

Association Between FOXP3/CD8 Lymphocyte Ratios and Tumor Infiltrating Lymphocyte Levels in Different Breast Cancer Subtypes

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Abstract. *Background/Aim:* Patients with non-luminal breast cancer subtypes with high levels of tumor infiltrating lymphocytes (TILs) have better prognosis than those with luminal subtype. We evaluated the role of TILs according to the subtype. *Materials and Methods:* An immunohistochemical analysis of 139 breast cancer cases was conducted to calculate the FOXP3+/CD8+ T cell ratios and their relationships with TILs and disease-free survival (DFS) were evaluated. *Results:* FOXP3+/CD8+ T cell ratios were significantly associated with TIL levels only in luminal breast cancers ($p=0.0001$). Low FOXP3+/CD8+ T cell ratio was significantly associated with longer DFS ($p=0.017$). All luminal subtype patients with high TIL levels had high FOXP3+/CD8+ T cell ratios compared to only half of non-luminal subtype patients with high TIL levels. *Conclusion:* High FOXP3+/CD8+ T cell ratios in breast cancers may partly explain the worse prognosis of luminal breast cancers, but not that of non-luminal breast cancers with high TIL levels.

Immune responses play a significant role in the prognosis and treatment efficacy of breast cancer patients. In particular, tumor infiltrating lymphocytes (TILs) have been reported to

be clinically useful (1) and are easily measurable in a clinical setting. In fact, in an analysis of 13100 patients across 23 studies investigating neoadjuvant chemotherapy (NAC), TIL levels were correlated positively with improved disease-free survival (DFS) [relative risk (RR)=0.61; 95% confidence interval (CI)=0.51-0.73; $p<0.001$] (2). Moreover, TIL levels correlated particularly well with DFS in human epidermal growth factor receptor 2 (HER2)-positive (RR, 0.67; 95%CI=0.50-0.89, $p=0.005$) and triple negative (TN; estrogen receptor (ER)-negative and HER2-negative) breast cancers (RR=0.54; 95%CI=0.43-0.70, $p<0.001$). Furthermore, in a meta-analysis of 22964 patients from 25 studies, high TIL levels were associated with better DFS [hazard ratio (HR)=0.82; 95%CI=0.76-0.88] and overall survival (OS) (HR=0.79; 95%CI=0.71-0.87) in TN breast cancer (3). However, in ER-positive breast cancer, no correlation was observed between TILs and prognosis in terms of either DFS (HR=1.01; 95%CI=0.94-1.07) or OS (HR=1.09; 95%CI=0.98-1.21) (3). In an analysis of 3771 cases of breast cancer treated with NAC, while high TIL levels significantly prolonged DFS in both TN (HR=0.93; 95%CI=0.87-0.98; $p=0.011$) and HER2-positive (HR, 0.94; 95%CI=0.89-0.99; $p=0.017$) breast cancers, no significant correlation was observed with hormone receptor-positive (HR)/HER2-negative breast cancer (HR=1.02; 95%CI=0.96-1.09; $p=0.46$) (4). Moreover, in an analysis of 318 cases of ER-positive/HER2-negative (luminal) breast cancer, a significantly shorter survival period was observed ($p=0.026$) (5). Thus, despite the fact that the significance of TIL levels as a prognostic factor varies depending on breast cancer subtypes, the mechanisms causing the association between high TIL levels and poor prognoses in luminal breast cancers remain unclear.

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There are several TIL subsets, including cluster of differentiation (CD)8-positive T cells, which exhibit anti-tumor effects, and regulatory T cells (Tregs), which express forkhead box protein 3 (FOXP3) and negatively regulate immune responses; host immune responses to breast cancers are believed to be controlled by the functions of both of these cell types (6). Thus, the prognosis of breast cancer patients cannot be predicted solely according to their TIL levels; an examination of the balance between CD8+ and FOXP3+ T cells present is needed. According to a report by Peng *et al.*, CD8+ T cells were correlated with good prognoses whereas FOXP3+ T cells were correlated with poor prognoses, and low FOXP3+/CD8+ T cell ratios were significantly associated with better prognoses (7). Another report by Liu *et al.* has indicated that low FOXP3+ T cell levels and a high CD8+/FOXP3+ T-cell ratio were positively associated with good DFS and OS (8). All these results suggest that good prognosis is expected in patients with high levels of CD8+ T cells and low levels of FOXP3+ T cells.

Liu *et al.* have also found that low FOXP3+ T cell levels and high CD8+/FOXP3+ T cell ratios were significantly associated with good prognoses in both ER-negative and ER-positive cancers (8). In addition, a report by Chung *et al.* on HR-positive breast cancer has indicated that high FOXP3+/CD8+ T cell ratios were significantly correlated with poor DFS ($p=0.032$) (9). However, after examining 8978 samples, CD8+ T cell levels were significantly correlated with breast cancer-specific mortality in ER-negative and ER-positive/HER2-positive breast cancers, but such a correlation was not observed for luminal breast cancers (10). According to Stanton, CD8+ T cells are a favorable prognostic factor for TN breast cancer, whereas FOXP3+ T cells are a poor prognostic factor for HR-positive breast cancer (11). A report by Papaioannou *et al.* has indicated that in ER-negative breast cancers, CD8+ and FOXP3+ T cells were significantly correlated with DFS ($p=0.000409$) and a trend was observed for DFS ($p=0.071$), respectively. However, both CD8+ and FOXP3+ T cells were not correlated with DFS in ER-positive breast cancers (12).

Thus, while it seems evident that with respect to the types of TILs, high levels of CD8+ T cells and low levels of FOXP3+ T cells are generally associated with good prognoses, their prognostic role in luminal breast cancer remains unclear. Furthermore, no reports examining the correlation between TIL levels and CD8+/FOXP3+ T cell ratios in luminal breast cancers exist. In this study, we evaluated FOXP3+ to CD8+ ratios and positive T cell levels using immunohistochemical staining in patients who had undergone surgical resection for primary breast cancer and examined their correlation with TIL levels considering subtypes in order to clarify the mechanisms behind the association between high TIL levels and poor prognoses in luminal breast cancer.

Patients and Methods

Patient eligibility. We reviewed the records of 250 patients recruited for a previous study in which formalin-fixed, paraffin-embedded tissues of adequate cancer cells were available (13), for potential enrolment in the present study. Of these 250 participants, 139 patients who had available clinical information as well as FOXP3 and CD8 immunohistochemical staining and TIL data were enrolled in this study. Any patients with non-invasive breast cancer, male breast cancer, or metastatic breast cancer were excluded.

The present study was approved by the ethics committee of the Hyogo College of Medicine (No. 106) in accordance with the Declaration of Helsinki and written informed consent was obtained from all patients. Data from individual participants are unavailable because the ethics committee did not permit their publication.

Definitions. DFS was defined as the time from the operation to the first event including distant metastasis, locoregional recurrence, contralateral breast cancer, and death due to any reason. OS was calculated from the time of the operation to death due to any reason.

Classification of subtypes and evaluation of Ki-67 expression levels. ER-positivity was defined as nuclear staining of $\geq 1\%$ cancer cells; HER2-positivity was defined as an immunohistochemical (IHC) membrane staining score of 3+, or IHC 2+ with a positive fluorescence *in situ* hybridization test (14). Ki-67 expression levels were determined by immunohistochemistry in the nuclei of cancer cells as described previously (14). Breast cancers were classified into the luminal type (ER-positive/HER2-negative; $n=110$) and non-luminal type (ER-negative/HER2-negative and HER2-positive; $n=15$ and $n=14$, respectively). The cutoff value of Ki-67 was set at 25% and classified into Ki-67-high ($>25\%$) and -low ($\leq 25\%$).

Determination of tumor infiltrating lymphocyte (TIL) levels. TIL levels were determined in hematoxylin and eosin stained samples obtained during operation ($n=132$) or biopsy samples for patients treated with preoperative chemotherapy ($n=7$). Hotspots within the tumor, as observed microscopically using the lowest power, were first selected; then, the percentage of lymphocytes and plasma cells in both stromal and intratumoral regions were evaluated under a medium-power field ($\times 100$). TIL scores were defined as follows; low ($<10\%$), intermediate ($\geq 10\%$ to $<50\%$), and high ($\geq 50\%$) as described previously (15). Under this classification TIL levels were divided into low ($n=64$), intermediate ($n=63$), and high ($n=12$).

Immunohistochemical examination of FOXP3 and CD8. Formalin-fixed, paraffin-embedded tumor samples were obtained during operation ($n=132$) or biopsy prior to preoperative chemotherapy ($n=7$). BOND Epitope Retrieval Solution 2 (Leica Microsystems, Tokyo, Japan) was used for 20 min for FOXP3 antigen retrieval and Cell Conditioning Solution (Ventana Medical Systems, Inc., Basel, Switzerland) was used for 64 min for CD8 antigen retrieval. Primary antibodies for FOXP3 (1:500; 236A/E7 antibody ab20034, mouse monoclonal, Abcam, Cambridge, UK) and CD8 (undiluted; CONFIRM anti-CD8 SP57 rabbit monoclonal antibody, Roche Diagnostics K.K., Tokyo, Japan) were used. All procedures were performed using the BOND Polymer Refine Detection kit (Leica Microsystems, Tokyo, Japan) on the automated immunostainer BOND-MAX or BOND-III (Leica Microsystems) for FOXP3 and using I-VIEW DAB universal kit (Roche Diagnostics K.K.) on the

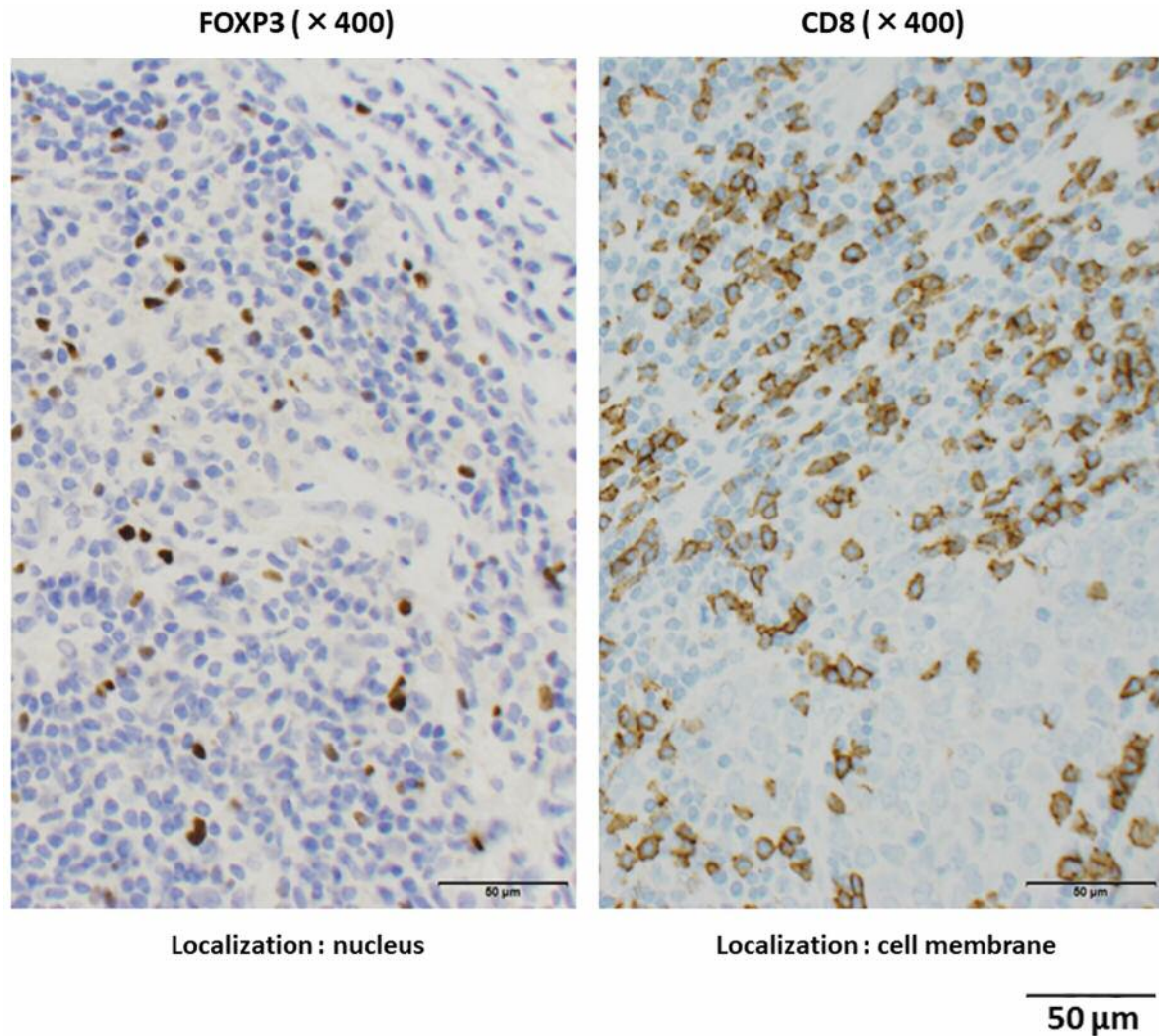


Figure 1. Positive immunohistochemical staining of FOXP3 and CD8. Nuclear staining in FOXP3 and membrane staining in CD8 were evaluated. Scale bar represents 50 µm.

immunostainer VENTANA BenchMark ULTRA (Roche Diagnostics K.K.) for CD8. Nuclear staining and cell membrane staining of lymphocytes were considered to be positive for FOXP3 and CD8, respectively (Figure 1). Positive cells were counted at a magnification of $\times 400$ and average counts of four fields were used to determine cell counts in each sample as previously reported (8, 16).

Ethics approval and informed consent. This study was approved by the ethics committee of the Hyogo College of Medicine (No. 106) and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients.

Statistical analysis. The relationship between FOXP3/CD8 ratios and clinicopathological characteristics or TIL levels were analyzed using Fisher's exact test. Differences in expression levels of FOXP3, CD8, and FOXP3+/CD8+ T cell ratios according to TIL levels were calculated by the Kruskal–Wallis test. Kaplan–Meier plots were compared between high and low FOXP3+/CD8+ T cell ratio groups

for DFS and log-rank tests were used for OS analysis. Unadjusted hazard ratios (HRs) and 95% CIs for DFS in each subgroup were calculated using a Cox proportional-hazards model. All statistical analyses were conducted using JMP® Pro Version 13 (SAS Institute Inc., Cary, NC, USA) and statistical significance was set at $p < 0.05$.

Results

Determination of FOXP3+ T cell counts, CD8+ T cell counts, and FOXP3+/CD8+ T cell ratio according to TIL levels. The median cell counts of FOXP3+ T cells and CD8+ T cells were 1.5 (range, 0–108.5) and 60.75 (range, 4.5–358.75), respectively and the median FOXP3+/CD8+ T cell ratio was 0.02554 (range=0–1.103). FOXP3+ T cell counts were significantly associated with TIL levels ($p < 0.0001$) and median cell counts in the high, intermediate, and low groups were 17.38 (range=0.5–

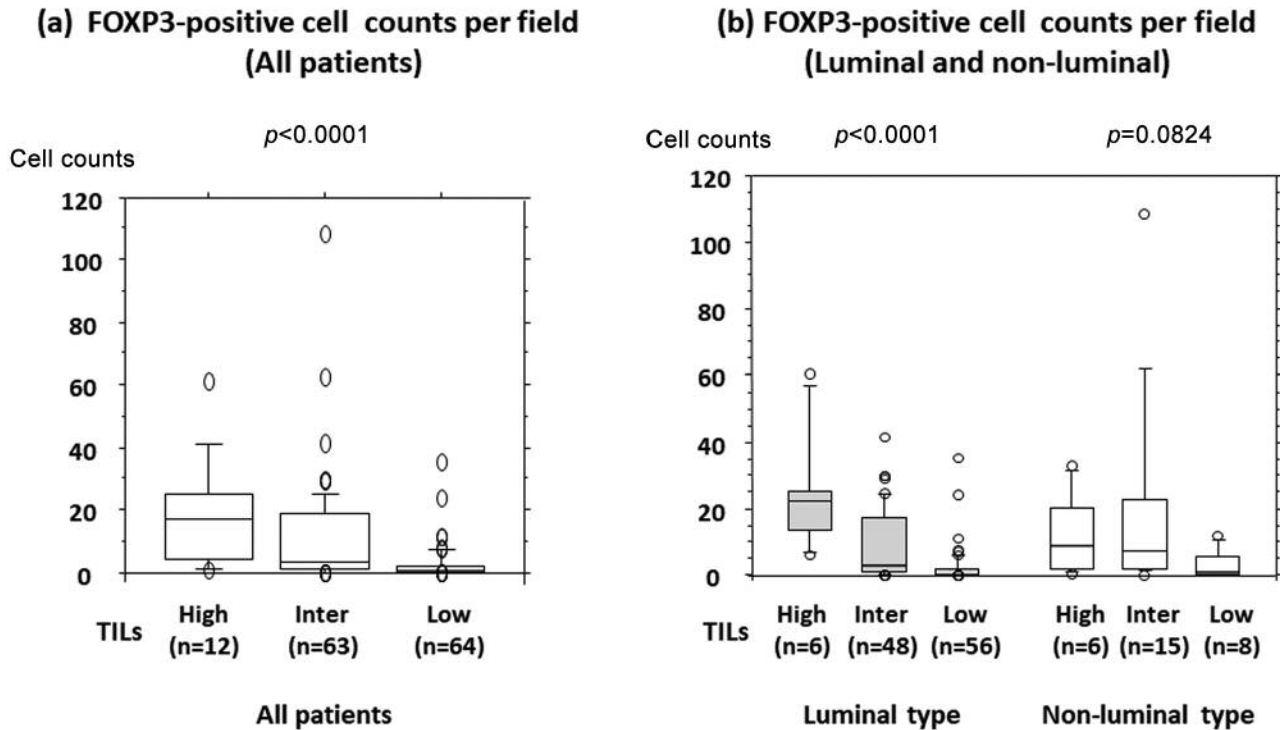


Figure 2. Association between FOXP3-positive cell counts and tumor infiltrating lymphocyte (TIL) levels. (a) FOXP3-positive cell counts per field according to the TIL levels in all patients and (b) FOXP3-positive cell counts per field according to the TIL levels in patients with luminal and non-luminal subtypes. TILs: low (<10%), intermediate (≥10% to <50%), and high (≥50%). Luminal type: estrogen receptor (ER)-positive/HER2-negative; non-luminal type: HER2-positive or ER-negative/HER2-negative.

60.75), 3.5 (range=0-108.5), and 0.25 (range=0-35.25), respectively (Figure 2a). The relationship between FOXP3+ T cell counts and TIL levels was consistently significant in the luminal subtype ($p<0.0001$) and not significant in the non-luminal subtype ($p=0.0824$) (Figure 2b). CD8+ T cell counts were significantly associated with TIL levels ($p<0.0001$) and the median cell counts in the high, intermediate, and low groups were 235.38 (range=37.75-358.75), 76.5 (range=12.0-316.0), and 42.38 (range=4.5-211.5), respectively (Figure 3a). The positive associations between CD8+ T cell counts and TIL levels were consistently significant in both luminal and non-luminal subtypes ($p<0.0001$ and $p=0.0027$, respectively) (Figure 3b). The FOXP3+/CD8+ T cell ratios were significantly associated with TIL levels ($p<0.0001$) (Figure 4a). The median cell counts in the high, intermediate, and low TIL level groups were 0.074 (range=0.002-0.536), 0.047 (range, 0-0.836), and 0.007 (range=0-1.103), respectively. Interestingly, the positive association between FOXP3+/CD8+ T cell ratios and TIL levels was consistent in the luminal subtype ($p=0.0001$) but not in the non-luminal subtype ($p=0.515$) (Figure 4b).

The cutoff value of FOXP3+/CD8+ T cell ratio for disease-free survival. In order to identify the optimal cutoff value of

FOXP3+/CD8+ T cell ratio for DFS, HRs and 95% CIs were first calculated using several cutoff values. The optimal cutoff value was determined to be 0.02 (Figure 5) (HR=0.286; 95%CI=0.082-0.774). Using this cutoff value, we further analyzed the prognostic significance of the FOXP3+/CD8+ T cell ratio. The DFS of patients with low FOXP3+/CD8+ T cell ratio ($n=58$) was significantly longer than those with high FOXP3+/CD8+ T cell ratio ($n=81$, $p=0.017$). Although the OS of patients with low FOXP3+/CD8+ T cell ratio was better than that with high FOXP3+/CD8+ T cell ratio, this difference was not statistically significant ($p=0.364$). The longer DFS of patients with low FOXP3+/CD8+ T cell ratio was consistently significant in the luminal subtype ($p=0.020$; HR=0.246; 95%CI=0.056-0.790) (Figure 6a). However, there was no significant association between FOXP3+/CD8+ T cell ratio and DFS in the non-luminal subtype ($p=0.835$) (Figure 6b). No significant differences in OS were observed in both luminal ($p=0.206$) and non-luminal subtypes ($p=0.496$) (Figure 6c, d).

Associations between FOXP3+/CD8+ T cell ratio and clinicopathological characteristics or TIL levels. Patients with high FOXP3+/CD8+ T cell ratios had significantly higher proportions of tumors >2 cm in size ($p=0.035$),

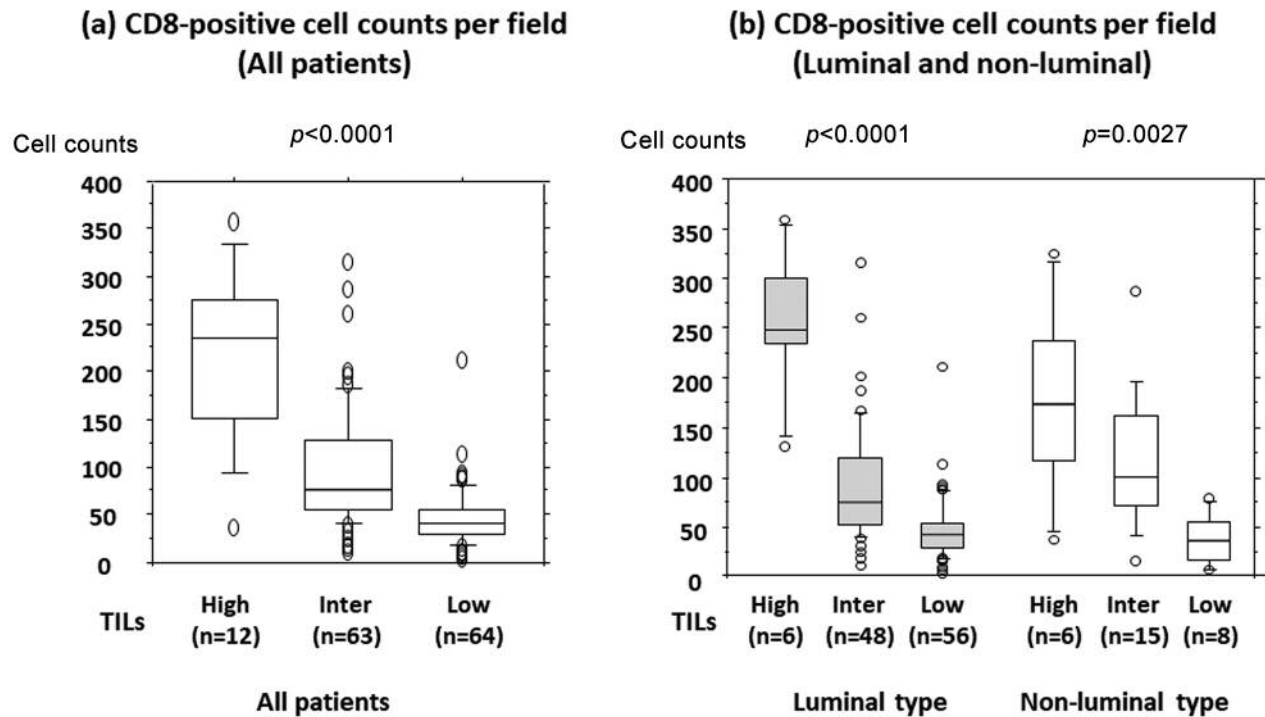


Figure 3. Association between CD8-positive cell counts and tumor infiltrating lymphocyte (TIL) levels. (a) CD8-positive cell counts per field according to the TIL levels in all patients and (b) CD8-positive cell counts per field according to the TIL levels in patients with luminal and non-luminal subtypes. TILs: low (<10%), intermediate ($\geq 10\%$ to <50%), and high ($\geq 50\%$). Luminal type: estrogen receptor (ER)-positive/HER2-negative, non-luminal type: HER2-positive or ER-negative/HER2-negative.

positive lymph node metastasis ($p=0.040$), non-luminal subtype ($p=0.035$), and high expression levels of Ki-67 ($p=0.005$) (Table I). There was significant association between FOXP3+/CD8+ T cell ratio and TIL levels in all patients ($p<0.0001$) and the luminal subtype ($p<0.0001$), but not in the non-luminal subtypes ($p=0.0518$) (Table II). In the luminal subtype, frequencies of breast cancers with high FOXP3+/CD8+ T cell ratio were lowest in the low TIL (35.7%) and highest in the high TIL (100%) groups (Table II). In contrast, 50% of breast cancers with high FOXP3+/CD8+ T cell ratio were half of the TIL-high group in the non-luminal subtype (Table II).

Discussion

In this study, we demonstrated that in luminal breast cancer, high FOXP3+/CD8+ T cell ratios were significantly associated with shorter DFS (HR=0.246; 95%CI=0.056-0.790) (Figure 6a). Moreover, higher TIL levels were associated with significantly higher FOXP3+/CD8+ T cell ratios in luminal breast cancer ($p=0.0001$) but no such correlation was observed in non-luminal breast cancer ($p=0.515$) (Figure 4), suggesting that the reason why high TIL levels are associated with a worse prognosis among

patients with luminal breast cancer could be partly due to an increase in FOXP3+/CD8+ T cell ratios. Similarly, Chung *et al.* have reported that in HR-positive breast cancer, high FOXP3+/CD8+ T cell ratios were significantly associated with worse DFS (HR=2.579; 95%CI=1.118-5.950) (9) and Mao *et al.* have reported that high CD8+/FOXP3+ T cell ratios were associated with favorable OS (HR=0.65; 95%CI=0.43-0.96) (3). Chung *et al.* used the median as cutoff value set, and we investigated several cutoff values and selected the optimal one (Figure 5). Further studies are required to conclusively identify the best cutoff value.

Our results also indicated that CD8+ and FOXP3+ T cell levels are lower in luminal breast cancer in comparison to non-luminal types (data not shown), and that breast cancers with low FOXP3+/CD8+ T cell ratios are more commonly found in the luminal subtype (46.4% for luminal and 24.1% for non-luminal) (Table I). Liu *et al.* have reported that FOXP3+ T-cell levels and CD8+/FOXP3+ T-cell ratios were significantly lower and higher in luminal breast cancers, respectively (8). It has also been reported that CD8+ T-cell percentages are lower (11) and the numbers of CD8+ and FOXP3+ T cells are fewer in luminal breast cancer in comparison to TN breast cancer (17). It has been reported that the number of FOXP3+ T cells in luminal breast cancer

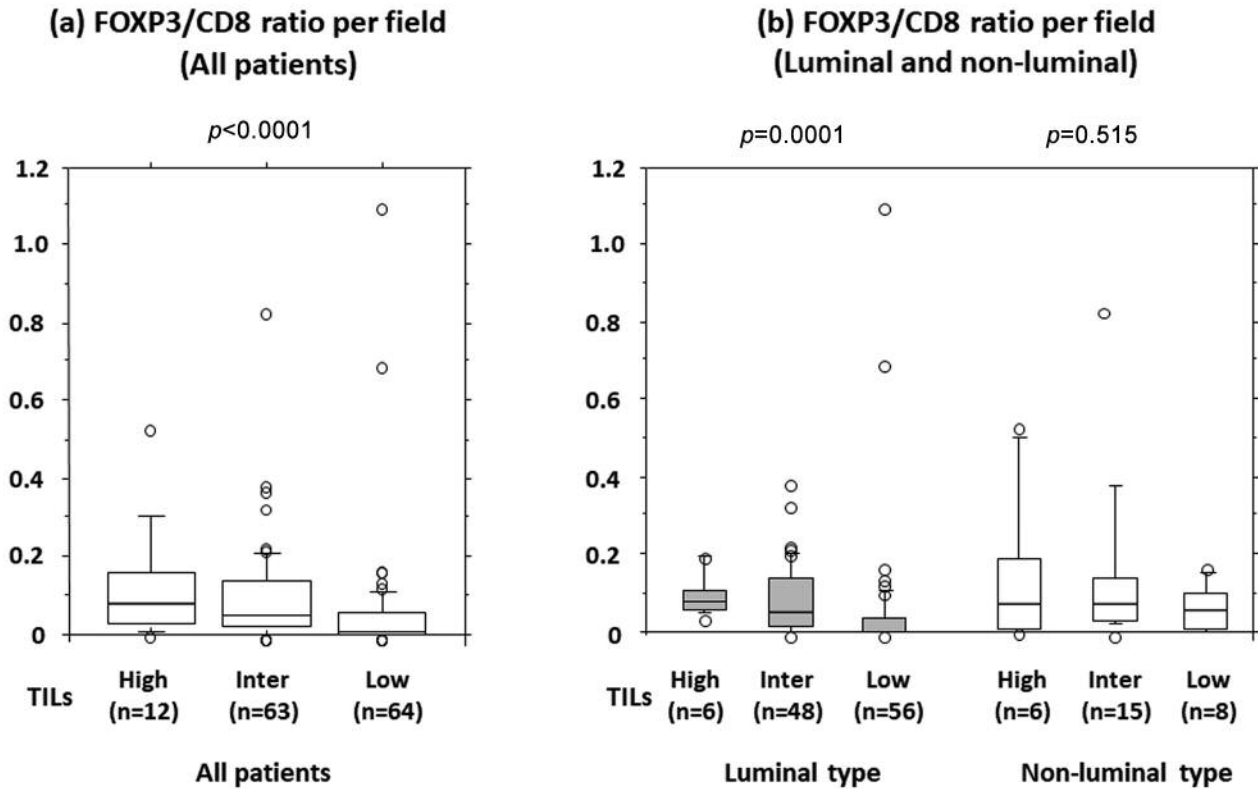


Figure 4. Association between the FOXP3+/CD8+ ratio and tumor infiltrating lymphocyte (TIL) levels. (a) FOXP3+/CD8+ ratio per field according to the TIL levels in all patients and (b) FOXP3+/CD8+ ratio per field according to the TIL levels in patients with luminal and non-luminal subtypes. TILs: low (<10%), intermediate ($\geq 10\%$ to <50%), and high ($\geq 50\%$). Luminal type: estrogen receptor (ER)-positive/HER2-negative, non-luminal type: HER2-positive or ER-negative/HER2-negative.

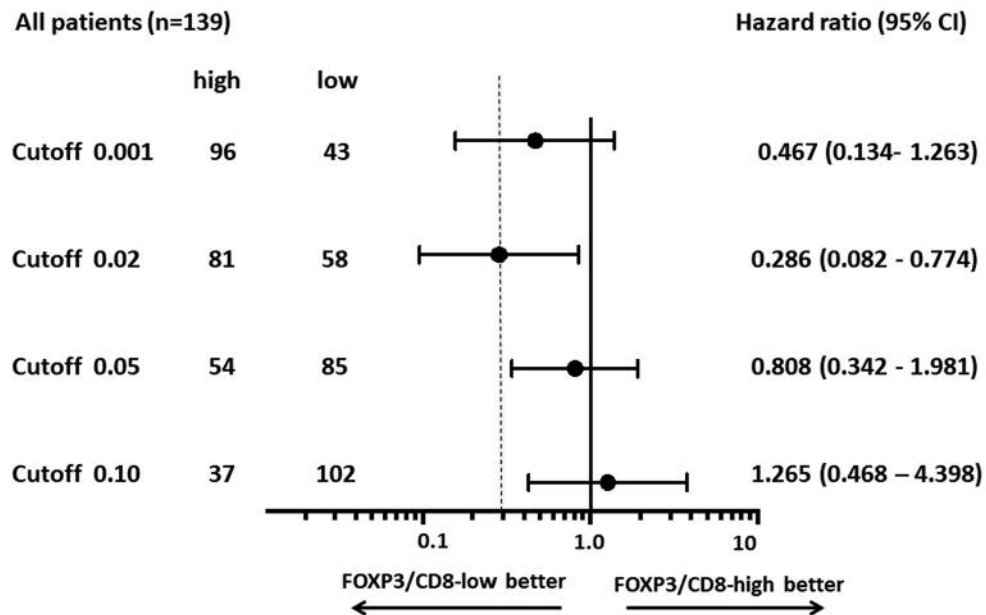


Figure 5. Forest plots of FOXP3+/CD8+ ratio for disease-free survival using several cutoff values. The dashed line shows the optimal cutoff value, with a hazard ratio (HR)=0.286 and 95% confidence interval (CI)=0.082-0.774.

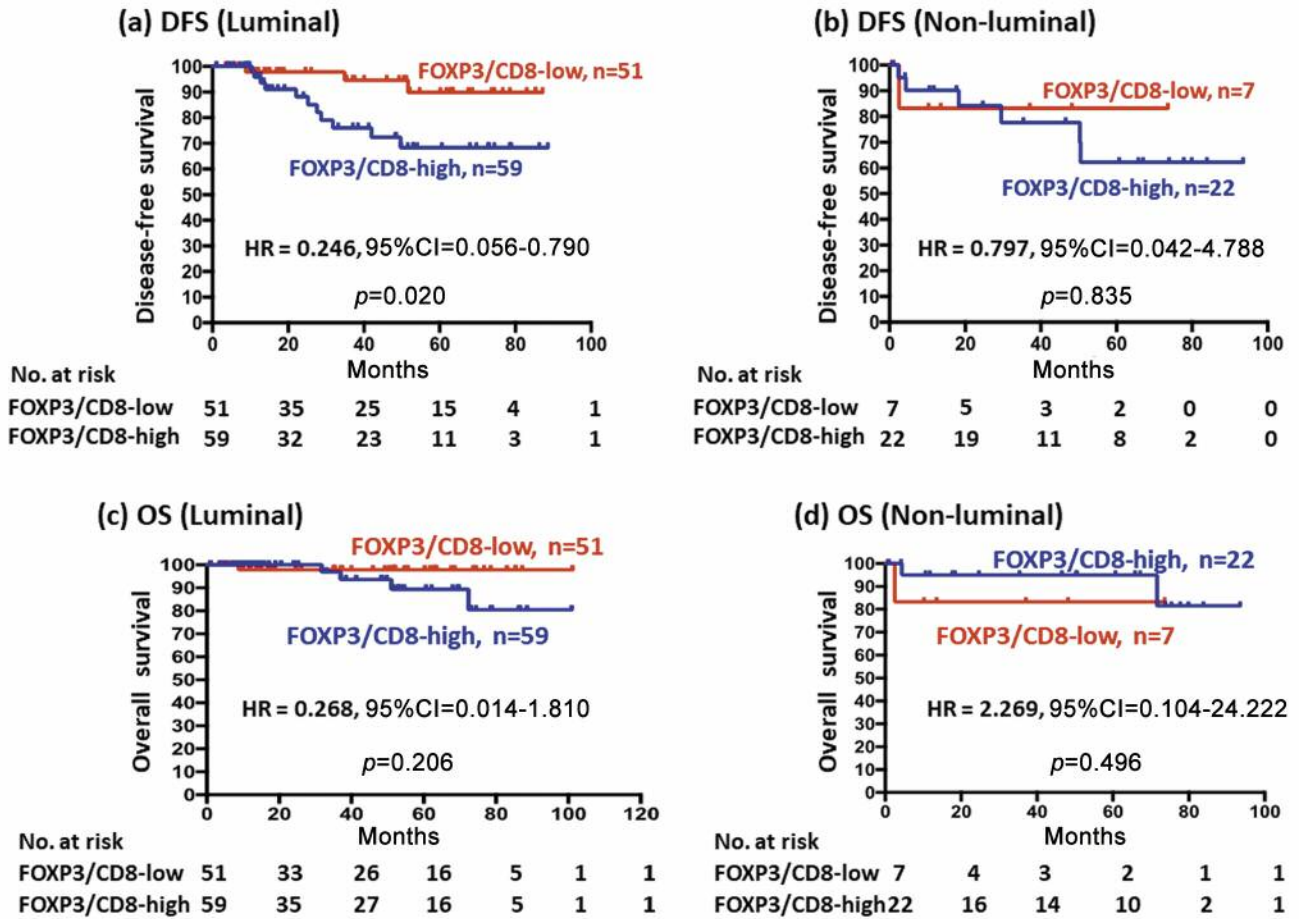


Figure 6. Disease-free survival and overall survival of patients with high and low FOXP3+/CD8+ ratios in the (a) luminal and (b) non-luminal subtypes. The cutoff for the FOXP3+/CD8+ ratio was 0.02.

is low (7), but no correlations between the subtypes and CD8+ T-cell levels or FOXP3+/CD8+ T cell ratios were observed (11). Interestingly, in both luminal and non-luminal subtypes, high TIL levels were associated with significant increases in CD8+ T-cell counts ($p<0.0001$) (Figure 3). In contrast, FOXP3+ T-cell levels were significantly increased when TIL levels were high in the luminal subtype ($p<0.0001$) but were not correlated with TIL levels in the non-luminal subtype ($p=0.0824$) (Figure 2). Thus, while FOXP3+/CD8+ T cell ratios increase with TIL levels in luminal breast cancers, FOXP3+/CD8+ T cell ratios did not increase in non-luminal breast cancers. According to a report by Liu *et al.*, in the absence of CD8+ T cells, FOXP3+ T cells were significantly associated with poor breast cancer-specific survival in ER-positive breast cancer (HR=1.30; 95%CI=1.02-1.66; $p=0.032$), but in the presence of CD8+ T cells, FOXP3+ T cells were associated with favorable breast cancer-specific survival in ER-negative/HER2-positive breast

cancer (HR=0.48; 95%CI=0.23-0.98; $p=0.047$) (18). Thus, when considering the correlation between FOXP3 and prognosis, CD8 levels also need to be considered. In the luminal subtype, FOXP3+ T-cell counts seem to affect the anti-tumor immune response and the ratio of FOXP3+ to CD8+ T cells may be clinically useful.

In terms of correlations with clinicopathological factors, high histological grade was significantly associated with increased FOXP3+ and CD8+ T cell counts (8). In addition, nodal metastasis-positive, high-grade, high-Ki-67 cancers were associated with significant increases in FOXP3+ T cell counts and FOXP3+/CD8+ T-cell ratios (7). Our investigations also revealed that high FOXP3+/CD8+ T-cell ratios were more common in a group of large tumor, nodal metastasis-positive, high-Ki-67 cancers (Table I). Thus, it is possible that breast cancers with high malignancy are concentrated in FOXP3+/CD8+ T-cell ratio-high group and poor prognoses may be due to the suppression of the immune

Table I. Relationship between FOXP3+/CD8+ T cell ratio and clinicopathological characteristics in patients with breast cancers.

	n	FOXP3/CD8-high ^a	FOXP3/CD8-low ^a	p-Value
Menopausal status ^b				
Pre-	41	22 (53.7%)	19 (46.3%)	0.573
Post-	97	58 (59.8%)	39 (40.2%)	
Tumor size				
≤2 cm	86	44 (51.2%)	42 (48.8%)	0.035
>2 cm	53	37 (69.8%)	16 (30.2%)	
Lymph node metastasis				
Negative	96	50 (52.1%)	46 (47.9%)	0.040
Positive	43	31 (72.1%)	12 (27.9%)	
Tumor grade ^c				
1	80	42 (52.5%)	38 (47.5%)	0.075
2 and 3	54	37 (68.5%)	17 (31.5%)	
Estrogen receptor status				
Positive	118	65 (55.1%)	53 (44.9%)	0.093
Negative	21	16 (76.2%)	5 (23.8%)	
HER2 status				
Negative	125	70 (56.0%)	55 (44.0%)	0.153
Positive	14	11 (78.6%)	3 (21.4%)	
Subtype ^d				
Luminal type	110	59 (53.6%)	51 (46.4%)	0.035
Non-luminal type	29	22 (75.9%)	7 (24.1%)	
Ki67 expression levels ^e				
Low	84	43 (51.2%)	41 (48.8%)	0.005
High	47	36 (76.6%)	11 (23.4%)	
Chemotherapy administration ^f				
No	94	53 (56.4%)	41 (43.6%)	0.462
Yes	44	28 (63.6%)	16 (36.4%)	

^aHigh: >0.02, low: ≤0.02. ^bOne patient was unknown; ^cFive patients were undetermined; ^dLuminal type: estrogen receptor (ER)-positive/HER2-negative, non-luminal type: HER2-positive or ER-negative/HER2-negative; ^eKi67 low: ≤25%, high: >25%; eight patients were unknown; ^fOne patient was unknown.

response. In this study, we found that elevated TIL levels were associated with increased CD8+ T-cell levels rather than increased FOXP3+ T-cell levels in the non-luminal subtype. In contrast, in the luminal subtype, greater increases in FOXP3+ T cells in comparison to CD8+ T cells were accompanied by higher TIL levels, resulting in higher increase of FOXP3+/CD8+ T-cell ratios, although the mechanisms for this increase remain unclear.

In the microenvironment of breast cancer, estrogen is known to promote the proliferation of Tregs and myeloid-derived suppressor cells (MDSCs), suppressing the immune response to cancer (19). Furthermore, an analysis of the gene signature of TILs revealed that in comparison to ER-negative cancer, populations of Tregs and MDSCs are higher in ER-positive breast cancers (20). On the basis of these reports, we can hypothesize that the estrogen enriched microenvironment of ER-positive breast cancers is immune suppressive due to activation of Tregs and MDSCs. Three out of six high-TIL cases in the non-luminal subtype had low FOXP3+/CD8+ T-cell ratios whereas all six cases of high-TIL in the luminal subtype had high FOXP3+/CD8+ T-cell ratios. Thus, even if

Table II. Relationship between FOXP3+/CD8+ T cell ratio and tumor infiltrating lymphocyte (TIL) levels.

	FOXP3/CD8-high ^a	FOXP3/CD8-low ^a	p-Value
All patients			
TIL-high ^b	9 (75.0%)	3 (25.0%)	<0.0001
TIL-intermediate	47 (74.6%)	16 (25.4%)	
TIL-low	25 (39.1%)	39 (60.9%)	
Luminal subtype ^c			
TIL-high	6 (100%)	0 (0%)	<0.0001
TIL-intermediate	33 (68.8%)	15 (31.2%)	
TIL-low	20 (35.7%)	36 (64.3%)	
Non-luminal subtype ^d			
TIL-high	3 (50.0%)	3 (50.0%)	0.0518
TIL-intermediate	14 (93.3%)	1 (6.7%)	
TIL-low	5 (62.5%)	3 (37.5%)	

^aHigh, >0.02; low, ≤0.02; ^bLow (<10%), intermediate (≥10% to <50%), and high (≥50%); ^cLuminal: estrogen receptor (ER)-positive/human epithelial growth factor receptor 2 (HER2)-negative; ^dNon-luminal: HER2-positive or ER-negative/HER2-negative.

the immune response is induced and TIL levels rise in luminal breast cancers, a relatively increased number of FOXP3+ T cells may suppress the immune response against breast cancers. We suggest that the increase in TIL levels accompanied by an increase in the FOXP3+/CD8+ T-cell ratios resulted in poor prognoses in the luminal subtype. However, our results were obtained from a single institute and this was a retrospective study including a relatively small number of cases; hence, further research incorporating more cases is necessary to verify these results.

In conclusion, while no correlation between TILs and FOXP3+/CD8+ T-cell ratios was observed in non-luminal breast cancer, an increase in TILs was significantly associated with an increase in FOXP3+/CD8+ T-cell ratios in the luminal breast cancer subtype. These results suggest that the poor prognosis in luminal breast cancers with high TIL levels are partly generated from the relative increase in FOXP3+ T cells to CD8+ T-cells.

Conflicts of Interest

YM received research funding and honoraria from Chugai, AstraZeneca, Eli Lilly, Pfizer, MSD, Kyowa-Kirin, Taiho, and Esai. The other Authors declare that they have no conflicts of interest.

Authors' Contributions

NI and KM performed immunohistochemical staining. RF, YF, and TW evaluated expression levels in immunohistochemical staining. RF, AB, TH, and MI were involved in data collection. TH and YM performed the statistical analyses. YM designed the study and SH supervised the study. RF and YM prepared the manuscript. All Authors read and approved the final manuscript.

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References

- 1 Luen SJ, Savas P, Fox SB, Salgado R and Loi S: Tumour-infiltrating lymphocytes and the emerging role of immunotherapy in breast cancer. *Pathology* 49: 141-155, 2017. PMID: 28049579. DOI: 10.1016/j.pathol.2016.10.010
- 2 Wang K, Xu J, Zhang T and Xue D: Tumor-infiltrating lymphocytes in breast cancer predict the response to chemotherapy and survival outcome: A meta-analysis. *Oncotarget* 7(28): 44288-44298, 2016. PMID: 27329588. DOI: 10.18632/oncotarget.9988
- 3 Mao Y, Qu Q, Zhang Y, Liu J, Chen X and Shen K: The value of tumor infiltrating lymphocytes (TILs) for predicting response to neoadjuvant chemotherapy in breast cancer: a systematic review and meta-analysis. *PloS One* 9: e115103, 2014. PMID: 25501357. DOI: 10.1371/journal.pone.0115103
- 4 Denkert C, von Minckwitz G, Darb-Esfahani S, Lederer B, Heppner BI, Weber KE, Budczies J, Huober J, Klauschen F, Furlanetto J, Schmitt WD, Blohmer JU, Karn T, Pfitzner BM, Kümmel S, Engels K, Schneeweiss A, Hartmann A, Noske A, Fasching PA, Jackisch C, van Mackelenbergh M, Sinn P, Schem C, Hanusch C, Untch M and Loibl S: Tumour-infiltrating lymphocytes and prognosis in different subtypes of breast cancer: a pooled analysis of 3771 patients treated with neoadjuvant therapy. *Lancet Oncol* 19(1): 40-50, 2018. PMID: 29233559. DOI: 10.1016/S1470-2045(17)30904-X
- 5 Miyoshi Y, Shien T, Ogiya A, Ishida N, Yamazaki K, Horii R, Horimoto Y, Masuda N, Yasojima H, Inao T, Osako T, Takahashi M, Tomioka N, Wanifuchi-Endo Y, Hosoda M, Doihara H and Yamashita H: Associations in tumor infiltrating lymphocytes between clinicopathological factors and clinical outcomes in estrogen receptor-positive/human epidermal growth factor receptor type 2 negative breast cancer. *Oncol Lett* 17(2): 2177-2186, 2019. PMID: 30675282. DOI: 10.3892/ol.2018.9853
- 6 Yu P and Fu YX: Tumor-infiltrating T lymphocytes: friends or foes? *Lab Invest* 86(3): 231-245, 2006. PMID: 16446705.
- 7 Peng GL, Li L, Guo YW, Yu P, Yin XJ, Wang S and Liu CP: CD8+ cytotoxic and FoxP3+ regulatory T lymphocytes serve as prognostic factors in breast cancer. *Am J Transl Res* 11(8): 5039-5053, 2019. PMID: 31497220.
- 8 Liu F, Lang R, Zhao J, Zhang X, Pringle GA, Fan Y, Yin D, Gu F, Yao Z and Fu L: CD8+ cytotoxic T cell and FOXP3+ regulatory T cell infiltration in relation to breast cancer survival and molecular subtypes. *Breast Cancer Res Treat* 130(2): 645-655, 2011. PMID: 21717105. DOI: 10.1007/s10549-011-1647-3
- 9 Chung YR, Kim HJ, Jang MH and Park SY: Prognostic value of tumor infiltrating lymphocyte subsets in breast cancer depends on hormone receptor status. *Breast Cancer Res Treat* 161(3): 409-420, 2017. PMID: 27913931. DOI: 10.1007/s10549-016-4072-9
- 10 Ali HR, Provenzano E, Dawson SJ, Blows FM, Liu B, Shah M, Earl HM, Poole CJ, Hiller L, Dunn JA, Bowden SJ, Twelves C, Bartlett JM, Mahmoud SM, Rakha E, Ellis IO, Liu S, Gao D, Nielsen TO, Pharoah PD and Caldas C: Association between CD8+ T-cell infiltration and breast cancer survival in 12,439 patients. *Ann Oncol* 25(8): 1536-1543, 2014. PMID: 24915873. DOI: 10.1093/annonc/mdu191
- 11 Stanton SE, and Disis ML: Clinical significance of tumor-infiltrating lymphocytes in breast cancer. *J Immunother Cancer* 4: 59, 2016. PMID: 27777769
- 12 Papaioannou E, Sakellakis M, Melachrinou M, Tzoracoleftherakis E, Kalofonos H and Kourea E: A standardized evaluation method for FOXP3+ Tregs and CD8+ T-cells in breast carcinoma: association with breast carcinoma subtypes, stage and prognosis. *Anticancer Res* 39(3): 1217-1232, 2019. PMID: 30842152. DOI: 10.21873/anticancer.13232
- 13 Fujimoto Y, Inoue N, Morimoto K, Watanabe T, Hirota S, Imamura M, Matsushita Y, Katagiri T, Okamura H and Miyoshi Y: Significant association between high serum CCL5 levels and better disease-free survival of patients with early breast cancer. *Cancer Sci* 111(1): 209-218, 2020. PMID: 31724785. DOI: 10.1111/cas.14234

- 14 Fujimoto Y, Watanabe T, Hida AI, Higuchi T, Miyagawa Y, Ozawa H, Bun A, Fukui R, Sata A, Imamura M, Hirota S and Miyoshi Y: Prognostic significance of tumor-infiltrating lymphocytes may differ depending on Ki67 expression levels in estrogen receptor-positive/HER2-negative operated breast cancers. *Breast Cancer* 26(6): 738-747, 2019. PMID: 31098866. DOI: 10.1007/s12282-019-00977-0
- 15 Watanabe T, Hida AI, Inoue N, Imamura M, Fujimoto Y, Akazawa K, Hirota S and Miyoshi Y: Abundant tumor infiltrating lymphocytes after primary systemic chemotherapy predicts poor prognosis in estrogen receptor-positive/HER2-negative breast cancers. *Breast Cancer Res Treat* 168(1): 135-145, 2018. PMID: 29168063. DOI: 10.1007/s10549-017-4575-z
- 16 Takada K, Kashiwagi S, Goto W, Asano Y, Takahashi K, Takashima T, Tomita S, Ohsawa M, Hirakawa K and Ohira M: Use of the tumor-infiltrating CD8 to FOXP3 lymphocyte ratio in predicting treatment responses to combination therapy with pertuzumab, trastuzumab, and docetaxel for advanced HER2-positive breast cancer. *J Transl Med* 16(1): 86, 2018. PMID: 29615076. DOI: 10.1186/s12967-018-1460-4
- 17 Glajcar A, Szpor J, Hodorowicz-Zaniewska D, Tyrak KE and Okoń K: The composition of T cell infiltrates varies in primary invasive breast cancer of different molecular subtypes as well as according to tumor size and nodal status. *Virchows Arch* 475(1): 13-23, 2019. PMID: 31016433. DOI: 10.1007/s00428-019-02568-y
- 18 Liu S, Foulkes WD, Leung S, Gao D, Lau S, Kos Z and Nielsen TO: Prognostic significance of FOXP3+ tumor-infiltrating lymphocytes in breast cancer depends on estrogen receptor and human epidermal growth factor receptor-2 expression status and concurrent cytotoxic T-cell infiltration. *Breast Cancer Res* 16(5): 432, 2014. PMID: 25193543. DOI: 10.1186/s13058-014-0432-8
- 19 Rothenberger NJ, Somasundaram A and Stabile LP: The Role of the Estrogen Pathway in the Tumor Microenvironment. *Int J Mol Sci* 19(2): E611, 2018. PMID: 29463044. DOI: 10.3390/ijms19020611
- 20 Hammerl D, Smid M, Timmermans AM, Sleijfer S, Martens JWM and Debets R: Breast cancer genomics and immunological markers to guide immune therapies. *Semin Cancer Biol* 52(Pt 2): 178-188, 2018. PMID: 29104025. DOI: 10.1016/j.semcancer.2017.11.003

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