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PRECLINICAL STUDY

Diffuse distribution of tumor-infiltrating lymphocytes is a marker for better prognosis and chemotherapeutic effect in triple-negative breast cancer

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Abstract

Purpose High-density tumor-infiltrating lymphocytes (TILs) are a prognostic marker for triple-negative breast cancer (TNBC). However, lymphocytic infiltration is heterogeneous in its pattern. We aimed to explore the utility of TIL distribution patterns against TIL density for predicting TNBC prognosis and chemotherapeutic effects.

Methods Primary invasive TNBC cases were retrieved from a single institutional cohort, and archived samples were reviewed by two board-certificated pathologists. We used 154 consecutive surgical specimens from patients with standard adjuvant therapy, and 80 biopsies taken before primary systemic chemotherapy. The average density of stromal TILs was scored at 10% intervals, while the distribution pattern of TILs was evaluated as diffuse or non-diffuse. The association between TILs and prognosis or pathological complete response (pCR) was statistically analyzed.

Results A diffuse pattern of TILs at primary surgery correlated with better prognosis (relapse-free survival [RFS], hazard ratio [HR] 3.71, 95% confidence interval [CI] 1.60–8.57; overall survival [OS], HR 3.87, 95% CI 1.46–10.27), as well as high TIL density (≥ 50%; RFS, HR 4.51, 95% CI 2.06–9.90; OS, HR 3.28, 95% CI 1.32–8.14). Diffuse TIL pattern and nodal status were independent prognostic factors in multivariate analysis. Diffuse TIL pattern upon biopsy was associated with higher pCR rate (diffuse, 46%; non-diffuse, 21%; $P = 0.032$). All high TIL cases had diffuse patterns and the best outcome. Interobserver concordance was moderate ($k = 0.53$–0.55; distribution pattern) to good (weighted $k = 0.67$–0.69; density), and it was faster to assess the distribution pattern than to assess the density of TIL.

Conclusions Showing similar clinical impacts to the TIL density, diffuse TILs could be a predictive marker for better prognosis and higher pCR. The assessment of TIL distribution pattern is simple, faster, and practical. Heterogeneous tumor immunity may contribute to further stratification of TNBC treatment.

Keywords Tumor-infiltrating lymphocytes · Triple-negative breast cancer · Heterogeneous tumor immunity · Pathological complete response · Interobserver concordance

Abbreviations

TIL	Tumor-infiltrating lymphocyte
TNBC	Triple-negative breast cancer
ER	Estrogen receptor
PgR	Progesterone receptor
HER2	Human epithelial growth factor receptor 2

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RFS Relapse-free survival
OS Overall survival
pCR Pathological complete response
HE Hematoxylin–eosin
PSC Primary systemic chemotherapy
LPBC Lymphocytic-predominant breast cancer

Introduction

There is accumulating evidence that tumor-infiltrating lymphocytes (TILs) have both prognostic and predictive values in breast cancer (BC) [1, 2], especially for triple-negative (TN) BC [3–5]. Lacking overexpression of estrogen receptor (ER), progesterone receptor (PgR), and human epithelial growth factor receptor 2 (HER2), TNBC patients usually receive cytotoxic chemotherapy as the standard treatment. In addition to the lack of specific targeted therapies, the aggressive nature of TNBC prevents cure in many patients. TNBC tends to occur at younger age, have a higher histological grade, and relapse in a short period [6, 7]. Much research has been focused on these issues to improve the outcome of TNBC. In recent years, poly ADP-ribose polymerase (PARP) inhibitors have been used for *BRCA*-mutant tumors [8], and anti-androgen drugs are candidates for the luminal androgen receptor (LAR) subtype based on molecular classification of TNBC [9].

For more detailed treatment stratification and relevant new strategies, tumor immunity in the microenvironment is an attractive factor in the era of immunotherapy [10–12]. Harboring stronger TIL reaction among BC, the TN subtype is a candidate target of immune-checkpoint inhibitors such as those that induce PD-1/PD-L1 blockade [13]. At the same time, a number of studies have shown that TILs are an independent favorable prognostic factor [3, 14] and are associated with better response to chemotherapy in TNBC [2, 15]. Most previous studies in the field evaluated the abundance of TILs, such as stromal TILs or intratumoral TILs under a high-power field. The International TIL Working Group proposed a protocol to evaluate TILs by hematoxylin–eosin (HE) staining [16], and recommended estimating the density of TILs in the stroma and taking the average in a continuous percentile. Many authors follow their recommendation, and this analytical method has been validated [4, 17]. These circumstances led the discussion as to whether we should assess TILs in routine practice [5].

Regarded as a gold standard to assess TILs, the proposed method still has some issues that need to be resolved before it can be applied in daily practice. First, evaluating the TIL average under a high-power field (×400) is a huge task for pathologists. A simpler method would be widely accepted with rapid uptake [18]. Second, there are questions about the heterogeneity of TILs in breast cancer as well as ER, PgR, HER2, and Ki-67 expression [19–21]. The spatial heterogeneity of immune infiltration is reported to have a critical role not only in ER-negative [22] but also in ER-positive BC [23]. We have also noted TIL heterogeneity (different intensities of lymphocytes in different areas) in previous studies. Through our experiences of assessing many BC cases for TIL density [24, 25], we recognized two distribution patterns of TILs, diffuse and non-diffuse.

In this study, we compared the density method proposed by the International TIL Working Group [16] and our original diffuse method [26]. The diffuse method is based on the simplest two-grade scale, diffuse or non-diffuse, which seems to be fast and easy without the need to estimate the average percentile. We defined the method in a protocol and investigated the analytical validity and clinical utility in retrospective cohorts of TNBC. Interobserver concordance and time needed to evaluate TILs were compared between the two methods of TIL assessment.

Methods

Patients and samples

This retrospective study was conducted at Hakuaikai Sagara Hospital with ethics committee approval (#14-06 and #17-33). Clinicopathological information was obtained from the hospital database and included age, sex, tumor size, histological classification, grade, lymphovascular invasion, nodal status, and biomarker profile. We searched for patients with primary BC who had been diagnosed with TN-subtype invasive ductal carcinoma (ER-, PgR-, and HER2-). The thresholds for ER positivity and PgR positivity were set at 1% using immunohistochemical staining. The overexpression and amplification of *HER2* were examined according to the guidelines of the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) [27, 28]. Two different cohorts were selected using inclusion and exclusion criteria described previously [24].

Briefly, the first cohort consisted of patients who underwent curative surgery and standard postoperative therapy between 2007 and 2014. Among 3547 invasive carcinomas identified during this period, 2,899 cases (82%) were ER+ and 568 cases (16%) were HER2+. TN subtype accounted for 381 (11%) cases, and 73 patients had received primary systemic chemotherapy. Among the rest, small cancers (pT1mi [micro-invasion]: 2, pT1a [<5 mm]: 16), locally advanced cancers (pT4: 5), special histology (ex. lobular carcinoma, apocrine carcinoma, metaplastic carcinoma, micro-papillary carcinoma: 42), incomplete surgeries without lymph node dissection (6), and lack of radiation after breast-conserving surgery (12) were excluded. As we regarded chemotherapy regimens including anthracycline, docetaxel, or paclitaxel as a standard, 64 patients with insufficient chemotherapy were also excluded. Seven patients were lost in the follow-up, and the remaining 154 female patients were retrieved for this adjuvant setting to avoid potential biases. The detailed distributions of age, tumor size, histological grade, nodal status, and regimens of systemic therapy are summarized in a previous report [24]. They received no prior therapy, and the pathological stage upon primary surgery varied as follows: I A; 67 (44%), I B; 8 (5.2%), II A; 45 (29%), II B; 22 (14%), III A; 7 (4.5%), III B; 0 (0%), III C; 5 (3.2%).

In the second cohort, patients treated with standard primary systemic chemotherapy (PSC) and subsequent surgery were selected between 2007 and 2016. These patients were all women aged 28–75 years who received an anthracycline and taxane at the standard dose. Eighty consecutive TN cases were identified and 28 patients (35%) had achieved pathological complete response (pCR) in this PSC setting; their detailed characteristics are summarized in Table 1.

Stored HE slides were retrieved for TIL assessment for most cases (all 154 surgical specimens in the adjuvant cohort and 78 biopsies) and scanned whole slide images with HE staining were used for two biopsy samples among the PSC cohort. The REMARK (REporting of tumor MARKer Studies) guidelines were followed [29].

Assessment of tumor-infiltrating lymphocytes (TILs)

In the first cohort of primary surgery and adjuvant chemotherapy (adjuvant cohort), a representative slide containing relatively high amounts of both invasive cancer and TILs was selected from resected specimens for each case. In the second cohort of primary systemic chemotherapy (PSC cohort), needle biopsy samples obtained before treatment were used for the assessment. All HE samples were evaluated by an experienced pathologist (A.H.), whose TIL score was compared with clinical outcomes throughout the study. Many of the slides were reviewed by another breast pathologist (T.W.) for a concordance analysis. He

Table 1 Patient characteristics of the primary systemic chemotherapy cohort ($n = 80$)

Age	
Median (range)	50.5 (28–75)
≤ 50 years	40 (50%)
> 50 years	40 (50%)
Clinical stage before systemic therapy	
1	1 (1.3%)
2A	14 (18%)
2B	19 (24%)
3A	21 (26%)
3B	11 (14%)
3C	13 (16%)
4	1 (1.3%)
Pathological response (Chevalier criteria)	
Grade 1 (No residual cancer in breast or lymph node)	22 (28%)
Grade 2 (Breast carcinoma in situ and node-negative)	5 (6.3%)
Grade 3 (Invasive carcinoma with chemotherapy-induced change)	23 (29%)
Grade 4 (Minimally modified carcinoma and/or node-positive)	30 (38%)
Histological grade of non-pCR cases ($n = 52$)	
Grade 1	1 (1.3%)
Grade 2	15 (19%)
Grade 3	35 (44%)
Undetermined[a]	1 (1.3%)[a]
Lymph node status after systemic therapy	
Negative	56 (80%)
Positive	24 (30%)
Primary systemic therapy	
Anthracycline → taxane	72 (90%)
Taxane → anthracycline	8 (10%)

pCR was defined as no residual invasive carcinoma in the breast regardless of lymph node status

[a]One case had so tiny invasion that we could not determine the histological grade

performed the assessment about a year after A.H. and skipped oldest 26 samples (25 surgical specimens and 1 biopsy) which had lost its color. Four biopsies in the PSC cohort were diagnosed at other institutions, and not available for T.W. The two pathologists scored independently without knowing the other's scores or patients' profiles. Time required to evaluate the whole samples by T.W. was recorded.

We applied two different methods to assess TILs. One is a widely accepted method proposed by the International Working Group [16]. Briefly, we screened samples within the tumor border under a high-power field (×200) and estimated the average density of TILs in the stroma. The other is our original method estimating the distribution pattern of TILs as 'diffuse' or 'non-diffuse' (Fig. 1). Both methods followed basic instructions described by Salgado et al. [16], such as shaping the region of interest within the invasive tumor border, counting lymphocytes and plasma cells only, and not focusing on hotspots. We defined TIL distribution as diffuse when TILs were observed in both peripheral and central areas under a low-power field (×40). If the central area was not affected by TILs or lymphocytes in the center were too few to be counted, the distribution pattern was estimated as non-diffuse. When confused, pathologists were advised to scan a representative line in resected samples or biopsy cores under a medium-power field (×100). If more than half of the scanned line/core had recognizable TILs, it was defined as diffuse.

Outcome measures of prognosis and pathological complete response

Prognostic information and response to PSC was obtained from the clinical database and electronic charts updated in March 2018. Relapse-free survival (RFS) was defined as the period from surgery to the latest follow-up, relapse of breast cancer, or death from any cause. Overall survival (OS) was defined as the period from surgery to the latest follow-up, or death from any cause. Pathological complete response (pCR) was defined as no residual invasive carcinoma at the primary site, such as ypT0 or ypTis, regardless of lymph node status.

Statistical analysis

Survival curves were drawn using the Kaplan–Meier method and analyzed by log-rank tests. The proportions of pCR were compared using Fisher's exact or Chi-square test. The association of clinicopathological variates, including TILs, with prognosis was examined using a Cox proportional hazards model in the adjuvant setting. We repeated the multivariate analyses since we used two different TIL assessment methods and needed to avoid the

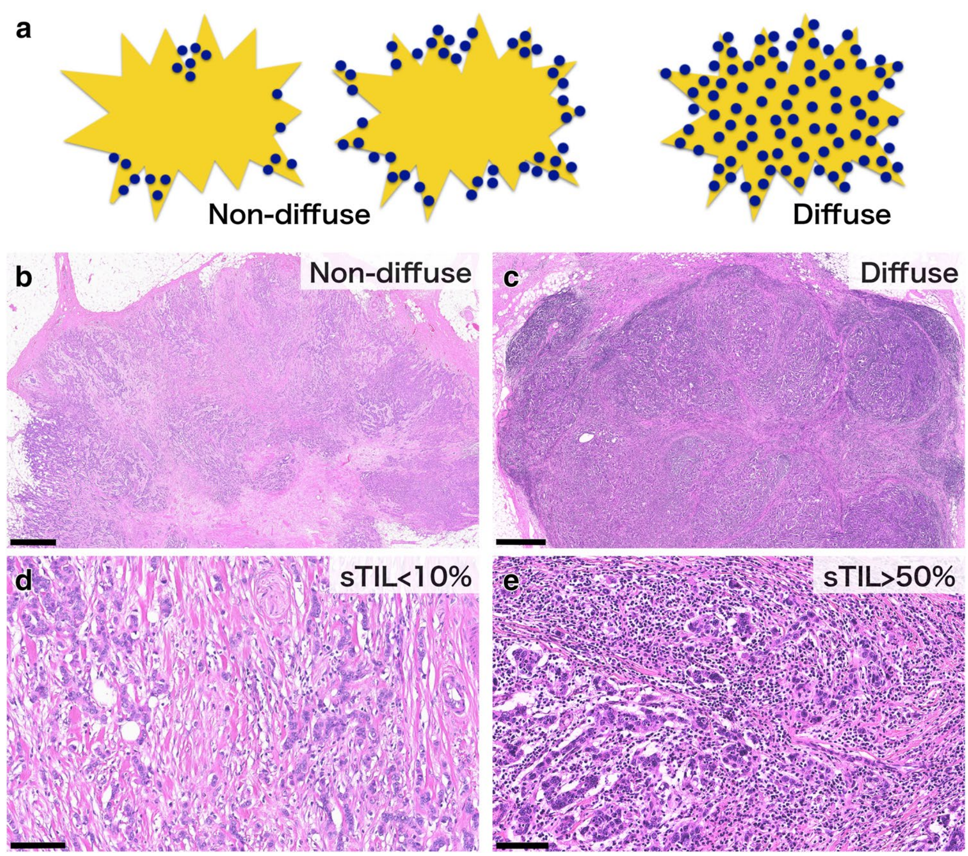

Fig. 1 Schematic illustration and representative photomicrographs of HE-stained tissue showing tumor-infiltrating lymphocytes (TILs). Compared with non-diffuse patterns with partial or peripheral infiltration (**a**; left), diffuse patterns were defined as having lymphocytic infiltration (navy dots) throughout an invasive tumor (**a**; right). The example cases were estimated as non-diffuse (**b**) or diffuse (**c**) under a low-power field (black bar in the lower left indicates 1 mm: **b**, **c**). The images in the lower panel represent the two cases. The average density of stromal TILs was estimated as less than 10% in the left-hand case (**d**) and 80% in the right-hand case (**e**) under a high-power field (black bar indicates 0.1 mm: **d**, **e**)

multicollinearity of TILs. Interobserver concordance was estimated by *kappa* value or weighted *kappa* value. Data were analyzed using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA) and JMP 14 (SAS Institute Inc., Cary, NC, USA).

Results

Interobserver concordance and duration of TIL assessment

The interobserver concordance of TIL assessment is summarized in Table 2. Two board-certified pathologists (A.H. and

Table 2 Interobserver concordance for the assessment of tumor-infiltrating lymphocytes in different methods

a	Non-diffuse (by TW)	Diffuse (by TW)	Total
Non-diffuse (by AH)	**66**	23	89
Diffuse (by AH)	5	**35**	40
Total	71	58	129
Kappa = 0.549			

b	< 10%	10%	20%	30%	40%	50%	60%	70%	80%	90%	Total
< 10%	**5**	2	0	0	0	0	0	0	0	0	7
10%	9	**11**	8	1	0	1	0	0	0	0	30
20%	0	3	**6**	4	2	0	1	0	0	0	16
30%	0	0	3	**3**	2	3	1	1	0	0	13
40%	0	2	0	6	**2**	4	3	2	2	0	21
50%	0	0	1	0	1	**1**	2	3	0	0	8
60%	0	0	0	0	0	1	**2**	5	3	0	11
70%	0	0	0	0	0	0	2	**3**	4	0	9
80%	0	0	0	0	0	0	0	0	**3**	5	8
90%	0	0	0	0	0	0	0	1	2	**3**	6
Total	14	18	18	14	7	10	11	15	14	8	129
(by AH)	Kappa = 0.220, weighted Kappa = 0.673										

c	Non-diffuse (by TW)	Diffuse (by TW)	Total
Non-diffuse (by AH)	**28**	15	43
Diffuse (by AH)	3	**29**	32
Total	31	44	75
Kappa = 0.532			

d	< 10%	10%	20%	30%	40%	50%	60%	70%	80%	90%	Total
< 10%	**10**	0	0	0	0	0	0	0	0	0	10
10%	11	**6**	1	0	0	0	0	0	0	0	18
20%	4	3	**4**	1	0	0	0	0	0	0	12
30%	1	4	2	**1**	0	1	0	0	0	0	9
40%	0	0	1	2	**0**	2	0	0	0	0	5
50%	0	0	1	0	0	**0**	1	1	0	0	3
60%	0	0	0	0	1	0	**1**	1	2	0	5
70%	0	0	0	2	0	0	0	**4**	1	0	7
80%	0	0	0	0	0	0	1	1	**2**	1	5
90%	0	0	0	0	0	0	0	0	1	**0**	1
Total	26	13	9	6	1	3	3	7	6	1	75
(by AH)	Kappa = 0.275, weighted Kappa = 0.685										

Resected specimens at the primary surgery (a, b) and biopsy samples before primary systemic chemotherapy (c, d) were assessed by two pathologists (AH and TW) independently. They evaluated TIL based on distribution pattern (a, c) and average density (b, d)

Bold values indicate the matched numbers by two pathologists

T.W.) evaluated 129 resected specimens in the adjuvant setting (Table 2a–b), and 75 needle biopsies in the PSC setting (Table 2c–d). The concordance of the diffuse method was moderate (*kappa* = 0.532–0.549; Table 2a, c), and that of the density method was good (weighted *kappa* = 0.673–0.685; Table 2b, d). It took a pathologist (T.W.) 54 min and 33 s to assess needle biopsies using the diffuse method, and 92 min and 50 s using the density method. Furthermore, it took 55 min and 30 s to assess surgical specimens using the diffuse method, and 128 min and 9 s using the density method.

Prognostic value of TILs in the adjuvant setting

Twenty-seven recurrences and 20 deaths were observed after curative surgery and standard postoperative therapy during a median follow-up period of 70.5 months (interquartile range, 46–96.8). Based on the density of TILs, 49 tumors (32%) among 154 were estimated as containing high TILs (stromal TIL ≥ 50%), for whom prognosis was significantly better than the low TIL group regarding both RFS ($P = 0.0067$; hazard ratio [HR] 4.51; 95% confidence interval [CI] 2.06–9.90) and OS ($P = 0.044$; HR 3.28; 95% CI 1.32–8.14) (Fig. 2a, c). Similarly, a diffuse pattern of TILs was also a favorable prognostic factor. We estimated 104 tumors (68%) as having a diffuse TIL pattern, and 50 tumors (32%) as having a non-diffuse pattern. The prognostic differences were significant in both RFS ($P = 0.0003$; HR 3.71; 95% CI 1.60–8.57) and OS ($P = 0.0014$; HR 3.87, 95% CI 1.46–10.27) (Fig. 2b, d).

In multivariate analyses, the prognostic impact of TILs by both methods remained significant regarding RFS (density method: $P = 0.0062$; HR 4.12; 95% CI 1.43–17.4; diffuse method: $P = 0.0005$; HR 3.91; 95% CI 1.82–8.71), as did nodal status (Table 3). We repeated the analysis separately to avoid the multicollinearity of TILs. Two different methods for TIL assessment, the diffuse method and the density method, showed a strong correlation ($P < 0.0001$, Chi-square test). Other factors including age, tumor size, histological grade, and lymphovascular invasion had no significant association with RFS. The analysis was applied not only to RFS

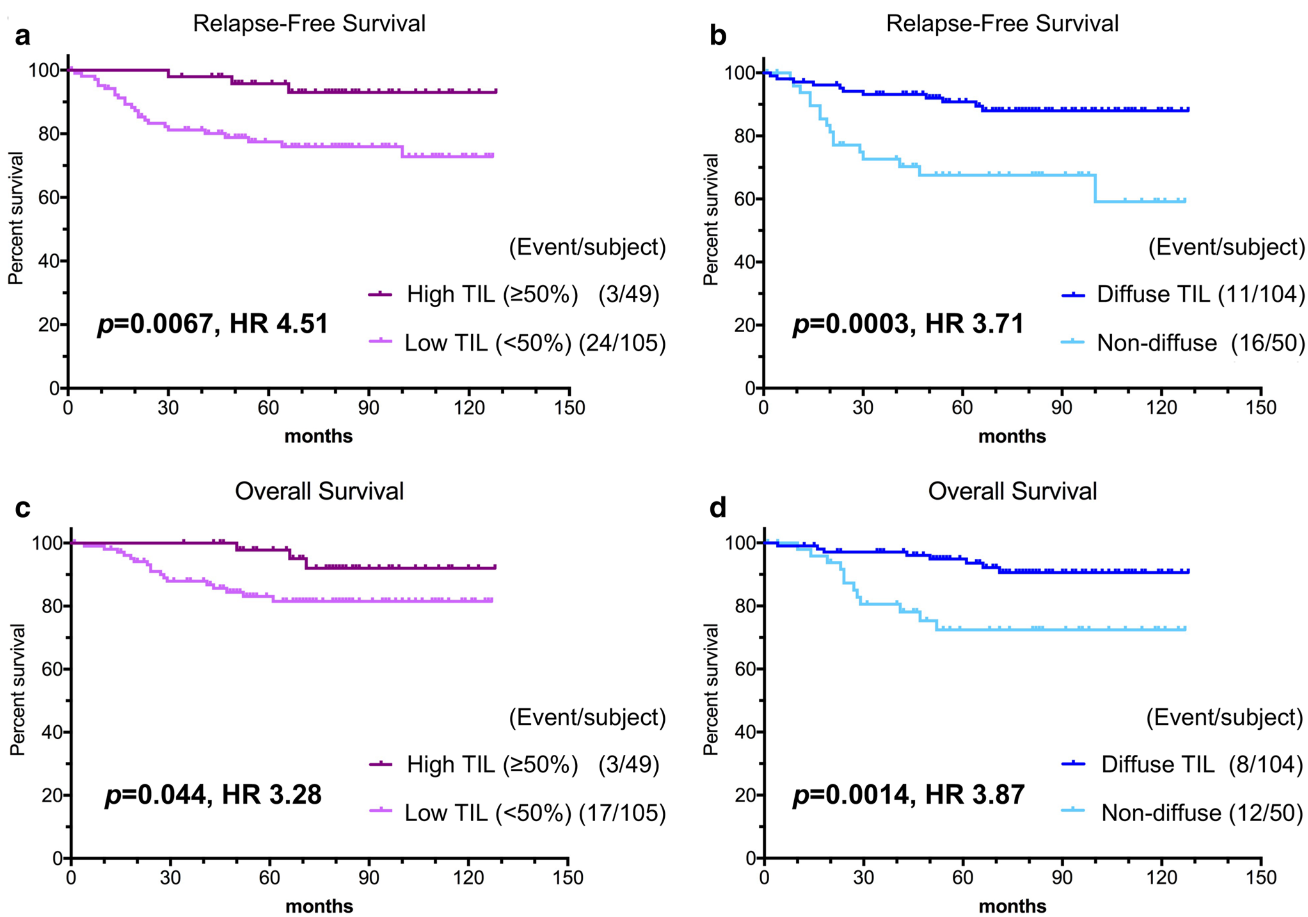

Fig. 2 Prognostic value of tumor-infiltrating lymphocytes (TILs). Relapse-free survival (**a**, **b**) and overall survival (**c**, **d**) were analyzed for 154 TNBCs based on the average density (**a**, **c**) and distribution pattern (**b**, **d**) of TILs. Stored samples were obtained from primary surgery for TIL assessments. All patients received standard adjuvant therapy after curative surgery. *HR* hazard ratio

Table 3 Association of tumor-infiltrating lymphocytes and other variates with survival

Variate	Univariate analysis		Multivariate analysis-1		Multivariate analysis-2	
	HR (95% CI)	*p* value	HR (95% CI)	*p* value	HR (95% CI)	*p* value
Relapse-free survival						
TIL						
Low vs. high (≥ 50%)	**4.54 (1.58–19.1)**	**0.0030**	**4.12 (1.43–17.4)**	**0.0062**	– *	– *
Non-diffuse vs. diffuse	**3.73 (1.74–8.29)**	**0.0007**	– *	– *	**3.91 (1.82–8.71)**	**0.0005**
Age						
≤ 50 years vs. > 50 years	1.22 (0.54–2.61)	0.627	–	–	–	–
Tumor size						
> 2 cm vs. ≤ 2 cm	1.44 (0.67–3.09)	0.344	–	–	–	–
Histological grade						
3 vs. 1/2	1.61 (0.69–4.38)	0.287	–	–	–	–
Lymphovascular invasion						
Present vs. absent	2.02 (0.94–4.36)	0.071	–	–	–	–
Nodal metastasis						
Positive vs. negative	**3.01 (1.40–6.84)**	**0.0048**	**2.74 (1.27–6.23)**	**0.0098**	**3.16 (1.47–7.18)**	**0.0032**
Overall survival						
TIL						
Low vs. high (≥ 50%)	**3.29 (1.10–14.1)**	**0.031**	2.94 (0.98–12.6)	0.055	– *	– *
Non-diffuse vs. diffuse	**3.90 (1.61–9.99)**	**0.0027**	– *	– *	**4.62 (1.87–12.0)**	**0.0010**
Age						
≤ 50 years vs. > 50 years	1.07 (0.40–2.62)	0.882	–	–	–	–
Tumor size						
> 2 cm vs. ≤ 2 cm	1.59 (0.65–3.88)	0.302	–	–	–	–
Histological grade						
3 vs. 1/2	1.77 (0.65–6.19)	0.281	–	–	–	–
Lymphovascular invasion						
Present vs. absent	**2.93 (1.21–7.48)**	**0.017**	1.31 (0.45–4.24)	0.632	1.70 (0.57–5.54)	0.349
Nodal metastasis						
Positive vs. negative	**4.27 (1.71–12.1)**	**0.0016**	**3.33 (1.02–11.4)**	**0.046**	**3.34 (1.03–11.4)**	**0.045**

TIL Tumor-infiltrating lymphocytes, *HR* hazard ratio, *CI* confidence interval. *Multivariate analyses were repeated twice to avoid the multicollinearity of TILs

Bold values indicate statistical significance

but also to OS. TIL density (low vs. high) did not reach a significant difference regarding OS ($P = 0.055$; HR 2.94; 95% CI 0.98–12.6) although the distribution pattern of TILs (non-diffuse vs. diffuse) remained significant ($P = 0.0010$; HR 4.62; 95% CI 1.87–12.0).

Association between pre-therapeutic TILs and pCR

Biopsy samples taken before PSC were available for 80 TNBCs. We assessed TILs using the aforementioned methods, then compared with pCR. The density method identified 23 high TILs (≥ 50%) cases and 57 low TILs (< 50%) cases. The high TILs group tended to achieve pCR more than the low TILs group, though the differences were not statistically significant ($P = 0.068$) (Fig. 3a). The diffuse method identified 46 cases as having a diffuse pattern and 34 cases with a non-diffuse pattern. The diffuse TIL group showed a significantly higher proportion of pCR than the non-diffuse group ($P = 0.032$) (Fig. 3b).

Combining two methods of TIL assessment in association with prognosis and pCR

Combining these two methods of TIL assessment yielded three subgroups: high and diffuse, low and diffuse, and low and non-diffuse. Among 154 cases in the adjuvant setting, there were 49 (32%), 55 (36%), and 50 (32%) cases, respectively. Among 80 cases in the PSC setting, there were 23 (29%), 23 (29%), and 34 (43%) cases, respectively. In other words, every single case with a high density of TILs (≥ 50%) was found to be correspondent with a diffuse pattern among 234 TNBCs.

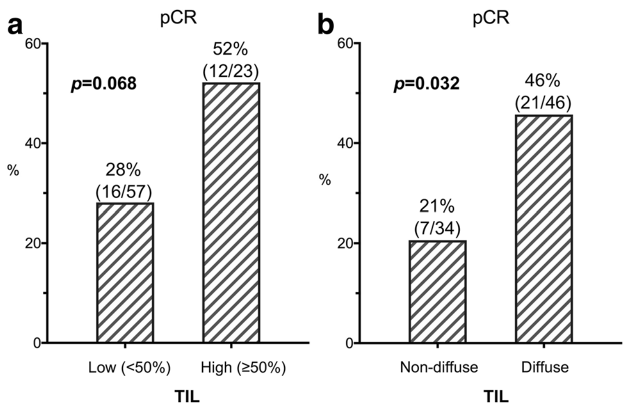

Fig. 3 Association between tumor-infiltrating lymphocytes (TIL) and pathological complete response (pCR). Needle biopsy samples taken prior to primary systemic chemotherapy were retrieved for TIL assessment. The proportion of cases achieving pCR was compared based on the average density (**a**) and distribution pattern (**b**) of TILs

These three subgroups of TILs also had a prognostic value in the adjuvant setting for RFS ($P=0.0008$) (Fig. 4a), as well as for OS ($P=0.0050$). The high and diffuse subgroup showed the best outcome, and diffuse pattern had a significantly favorable prognostic value among low TIL tumors not only for RFS ($P=0.0269$; HR 2.49; 95% CI 1.11–5.60) but also for OS ($P=0.0307$; HR 2.88; 95% CI 1.10–7.51). Additionally, the three subgroups of TIL revealed a stepwise increase in pCR proportion in the PSC setting ($P=0.0438$) (Fig. 4b). The high and diffuse group showed the highest frequency of achieving pCR (52%, 12/23), while the low and diffuse and low and non-diffuse groups had intermediate (39%, 9/23) and lowest pCRs (21%, 7/34), respectively. The diffuse method could divide low TIL tumors (pCR: 28%) into low and non-diffuse (pCR: 21%) and low and diffuse (pCR: 39%) groups, but the difference was not statistically significant ($P=0.145$).

Discussion

The prognostic and predictive impacts of TILs in TNBC have been consistently demonstrated by many researchers, most of whom assessed the density of TILs as stromal TILs [30] or lymphocyte-predominant breast cancer (LPBC) [31]. Our definition of high TILs (stromal TIL ≥ 50%) is identical to LPBC and was verified to be a favorable prognostic factor in the adjuvant setting of this retrospective study. Although it seems that we detected relatively more patients with high TILs (31.8%, 49/154), the survival curves are similar to that of previous studies [1] [14]. The proportion of LPBC in randomized trials of TNBC varied from 4.4% (21/481) [3] to 18.4% (119/647) [32], and a bigger retrospective cohort study from the European Institute of Oncology estimated 21.9% as LPBC (197/897) [4]. Real-world data can differ from randomized trials and interobserver or interinstitutional variability may exist.

The median value of stromal TILs in surgical specimens was 30% in our study, which is higher than that determined by the European Institute of Oncology (20%) [4]. However, in the PSC setting, our data for high TIL cases (28.8%, 23/80) are very close to that of a pooled analysis (30.1%, 273/906) by the German Breast Group [15]. They showed a similar proportion of pCR (high TIL: 50% [136/273]; low–intermediate TIL: 31% [197/633]) compared with our

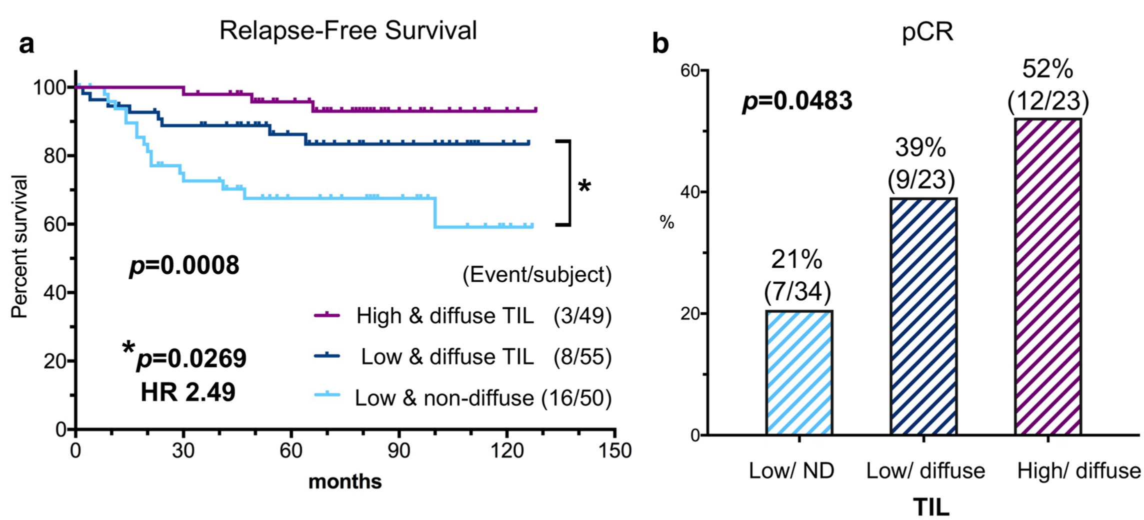

Fig. 4 Prognosis and chemotherapeutic effect in three subgroups of tumor-infiltrating lymphocytes (TILs). Relapse-free survival was analyzed for 154 TNBCs (**a**), and pathological complete response (pCR) was compared among 80 TNBCs (**b**). Every case harboring a high density of TILs had diffuse distribution of TILs, and this subgroup showed the best prognosis and pCR rate. The distribution pattern had a prognostic value among low TIL cases (*). *HR* Hazard ratio, *ND* non-diffuse

result (high TIL: 52% [12/23]; low TIL: 28% [16/57]). Small biopsy samples before systemic therapy might reduce the interobserver variability. As both our clinical outcomes are consistent with previous papers, it is regarded that our basic procedure for TIL assessment is reliable.

Our original approach to evaluate TIL distribution (diffuse method) proved to be an independent prognostic factor for both RFS and OS among TNBC. Compared with the 'density method' identifying one-third of high TIL patients with a good prognosis, our 'diffuse method' identifies one-third of non-diffuse pattern patients with poor prognosis. It is reasonable that patients with diffuse TIL patterns have a better prognosis since TILs are mainly composed of cytotoxic T-lymphocytes [33]. This result fits well with one study showing a critical role for spatial heterogeneity in ER-negative BC [22], and also an earlier study of colorectal cancer [34]. Nawaz et al. reported that the amount of co-localization of cancer and immune hotspots correlated with a better prognosis, which could further stratify high TIL BCs with disease-specific survival [22]. Similarly, our diffuse method had a prognostic impact among low TIL tumors in TNBC.

We also showed the association of diffuse TILs before primary systemic chemotherapy and higher probability of pCR with a significant difference. The density method identified one-third of high TIL patients with a greater pCR, and the diffuse method identified two-fifths of non-diffuse pattern tumors with a lower pCR. A detailed analysis of melanoma supported our concept by showing that acquired resistance to PD-1 blockade is associated with TIL restriction at the peripheral margin [35]. They revealed that intratumoral CD8+ cytotoxic T-cells had increased upon biopsy when the therapeutic effect was recognized. Although our biopsy samples were taken before chemotherapy, the presence of TILs within the tumor seems to have a predictive value to achieve pCR. A recent study showed a similar result; responders to neoadjuvant chemotherapy had significantly more TILs at the center of their primary breast cancer tumors [36]. Furthermore, the combined density and diffuse method of TILs yielded three subgroups with different probability of pCR, which is identical to the three subgroups with different prognosis in the adjuvant setting (Fig. 4). Although a large analysis from the German Breast Group showed no difference in pCR between low TILs (31% [80/260]) and intermediate TILs (31% [117/373]) [15], the addition of our diffuse method may enable stratification of these patients of no-LPBC.

We believe it is important to identify a group of patients who have lower expectations for survival or good response to standard chemotherapy because they need another strategy. If you can predict the prognosis after or the response to a kind of systemic therapy, you may be able to look for another drug or adjuvant chemotherapy ahead of an incurable relapse. A recent study (IMpassion130) revealed the efficacy of an immune-checkpoint inhibitor (atezolizumab) combined with chemotherapy (nab-paclitaxel) to treat TNBC, which is greater for PD-L1-positive subgroup [13]. Another possibility is adjuvant capecitabine for the residual HER2-negative cancer after preoperative chemotherapy (CREATE-X) [37]. Although the mechanism is not clear yet, metronomic oral administration of fluorouracil may have stimulated the immune microenvironment.

For the future development, a larger cohort with enough statistical power or combination of the immunohistochemical staining of PD-L1 would be required. The sample size is small in our study, and we have relatively short follow-up time for some patients. For example, 3 cases in the adjuvant setting had only 1, 4, and 6 months between the relapse and the latest follow-up. The present study has another limitation. As this is a retrospective cohort study, it could contain some unknown biases. Strict regulation of sample collection in prospective randomized trials could prevent this type of problem.

In terms of analytical validation, the robustness and reproducibility of our diffuse method was not satisfactory. Assuming that this simple method has a high concordance, we just used a printed document with a schema of Fig. 1 and the short instructions described in the methods. Interobserver concordance was moderate ($\kappa = 0.53$–0.55), and cases of total agreement totaled 76–78% (adjuvant setting: 101/129; PSC setting: 57/75). Necrosis, neutrophilic infiltration, faded staining on old slides, and fragmentation of biopsy samples were recognized as challenging conditions in our assessment. Although the κ value of the diffuse method (0.53–0.55) was lower than that of the density method (weighted $\kappa = 0.67$–0.69), it would not be fair to compare the two-grade scale and the detailed 10-grade scale. It should be also noticed that the latter value is weighted, and total agreement accounted for only 30–37% of cases (adjuvant setting: 39/129, PSC setting: 28/75). Interestingly, our previous investigation recruiting nine pathologists (eight general pathologists and one breast pathologist [A.H.]) showed an identical result for the diffuse method (mean of agreement and κ-value: 79% and 0.57, respectively; $n = 90$: ER-negative BC) using the same instructions [26]. In addition, the other authors validated the three-grade method proposed by an international working group and demonstrated a similar result (agreement: 87% [65/75]; $\kappa = 0.57$) [38]. Since we followed the supplemental instructions by Salgado et al. for the density method, a precise explanation of the diffuse method and/or short lectures with example cases would have improved the concordance. Nonetheless, these data imply that our diffuse method can be used equally by general pathologists as well as breast pathologists.

From a practical point of view, the simplicity of a pathological assessment is important for wide acceptance and adoption. The density method to assess stromal TILs in detail needed more time than the diffuse method; 1.7 times more for needle biopsies and 2.3 times more for surgical specimens. It took twice as long on average, and thus it cost twice as much. Our previous analysis showed that the diffuse method took 6 to 7 min per 10 resected samples on average [26], consistent with the present study. Nevertheless, interlaboratory or intraobserver concordances should be confirmed before utilization in clinical decision-making. Another possible advantage of the diffuse method is that we can generate a steady score representative of the whole tumor. There has been a lot of debate on the intratumoral heterogeneity of BC biomarkers [19–21]. However, it is easily imagined that the diffuse method of TIL assessment would not be influenced so much by intratumoral heterogeneity unless the whole biopsy cores came from the peripheral area [39]. Given the limitations on reproducibility by human pathologists, automated image analysis [23, 40, 41] or multi-gene assays [2, 9, 42] may represent possible future directions in the era of immunotherapy. As immune-checkpoint inhibitors have been developed in various kinds of solid cancers [43, 44], widespread use of TIL assessment in BC patients might be encouraged [17]. Diffuse patterns of TILs have potential as an additional prognostic marker, as well as an additional predictive marker to assume the risk and the benefit of treatment in patients with TNBC. This fast and practical method may contribute to understanding the nature of the tumor microenvironment and could be utilized in translational research or routine practice.

Conclusions

In summary, we propose an alternative method to evaluate TILs that is simple and fast. The diffuse method for TIL assessment is regarded to be practical since it is firmly associated with prognosis and chemotherapeutic effect among TNBC. As the diffuse pattern showed an additional impact on clinical outcome, more detailed discussion about tumor immunity would facilitate translational research. New insights into the distribution pattern of TILs may contribute to the understanding of the response to systemic chemotherapy or innovative immunotherapy to improve the outcome of TNBC.

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Authors' contributions AH, KA, and YO planned the study. AH and TW conducted pathological assessments. Yasuaki S., Yoshiaki S., and MK performed biopsies and/or surgeries, and provided oncological treatment. KA supervised the whole study. The manuscript was mainly written by AH, and amended by YO and AT. All authors read and approved the final manuscript.

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Data availability The datasets during and/or analyzed during the current study available from the corresponding author on reasonable request.

Compliance with ethical standards

Conflict of interest AH received personal fees as honoraria from Chugai Pharmaceutical, Taiho Pharmaceutical, and Novartis Pharma. YS received personal fees as honoraria from AstraZeneca, Chugai Pharmaceutical, Pfizer, Eisai, Novartis Pharma, Taiho Pharmaceutical, and Takeda Pharmaceutical. MK received personal fees as honoraria from Chugai Pharmaceutical, Eisai, AstraZeneca, Pfizer, Taiho Pharmaceutical, Novartis Pharma, Takeda Pharmaceutical, Daiichi Sankyo, Kyowa Hakko Kirin, Shionogi, and Asashi Kasei. KA received personal fees as honoraria from Chugai Pharmaceutical, Eisai, AstraZeneca, Taiho Pharmaceutical, Novartis Pharma, Daiichi Sankyo, Mochida Pharmaceutical, Ono Pharmaceutical, and Eli Lilly Japan, and his institution received research funds from Chugai Pharmaceutical, Eisai and Sanofi. The other authors have no competing interests to declare.

Ethical approval All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional research committee (No. 14-06 & 17-33) and with the 1964 Helsinki declaration and its later amendments.

Informed consent Formal consent was not required for this type of study.

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