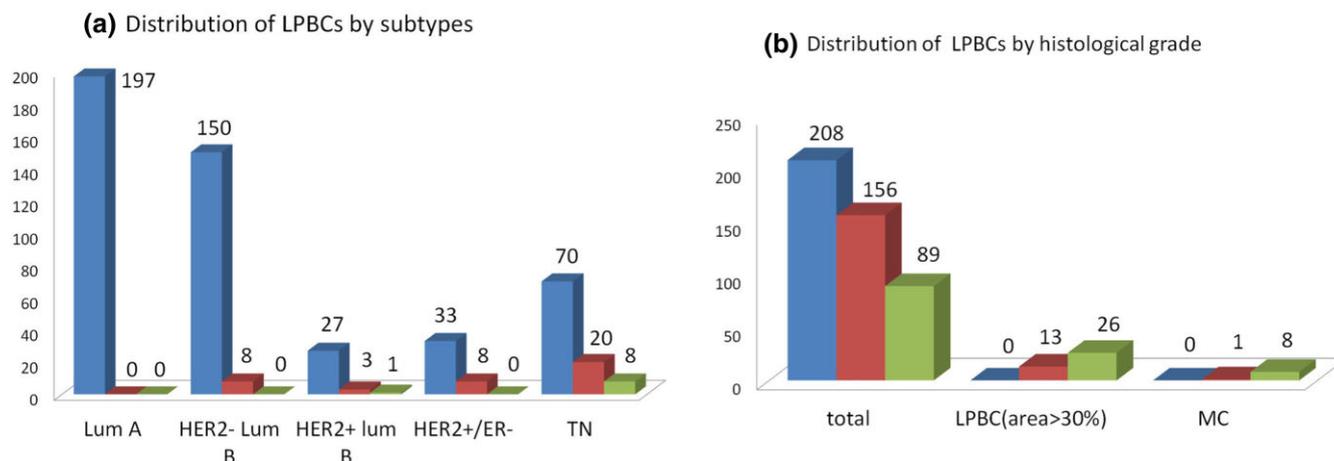


Figure 3 Occurrence of lymphocyte-predominant breast cancer (LPBC) by subtypes (a) and histological grade by LPBCs (b). Numbers indicate the number of cases in each group. Note that no grade 1-scored tumor is found in LPBC.

Table 1 Multivariate analyses (logistic regression) to analyze factors related to the formation of LPBCs in consecutive group ($N = 477$)

Co-variables	Univariate			Multivariate (backward stepwise elimination [Wald])			
	p-value	Odds ratio	95% CI	Co-variables	P-value	Odds ratio	95% CI
LPBC (area > 30%) ($N = 39$)							
ER (-)	<0.0005	13.4	6.3–28.8	ER (-)	<0.0005	6.0	2.5–14.5
Grade 3	<0.0005	13.5	6.5–27.9	Grade 3	<0.0005	5.4	2.4–12.6
HER2 (+)	0.001	3.6	1.7–7.7				
T (T1 vs T2–4)	0.49	0.79	0.41–1.5				
N ([+] vs [-])	0.15	1.7	0.84–3.3				
Medullary carcinoma (MC) ($N = 9$)							
ER (-)	0.002	26.9	3.3–221				
Grade 3	0.001	38.1	4.6–314	Grade 3	0.001	38.1	4.6–314
HER2 (+)	0.95	1.1	0.13–8.9				
T (T1 vs T2–4)	0.34	2.2	0.44–11				
N ([+] vs [-])	0.32	0.34	0.042–2.8				

ER-negativity and histological grade 3 scores significantly associated with the formation of LPBC. Bold fonts indicate co-variables significant by multivariate analysis.

CI, confidence interval; ER, estrogen receptor; LPBC, lymphocyte-predominant breast cancer; MC, medullary carcinoma.

far suggested that both the luminal A-like subtype and a histological grade 1 score were inhibitive to the formation of LPBC (area > 30%) as long as the statistical data are concerned.

Multivariate analysis of LPBCs to analyze factors related to its formation

We then analyzed which clinicopathological factors were closely related to the formation of LBPCs using the consecutive series. As co-variables, we analyzed ER-negativity, histological grade 3 score (vs. grade 1 + 2), HER2-positivity, pT (pT2–4 vs. pT1), and pN (pN1–3 vs pN0). Multivariate analysis by logistic regression with backward stepwise elimination (Table 1) showed that ER-negativity and grade 3 scores associated equally with LPBC (area > 30%). Only grade 3

scores were significantly related to MC. HER2-positivity was only significant to LPBC (area > 30%) by univariate analysis, but not by multivariate analysis.

The results of LPBC (area > 50%) were essentially the same as those of LPBC (area > 30%) as shown in Supplementary data S2C.

Improved disease-free survival of patients with LPBC

Survival analyses were performed in 39 cases of LPBC (area > 30%) without preoperative chemotherapy (age 58.7 ± 13.2 , range 39–86). The observation period was 59.7 ± 34.6 months (mean \pm SD; range 12–138 months). LPBC (area > 30%) showed improved disease-free survival compared with grade-matched controls ($P = 0.012$, log-rank test; Fig. 4a). Multivariate analyses (Cox proportional

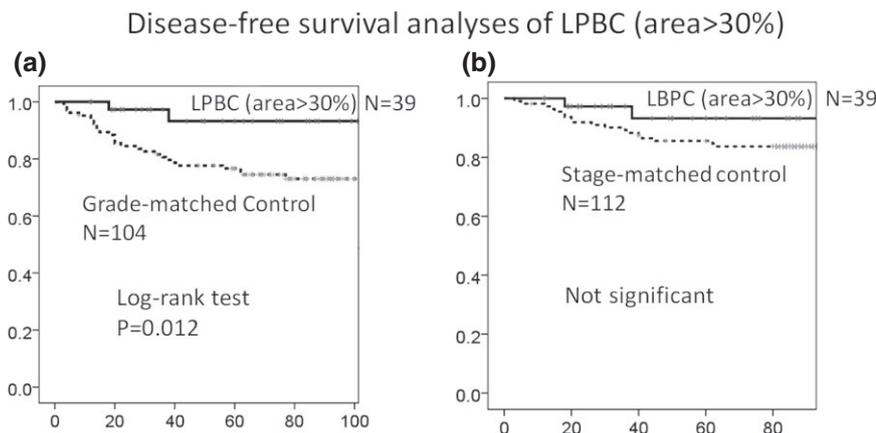


Figure 4 Survival analyses of lymphocyte-predominant breast cancer (LPBC) (area > 30%). (a) LPBC (area > 30%) (39 cases) shows a better disease-free survival than grade-matched controls (104 cases), but (b) no significant differences were noted with stage-matched controls (112 cases). Vertical axes, disease-free survival. Horizontal axes, months after the resection of breast cancer.

Table 2 Multivariate analysis to analyze disease-free survival (Cox proportional hazards regression) in the group of LPBC (area > 30%) (N = 39) and grade-matched control (N = 104)

Co-variables	Univariate			Co-variables	Multivariate (backward stepwise elimination [Wald])		
	P-value	Hazard ratio	95% CI		P-value	Hazard ratio	95% CI
LPBC (area > 30%)	0.024	0.19	0.044–0.80	LPBC (area > 30%)	0.030	0.20	0.046–0.86
N ([+] vs [-])	0.005	2.9	1.4–6.1	N ([+] vs [-])	0.008	2.7	1.3–5.7
ER (-)	0.29	1.5	0.72–3.0				
HER2 (+)	0.25	1.6	0.73–3.5				
T (T1 vs T2–4)	0.12	2.4	0.84–4.0				
Age	0.14	1.7	0.83–3.6				

LPBC (area > 30%) was an independent prognostic factor together with N-factor. Bold fonts indicate co-variables significant by multivariate analysis. Age, divided at the median value (age 54).

CI, confidence interval; ER, estrogen receptor; LPBC, lymphocyte-predominant breast cancer.

hazards regression with backward stepwise elimination) showed that LPBC (area > 30%) was an independent prognostic factor compared with grade-matched control (Table 2). No statistically significant difference was observed between LPBC (area > 30%) and stage-matched controls with both groups showing high survival rates (Fig. 4b).

None of the MC cases (11 cases) suffered disease recurrence or death with the observation period of 73.5 ± 40.9 months (mean ± SD), indicating 100% disease-free survival. Also LPBC (area > 50%) (27 cases) showed 100% disease-free survival with the observation period of 63.0 ± 35.3 months (mean ± SD). These two data suggest that the survival rate of LPBC (area > 50%) and that of MC were higher than that of LPBC (area > 30%). However, the exact survival analyses were not possible due to 100% disease-free survival. Collectively, our data indicate that breast cancers with pronounced lymphoid stroma predict a better survival as compared with the same high-grade breast cancers.

LPBC (area > 30%) effectively predicts complete pathological remission (pCR) after preoperative chemotherapy

In 59 cases, we analyzed the effect of LPBCs on the responses to neoadjuvant chemotherapy. Of the 59 cases

analyzed, 12 were LPBC (area > 30%) with the mean age of 51.8 years old. Of the 59 cases, 19 cases (32%) were judged to be pCR. LPBC (area > 30%) showed the pCR ratio of 80%. By multivariate analysis (logistic regression with backward stepwise elimination), LPBC (area > 30%) and ER-negativity significantly contributed to predicting pCR. None of the HER2-positivity, histological grade 3, T-factor, or N-factor variables significantly predicted pCR (Table 3). The results in this and previous sections indicated that our method of the definition of LPBC has its own clinicopathological significance.

DISCUSSION

To overcome considerable heterogeneity of distribution of lymphocytes in cancer stroma, the present study analyzed LPBCs defined by the proportion of lymphoid stroma. We dealt with three different cut-off points of lymphoid stroma; *i.e.*, LPBC (area > 30%), LPBC (area > 50%) and MC (=LPBC [area > 75%]). The disease-free survival rate of LPBC (area > 50%) and that of MC were 100%. However, this made detailed survival analysis difficult, and mainly the data of the LPBC (area > 30%) were presented. LPBC (area > 30%) denoted invasive breast cancer in which

Table 3 Multivariate analyses (logistic regression) to analyze factors that predict pathological complete remission after preoperative chemotherapy (N = 59)

Co-variables	Univariate			Co-variables	Multivariate (backward stepwise elimination [Wald])		
	P-value	Odds ratio	95% CI		P-value	Odds ratio	95% CI
LPBC (area > 30%)	<0.0005	21	3.9–114	LPBC (area > 30%)	0.001	32	4.2–240
ER (-)	0.002	7.4	2.1–25	ER (-)	0.004	11	2.1–58
HER2 (+)	0.011	4.5	1.4–14				
Grade 3	0.029	3.6	1.1–11				
T (T1 vs T2–4)	0.34	0.43	0.079–2.4				
N ([+] vs [-])	0.82	1.2	0.21–6.9				

LPBC (area > 30%) and ER-negativity are significant to predict pathological complete remission. Bold fonts indicate co-variables significant by multivariate analysis.

CI, confidence interval; ER, estrogen receptor; LPBC, lymphocyte-predominant breast cancer; N, lymph node metastasis; T, primary tumor.

lymphocyte-rich stroma exceeded 30% of the whole cancer stroma by semi-quantification (for details, see Supplementary data S1). Our results showed the predominance of TN subtype and grade 3 in LPBC (area > 30%), basically corroborating previous reports based on the TIL-density method.^{9,11,12} Our study also showed LPBCs do not contain any luminal A-like subtypes (including Ki-67 less than 15% as one of the criteria) or grade 1 scored tumors. Namely TILs are less prominent in well differentiated breast cancer (luminal A-like subtype or histological grade 1), which generally shows a favorable prognosis. This reinforces why TILs are not considered a prognostic factor in well-differentiated breast cancer, only being significant in the TN subtype.^{10–12} Multivariate analyses confirmed that a de-differentiated phenotype in breast cancer (ER-negativity or grade 3) is related to the formation of LPBCs. This is consistent with results identifying the number of CD8⁺ T cells in breast cancer.^{25,26} These discussions confirm the validity of the definition of LPBC in the present study.

We confirmed that patients with LPBC (area > 30%) showed a better disease-free survival than grade-matched control by both univariate and multivariate analyses. Patients with LPBC (area > 50%) and those with MC showed 100% disease-free survival. These data indicate a good prognosis of patients with LPBCs defined by the proportion of lymphoid stroma, suggesting that predominance of lymphocyte-rich stroma is important for the prognostication of high-grade breast cancer. Patients with LPBC (area > 30%) did not show significant difference as compared with stage-matched control. This may reflect the fact that LPBC (area > 30%) is composed mainly of triple-negative and HER2⁺ subtypes, and the effects of lymphocytic responses may be canceled or diminished by more-aggressive nature of cancer cells when compared with the same low-stage breast cancers.

Through these discussions, limitations of the present study are also noted. The present study is based on patients in one hospital, and the number of patients with LPBC may not be abundant. Therefore, a future study with a larger scale would be required to clarify which cut-off value (30%, 50% or other) is the most valuable in routine diagnosis.

The association we observed between TILs and de-differentiation status may explain why CD4⁺ or CD8⁺ T cells in breast cancer are associated with lymph node metastasis.²⁷ Our data showed that LPBCs (area > 30%) contained more lymph node metastasis than non-LPBCs. Despite these, follow-up analysis showed that LPBC (area > 30%) displayed better disease-free survival than grade-matched controls. This may be explained by the possibility that breast cancer in Japanese women is less aggressive than in Western countries.²⁸ However, large-scale analysis shows that there is no significant difference between the two.²⁹ This allows us to compare our data with studies performed in other countries, confirming the less aggressiveness of LPBCs in Japan.

Recently, Nawaz *et al.* reported that the degree of colocalization of immune cells (lymphocytes) and cancer cells is important for longer survival of patients with ER-negative breast cancer.³⁰ This viewpoint of colocalization is important. However their method required the use of an automated image analyzer. Our simple method to overcome the difficulty of heterogeneity of lymphocyte distribution would be worth further analyses.

Lymphocyte-predominant breast cancer (area > 30%) showed an improved response to preoperative chemotherapy, corroborating previous reports based on various quantitative and semi-quantitative TIL analyses.^{5–9} The improved responses are likely due to augmented immune responses to cancer cells induced by chemotherapy, hence this regime is designated as 'immunogenic chemotherapy'.³¹ Collectively, the results on LPBC based on our method were similar to those of LPBC defined by TIL-density method.^{9,11,12} This further confirms the usefulness of our method to define LPBC by the proportion of lymphoid stroma in routine diagnosis.

Previous reports showed that CD8⁺ T cells⁶ or cells positive for cytotoxic granules (granzymes or perforin)⁷ contributed to pCR after chemotherapy, and CD8⁺ T cells are associated with better survival.²⁵ Cytotoxic T-cells (CD8⁺ and granzyme B⁺) were reportedly increased in medullary breast carcinoma.³² These data show that de-differentiated cancer tends

to be associated with cytotoxic immune responses. This also indicates that de-differentiated breast cancers (grade 3, triple-negative, or HER2⁺ subtype) that lack TIL response would be regarded as a high-grade cancer without immune-mediated restriction. Although the recent St Gallen Consensus meeting 2015 reported that a clear majority of the panel did not accept the presence of TILs as either a prognostic or a predictive marker,³³ our results demonstrate that the prominence of TILs, or lymphoid stroma is important in breast cancer.

Cancers other than breast cancer also show the association between an abundance of TILs and the de-differentiation status of tumor cells, including lymphoepithelial carcinoma of the nasopharynx,³⁴ stomach cancer (gastric carcinoma with lymphoid stroma),¹⁶ colon cancer (medullary carcinoma),³⁵ and lung cancer (lymphoepithelioma-like carcinoma).³⁶ We have previously reported that high-grade renal cell carcinoma was associated with more pronounced infiltration by CD8 + T cells.³⁷ Considering this, the correlation between the de-differentiation status and prominence of TILs is likely to be common in various types of cancer.

To conclude, we would like to propose the definition of LPBC by the proportion of lymphoid stroma in routine histopathological diagnosis. This could aid in assessing prognosis of patients with high-grade breast cancer.

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DISCLOSURE STATEMENT

None declared.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Data S1 Details of the definition of LPBC.

Data S2 Additional data on LPBC.