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ORIGINAL ARTICLE

# Prognostic significance of tumor-infiltrating lymphocytes may differ depending on Ki67 expression levels in estrogen receptor-positive/HER2-negative operated breast cancers

Yukie Fujimoto[1] · Takahiro Watanabe[2] · Akira I. Hida[3] · Tomoko Higuchi[1] · Yoshimasa Miyagawa[1] · Hiromi Ozawa[1] · Ayako Bun[1] · Reiko Fukui[1] · Atsushi Sata[1] · Michiko Imamura[1] · Seiichi Hirota[2] · Yasuo Miyoshi[1]

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## Abstract

**Background** The prognostic significance of tumor-infiltrating lymphocytes (TILs) has been established in breast cancers with estrogen receptor (ER)-negative and human epithelial growth factor receptor 2 (HER2)-negative or HER2-positive subtypes; however, its utility concerning the ER + /HER2 − subtype remains unclear.

**Methods** We evaluated the prognostic value of TILs by analyzing 717 invasive breast cancer operation cases. TILs were classified into three groups based on the proportion of area within the tumor: low (< 10%), intermediate (10–50%), and high (> 50%). Disease-free survival (DFS) and overall survival (OS) were calculated according to TIL levels.

**Results** Although there was no significant association between TIL levels and DFS or OS in all patients, high TILs were significantly associated with favorable DFS in Ki67-high ($n = 238$, $p = 0.035$) but not in Ki67-low ($n = 470$, $p = 0.46$) breast cancers. Multivariable analysis showed that high TILs were a significant and independent factor for DFS (HR 0.34; 95% CI 0.10–0.87; $p = 0.023$) among the Ki67-high group. In the ER + /HER2 − subtype, high-TILs showed favorable DFS in the Ki67-high group, although this was not statistically significant ($p = 0.48$); in contrast, unfavorable DFS was observed in the Ki67-low group ($p = 0.027$).

**Conclusions** In Ki67-high breast cancers, high TILs were associated with favorable DFS, irrespective of subtype, but increasing TIL levels correlated with worse DFS in the Ki67-low group with the ER + /HER2 − subtype. These results highlight variation in TIL prognostic significance between Ki67-high and -low breast cancers, particularly for the ER + /HER2 − subtype.

**Keywords** Breast cancer · Tumor-infiltrating lymphocytes · Ki67 · Prognosis

## Introduction

Cancer immunology as a modern discipline is focused on understanding the biology of cancer progression and developing treatment strategies for diseases such as breast cancer [1]. Tumor-infiltrating lymphocytes (TILs) are a widely accepted indicator for local adaptive immune activation in clinical samples [1]. Assessment of TILs is useful for predicting sensitivity to chemotherapies and patient prognosis and TILs can be a potential biomarker for immune checkpoint inhibitors.

It is recognized that a high-TIL level is significantly and independently associated with pathological complete response (pCR) in breast cancers treated with neoadjuvant chemotherapy (NAC) [2]. The positive association between TILs and pCR seems to be recognized in triple negative (TN; estrogen receptor (ER)-negative/progesterone receptor-negative/human epidermal growth factor receptor 2 (HER2)-negative) and HER2-positive breast cancers [3, 4]. A meta-analysis of 13,100 patients across 23 studies showed that a high-TIL level is associated with a significantly improved pCR rate compared with a low-TIL level (odds ratio (OR), 2.81; 95% CI 2.02–3.91; $p < 0.001$) [5]. According to the

✉ Yasuo Miyoshi
ymiyoshi@hyo-med.ac.jp

[1] Department of Surgery, Division of Breast and Endocrine Surgery, Hyogo College of Medicine, Mukogawa-cho 1-1, Nishinomiya City, Hyogo 663-8501, Japan

[2] Department of Surgical Pathology, Hyogo College of Medicine, Mukogawa-cho 1-1, Nishinomiya City, Hyogo 663-8501, Japan

[3] Department of Pathology, Matsuyama Shimin Hospital, 2-6-5 Otemachi, Matsuyama, Ehime 790-0067, Japan

subset analysis of this meta-analysis, positive associations between TILs and pCR were consistently significant in TN (OR 4.67; 95% CI 2.14–10.19, $p < 0.001$) and HER2-positive breast cancers (OR 4.08; 95% CI 1.45–11.49, $p = 0.004$). Similarly, marginal significance between higher levels of TILs and increased pCR was reported for the ER-positive breast cancers (OR 6.21; 95% CI 0.86–45.15; $p = 0.071$) [6]. These data may indicate that TILs are essential to achieving pCR in chemotherapy treatment irrespective of subtype.

Significant positive association between increased TILs and favorable disease-free survival (DFS) was also reported in the meta-analysis [relative risk (RR), 0.61; 95% CI 0.51–0.73] [5]. Although consistently improved prognosis was reported in high-TILs patients with TN breast cancers, this association was not significant in HER2 + and ER + / HER2 − breast cancers [7, 8]. In another study consisting of 3771 breast cancer patients treated with NAC, high TILs were significantly associated with favorable DFS for TN and HER2 + breast cancers but not for the hormone receptor-positive (luminal)/HER2-negative breast cancers [9]. Similar to other reports, a significant association between increasing TILs and increased pCR was consistently observed across all breast cancer subtypes including luminal/HER2-breast cancers. Thus, the prognostic significance of TILs may not be consistent across breast cancer subtypes.

On the basis of these reports, the clinical utility of TILs for predicting response to chemotherapy and prognosis appears to depend on breast cancer subtype. ER + / HER2 − breast cancers with high levels of TILs are notably unlikely to result in favorable prognosis, despite the higher chance of achieving pCR. These inconsistencies may indicate differences in immunological microenvironment for each breast cancer subtype. However, a definitive explanation for this mechanism is currently unknown, and the prognostic significance of TILs in the clinical setting in the ER + /HER2 − subtype remains unclear. To find mechanisms that improve our understanding of the immunological microenvironment and to help develop treatment strategies, we designed this study to identify a subgroup among operated breast cancers for which TILs have a prognostic impact, focusing specifically on the ER + /HER2 − subtype.

## Patients and methods

### Patient eligibility

We used retrospective data from consecutively recruited patients with invasive breast cancers resected at the Hyogo College of Medicine Hospital between October 2008 and March 2017. Clinicopathological characteristics of 717 total patients were retrieved from electronic chart and pathological reports. Preoperative and postoperative chemotherapies were administered in 171 and 135 patients, respectively. Adjuvant endocrine therapies were administered in 537 patients out of 583 patients with ER-positive cancers. Out of 106 patients with HER2-positive cancers, 95 were given trastuzumab. Adjuvant treatments were determined based on the St. Gallen guidelines from that time [10–15].

During the follow-up (median, 35.1 months; range 1–100.6 months), 88 patients had recurrence (distant 55, locoregional 13, and ipsilateral breast 4). Contralateral breast cancers and death due to any cause occurred in seven and nine patients, respectively. The DFS and overall survival (OS) were defined as the time from the operation to the first events. This study was approved by the ethics committee of the Hyogo College of Medicine (no. 1886) in accordance with the Declaration of Helsinki. As this study collected only retrospective clinical data and offered no risk to the participants, the ethics committee did not require written informed consent.

### Immunohistochemistry

We determined the expression levels of ER by immunohistochemical staining using the antibodies 1D5 (Dako, Glostrup, Denmark) or 6F11 (Leica Biosystems, Wetzler, Germany). Samples were defined as ER-positive if immunohistochemical staining (IHC) was noted in ≥ 1% of the nuclei of cancer cells. The Hercep test (Dako) or the BOND Oracle HER2 IHC System (Leica Biosystems, Tokyo, Japan) was used for HER2 staining. Samples were defined as HER2-positive if an IHC score of 3 was noted or if a positive fluorescence in situ hybridization test was observed for patients with an IHC score of 2. Expression levels of Ki67 were detected using the antibody MIB1 (Dako), and the percentage of Ki67-positive cells in the nuclei of cancer cells was evaluated. We classified the samples into Ki67-high (> 25%) and -low (≤ 25%). All immunohistochemical stainings were performed using automated immunostainers (BOND-MAX, Leica Biosystems, or Autostainer, Dako).

### Measurements of TILs

TILs were evaluated within the tumor in biopsy specimens treated with preoperative chemotherapy ($n = 171$) or surgical specimens not exposed to preoperative chemotherapy ($n = 546$) by the method reported previously [8]. Briefly, we used representative hematoxylin and eosin-stained slides without applying immunostaining techniques. We detected a hot spot in a low-power field within the tumor and the areal percentage containing lymphocytes and plasma cells in both stromal and intratumoral regions was evaluated in a medium-power field (× 100). We classified TILs scores

into three groups; low (< 10%), intermediate (10–50%), and high (> 50%).

### Statistical analysis

The relationships between clinicopathological characteristics and TILs were analyzed using Fisher's exact test. Differences between Kaplan–Meier plots for DFS and OS in each group were calculated by log-rank tests. Unadjusted HRs and 95% CIs of increase of TILs for DFS in each subgroup were calculated using a Cox proportional-hazards model. Univariable and multivariable analyses of clinicopathological factors and TILs levels were performed using a Cox proportional-hazards model to obtain the HR and 95% CI. Statistical significance was set at $p < 0.05$ and all statistical calculations were performed using JMP® Pro Version 13 (SAS Institute Inc., Cary, NC).

## Results

### Clinicopathological characteristics and prognosis of patients according to TILs levels

We classified 486, 168, and 63 breast cancers into low, intermediate, and, high TILs, respectively. TILs frequencies (low/intermediate/high) were significantly higher among the nuclear grade 2 + 3 (46.5%/35.4%/18.1% vs 80.6%/15.5%/3.9%; $p < 0.0001$), ER- (41.0%/33.6%/25.4% vs 73.9%/21.1%/5.0%; $p < 0.0001$), HER2 + (52.8%/37.7%/9.4% vs 70.4%/20.9%/8.7%; $p = 0.0008$), Ki67-high (44.5%/35.7%/19.7% vs 78.9%/17.7%/3.4%; $p < 0.0001$), and chemotherapy administration (52%/36.7%/11.2% vs 74.1%/18.2%/7.7%; $p = 0.0001$) (Table 1). There was no significant association between TILs and menopausal status, tumor size, and lymph node metastasis. Patient DFS

**Table 1** Clinicopathological characteristics of breast cancer according to tumor-infiltrating lymphocytes (TILs)

| Characteristics | TILs-low[a] ($n = 486$) | TILs-intermediate ($n = 168$) | TILs-high ($n = 63$) | p value |
|---|---|---|---|---|
| Menopausal status | | | | |
| Premenopausal | 168 (65.9)[b] | 61 (23.9) | 26 (10.2) | 0.56 |
| Postmenopausal | 314 (68.9) | 105 (23.0) | 37 (8.1) | |
| Unknown | 4 (66.7) | 2 (33.3) | 0 (0) | |
| Tumor size | | | | |
| ≤ 2 cm | 296 (70.6) | 90 (21.5) | 33 (7.9) | 0.15 |
| > 2 cm | 190 (63.8) | 78 (26.2) | 30 (10.1) | |
| Lymph node metastasis | | | | |
| Negative | 333 (69.5) | 106 (22.1) | 40 (8.4) | 0.37 |
| Positive | 150 (64.4) | 60 (25.8) | 23 (9.9) | |
| Not examined | 3 (60.0) | 2 (40.0) | 0 (0) | |
| Nuclear grade | | | | |
| 1 | 332 (80.6) | 64 (15.5) | 16 (3.9) | < 0.0001 |
| 2 + 3 | 121 (46.5) | 92 (35.4) | 47 (18.1) | |
| Unknown | 33 (73.3) | 12 (26.7) | 0 (0) | |
| Estrogen receptor | | | | |
| Positive | 431 (73.9) | 123 (21.1) | 29 (5.0) | < 0.0001 |
| Negative | 55 (41.0) | 45 (33.6) | 34 (25.4) | |
| HER2 status | | | | |
| Negative | 430 (70.4) | 128 (20.9) | 53 (8.7) | 0.0008 |
| Positive | 56 (52.8) | 40 (37.7) | 10 (9.4) | |
| Ki67 status[c] | | | | |
| Low | 371 (78.9) | 83 (17.7) | 16 (3.4) | < 0.0001 |
| High | 106 (44.5) | 85 (35.7) | 47 (19.7) | |
| Unknown | 9 (100) | 0 (0) | 0 (0) | |
| Chemotherapy | | | | |
| No | 383 (74.1) | 94 (18.2) | 40 (7.7) | 0.0001 |
| Yes | 102 (52.0) | 72 (36.7) | 22 (11.2) | |
| Unknown | 1 (25.0) | 2 (50.0) | 1 (25.0) | |

[a]low: < 10%, intermediate: 10–50%, high: > 50%

[b](%),

[c]low: ≤ 25%, high: > 25%

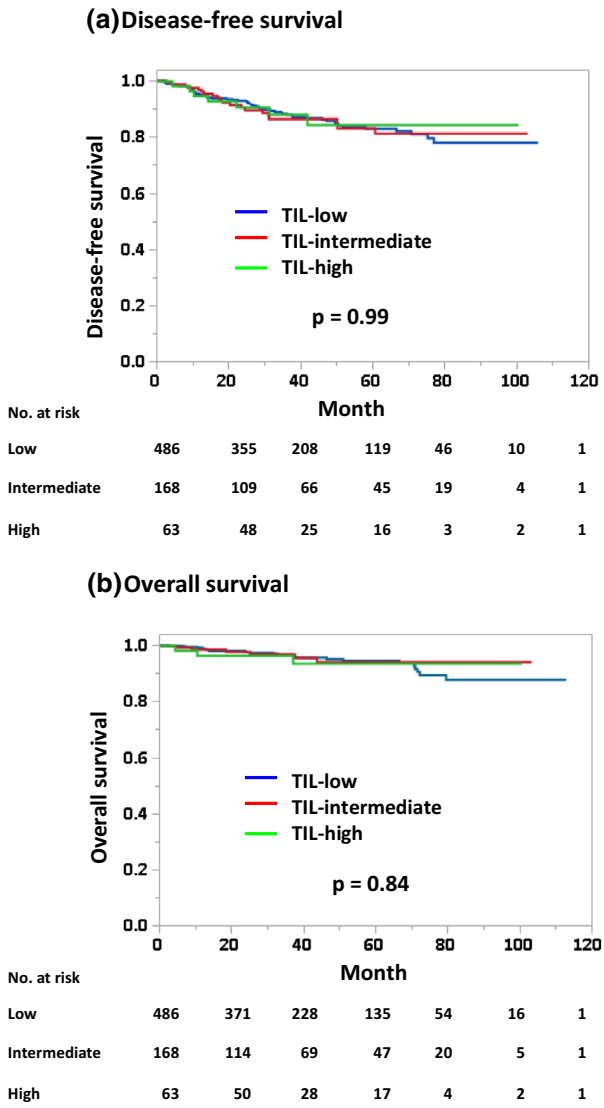


**Fig. 1** **a** Disease-free survival (DFS) and **b** overall survival (OS) of patients according to tumor-infiltrating lymphocytes (TILs) levels

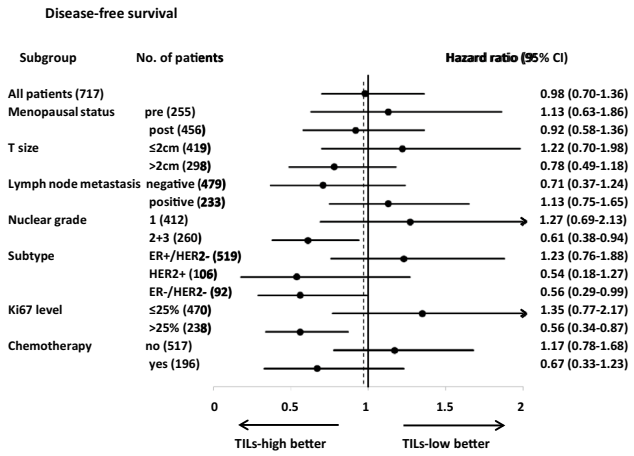


**Fig.2** Forest plots of continuous tumor-infiltrating lymphocytes (TILs) concentration for disease-free survival in each subgroup. The dashed line shows the hazard ratio 0.98 in all patients. 95% CI confidence interval

and OS according to TILs levels are shown in Fig. 1. There was no significant difference between TILs levels and DFS ($p=0.99$, Fig. 1a) or OS ($p=0.84$, Fig. 1b).

## Subgroup analysis of TILs for association with DFS

HR and 95% CI of continuous TILs in each subgroup are shown in Fig. 2. There was no significant association between TILs and DFS in all patients (HR 0.98; 95% CI 0.70–1.36; $p=0.91$). Subgroup analyses reveal increased TILs significantly associated with DFS in the nuclear grade 2+3 (HR 0.61; 95% CI 0.38–0.94; $p=0.024$) and Ki67-high groups (HR 0.56; 95% CI 0.34–0.87; $p=0.0088$). Increases in TILs were significantly (HR 0.56; 95% CI 0.29–0.99; $p=0.046$) associated with improved DFS in the ER−/HER2− subtype; in the HER2+ subtype, although an association with improved DFS was observed, it was not statistically significant (HR 0.54; 95% CI 0.18–1.27; $p=0.17$). In contrast, high TILs tended to be associated with worse DFS in the ER+/HER2− subtype (HR 1.23; 95% CI 0.76–1.88; $p=0.38$).

A significant association between increased TILs and better DFS was observed in the Ki67-high ($p=0.035$) (Fig. 3a) but not in the Ki67-low breast cancers ($p=0.46$) (Fig. 3b). The DFS was highest for the high TILs (5-year DFS, 0.90) and lowest for the low TILs (5-year DFS, 0.67) among Ki67-high cancers, while DFS of patients with high TILs (5-year DFS, 0.75) was worse than others (5-year DFS, 0.88 for low TILs and 0.83 for intermediate-) in the Ki67-low cancers. Although OS of low-TILs patients was inferior compared to high- or intermediate-TILs individuals, the difference was not significant in the Ki67-high ($p=0.21$; Fig. 3c) and -low groups ($p=0.74$; Fig. 3d).

## Univariable and multivariable analyses of DFS and OS in the Ki67-high breast cancers

Prognostic significance of TILs for DFS and OS including other clinicopathological factors of breast cancers was analyzed in the Ki67-high group (Table 2). Univariable analysis showed that lymph node metastasis, chemotherapy administration, and TILs were significant predictors for DFS. Multivariable analysis for these factors revealed lymph node metastasis-positive (HR 2.78; 95% CI 1.51–5.20;

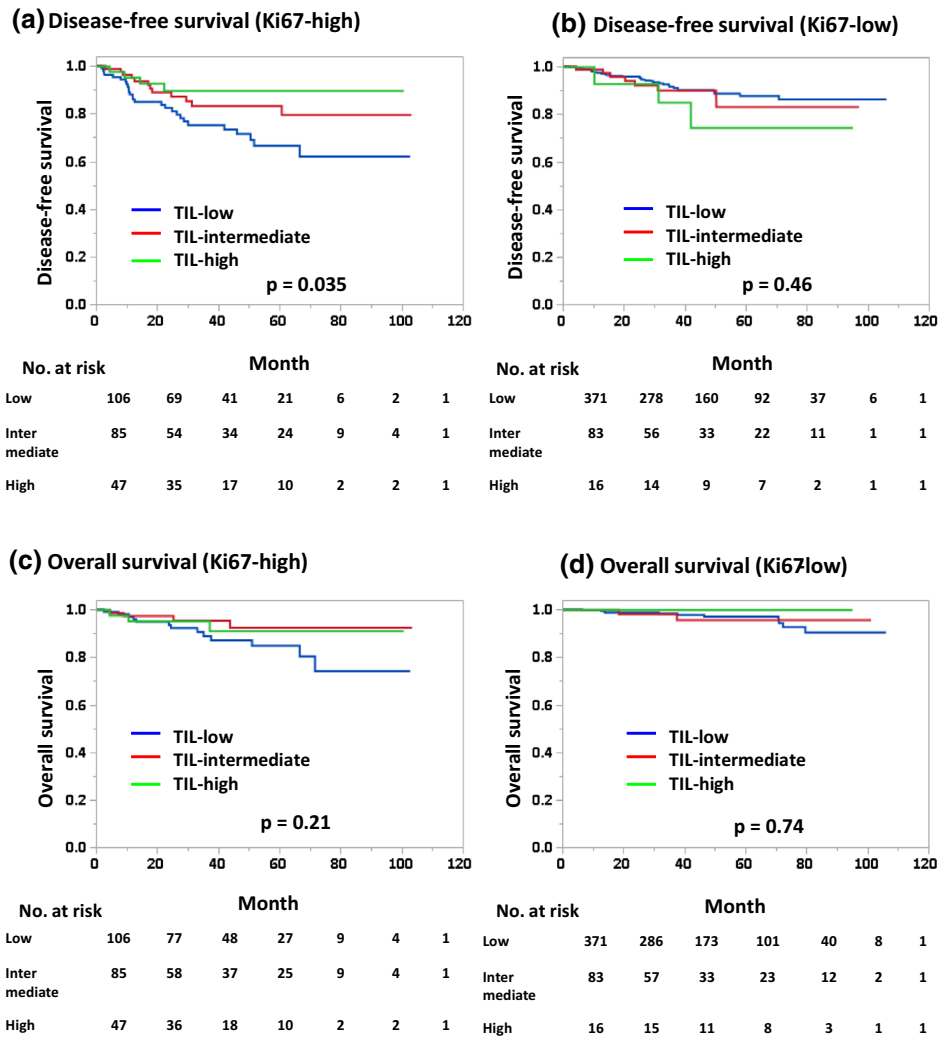


**Fig. 3** Disease-free survival of patients with Ki67-high (**a**) and Ki67-low (**b**) breast cancers according to the levels of tumor-infiltrating lymphocytes (TILs). Overall survival of patients with Ki67-high (**c**) and Ki67-low (**d**) breast cancers according to the levels of tumor-infiltrating lymphocytes (TILs)

$p = 0.0011$), chemotherapy administration (HR 0.37; 95% CI 0.17–0.75; $p = 0.0048$), and TILs-high (HR 0.34; 95% CI 0.10–0.87; $p = 0.023$) were significant and independent factors for DFS. Lymph node metastasis showed a significant association in the univariable analysis; however, there was no significant association of any other clinicopathological factors, including TILs and OS in breast cancers, with Ki67-high (Table 3).

## Relationships between TILs and DFS according to Ki67 expression levels in each subtype

Next, we analyzed DFS according to TIL levels, considering Ki67 expression levels in each subtype. The DFS of patients with high TILs was better than that of others, although this was not statistically significant (5-year 0.90 for high TILs, 0.80 for intermediate-TILs, and 0.72 for low TILs, $p = 0.48$) in the ER + /HER2 − subtype with high expression levels of Ki67 (Fig. 4a). In contrast, the DFS of patients with high TILs was significantly inferior to that of others (5-year 0.58 for high TILs, 0.89 for intermediate-TILs, and 0.88 for low TILs, $p = 0.027$) in the ER + /HER2 − subtype with low expression levels of Ki67 (Fig. 4b). Although there was no significant difference, the DFS of patients with high TILs tended to be better than that of others, irrespective of Ki67 expression levels in both HER2 (Fig. 4c, d) and TN (Fig. 4e, f) subtypes.

## Discussion

In the present study, we identified a significant association between favorable DFS and increased TILs in breast cancers with high expression levels of Ki67 but a negative correlation was found in the Ki67-low cancers. The association between DFS and TILs in the Ki67-high group seems

**Table 2** Univariable and multivariable analyses for disease-free survival in breast cancers with high expression levels of Ki67[a]

| | *n* | Univariable analysis HR (95% CI)[b] | *p* value | Multivariable analysis HR (95% CI)[b] | *p* value |
|---|---|---|---|---|---|
| Menopausal status | | | | | |
| Premenopausal | 93 | 1.00 | | | |
| Postmenopausal | 143 | 1.54 (0.83–3.00) | 0.18 | | |
| Tumor size | | | | | |
| ≤ 2 cm | 105 | 1.00 | | | |
| > 2 cm | 133 | 1.46 (0.79–2.77) | 0.23 | | |
| Lymph node metastasis | | | | | |
| Negative | 148 | 1.00 | | 1.00 | |
| Positive | 89 | 2.25 (1.23–4.14) | 0.0084 | 2.78 (1.51–5.20) | 0.0011 |
| Nuclear grade | | | | | |
| 1 | 51 | 1.00 | | | |
| 2 + 3 | 177 | 0.92 (0.47–1.91) | 0.80 | | |
| Estrogen receptor status | | | | | |
| Positive | 146 | 1.00 | | | |
| Negative | 92 | 1.33 (0.72–2.42) | 0.36 | | |
| HER2 status | | | | | |
| Negative | 177 | 1.00 | | | |
| Positive | 61 | 0.47 (0.18–1.03) | 0.061 | | |
| Chemotherapy | | | | | |
| No | 139 | 1.00 | | 1.00 | |
| Yes | 95 | 0.40 (0.18–0.80) | 0.0086 | 0.37 (0.17–0.75) | 0.0048 |
| Tumor-infiltrating lymphocytes[c] | | | | | |
| Low | 106 | 1.00 | | 1.00 | |
| Intermediate | 85 | 0.53 (0.26–1.03) | 0.060 | 0.63 (0.30–1.22) | 0.17 |
| High | 47 | 0.33 (0.099–0.85) | 0.020 | 0.34 (0.10–0.87) | 0.023 |

[a]Ki67 > 25%

[b]Hazard ratio (95% confidence interval)

[c]low: < 10%, intermediate: 10–50%, high: > 50%

to be consistent across breast cancer subtypes, including ER + /HER2 − . A noted observation was how increased TILs tended to associate with worse DFS in the ER + / HER2 − breast cancers with low levels of Ki67. These results may indicate that prognostic significance of TILs varies depending on biological feature of breast cancers evaluable by proliferative activity especially in the ER + / HER2 − subtype.

TILs serve as an indicator of survival benefit in patients with TN and HER2 + subtypes but not the ER + subtype [6]. Favorable DFS with increased TILs was reported in the HER2 + and TN but not in the luminal/HER2 − subtypes in patients treated with NAC [9]. Additionally, the study noted that increased TILs was significantly associated with shorter OS in luminal/HER2 − cancers (HR 1.10; 95% CI 1.02–1.19; $p = 0.011$). Our previous data consistently demonstrated a worse prognosis for patients with high TILs in the ER + / HER2 − breast cancers with non-pCR after chemotherapy [16]. Thus, the clinical significance of TILs as a tool of prognosis in the ER + /HER2 − subtype is complicated, with high-TIL levels linked to worse prognosis.

The detailed mechanism for poor prognosis of patients with high TILs in the ER + /HER2 − cancers is currently unknown. Higher grade and high Ki67 were observed in breast cancers with high TILs and seemed to result in poor prognosis due to such aggressive phenotypes of breast cancer (Table 1). However, this speculation does not explain how the observed association between increased TILs and worse prognosis was exclusive to the Ki67-low but not the Ki67-high breast cancers. Consistent with the present study, in patients treated with aromatase inhibitor TILs and immune-related gene expression were associated with poor response [17]. Blok et al. observed no significant difference of DFS between exemestane and tamoxifen groups within the high CD8 + TILs group [18]. However, among low CD8 + TILs, patients treated with exemestane had better DFS than patients treated with tamoxifen. According to these results, the association between poor patient prognosis

**Table 3** Univariable analysis for overall survival in breast cancers with high expression levels of Ki67[a]

| | *n* | Univariable analysis HR (95% CI)[b] | *p* value |
|---|---|---|---|
| Menopausal status | | | |
| Premenopausal | 93 | 1.00 | |
| Postmenopausal | 143 | 2.19 (0.85–6.75) | 0.11 |
| Tumor size | | | |
| ≤ 2 cm | 105 | 1.00 | |
| > 2 cm | 133 | 1.72 (0.70–4.59) | 0.24 |
| Lymph node metastasis | | | |
| Negative | 148 | 1.00 | |
| Positive | 89 | 3.62 (1.48–9.64) | 0.0048 |
| Nuclear grade | | | |
| 1 | 51 | 1.00 | |
| 2 + 3 | 177 | 0.58 (0.23–1.56) | 0.27 |
| Estrogen receptor status | | | |
| Positive | 146 | 1.00 | |
| Negative | 92 | 1.16 (0.45–2.81) | 0.75 |
| HER2 status | | | |
| Negative | 177 | 1.00 | |
| Positive | 61 | 0.75 (0.22–2.05) | 0.60 |
| Chemotherapy | | | |
| No | 139 | 1.00 | |
| Yes | 95 | 0.41 (0.12–1.12) | 0.084 |
| Tumor-infiltrating lymphocytes[c] | | | |
| Low | 106 | 1.00 | |
| Intermediate | 85 | 0.38 (0.11–1.08) | 0.072 |
| High | 47 | 0.58 (0.13–1.80) | 0.37 |

[a]Ki67 > 25%

[b]Hazard ratio (95% confidence interval)

[c]Low: < 10%, intermediate: 10–50%, high: > 50%

with endocrine therapy and TILs may not be mediated by breast cancer aggressiveness but at least in part by effects of the therapy itself. Although exemestane and tamoxifen both block estrogen signaling in breast cancer, systemic depletion of estrogen by exemestane and selective ER modulation induced by tamoxifen indicate different potential influences on non-cancerous cells, including immune-related cells, and altered treatment efficacy between exemestane and tamoxifen.

According to Chan et al. [19], the ratio of CD8 + T-cells and regulatory T-cells (Tregs) significantly increased in response to neoadjuvant endocrine therapy using aromatase inhibitor. ER and aromatase expression are also identified in the cancer-associated fibroblasts (CAFs), tumor-associated macrophages (TAMs), and myeloid-derived suppressor cells (MDSCs) that are involved in breast cancers [20]. These results suggest enhancement of cancer immunity using endocrine therapies such as aromatase inhibitor resulted in a favorable prognosis. We have demonstrated that the prognostic significance of high TILs for ER + /HER2 − breast cancers was inconsistent between Ki67-high and -low groups. It is unclear whether the association between endocrine therapy efficacy and TIL levels is different between Ki67-high and -low groups. A possible explanation is that a varying composition of immune cells is present between Ki67-high and -low cancers. Usually, higher levels of TILs contain cytotoxic T cells and B cells, directing tumor suppression, as well as immune suppressive factors including Tregs, CAFs, TAMs, or MDSCs. If the ratio of these immune stimulate and suppressive factors is different between high and low Ki67 groups in ER + /HER2 − cancers, prognostic impact may be altered. Denkert et al. [9] reported that in ER-/HER2 − subtype, presence of T cells, B cells, natural killer (NK) cells and monocytes were significantly associated with improved prognosis. In contrast, only an abundance of B cells was linked with improved prognosis, while monocytes were associated with worse prognosis in the luminal/HER2 − subtype. Since high TILs and favorable prognosis was consistent in ER-/HER2 − and ER + /HER2 − breast cancers among the Ki67-high group, the immune profiling seemed to be identical for these groups. However, the ER + /HER2 − breast cancers with Ki67-low were different as reported by Denkert et al. The precise mechanism of poor prognosis in patients with high TILs in the Ki67-low group remains unexplained.

In the ER + /HER2 − subtype, more chemotherapy was administered in the Ki67-high breast cancers. Sensitivity to chemotherapy is different between high and low TILs [16] and may affect prognosis of this subtype. However, among breast cancers not treated with chemotherapy, DFS of patients with high TILs was worse in the ER + /HER2 − subtype but favorable in other subtypes (data not shown). We think chemotherapy has no influence on the differing prognoses of ER + /HER2 − subtype between Ki67-high and -low breast cancers. Recently, the efficacy of immune checkpoint inhibitors (ICIs) in breast cancers has been reported. The efficacy of ICIs seems to be lower for the ER + /HER2 − subtype than for the TN subtype [21]. Higher benefits are obtained in tumors with high TILs [22] or a high expression of programmed cell death ligand 1 (PD-L1) [23]. Higher TIL levels are associated with higher expression levels of PD-1 and PD-L1 in early breast cancer [24], and PD-L1 expression in immune cells is a biomarker of the efficacy of atezolizumab, an ICI in advanced TN breast cancers [25]. Thus, TILs, PD-1, and PD-L1 may predict the efficacy of ICIs in breast tumors, including the ER + /HER2 − subtype. If the immune microenvironment of tumors with high TILs differs between Ki67-high and -low breast cancers in the ER + /HER2 − subtype, the treatment efficacy of ICIs in this subtype will be altered depending on the different immune microenvironments. Thus, an analysis of the treatment

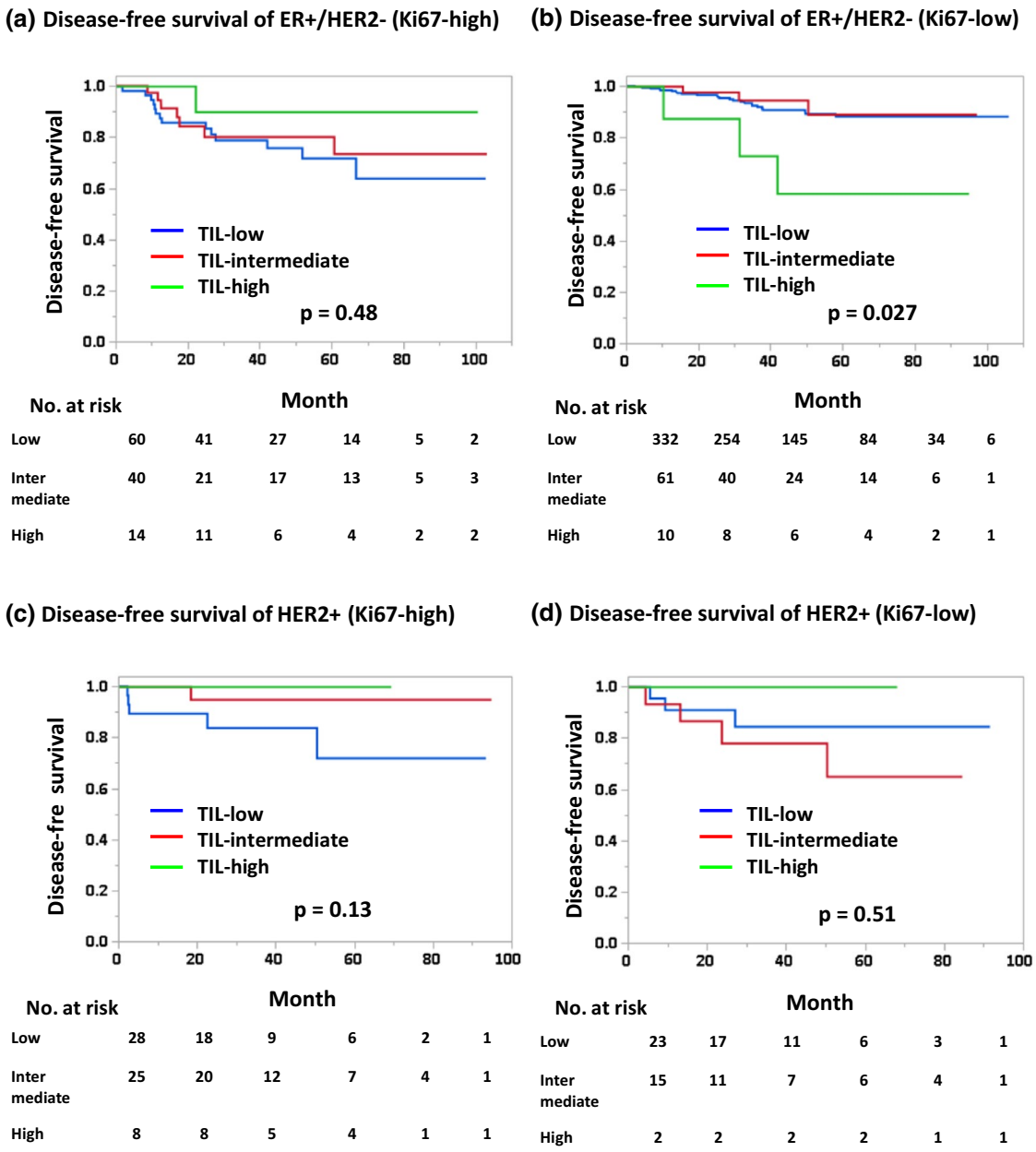


**Fig. 4** Disease-free survival of patients with Ki67-high (**a**, **c**, **e**) and Ki67-low (**b**, **d**, **f**) breast cancers according to the levels of tumor-infiltrating lymphocytes (TILs) in estrogen receptor (ER)-positive/human epithelial growth factor receptor 2 (HER2)-negative (**a**, **b**), HER2-positive (**c**, **d**), and TN (ER-negative/HER2-negative) (**e**, **f**) subtypes of breast cancer

efficacy of ICIs in the ER + /HER2 − subtype with consideration for Ki67 expression levels is interesting.

According to Denkert et al., even in the ER + / HER2 − cancers, prognosis was favorable in the subgroup of increased TILs with grade 3. Consistent with this report, our data also demonstrated a link between high TILs and favorable prognosis exclusively in nuclear grade 2 + 3 cancers. Although Ki67 expression levels distinguished prognostic differences of TILs more precisely than grade in the present study (data not shown), ongoing investigation is necessary to determine the dominant factor. In the present study, we set the cut-off value for Ki67 expression levels at 25%, since this cut-off value was associated with the greatest risk of death, as revealed in the meta-analysis [26]. Although the optimal cut-off value for Ki67 expression levels remains undetermined, data obtained for the present study were consistent when using variable cut-off values ranging from 20 to 30% (data not shown). Our data indicate that the prognostic significance of TILs seems to be different depending on Ki67 expression levels for the

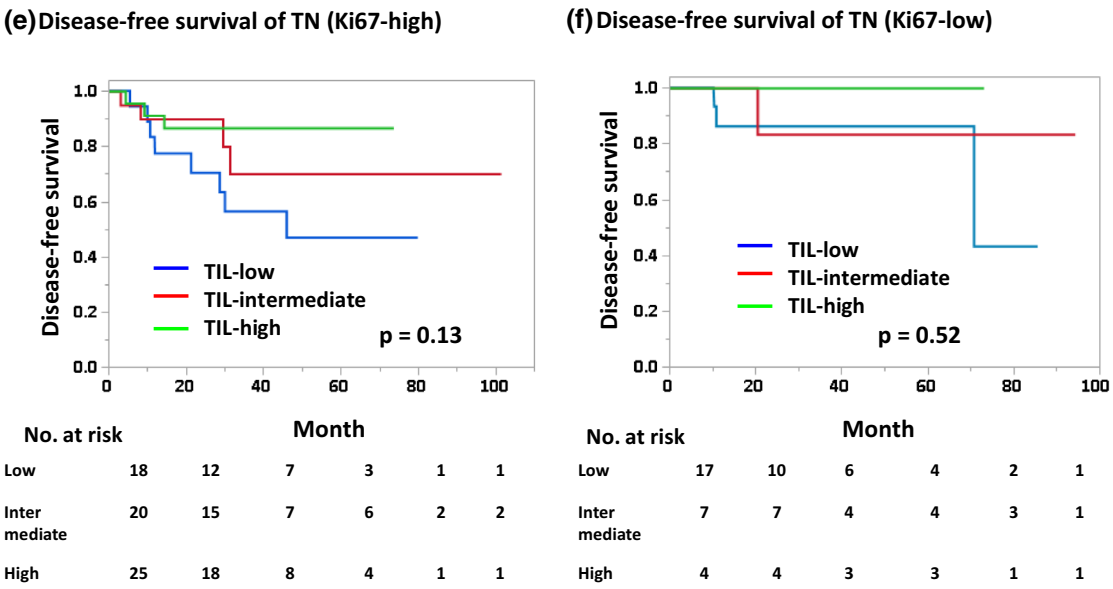


**Fig. 4** (continued)

ER + /HER2 − subtype; nevertheless, it remains inconclusive owing to the subgroup analyses. Further confirmation of results obtained here, including large number of patients, is also required.

In conclusion, we have demonstrated that prognostic significance of TILs depends on Ki67 expression levels for the ER + /HER2 − subtype. Although increased TILs were significantly associated with improved DFS in the Ki67-high group, even among the ER + /HER2 − subtype, the reverse was observed in the Ki67-low group. The results presented here may be useful not only for understanding differences in the immune system at the microenvironment level of breast cancers depending on biology of cancers such as Ki67 expression levels, but also for considering the prediction of treatment efficacy of ICIs in the ER + / HER2 − subtype.

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**Data availability** The datasets analyzed during the current study are not publicly available because the Ethics Committee of the institute did not permit to provide data of individual participants.

## Compliance with ethical standards

**Conflict of interest** AH received personal fees as honoraria from Chugai Pharmaceutical, Taiho Parmaceutical, and Novartis Pharma. YaM received lecture fees or grants from Chugai Pharmaceutical, AstraZeneca, Eli Lily, Phizer, Eizai, MSD, and Taiho Pharmaceutical. Other authors declare that they have no conflict of interest.

**Ethics approval** This study was approved by the ethics committee of the Hyogo College of Medicine (No. 1886) in accordance with the Declaration of Helsinki.

**Informed consent** As this study collected only retrospective clinical data and offered no risk to the participants, written informed consent was not required.

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