Prognostic significance of tumour-infiltrating lymphocytes for oestrogen receptor-negative breast cancer without lymph node metastasis

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Abstract. Tumour-infiltrating lymphocytes (TILs) are regarded as significant prognostic markers in patients with breast cancer. However, the prognostic utility of TIL expression based on the intrinsic subtypes has just been identified. The present study investigated the relationship between TIL grades and prognosis in 294 Japanese paitents with breast cancer stratified based on the intrinsic subtypes and clinico-pathological characteristics. Stromal TIL status was evaluated using haematoxylin and eosin staining, and TIL grades were categorised into low (<10%), intermediate (≥10 and ≤40%) and high (>40%) groups. The relationship between TIL expression and the intrinsic subtypes, clinicopathological characteristics and patient prognosis was analyzed. It was revealed that high TIL expression was correlated with negative oestrogen receptor (ER) expression and high histological grade (P<0.001). Among the ER-negative patients, the relapse-free survival (RFS) rate of the high-grade TIL group was significantly higher than that of the low-grade TIL group (P=0.04). Among the ER-negative patients without lymph node metastasis, RFS and cancer-specific survival (CSS) rates of patients with high-grade TILs were significantly higher than the RFS and CSS rates of patients with low-grade TILs (P=0.01). However, among ER-positive patients, RFS was significantly higher in the low-grade TIL group than in the high-grade TIL group (P=0.02). In conclusion, TIL expression correlated with ER status and tumour proliferation. High TIL expression was a poor prognostic marker in ER-positive patients but was a good prognostic marker in ER-negative patients. Therefore, the biological association between TILs and primary breast tumours may differ between ER-positive and ER-negative breast cancer.

Introduction

In immune cell infiltration in breast cancer tissue, the expression of tumour-related immune cells differed greatly among different breast cancer subtypes and patients (1). Lymphocyte-predominant breast cancer (LPBC) is defined as a presentation wherein at least 50% of the tumour tissue is invaded by tumour-infiltrating lymphocytes (TILs). LPBC was observed in 20% of triple-negative breast cancers, 16% of HER2-positive tumours and 6% of the hormonal receptor (HR)-positive tumours (1). Since the 1970s, studies have attempted to understand the basic function of TILs in cancer as immune response in cancer tissue has been driven by the specific functions of TILs (2). Moreover, the variety of immune cells is believed to contribute to drug sensitivity and prognosis in breast cancer patients. In 2006, Galon *et al* were the first to report that *in situ* TIL expression could serve as a strong prognostic marker for colorectal and breast cancer (3). Thereafter, many retrospective studies reported that TIL expression in breast cancer could predict the efficacy of drug therapy and prognosis (4-9). Although the methods to quantify TIL expression and cut-off TIL values in breast cancer tissues varied among studies and have not been clearly standardised, the International Working Group of TILs published the first guidelines for a TIL evaluation in 2014 (10). Accordingly, mononuclear immune cells located between tumour nests, i.e., within the tumour stroma, are defined as stromal TILs (str-TILs). The International TILs Working Group recommended that str-TIL expression should be graded as low (str-TILs: <10%), intermediate (str-TILs: ≥10 and ≤40%) and high (str-TILs: >40%) based on their relative abundance within the tumour stroma. However, there is no sufficient evidence to

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support the efficacy of this classification. In the present study, we evaluated the relationship of TIL grades with clinicopathological characteristics of breast cancer patients and prognosis based on the guidelines of the International TILs Working Group.

Patients and methods

Patient background. A total of 294 consecutive female patients, diagnosed as invasive breast cancer who underwent breast-conserving surgery or modified radical mastectomy without neoadjuvant treatment at Saitama Cancer Centre between January 2000 and December 2001, were enrolled in this retrospective study. After the evaluation of intrinsic subtypes, patients with bilateral breast cancer were excluded from the study. Clinicopathological data on pathological tumour size, the status of pathological lymph node metastasis and clinical course were extracted from the patient medical records. This study was conducted in accordance with the Declaration of Helsinki and after the approval by the Institutional Review Board of the Saitama Cancer Centre (nos. 231, 483 and 534). All patients enrolled provided written comprehensive informed consent.

Evaluation of str-TILs. Surgical specimens were fixed in buffered formalin solution, cut to 4-μm-thick slices and stained with haematoxylin and eosin. Using an optical microscope with x200 and x400 magnification, a surgical pathologist specialising in breast pathology (MK) quantified str-TILs. Str-TIL expression was classified into the following three grades per the International TILs Working Group (10) criteria: Low (str-TILs: <10%), intermediate (str-TILs: ≥10 and ≤40%) and high (str-TILs: >40%; Fig. 1A-C).

Procedures and evaluation of the expression of oestrogen receptor (ER), progesterone receptor and HER2. The following antibodies were used for immunostaining: 1D5 (Dako, Glostrup, Denmark) for ER, PgR636 (Dako) for progesterone receptor (PgR) and Hercep Test (Dako) for HER2. For evaluation of HER2 gene amplification, dual *in situ* hybridisation (DISH) was performed with INFORM HER2 Dual ISH DNA Probe Cocktail assay (Ventana Medical Systems, Inc., Tuscon, AZ, USA). ER, PgR and HER2 expression were evaluated in accordance with the American Society of Clinical Oncology and College of American Pathologists (11,12) criteria. In addition, the degree of ER and PgR staining ≥1% and the specimen was determined as positive. The proportional scores of cells membrane HER2 staining intensity were as follows: scores 0, 1+, 2+ and 3+. HER2 immunostaining with a score of 2+ was subjected to a DISH assay to assess the gene amplification of HER2. A HER2 score of 2+/DISH positive or 3+ was defined as HER2-positive cancer. Patients with ER-positive and/or PgR-positive breast cancer were defined as a hormonal receptor (HR)-positive breast cancer.

Statistical analysis. The relationship between TIL grades and various clinicopathological factors, including ER, PgR and HER2 expression was analysed by Chi-square and Fisher's exact tests. The log-rank test and Kaplan-Meier method were used to estimate relapse-free survival (RFS) and cancer-specific
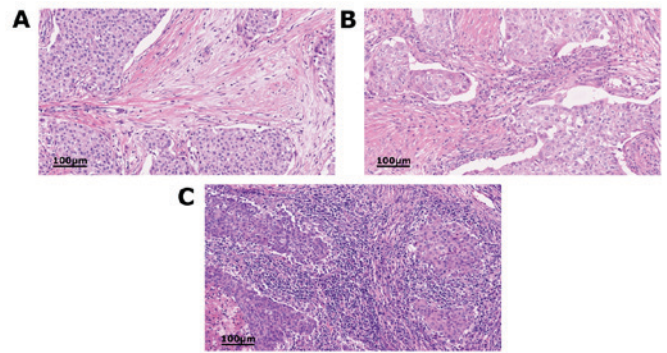

Figure 1. Classification of tumour-infiltrating lymphocytes (TILs) using the criteria of the International TILs Working Group. (A) Low-grade TILs: A few lymphocytes are present in tissue surrounding the cancer nests. (B) Intermediate-grade TILs. (C) High-grade TILs: Numerous lymphocytes are distributed adjacent to the cancer nests.

survival (CSS). RFS was defined as the length of time from the day of surgery to any tumour recurrence (including locoregional recurrence). CSS was defined as the period from the day of surgery until the time of death caused by the progression of breast cancer. In the multivariate analysis, 95% confidence intervals for the relationship between TIL grades and clinicopathological factors were obtained using a logistic regression test. The relationship between TIL grades and prognosis were obtained using a Cox proportional hazards regression model. Statistical analyses were performed using the SPSS v22.0 software (IBM Corp., Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Patient and tumour characteristics. The median age of the 294 patients enrolled in the study was 55 years (age range, 25-87 years). Of these patients, 171 (58.2%) were in postmenopausal state, 134 (45.6%) had positive pathological nodes (pN), 162 (55.1%) had a histological grade 3 tumour, 213 (72.4%) had HR-positive breast cancer, 176 (59.8%) underwent adjuvant endocrine therapy, 158 (53.7%) underwent adjuvant chemotherapy and 47 (16.0%) were diagnosed with HER2-positive breast cancer (Table I). There were no patients with HER2-positive who received adjuvant trastuzumab therapy since adjuvant trastuzumab administration had not been approved in Japan before 2008.

Relationship between TIL expression and clinicopathological characteristics. Of the patients assessed, 32 (10.9%), 44 (15.0%) and 218 (74.1%) patients have high, intermediate and low TIL expression, respectively. In both the HER2-negative and HER2-positive patients, only 3.3% had high TIL expression. In the HR-negative group, 30.9% had high TIL expression (Table II). The rate of high TIL expression was significantly higher in the HR-negative group than in the HR-positive group (P<0.001). A univariate analysis identified that the ER (P<0.001), PgR (P<0.001) and HER2 (P=0.004) status and histological grade (P<0.001) were significant (Table III). High TIL expression was significantly associated with ER-negative, PgR-negative, HER2-positive and histological grade 3 tumours. On the other hand, multivariate analysis confirmed

Table I. Patient characteristics.

Characteristics	Total
Age, years	
<40	26
≥40 and <60	158
≥60	110
Menopause status	
Pre-	123
Post-	171
Type of breast surgery	
Breast-conserving surgery	227
Mastectomy	67
Axillary surgery	
Sentinel lymph node (biopsy alone)	149
Axillary lymph node (dissection)	142
No surgery	4
Pathological tumour size	
pT1	152
pT2	116
pT3	17
pT4	9
Pathological nodal status	
pN0	156
pN1	78
pN2	34
pN3	22
No surgery	4
Pathological TNM stage	
I	98
IIA	96
IIB	35
IIIA	30
IIIB	9
IIIC	22
Not evaluated	4
Histological grade	
Grade 1	45
Grade 2	87
Grade 3	162
Oestrogen receptor	
Positive	207
Negative	87
Progesterone receptor	
Positive	179
Negative	115
HER2 status	
Positive	47
Negative	247

TNM, tumor-node-metastasis.

that TIL expression was significantly higher in patients with ER-negative ($P<0.001$) and histological grade 3 ($P=0.03$) tumours (Table III).

Prognostic significance of TIL expression. The analysis identified that the median of RFS was 127 (range, 1-147 months), and median of CSS was 130 (range, 4-149 months). TIL expression was not a significant prognostic factor when the analysis included the entire study cohort of breast cancer patients. Multivariate analysis confirmed that high TIL expression was not an independent prognostic marker (Table IV). TIL expression was not a significant prognostic marker in the HER2-positive breast cancer patient (RFS, $P=0.67$; CSS, $P=0.87$; Fig. 2A and B). Additionally, in the HR-positive and HER2-negative breast cancer patients, no significant difference in survival was recognised between the high- and low-grade TIL patients (RFS, $P=0.50$; CSS, $P=0.94$; Fig. 2C and D). Among the triple-negative breast cancer patients, the RFS and CSS were higher in the high-grade TIL group compared with the low-TIL group, although the difference was not significant (RFS, $P=0.16$; CSS, $P=0.13$; Fig. 2E and F). By contrast, among the ER-negative breast cancer patients, the RFS was significantly higher in the high-grade TIL group than in the low-grade TIL group (RFS, $P=0.04$; CSS, $P=0.07$; Fig. 3A and B). Conversely, among the ER-positive breast cancer patients, the RFS was significantly higher in the low-grade TIL group than in the high-grade TIL group with nonsignificant difference in the CSS (RFS, $P=0.02$; CSS, $P=0.18$; Fig. 3C and D).

Among the pN-negative patients, none of the cases with a high TIL score had recurrence. Moreover, the RFS and the CCS tended to be higher in the high-grade TIL group than in the low-grade TIL group (RFS, $P=0.07$; CSS, $P=0.10$; Fig. 4A and B). However, TIL expression was not a prognostic indicator in the pN-positive patients (RFS, $P=0.17$; CSS, $P=0.14$; Fig. 4C and D). Among the ER-negative and pN-negative patients, the RFS and CCS were significantly higher in the high-grade TIL group than in the low-grade TIL group (RFS, $P=0.01$; CSS, $P=0.01$; Fig. 5A and B). Conversely, TIL expression was not recognised as a prognostic factor in ER-negative and pN-positive patients (RFS, $P=0.49$; CSS, $P=0.74$; Fig. 5C and D).

Discussion

It has been revealed that patients with invasive breast cancer and high TIL expression, those with ER-negative and high histological grade tumours were significantly more common. Moreover, TIL expression level was a strong prognostic marker for ER-negative breast cancer. Among different breast cancer subtypes, ER-negative breast cancer and a subtype with high-grade malignancy exhibit extremely high growth potential. In the Breast International Group (BIG) 2-98 trial, Loi et al reported that TIL expression was a prognostic marker for ER-negative patients, especially those with triple-negative breast cancer (13). Adams et al (14) demonstrated that TIL expression was a strong prognostic marker for triple-negative breast cancer in the Eastern Cooperative Oncology Group (ECOG) 2197 and ECOG 1199 trials. Loi et al (15) conducted an meta-analysis of the efficacy of TIL expression as a prognostic marker in triple-negative breast cancer patients

Table II. Patient distribution by TIL grades in each breast cancer subtype.

	Patients, no. (%)			
Subtype	High TIL group	Intermediate TIL group	Low TIL group	Total
HR+/HER2-	6 (3.1)	24 (12.2)	166 (84.7)	196
HR+/HER2+	1 (5.9)	5 (29.4)	11 (64.7)	17
HR-/HER2+	9 (30.0)	6 (20.0)	15 (50.0)	30
HR-/HER2-	16 (31.4)	9 (17.6)	26 (50.9)	51
Total	32 (10.9)	44 (15.0)	218 (74.1)	294

TIL, tumour-infiltrating lymphocyte; HR, hormonal receptor; HER2, human epidermal growth factor receptor type 2.

Table III. The association between TIL grades and clinicopathological characteristics.

	Patients, no.			Univariate analysis	Multivariate analysis
Clinicopathological factor	Low TIL group	Intermediate TIL group	High TIL group	P-value	P-value
ER status				<0.001	<0.001
Positive	175	27	5		
Negative	43	17	27		
PgR status				<0.001	0.31
Positive	148	26	5		
Negative	70	18	27		
HER2 status				0.004	0.61
Positive	26	11	10		
Negative	192	33	22		
Tumour size				0.16	
T1-2	191	38	24		
T3-4	27	6	8		
Nodal status				0.14	
Negative	117	19	12		
Positive	101	25	20		
Menopausal status				0.31	
Pre-	87	23	13		
Post-	131	21	19		
Histological grade				<0.001	0.03
1-2	116	11	5		
3	102	33	27		

TIL, tumour-infiltrating lymphocyte; ER, oestrogen receptor; PgR, progesterone receptor; HER2, human epidermal growth factor receptor type 2.

enrolled in large-scale trials, such as these trials and the Finland Herceptin Trial (FinHER) (16) and reported that TIL expression was a prognostic factor in triple-negative breast cancer. Therefore, most of the studies that showed the efficacy of TIL expression, as a predictor of prognosis and drug efficacy, were retrospective. We believe that a prospective clinical trial is necessary to clarify the real utility of TIL expression as a prognostic marker.

We found that survival was significantly higher in the low-grade TIL group than in the high-grade TIL group among ER-positive breast cancer patients. Recent studies on large cohorts reported that high TIL expression was a poor prognostic factor in ER-positive breast cancer (17). Consistent with these studies, morphological TIL heterogeneity is frequent in ER-positive patients compared with ER-negative patients (18). It is possible that this intra-tumour heterogeneity

Table IV. Results of univariate and multivariate survival analyses on the influence of clinicopathological variables, including the expression of TILs.

A, RFS

Clinicopathological factor	Univariate analysis		Multivariate analysis		
	Hazard ratio	P-value	Hazard ratio	95% CI	P-value
TIL expression					
Low	Reference		Reference		
Intermediate	0.050	0.82	0.88	0.49-1.61	0.69
High	0.017	0.90	0.61	0.28-1.30	0.20
ER status					
Positive	Reference		Reference		
Negative	4.68	0.03	1.05	0.55-2.02	0.88
PgR status					
Positive	Reference		Reference		
Negative	8.01	0.005	1.68	0.92-3.07	0.09
HER2 status					
Negative	Reference		Reference		
Positive	9.49	0.002	1.52	0.88-2.61	0.13
Tumour size					
T 1-2	Reference		Reference		
T 3-4	15.13	<0.001	1.79	0.99-3.23	0.05
Nodal status					
Negative	Reference		Reference		
Positive	29.80	<0.001	3.04	1.88-4.92	<0.001
Histological grade					
Grade 1-2	Reference		Reference		
Grade 3	5.80	0.02	1.19	0.71-2.00	0.51

B, CSS

Clinicopathological factor	Univariate analysis		Multivariate analysis		
	Hazard ratio	P-value	Hazard ratio	95% CI	P-value
TIL expression					
High	Reference		Reference		
Intermediate	0.60	0.44	0.56	0.26-1.21	0.14
Low	0.11	0.74	0.54	0.24-1.22	0.14
ER status					
Positive	Reference		Reference		
Negative	9.93	0.002	1.51	0.72-3.16	0.28
PgR status					
Positive	Reference		Reference		
Negative	11.28	<0.001	1.60	0.78-3.29	0.20
HER2 status					
Negative	Reference		Reference		
Positive	4.84	0.03	1.09	0.59-2.02	0.79
Tumour size					
T1-2	Reference		Reference		
T3-4	11.51	0.001	1.95	1.00-3.81	0.05

Table IV. Continued.

Clinicopathological factor	Univariate analysis (CSS)		Multivariate analysis (CSS)		
	Hazard ratio	P-value	Hazard ratio	95% CI	P-value
Nodal status					
Negative	Reference		Reference		
Positive	17.93	<0.001	2.60	1.49-4.52	<0.001
Histological grade					
Grade 1-2	Reference		Reference		
Grade 3	9.06	0.003	1.56	0.85-2.87	0.15

TILs, tumour-infiltrating lymphocytes; ER, oestrogen receptor; PgR, progesterone receptor; HER2, human epidermal growth factor receptor type 2; RFS, relapse-free survival; CSS, cancer-specific survival; CI, confidence interval.
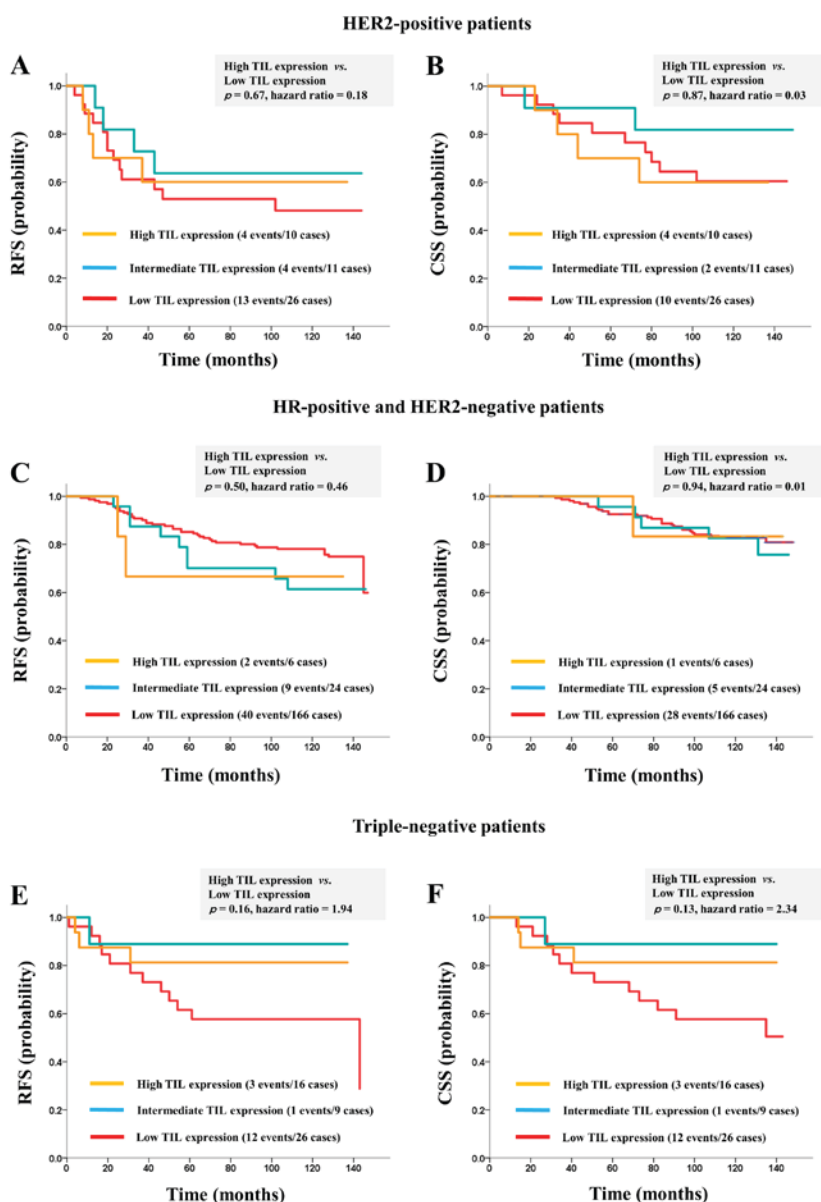
Figure 2. Survival curves of breast cancer subtypes stratified by TIL expression. (A) RFS and (B) CSS curves stratified by TIL expression in HER2-positive patients. (C) RFS and (D) CSS curves stratified by TIL expression in HR-positive and HER2-negative patients. (E) RFS and (F) CSS curves stratified by TIL expression in triple-negative patients. TIL, tumour-infiltrating lymphocyte; RFS, relapse-free survival; CSS, cancer-specific survival; HER2, human epidermal growth factor receptor type 2; HR, hormonal receptor.
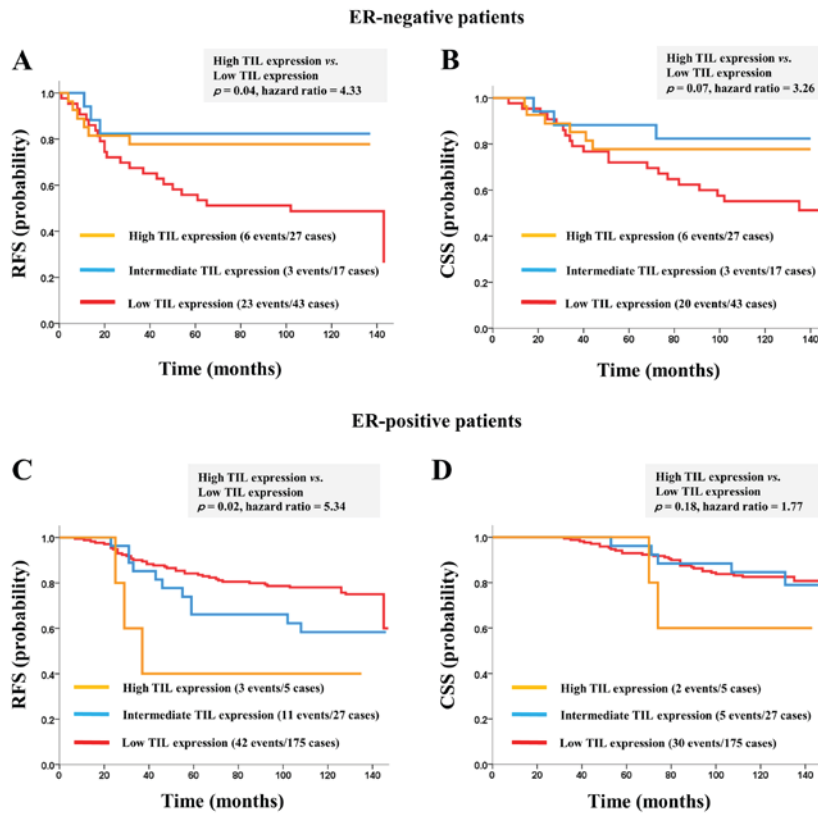
Figure 3. Survival curves stratified by TIL expression and ER status. Comparisons of (A) RFS and (B) CSS between high-grade and low-grade TILs groups in ER-negative patients. The relationship between TIL expression and (C) RFS and (D) CSS in ER-positive breast cancer patients. TIL, tumour-infiltrating lymphocyte; ER, oestrogen receptor; RFS, relapse-free survival; CSS, cancer-specific survival.
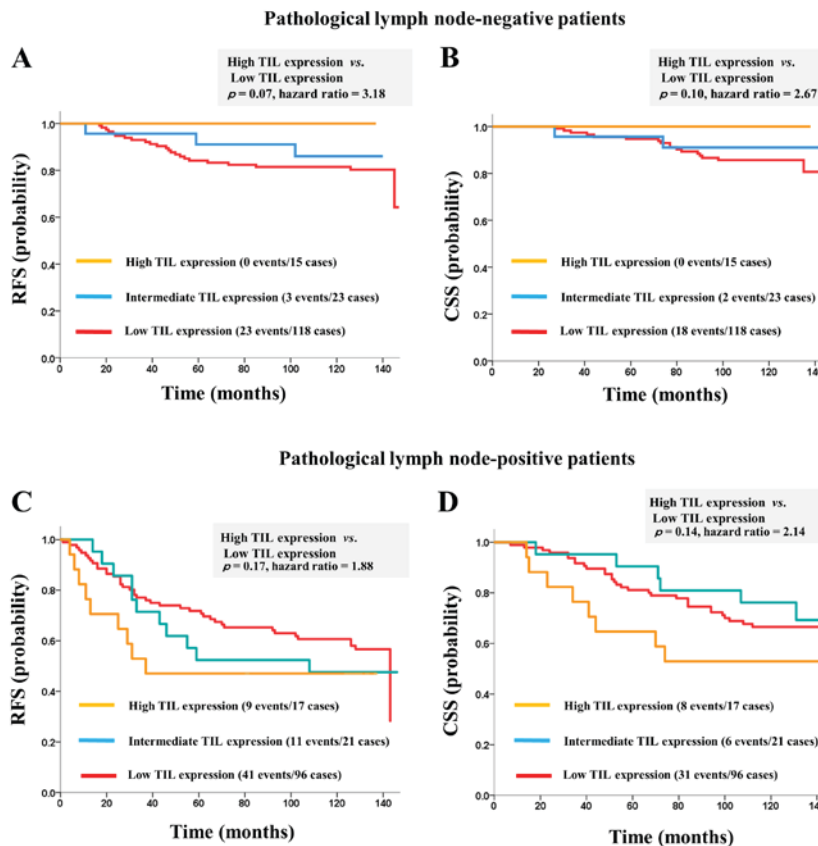

Figure 4. Survival curves stratified by TIL expression and pathological nodal status. Comparisons of (A) RFS and (B) CSS between high-grade and low-grade TIL groups in pathological node-negative patients. The relationship between TIL expression and (C) RFS and (D) CSS in pathological node-positive patients. TIL, tumour-infiltrating lymphocyte; RFS, relapse-free survival; CSS, cancer-specific survival.
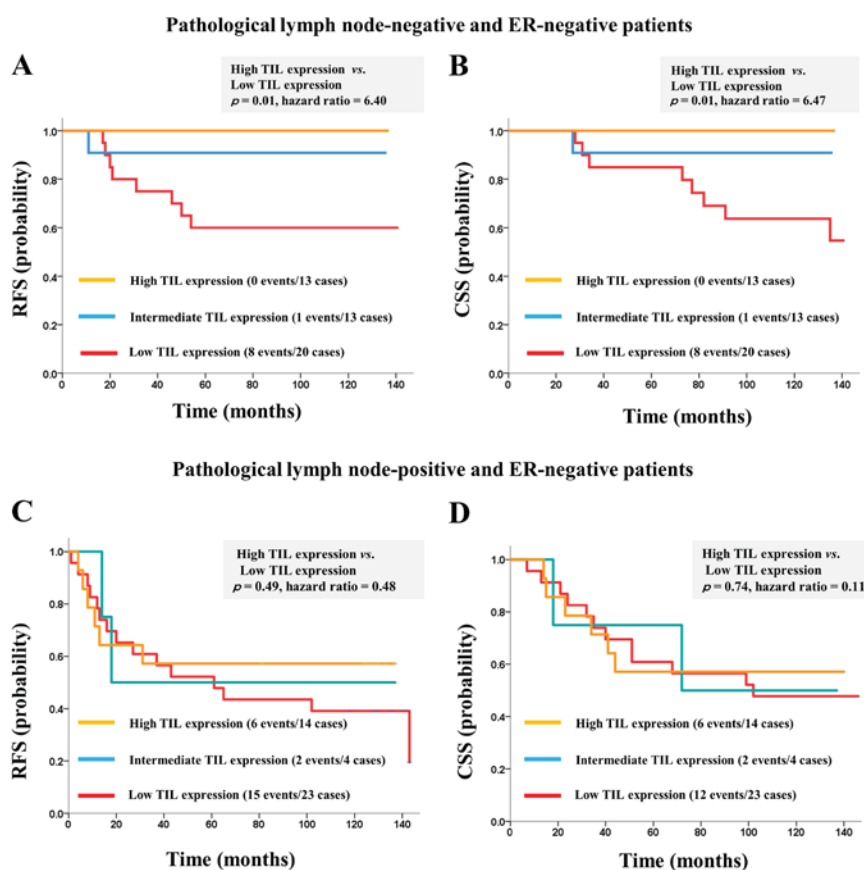
Figure 5. Survival curves stratified by TIL expression and pathological nodal status in ER-negative patients. Comparisons of (A) RFS and (B) CSS between high-grade and low-grade TIL groups in ER-negative and pathological node-negative patients. The relationship between TIL expression and (C) RFS and (D) CSS in ER-negative and pathological node-positive patients. TIL, tumour-infiltrating lymphocyte; ER, oestrogen receptor; RFS, relapse-free survival; CSS, cancer-specific survival.

may affect the expression patterns of immune cells, thereby underlying the difference in the prognostic significance of TIL expression between ER-positive and ER-negative breast cancer patient. To ascertain different roles associated with various TIL types, immunohistochemical analysis of TILs is necessary to determine the distribution of immune cells (19). In addition, the tumour mutational burden is associated with tumour immunity (20) and a high level of the mutational load is associated with poor outcomes in patients with ER-positive tumours (21,22). ER is considered to play an important role in tumour immunosuppression (23) as treatment options for ER-positive and ER-negative breast cancer are distinct. Several clinical studies demonstrated an association between TIL expression and response to chemotherapy (19). Endocrine therapy may modulate immune microenvironment of primary tumours (24); however, relationship of TILs with the response to endocrine therapy in ER-positive breast cancer remains controversial. A grade of ER expression and therapeutic options may affect the extent and the pattern of infiltrating immune cells in the tumour tissue as TIL expression may contribute to different prognostic rates between ER-positive and ER-negative breast cancers. However, there is no clear evidence to support this biological mechanism and the prognostic power of TILs in ER-positive patients with the luminal A and B subtypes. Therefore, further investigation is needed to determine the prognostic utility of TIL expression in ER-positive breast cancer.

It is noted that TIL expression was not a prognostic marker in pN-positive patients. However, among pN-negative patients, the prognosis was better in the high-grade TIL group than in the low-grade TIL group. Lymph node metastasis is an important factor in determining the prognosis of breast cancer patients and the mechanism underlying lymph node metastasis in breast cancer involves various factors. Nonetheless, we propose that a reduced ability of immune cells within the tumour microenvironment, which inhibits cancer cell metastasis is responsible for lymph node metastasis. Programmed cell death protein-1 (PD-1) contributes to the coexistence of cancer cells and immune cells. Moreover, programmed cell death-ligand 1 (PD-L1) inhibits killer T cell activity (25). PD-1 and PD-L1 inhibitors suppress these immune checkpoints, thereby promoting tumour immune response by killer T cells and exhibiting an anti-tumour effect (26-31). Recent studies were conducted to determine the mechanism by which these molecules interact with cancer invasion and metastasis (32). The expression levels of TILs and immune checkpoint markers in ductal carcinoma *in situ* provide important information on tumour microenvironment before breast cancer invasion and metastasis. Hendry *et al* (33) suggested that ductal carcinoma *in situ* progression into invasive breast cancer has a reduced ability of immune cells in the tumour microenvironment, which inhibits cancer cell invasion caused by PD-1 and PD-L1 activation. Furthermore, Tarhini *et al* (34) reported

that PD-L1 expression in melanoma tumour cells contributed to the degree of micrometastasis and sentinel lymph nodes. However, there is still no clear evidence to support whether immune checkpoint factors, such as PD-1 and PD-L1, are involved in axillary lymph node metastasis of breast cancer. Thus, further investigation is required to determine the relationship between lymph node metastasis and tumour immunity in breast cancer.

In conclusions, high TIL expression correlated with negative ER and high histological grade. Among ER-negative patients, survival rate was higher in the high-grade TIL group than the low-grade TIL group. Moreover, TIL expression might be a potent prognostic marker in ER-negative and pN-negative patients. Conversely, among the ER-positive breast cancer patients, survival rate was significantly better in the low-grade TIL group than in the high-grade TIL group. Thus, the biological association of TILs with primary breast tumour might differ between ER-positive and ER-negative breast cancer. The current study revealed that the RFS and the CSS of triple-negative patients tended to be higher in the high-grade TIL group than in the low-grade TIL group without statistical significance. This cause might be due to several factors, which include differences in patient background (race, residence, and familial history) and differences in TIL evaluation method. The number of triple-negative patients was not large in this study. As such, a further study to validate the prognostic utility of TILs based on breast cancer subtypes in large Japanese cohorts is required.

Acknowledgements

Funding

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

All authors participated in the study design. SK mainly performed image acquisition and statistical analyses. MK assisted SK in histological examinations and evaluating results. KI, HM and MK assisted in the production of the study design and evaluating the results obtained. TF, JH, KS, TO and HK contributed toward the statistical evaluation of results and theoretical organization of the manuscript.

Ethics approval and consent to participate

The present study was performed following approval from the Institutional Review Board in Saitama Cancer Centre (nos. 231, 483 and 534). Furthermore, the study was performed in accordance with the Declaration of Helsinki, and written informed consent was obtained from all patients prior to inclusion in the study.

Patient consent for publication

Written informed consent was obtained from all patients prior to inclusion in the study.

Competing interests

JH received research funding from CHUGAI Pharmaceutical Co., Ltd. and Taiho Pharmaceutical Co., Ltd. JH received an honoraria from Astra Zeneca K.K. in Japan. All remaining authors have declared that they have no competing interests.

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