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PRECLINICAL STUDY

Tumor-infiltrating CD8+ and FOXP3+ lymphocytes in triple-negative breast cancer: its correlation with pathological complete response to neoadjuvant chemotherapy

Minoru Miyashita · Hironobu Sasano · Kentaro Tamaki · Monica Chan · Hisashi Hirakawa · Akihiko Suzuki · Hiroshi Tada · Go Watanabe · Noriko Nemoto · Saki Nakagawa · Takanori Ishida · Noriaki Ohuchi

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Abstract The anti-tumor immune response was recently reported to play a critical role in the chemotherapeutic sensitivity of breast cancer. Therefore, we investigated the correlation between CD8+ and FOXP3+ tumor-infiltrating lymphocytes and the pathological complete response (pCR) following neoadjuvant chemotherapy (NAC) in triple-negative breast cancer (TNBC), in conjunction with neoangiogenesis, basal and proliferation markers. CD8+ and FOXP3+ lymphocytes were assessed in biopsy specimens by double-staining immunohistochemistry, in combination with immunostaining of vasohibin-1, CD31, EGFR, CK5/6, and Ki-67. Earlier age, pre-menopausal status, smaller tumor size, and high Ki-67 were significantly associated with pCR, as in high CD8+, high CD8+/FOXP3+ ratio, and low vasohibin-1 positive ratio. Multivariate analysis did reveal that a high CD8+/FOXP3+ ratio was a strong predictor of pCR with an odds ratio of 5.32 ($P = 0.005$). High Ki-67 was also significantly associated with pCR ($P = 0.002$). TNBCs with a high CD8+/FOXP3+ ratio and high Ki-67 had the highest pCR rate (70 %) following NAC. However, the pCR rate of the patients with low CD8+/FOXP3+ ratio and low Ki-67 was only 5 %. The pCR rates of a high CD8+/FOXP3+ ratio and low Ki-67 patients and those with a low CD8+/FOXP3+ ratio and high Ki-67 were 24 and 21 %, respectively. TNBCs with a high CD8+/FOXP3+ ratio were more sensitive to anthracycline and taxane-based chemotherapeutic regimens, and the CD8+/FOXP3+ ratio in conjunction with Ki-67 could predict pCR following NAC in TNBC. This predictor may represent a new surrogate for testing the efficacy of investigational agents in the neoadjuvant setting.

Keywords Triple-negative breast cancer · Chemo-sensitivity · pCR · CD8 · FOXP3 · Ki-67

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M. Miyashita (✉) · A. Suzuki · H. Tada · G. Watanabe · N. Nemoto · S. Nakagawa · T. Ishida · N. Ohuchi
Department of Surgical Oncology, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan
e-mail: atihsayim8m8@med.tohoku.ac.jp

M. Miyashita · H. Sasano · K. Tamaki · M. Chan · N. Nemoto · S. Nakagawa
Department of Pathology, Tohoku University Hospital, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan

K. Tamaki
Department of Breast Surgery, Nahanishi Clinic, 2-1-9 Akamine, Naha 901-0154, Japan

H. Hirakawa
Department of Breast Surgery, Tohoku Kosai Hospital, 2-3-11 Kokubuncho, Aoba-ku, Sendai 980-0803, Japan

Introduction

Breast cancer is a heterogeneous disease according to the results of gene expression profiling using microarray analysis [1–3]. Based on the differences in sensitivity to therapeutic drugs and in clinical outcomes, the implementation of personalized medicine is required for treating breast cancer. Triple-negative breast cancer (TNBC), which lacks estrogen receptor (ER), progesterone receptor (PgR) and human epidermal growth factor receptor-2 (HER2) expression, is the breast cancer subtype associated with the worst clinical outcome. Chemotherapy with anthracyclines and taxanes usually constitute the backbone

of the treatment of this aggressive subtype, and a pathological complete response (pCR) following neoadjuvant chemotherapy (NAC) is considered a reliable surrogate marker for survival of TNBC [4]. Approximately, 30 % of the pCR rate was reported to be achieved by NAC administering both anthracyclines and taxanes in TNBCs, and achieving a pCR associated with a good prognosis [4]. In contrast, TNBC with a non-pCR was reported to display a markedly worse prognosis and are required to undergo treatment with new investigational therapeutic agents [4, 5]. Therefore, the reliable prediction of pCR should be made as early as possible before drugs with unknown efficacies are administered. Many studies have focused on predicting pCR from biopsy specimens but there have been no reliable markers associated with pCR in TNBC with the exception of the Ki-67 [6, 7]. Gene expression analyses have been proposed useful for predicting pCR [8, 9] but these techniques are not necessarily applicable in clinical settings, and sufficient validation has not been performed. Therefore, the development of the ability to predict pCR from biopsy specimens has remained a major challenge in clinical research.

Preclinical studies were recently reported that cytotoxic agents could partially exert their antitumor activities by inducing an immune response against tumor cells [10, 11]. The demise of immunogenic cells induced by cytotoxic agents allows cross-presentation of antigens and induction of tumor-specific cytotoxic T cells. Cytotoxic T cells (CD8+ T cells) have been reported to be associated with a higher pCR rate following NAC and a better outcome in the patients with breast cancer [12–14]. In addition, the regulatory T cells defined as forkhead box protein 3 (FOXP3)+ T cells have a critical role in suppressing antitumor immunity [15, 16]. However, it is also true that the prognostic and predictive roles of FOXP3 have remained in dispute; breast cancer tumors infiltrated with FOXP3+ T cells were reported to be less sensitive to cytotoxic chemotherapy and have a worse prognosis by some investigators [12, 17] but others indicated that breast tumors with FOXP3+ T cells achieved a higher pCR rate after NAC and had a better prognosis [18, 19]. These discrepancies could be due to the differences in the studied breast cancer subtypes and therapeutic regimens. The density of CD8+ and FOXP3+ T cells has previously been demonstrated to depend on the breast cancer subtype [12]. In addition, the therapeutic regimens and sensitivity to drugs could enormously vary across breast cancer subtypes. Therefore, the predictive roles of CD8 and FOXP3 should be investigated in a relatively homogeneous patient cohorts, namely in only one subtype and in those treated with current standard regimen for the subtype.

As in the immune response by T cells, neoangiogenesis has been considered important in breast cancer [20–22]. Neoangiogenesis is frequently co-regulated with tumor-infiltrating lymphocytes and increased neoangiogenesis in responders to neoadjuvant aromatase inhibitor, as reported by increase in the vasohibin-1 positive ratio (VPR) derived from the CD31 to vasohibin-1 ratio [23]. Therefore, the evaluation of neoangiogenesis combined with CD8+ and FOXP3+ infiltration is required for assessing the prediction of pCR.

Here we studied the potential roles of CD8 and FOXP3 via immunohistochemical double-staining in predicting pCR of the patients, together with analyses of neoangiogenesis, basal and proliferation markers, in a relatively larger TNBC cohort than previous studies and in a cohort treated with the current standard regimen of NAC.

Materials and methods

Patients and sample selection

In this retrospective study, 110 unselected TNBC patients who received NAC at three Japanese institutions (Tohoku University Hospital, Sendai, Japan; Tohoku Kosai Hospital, Sendai, Japan; and Nahanishi Clinic, Okinawa, Japan) between 2009 and 2012 were consecutively included. All biopsy specimens prior to NAC were fixed in 10 % neutral-buffered formalin and embedded in paraffin. The three institutional review boards approved the protocol of this study, which was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients.

Immunohistochemical double-staining and quantification of CD8 and FOXP3

The formalin-fixed paraffin-embedded specimens were cut into 4 μm thick sections for immunohistochemistry. Briefly, the antigen retrieval of FOXP3 was performed by autoclaving, and the anti-FOXP3 antibody reaction (clone: 236A/E7, Abcam) was performed. Next, CD8 antigen retrieval was performed and the anti-CD8 antibody reaction (clone: C8/144B, Nichirei) was performed. After the procedure for the biotin-streptavidin reaction, Vector Blue® was used to visualize the binding of the anti-CD8 antibody (blue), in contrast to FOXP3 (brown) [24].

To quantify the infiltration of CD8+ or FOXP3+ T cells, four non-overlapping fields with high numbers of tumor-infiltrating lymphocytes on hematoxylin–eosin-stained glass slides were selected. In the same fields of double-staining with CD8 and FOXP3 as the above four fields, the number of CD8+ or FOXP3+ lymphocytes was counted under high power magnification (×400). The mean number of CD8+ or FOXP3+ lymphocytes per field was

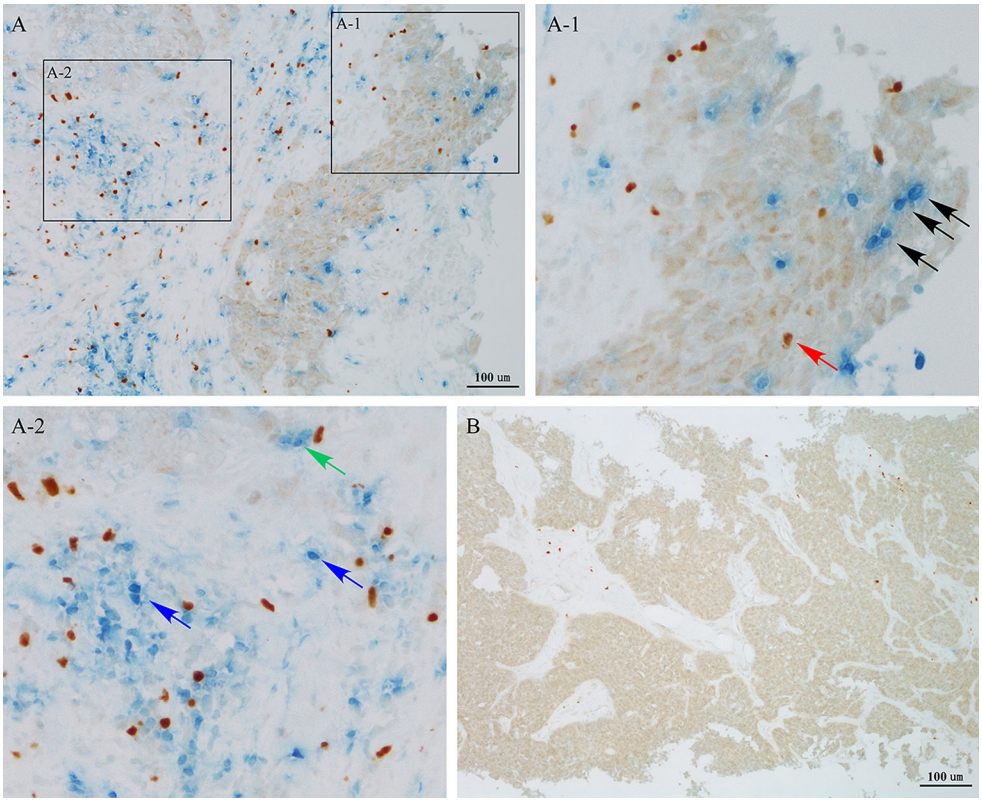

Fig. 1 Immunohistochemical double staining of tumor-infiltrating CD8 (*blue*) and FOXP3 (*brown*). The representative tumor tissue of high (**a**) and low infiltration (**b**) of CD8 and FOXP3. Tumor-infiltrating lymphocytes were counted in the three compartments of each tumor: the intra-tumoral compartment (*black arrow* points to CD8+ lymphocytes and *red arrow* points to CD8+ lymphocytes), adjacent stromal compartment (*green arrow* points to CD8+ lymphocytes) and distant stromal compartment (*blue arrow* points to CD8+ lymphocytes)

counted, and the ratio of CD8+ to FOXP3+ was calculated [17, 25]. These procedures were performed in the following three compartments of each tumor: the intra-tumoral compartment (within the tumor nests), the adjacent stromal compartment (the distance between the lymphocytes and tumor nest is less than or equal to the size of one tumor cell), and the distant stromal compartment (the distance between the lymphocytes and tumor nest is greater than the size of a single tumor cell) [17] (Fig. 1).

Immunohistochemistry for vasohibin-1 and CD31

We performed immunohistochemical staining for vasohibin-1 and CD31 on the biopsy specimens. Microvessels were identified as the lumen lined by endothelial cells stained with anti-CD31 antibody (code M0823, Dako). We counted the microvessels in one high power field (×200) after the areas with the greatest number of microvessels had been selected from low magnifications (×40 and ×100) [20, 21]. Vasohibin-1 signals were counted in the same hot spot and in the same field in which the highest number of anti-CD31+ vessels was identified. We defined the vasohibin-1-positive ratio (VPR) as the number of vasohibin-1-positive signals divided by the number of CD31-positive signals [26, 27].

Evaluation of ER, PgR, HER2, Ki-67, EGFR, and CK5/6

The ER and PgR statuses were evaluated by immunostaining using monoclonal antibodies (code 107925 and 102333, Roche Diagnostics), and nuclear staining of more than 1 % was considered positive. The HER2 status was evaluated by immunohistochemistry (HercepTest, code K5204, Dako) or by fluorescence in situ hybridization (the PathVysion Kit; Abbott). HER2 positivity was defined in accordance with the American Society of Clinical Oncology/College of American Pathologists Guideline [28]. The Ki-67 was determined with an anti-MIB-1 monoclonal antibody (code M7240, Dako) by counting 1,000 tumor cells in the hot spots [29, 30]. EGFR was interpreted as positive if the membranes of 10 % or more carcinoma cells were stained using monoclonal antibodies (code K1492, Dako). CK5/6 was interpreted as positive if 10 % or more

carcinoma cells showed monoclonal antibody binding in the cytoplasm (code M7237, Dako) [31, 32]. The basal-like type was defined as tumors expressing either EGFR or CK5/6. Two pathologists performed all of the pathological diagnoses and staining assessments of individual cases.

Clinical information and pathological response

We collected clinical information on TNBCs from the individual breast cancer databases of the three institutions. The pathological therapeutic response of the surgically resected tumor was evaluated after NAC. The surgical specimens were cut into 5 mm slices and processed with hematoxylin–eosin staining. A pCR was defined as the absence of all invasive cancer cells and lymph node metastasis, regardless of the presence or absence of non-invasive cancer cells.

Statistical analyses

All statistical analyses were performed using SAS software, JMP Pro 11 (Tokyo, Japan). Associations among variables were evaluated using Fisher's exact test or the χ^2 test. The Mann–Whitney U and Spearman's correlation tests were used to compare non-continuous parameters. Logistic regression analyses were performed for univariate and multivariate analyses to determine the independent variables for the prediction of pCR. All tests were two sided, and a P value of less than 0.05 was considered statistically significant.

Results

Clinicopathological factors and their association with CD8 and FOXP3 and the ratio of CD8+/FOXP3+

After immunohistochemical re-examination of ER, PgR and HER2 status, eight patients who presented with low ER status of between 1 and 10 % were excluded in this study. An evaluation could not be performed in fourteen patients because of little tumor specimens in the biopsy materials. Immunohistochemical analyses of CD8, FOXP3, vasohibin-1, CD31, EGFR, CK5/6, and Ki-67 and the CD8+/FOXP3+ ratio by the double-staining method were subsequently performed in the tumors from 88 TNBCs treated with NAC. Seventy-eight patients (89 %) were treated with NAC regimens containing both anthracyclines and taxanes, and almost all of the other patients were treated with anthracyclines. Twenty-six patients were diagnosed as having a pCR according to the detailed pathological examination, and the pCR rate was 29.5 % (26/88).

Representative images of the immunohistochemistry were presented in Fig. 1. The clinicopathological factors, including vasohibin-1, CD31 and VPR, were examined for the patients with high or low infiltration of CD8+ and FOXP3+ T cells, and the CD8+/FOXP3+ ratio was determined. The cut-offs of high or low infiltration were defined as the median number of infiltrating cells per field as follows: CD8, 86.75 (the numbers of infiltrating cells per field); FOXP3, 73.25 (the numbers of infiltrating cells per field) and CD8+/FOXP3+ ratio, 1.0 (Table 1). TNBCs with high CD8+/FOXP3+ ratios were significantly more frequent in the patients with premenopausal status ($P = 0.002$) and those diagnosed at an earlier age ($P = 0.003$). A significant inverse correlation was detected between the CD8+/FOXP3+ ratio and the VPR in these tumors ($P = 0.021$). There were no clinicopathological factors associated with TNBCs with a high infiltration of CD8+ and FOXP3+ T cells (Table 1).

Correlations between CD8, FOXP3, the CD8+/FOXP3+ ratio and pCR

We evaluated the infiltrating CD8+ and FOXP3+ lymphocytes and the CD8+/FOXP3+ ratio by double-immunostaining in the three compartments of each tumor. In this TNBC cohort, positive correlations were detected between CD8+ lymphocyte infiltration and FOXP3+ lymphocyte infiltration in the intra-tumoral ($r = 0.5583$, $P < 0.0001$), adjacent stromal ($r = 0.5834$, $P < 0.0001$) and distant stromal compartment ($r = 0.4803$, $P < 0.0001$; Fig. 2a–c). The statistically positive correlation was detected between the total CD8 and FOXP3 levels in the entire field, which represents the summation of CD8 and FOXP3 levels in these three compartments ($r = 0.4798$, $P < 0.0001$; Fig. 2d).

The TNBCs were classified into high and low groups for CD8, FOXP3 and the CD8+/FOXP3+ ratio using the cut-off values defined as the median number of infiltrating cells per field as described in Fig. 3. In the intra-tumoral compartment, the patients whose tumors had a high CD8 level or high CD8+/FOXP3+ ratio had a significantly higher pCR rate than those with a low CD8 level or low CD8+/FOXP3+ ratio (46 vs. 15 %, $P = 0.002$ and 52 vs. 19 %, $P = 0.003$, respectively; Fig. 3a, c). In the adjacent stromal compartment, the patients whose tumors had a high CD8 or high CD8+/FOXP3+ ratio also had a significantly higher pCR rate than those with weakly infiltrated tumors (49 vs. 13 %, $P < 0.001$ and 45 vs. 17 %, $P = 0.005$, respectively; Fig. 3a, c). The pCR rate in high CD8 tumors was not significantly different from that in low CD8 tumors (40 vs. 22 %, $P = 0.097$) but the patients whose tumors had a high CD8+/FOXP3+ ratio were associated with a significantly higher pCR rate than did those with weakly

Table 1 Clinicopathological factors and association with CD8, FOXP3, the ratio of CD8/FOXP3 in TNBC

	CD8[a]			FOXP3[b]			CD8/FOXP3[c]		
	High	Low	*P* value	High	Low	*P* value	High	Low	*P* value
Age (years)			0.198			0.668			0.003*
≤50	23	16		21	18		27	12	
>50	21	28		23	26		18	31	
Menopausal status			0.081			1.000			0.002*
Premenopausal	22	13		18	17		25	10	
Postmenopausal	22	31		26	27		20	33	
Tumor size			0.051			1.000			0.469
≤5.0 cm	37	28		32	33		35	30	
>5.0 cm	7	16		12	11		10	13	
Nodal status			1.000			0.783			0.784
Negative	8	8		7	9		9	7	
Positive	36	36		37	35		36	36	
Histological grade			0.087			0.087			1.000
I, II	16	25		16	25		21	20	
III	28	19		28	19		24	23	
Basal-like type			0.372			0.372			0.824
Basal-like	26	31		26	31		30	27	
Non basal-like	18	13		18	13		15	16	
Ki-67 LI (cut-off 57.5)			1.000			0.831			0.394
Low	22	22		21	23		25	19	
High	22	22		23	21		20	24	
Vasohibin-1 (cut-off 20)			0.199			1.000			0.134
Low	27	20		23	24		28	19	
High	17	24		21	20		17	24	
CD31 (cut-off 40)			0.670			0.201			0.831
Low	24	21		26	19		24	21	
High	20	23		18	25		21	22	
VPR (vasohibin-1/CD31; cut-off 0.6)			1.000			0.831			0.021*
Low	21	21		20	22		27	15	
High	23	23		24	22		18	28	

LI labeling index, *VPR* vasohibin-1 positive ratio

* The *P* value is significant

[a] The cut-off of CD8 infiltration is 86.75

[b] The cut-off of FOXP3 infiltration is 73.25

[c] The cut-off of CD8/FOXP3 ratio is 1.0

infiltrated tumors in the distant stromal compartment (45 vs. 12 %, $P = 0.001$; Fig. 3a, c). No differences were detected between tumors infiltrated with high levels of FOXP3+ lymphocytes and those infiltrated with low levels, in the intra-tumoral, adjacent stromal and distant stromal compartments (38 vs. 23 %, $P = 0.163$; 37 vs. 23 %, $P = 0.242$; and 30 vs. 29 %, $P = 0.816$, respectively; Fig. 3b). In the entire carcinoma areas, the patients whose TNBC tumors had a high total CD8 level or total CD8+/FOXP3+ ratio achieved significantly higher pCR rates than those with a low total CD8 level or total CD8+/FOXP3+ ratio (41 vs. 18 %, $P = 0.034$ and 44 vs. 14 %, $P = 0.002$, respectively; Fig. 3a, c).

Correlation between vasohibin-1, CD31 and pCR

The microvessel density was counted at the hot spot of CD31 staining, and the median was 39 (range 10–90) per field. Vasohibin-1 immunoreactivity was only detected in endothelial cells, and the median number of vasohibin-1-positive microvessels in the hot spot was 20 (range 6–60) per field. The median of VPR calculated was 0.618 (range

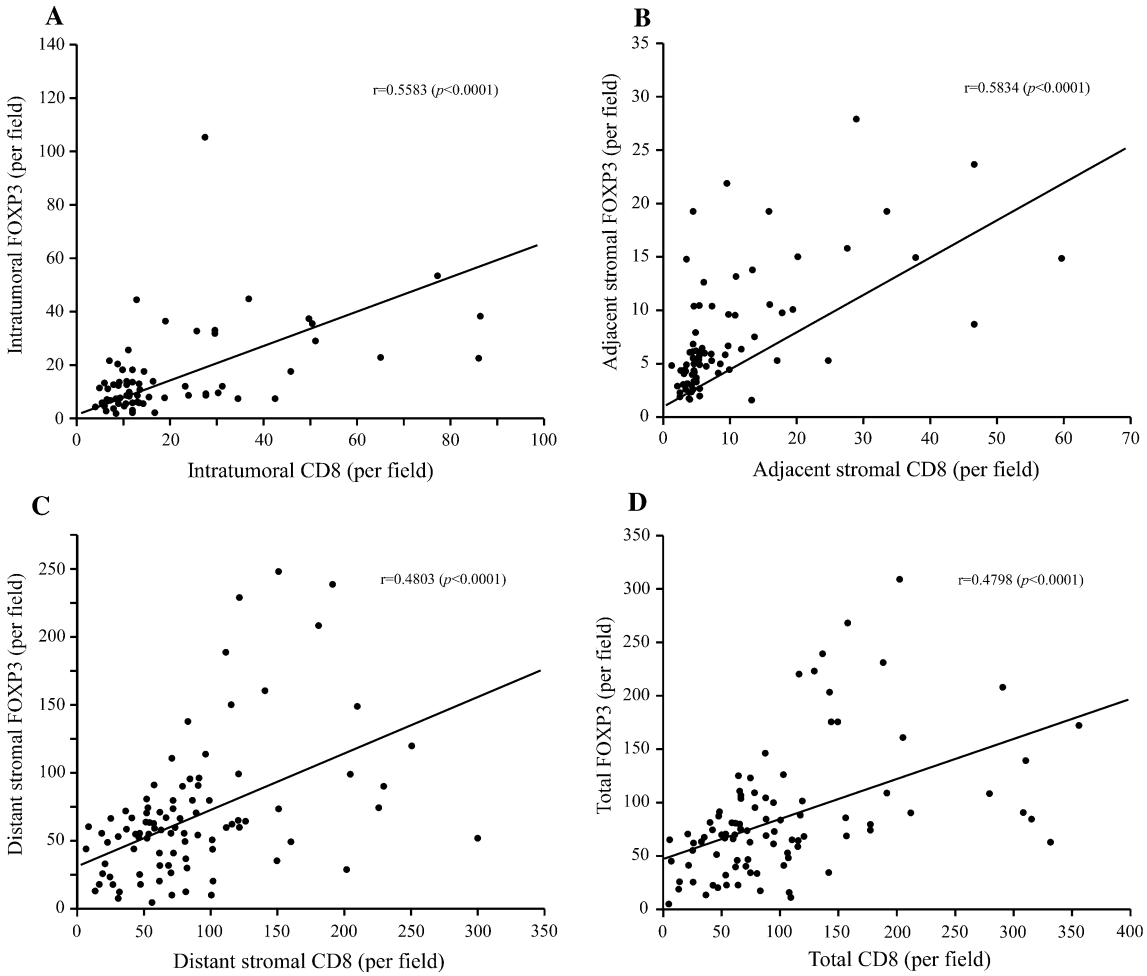

Fig. 2 The correlation diagrams of the infiltrating CD8+ and FOXP3+ lymphocytes in the compartments of each tumor: **a** the intra-tumoral compartment, **b** adjacent stromal compartment, **c** distant stromal compartment and **d** entire field. In this TNBC cohort, moderate positive correlations were observed between CD8+ lymphocyte infiltration and FOXP3+ lymphocyte infiltration in all of the compartments

0.152–0.917). Breast tumors were classified into high and low groups for vasohibin-1, CD31 and VPR, with the median. No significant differences in the vasohibin-1 or CD31 levels were detected between the pCR and non-pCR groups. However, low VPR tumors were significantly correlated with a higher pCR rate than high VPR tumors in the univariate analysis (odds ratio = 0.36, 95 % CI 0.14–0.92, $P = 0.031$; Table 2; Supplementary Figure).

Predictive value of the ratio of CD8+/FOXP3+ and the Ki-67 for pCR in multivariate analysis

The variables, including clinicopathological factors, CD8, FOXP3, vasohibin-1, CD31 and Ki-67, were investigated for their association with pCR after NAC, using multivariate analyses. Among these variables, earlier age at diagnosis ($P = 0.009$), pre-menopausal status ($P = 0.027$), smaller tumor size ($P = 0.004$) and a high Ki-67 ($P = 0.003$) were all significantly associated with pCR, as were a high CD8 level ($P = 0.013$), a high CD8+/FOXP3+ ratio ($P = 0.001$) and a low VPR level ($P = 0.031$; Table 2; Supplementary Figure). These significant variables and the suggestive variable "basal-like type" ($P = 0.075$) were all assessed in together to verify their predictive value for pCR in multivariate analysis. Results indicated that a high CD8+/FOXP3+ ratio was a markedly powerful predictor of pCR, with an odds ratio (OR) of 5.32 (95 % CI 1.62–19.98, $P = 0.005$). A high Ki-67 LI was also significantly associated with pCR (OR = 5.69, 95 % CI 1.83–20.24, $P = 0.002$; Table 2).

In addition, ROC curve analysis was performed for two independent factors: the CD8+/FOXP3+ ratio and the Ki-67. The area under the curve (AUC) was 0.708 for the CD8+/FOXP3+ ratio ($P = 0.012$) and 0.702 for Ki-67 ($P = 0.006$; Fig. 4). The sensitivity and specificity of the CD8+/FOXP3+ ratio and Ki-67 for pCR were summarized in Fig. 4. We also investigated the pCR rates of

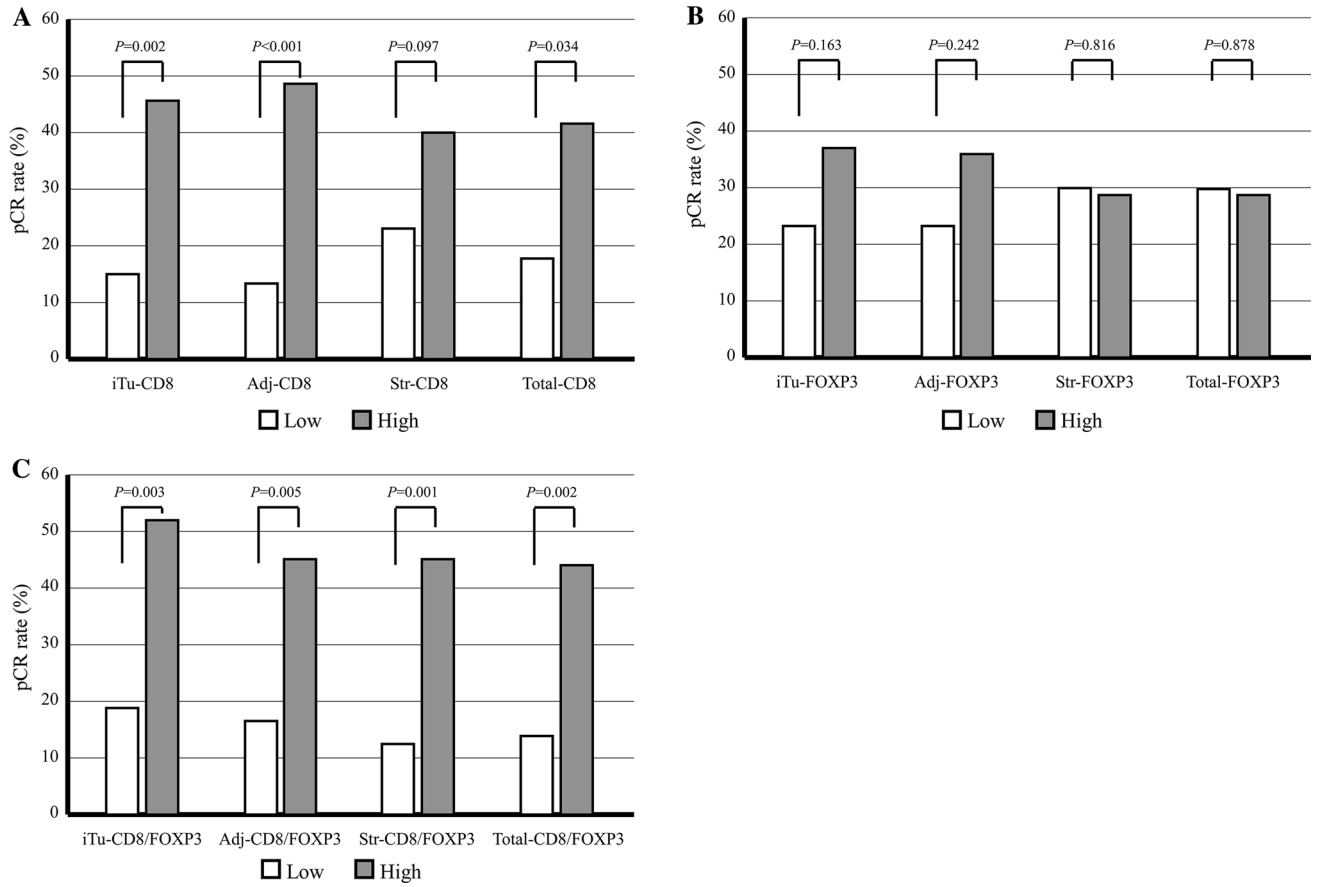

Fig. 3 Pathological complete response (pCR) rates between high and low groups for **a** CD8, **b** FOXP3 and the **c** CD8+/FOXP3+ ratio using the cut-offs of the each compartment was defined as the median number of infiltrating cells per field as follows: CD8, 4; FOXP3, 7 and CD8+/FOXP3+ ratio, 1 in the intra-tumoral (iTu) compartment: CD8, 4; FOXP3, 5 and CD8+/FOXP3+ ratio, 1 in the adjacent stromal (Adj) compartment: CD8, 70; FOXP3, 60 and CD8+/FOXP3+ ratio, 1 in the distant stromal (Str) compartment: CD8, 86.75; FOXP3, 73.25 and CD8+/FOXP3+ ratio, 1 in the entire field

TNBCs using the combination of the CD8+/FOXP3+ ratio and the Ki-67. Results demonstrated that TNBCs could be classified into several types that had extremely different pCR rates. The patients with breast tumors with a high CD8+/FOXP3+ ratio and a high Ki-67 achieved the highest pCR rate (70 %) following NAC. In contrast, the pCR rate for patients whose tumors had a low CD8+/FOXP3+ ratio and a low Ki-67 was only 5 % (Fig. 5). The pCR rate for patients whose tumors had a high CD8+/FOXP3+ ratio and a low Ki-67 and that had a low CD8+/FOXP3+ ratio and a high Ki-67 tumor were 24 and 21 %, respectively (Fig. 5).

Discussion

Results of this study indicated that a high CD8+/FOXP3+ ratio in biopsy specimens is an accurate predictor of pCR following NAC in TNBCs. Breast tumors with relatively high numbers of intra-tumoral CD8+ lymphocytes were reported to have favorable clinical outcomes [12–14] but the presence of FOXP3+ lymphocytes in breast tumors has been reported to be associated with paradoxically both reduced survival [12, 17] and improved survival [19]. Liu et al. reported that the density of intra-tumoral FOXP3+ infiltrates and the peritumoral CD8+/FOXP3+ ratio could represent independent prognostic factors correlated with the prognosis or clinical outcome of ER-positive or -negative breast cancer patients [12]. However, West et al. reported that intra-tumoral FOXP3+ lymphocytes were associated with robust anti-tumor immunity and a favorable prognosis in ER-negative breast cancer patients [19]. It is also important to note that the status of FOXP3+ was also by no means associated with breast cancer-specific survival after adjusting for known prognostic factors [14]. These discrepancies could be derived from the heterogeneity of the studied breast cancer patients. These studies were conducted for several breast cancer subtypes with different biological characteristics and treated with different therapeutic drugs; for example, endocrine therapy for

Table 2 Univariate and multivariate analyses of variables in the prediction for pCR in TNBC

	Univariate analysis			Multivariate analysis		
	OR	95 % CI	*P* value	OR	95 % CI	*P* value
Age (≤50 vs. 50<)	3.43	1.34–9.31	0.009*	1.66	0.11–8.75	0.706
Menopausal status (pre vs. post)	2.86	1.13–7.52	0.027*	1.42	0.09–11.48	0.797
Tumor size (≤5.0 vs. 5.0 cm<)	4.01	1.75–12.01	0.004*	2.91	0.47–12.1	0.137
Nodal status (pos vs. neg)	1.78	0.51–8.36	0.383			
Grade (III vs. I, II)	1.32	0.52–3.47	0.571			
Basal-like versus non-basal-like	2.60	0.92–8.64	0.075	3.37	0.91–13.54	0.062
Ki-67 LI (high vs. low)	4.46	1.62–13.58	0.003*	5.69	1.83–20.24	0.002*
CD8 (high vs low)	3.52	1.29–10.42	0.013*	1.94	0.51–7.73	0.329
FOXP3 (high vs. low)	0.79	0.31–2.04	0.632			
CD8/FOXP3 (high vs. low)	4.93	1.82–15.09	0.001*	5.32	1.62–19.98	0.005*
Vasohibin-1 (high vs. low)	0.78	0.31–1.97	0.601			
CD31 (high vs. low)	0.91	0.09–9.06	0.939			
VPR (vasohibin-1/CD31; high vs. low)	0.36	0.14–0.92	0.031*	0.72	0.22–2.36	0.582

A logistic regression analyses were performed for univariate and multivariate analyses

OR odds ratio, *95 % CI* 95 % confidence interval, *LI* labeling index, *VPR* vasohibin-1 positive ratio

* The *P* value is significant

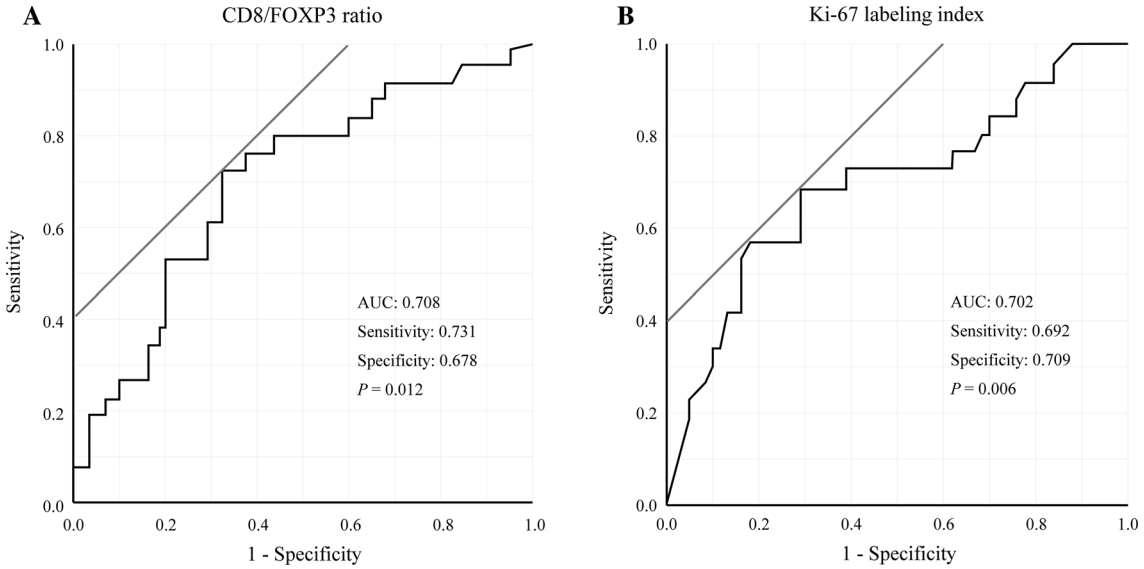

Fig. 4 ROC curve analysis for two independent factors: the CD8+/FOXP3+ ratio and the Ki-67 labeling index. *AUC* area under the curve

ER-positive breast cancers and anti-HER2 therapy for HER2-positive breast cancers, and the sensitivities to the drugs and the prognosis could markedly vary among different subtypes. The possible association between the prognostic significance of infiltrating lymphocytes and the breast cancer subtype have been explored by several investigators [33, 34]. Significant differences in the status of FOXP3+ infiltration and the CD8+/FOXP3+ ratio have been reported in the five molecular subtypes of breast cancer [12]. Different types of immune responses in different subtypes of breast cancer could, therefore, explain the contradictory results. Therefore, we conducted an immunohistochemical study of CD8 and FOXP3 only in the TNBC subtype, generally treated with current standard regimens containing anthracyclines and taxanes.

Biopsy specimens in the neoadjuvant setting that were not affected by treatment can be used to estimate the efficacy of drugs. This design is possible for TNBC, for which pCR after NAC has been validated as a reliable surrogate marker for survival. To the best of our knowledge, two studies evaluated the roles of CD8 and FOXP3 in biopsy specimens to predict pCR following NAC [18, 35]. Ladoire et al. reported that significantly decreased levels of FOXP3+ lymphocytes after NAC were more likely to generate strong anti-tumor immunity and achieved a high pCR rate in breast tumors harboring high levels of CD8+ lymphocytes remaining unchanged [35]. Oda et al. reported that FOXP3+ lymphocyte and the Ki-67 were both independent predictors of pCR [18]. However, these studies evaluated all breast cancer subtypes, including

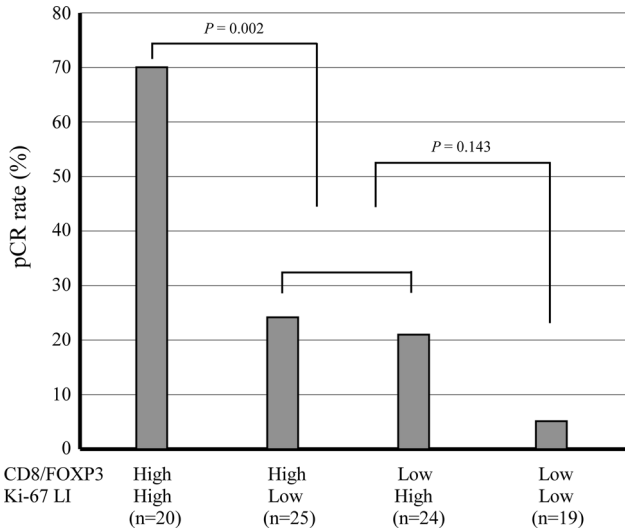

Fig. 5 Pathological complete response (pCR) rates of TNBC tumors using the combination of the CD8+/FOXP3+ ratio and the Ki-67 labeling index (LI)

approximately 20–30 TNBCs. In our present study, the predictive roles of status of intra-tumoral CD8 and FOXP3, together with neoangiogenesis, basal and proliferation markers, in biopsy specimens was investigated in a relatively large number of TNBC samples for NAC cohorts (slightly fewer than 100 patients). We set the cut-off value of high or low Ki-67 as the median number. For the reason, the statistical analyses could not be performed using the cut-off of 20 % that is recommended at the 13th St Gallen International Breast Cancer Conference 2013 because there were only eight cases (9 %) classified into low Ki-67 group in our cohort of aggressive triple-negative breast cancer [36]. Results of our present study did indicate that a high CD8+/FOXP3+ ratio and a high Ki-67 were significantly associated with pCR in a multivariate analysis. In addition, the patients whose breast tumors had a high CD8+/FOXP3+ ratio and a high Ki-67 achieved a 70 % pCR rate; therefore, these markers could be clinically valuable predictors of the response to NAC.

A few studies have recently examined the localization of CD8+ and FOXP3+ infiltrating lymphocytes to evaluate the microenvironment and expansion of lymphocytes in breast tumors [17, 19]. Mahmoud et al. reported that an association with adverse clinical outcome was detected in intra-tumoral and tumor-adjacent stromal FOXP3+ lymphocytes [17]. West et al. reported that the number of FOXP3+ lymphocytes was correlated with the number of CD8+ lymphocytes regardless of whether CD8+ cells were present in the tumor or stroma and that these levels were associated with prolonged survival [19]. In the present study, we performed the assessments of the localization using the immunohistochemical double-staining method for CD8 and FOXP3, allowing evaluation of the balance between CD8+ and FOXP3+ infiltrating lymphocytes in the same field of the same section. Statistically positive correlations were detected between CD8+ and FOXP3+ in each of the three compartments. Therefore, regardless of the localization in breast tumors, a high CD8 level or a high CD8+/FOXP3+ ratio was associated with a significantly higher pCR rate, and there was no difference between tumors that were infiltrated with high numbers and low numbers of FOXP3+ T cells. Based on these data, the same degree of anti-tumor immune response could be induced in each region.

We also investigated the VPR which was reported to be associated with neovascularization and breast cancer survival [26, 27]. The inverse correlation between the CD8+/FOXP3+ ratio and the VPR was detected in our TNBCs. Neoangiogenesis factors, including vasohibin-1, which is a negative feedback suppressor induced by vascular endothelial growth factor (VEGF)-A, were previously reported to suppress tumor-infiltrating lymphocytes and anti-tumor immune responses [37, 38]. Results of our study demonstrating the inverse correlation between the CD8+/FOXP3+ ratio and the VPR were of interest. The tumors with a low VPR were significantly correlated with a higher pCR than those with a high VPR in the univariate analysis and no significant association of VPR with pCR was detected in the multivariate analysis.

In summary, results of our present study demonstrated that both CD8+/FOXP3+ ratio and the Ki-67 were independent predictors of pCR in TNBC patients treated with the current standard regimens of NAC. By examining both the CD8+/FOXP3+ ratio and the Ki-67, which are conveniently available in the great majority of diagnostic laboratories, we could predict both the subgroup expected to have a high pCR rate and subsequently favorable prognosis and the subgroup that should be treated with new investigational drugs. However, results in our present retrospective study need to be validated in a prospective study.

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Conflict of interest None.

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