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^{18}F-fluorodeoxyglucose Positron Emission Tomography/Computed Tomography Predicts Tumor Immune Microenvironment Function in Early Triple-negative Breast Cancer

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Abstract. *Background/Aim: The maximum standardized uptake value (SUVmax) obtained using ^{18}F-fluorodeoxyglucose (FDG) positron emission tomography (PET)/computed tomography (CT) is presumed to indicate tumor and active immune cells in the tumor immune microenvironment (TIME) based on their glycolysis activity. Therefore, this study investigated whether the metabolic parameter SUVmax could provide information regarding TIME in triple-negative breast cancer (TNBC) patients. Patients and Methods: Fifty-four patients with TNBC underwent FDG PET/CT before neoadjuvant chemotherapy. Pretreatment biopsy specimens were pathologically evaluated. Expression statuses of CD8, forkhead box P3 (FOXP3), programmed cell death-1 (PD-1), and programmed cell death-ligand 1 (PD-L1) were assessed by immunohistochemistry. The relationships between immunological factors, including the tumor-infiltrating lymphocyte (TIL) grade and SUVmax or pathological complete response (pCR), were investigated. Results: CD8, FOXP3, PD-1, and PD-L1 were high in 15 (27.8%), 39 (72.2%), 18 (33.3%), and 26 (48.2%) patients, respectively. SUVmax was significantly correlated with tumor size, Ki-67 labeling index, and CD8/FOXP3 ratio. Multiple linear regression analysis indicated that tumor size and the CD8/FOXP3 ratio predicted SUVmax. Seventeen patients (31.5%) achieved a pCR; TILs, the CD8/FOXP3 ratio, PD-1, and PD-L1 were significantly correlated with pCR rate. Multivariate analysis indicated that the CD8/FOXP3 ratio was the only independent predictive factor for pCR. Conclusion: SUVmax could provide metabolic information regarding TIME for TNBC patients and might be beneficial for formulating a treatment strategy and predicting pCR after neoadjuvant chemotherapy.*

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Breast cancer consists of tumor cells and tumor immune microenvironment (TIME) components; the relationship between the immune system and breast cancer is complex (1, 2). A recent study showed that tumor-infiltrating lymphocytes (TILs), one of the major components of TIME, are substantially related to tumor progression and the response to chemotherapy administered for breast cancer (3, 4). In triple-negative breast cancer (TNBC), TILs have been reported as favorable prognostic factors and predictive biomarkers for a pathological complete response (pCR) following neoadjuvant chemotherapy; therefore, there is a need for evaluation of the tumor microenvironment, including TILs by pre-treatment findings (3-10). However, there is heterogeneous TIL expression and distribution in breast cancer tissues, which renders the accurate evaluation of TIME (using a few core needle biopsy specimens) difficult, especially the non-brisk pattern of TIL expression. Accordingly, accurate TIME evaluation requires an overall assessment of the tumor and its microenvironment.

Since ^{18}F-fluorodeoxyglucose (FDG) is transported into cells *via* active glucose metabolism, the maximum standardized uptake value (SUVmax) obtained using FDG positron emission tomography (PET)/computed tomography (CT) is presumed to be related to tumor cells and TILs that constitute the TIME. A

previous study reported that the SUVmax obtained using FDG PET/CT was related to the tumor size, axillary lymph node status, tumor growth potential, such as Ki-67 labeling index, systemic inflammation, and TIL concentration associated with invasive breast cancers (11-14). There are two types of TILs: functional TILs, which have immune functions, such as cytotoxic T lymphocytes (CTLs); and regulatory T (T-reg) cells and bystander TILs, which do not have immune functions (15). CD8 and forkhead box P3 (FOXP3) are immune-functional markers of TILs, and programmed cell death-1 (PD-1) is expressed on TILs activated in TIME (16, 17). Programmed cell death-ligand 1 (PD-L1) is a biomarker of immune checkpoint inhibitors correlated with the response to neoadjuvant chemotherapy for breast cancer (18-20). In particular, a high CD8/FOXP3 ratio has been linked to a high pCR rate after neoadjuvant chemotherapy and a favorable prognosis for patients with TNBC (21, 22). The correlation between the SUVmax obtained using FDG PET/CT and TIME factors has been associated with TILs and FOXP3 in gastric cancer; whereas, in clear renal cell carcinoma, this correlation has been associated with TILs. Moreover, in non-small-cell lung cancer, this correlation has been associated with TILs, CD8, FOXP3, PD-1, and PD-L1 (23-25). Although the association between the SUVmax obtained using FDG PET/CT and PD-L1 has been reported for breast cancer, other TIME factors have not been extensively studied (26). Additionally, previous studies did not adjust for clinicopathological factors affecting FDG uptake; therefore, their results might not adequately reflect immunological information regarding TIME.

We hypothesized that the FDG uptake on FDG PET/CT reflects the biology of tumor cells and the functionality of immunological factors in TIME with TNBC. Therefore, we investigated the relationship between SUVmax and immunological features of TIME. Additionally, the correlation between immunological factors and pCR was assessed in patients with TNBC.

Patients and Methods

Study population. This study involved 55 Japanese patients with primary TNBC who received neoadjuvant chemotherapy and underwent pre-treatment FDG PET/CT between August 2008 and May 2019 at Hiroshima University Hospital. All patients were diagnosed by core needle biopsy before neoadjuvant chemotherapy, an anthracycline-based and taxane-based chemotherapy. One patient was excluded because of an insufficient sample volume for immunostaining; finally, 54 patients were retrospectively assessed. The institutional review board approved this study (E-559). All procedures performed during studies involving human participants followed the institutional and national research committee's ethical standards, the 1964 Helsinki Declaration, and its later amendments or comparable ethical standards. Due to the retrospective nature of this study's design, formal consent was not required.

Pathological assessment and immunohistochemistry. The pathological evaluation was performed using pre-treatment biopsy specimens, excluding the assessment of pathological responses in the surgical specimens. Estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 were assessed using the American Society of Clinical Oncology/College of American Pathologists Guidelines (27, 28). Following the recommendations of the international TILs working group, two pathologists determined the proportion of stromal TILs, while the remaining clinical information was blinded. Moreover, patients were categorized into high and low groups using a cut-off value of 50% (29). A pCR was defined as no residual carcinoma at the primary site and regional lymph nodes (ypT0N0).

The following antibodies were used to assess the TIL subsets: anti-CD8 antibody (SP57, prediluted; Roche, Basel, Switzerland); anti-FOXP3 antibody (D2W8E, 1:50 dilution; Cell Signaling Technology, Danvers, MA, USA); and anti-PD-1 antibody (NAT105, prediluted; Cell Marque, Rocklin, CA, USA). High-power fields were randomly chosen for cells counted manually in 10 stromal compartments per sample, and the median was obtained in each case. According to the median number of positive cells infiltrating stromal compartments, these markers were subdivided into high and low groups. Anti-PD-L1 antibody (28-8, 1:400 dilution; Abcam, Cambridge, UK) was used to perform immunohistochemistry for tumor cell membrane staining. Expression was characterized according to the combined positive score, which was defined as the number of PD-L1-positive cells (tumor cells, lymphocytes, and macrophages) divided by the total number of tumor cells multiplied by 100; specimens with a combined positive score of 1 or more were considered PD-L1-high (30).

FDG PET/CT examination. Patients were told to fast for at least 4 h before intravenous injection of FDG (3-3.7 MBq/kg of body weight). PET/CT scanning was performed 1 h after FDG administration using a Discovery ST16 PET/CT scanner (GE Healthcare, Little Chalfont, UK). PET/CT were performed during normal tidal breathing. Low-dose non-enhanced CT images (slice thickness, 3 to 4 mm) for attenuation correction and localization of lesions identified using PET were obtained from the head to the pelvic floor based on a standard protocol. Immediately after CT, the identical axial field of view (FOV) (154 mm) was scanned using PET for 2-3 min per table position. The obtained data were reconstructed as 128×128 matrix images (pixel size, 4.7×3.25 mm) using Fourier rebinning and ordered subset expectation maximization algorithms.

PET image evaluation and quantification of SUVmax were performed using the Xeleris workstation version 1.1452 (GE Healthcare, Little Chalfont, UK). Regions of interest were first delineated within the primary tumor on attenuation-corrected FDG PET images and the ipsilateral normal breast tissue for the background uptake; then, SUVmax was measured. A nuclear medicine radiologist and a breast cancer specialist read all PET images.

Statistical analysis. The summarized data are presented as the median and interquartile range (IQR) for continuous variables and as number (%) for categorical variables. Frequencies were compared using Fisher's exact test for categorical variables, and correlation analyses were performed using Spearman's rank correlation coefficients. A linear regression analysis was performed to predict SUVmax; a logistic regression analysis was also performed to

predict pCR. The cut-off for the Ki-67 labeling index was defined based on the median value. Statistical significance was set at a *p*-value of <0.05. All statistical analyses were performed using JMP 14 (SAS Institute Inc., Cary, NC, USA).

Results

Patient characteristics. The characteristics of the 54 patients are presented in Table I. The median age was 56.6 years; all tumors were infiltrating duct carcinomas unless otherwise specified. The median SUVmax was 7.0, and 15 (27.8%) patients were classified as TIL-high. Moreover, 17 tumors (31.5%) achieved a pCR.

Immunohistochemistry. Figure 1 shows representative images of the TILs and immunohistochemical staining of CD8, FOXP3, PD-1, and PD-L1. The median numbers of CD8-positive, FOXP3-positive, and PD-1-positive cells were 132.5 (78.8-207.3), 28.0 (15.0-38.0), and 31.0 (12.0-56.5), respectively. Expression of CD8, FOXP3, PD-1, and PD-L1 was high in 15 (27.8%), 39 (72.2%), 18 (33.3%), and 26 (48.2%) patients, respectively.

Correlation with SUVmax and TIL subsets. SUVmax was significantly correlated with the tumor size (r=0.392; p=0.003), Ki-67 labeling index (r=0.293; p=0.043), and CD8/FOXP3 ratio (r=0.324; p=0.017) (Figure 2). Multiple linear regression analysis revealed that the tumor size and CD8/FOXP3 ratio were significant factors influencing SUVmax (p<0.001 and p=0.045, respectively) (Table II). However, the Ki-67 labeling index, PD-1 expression, and PD-L1 expression were unrelated to SUVmax.

Figure 3 shows the representative FDG PET/CT images and pathological findings according to the CD8/FOXP3 ratio in breast cancers with similar tumor biology. When the CD8/FOXP3 ratio was high, the SUVmax was also high; thus, a pCR was achieved (Figure 3A). However, when the CD8/FOXP3 ratio was low, the SUVmax was also low; hence, a pCR was not achieved (Figure 3B).

Relationship between the pCR rate and TIL subsets. According to Fisher's exact test, the pCR rate was significantly associated with high TILs [odds ratio (OR)=5.81; 95% confidence interval (CI)=1.60-21.2; p=0.009], CD8/FOXP3 ratio (OR=17.7; 95%CI=3.46-90.9; p<0.001), PD-1 expression (OR=7.86; 95%CI=2.16-28.6; p=0.002), and PD-L1 expression (OR=6.00; 95%CI=1.62-22.2; p=0.008) (Figure 4). Multivariate logistic regression analysis revealed that the CD8/FOXP3 ratio was an independent predictor of pCR (OR=32.2; 95%CI=2.26-458.2; p=0.010), whereas tumor size (≥T2), Ki-67 labeling index, PD-1 expression, and PD-L1 expression were not associated with pCR (Table III).

Table I. *Patient characteristics*.

Characteristic	Number (%)
Age (years), median (range)	56.6 (22-76)
T status	
1	17 (31.5)
2	29 (53.7)
3	3 (5.6)
4	5 (9.2)
N status	
0	20 (37.0)
1	24 (44.4)
2	1 (1.9)
3	9 (16.7)
Nuclear grade	
1	4 (8.9)
2	7 (15.6)
3	34 (75.5)
Ki-67 labeling index (%), median (IQR)	92.5 (66.5-99.0)
TILs	
Low	39 (72.2)
High	15 (27.8)
Pathological response	
pCR	17 (31.5)
No pCR	37 (68.5)
SUVmax, median (IQR)	7.0 (4.5-9.6)

HER2: Human epidermal growth factor receptor 2; IQR: interquartile range; pCR: pathological complete response; SUVmax: maximum standardized uptake value; TILs: tumor-infiltrating lymphocytes; T1: tumor ≤2 cm; T2: tumor >2-5 cm; T3: tumor >5 cm; T4: tumor extension to the chest wall or skin.

Discussion

This study demonstrated that the SUVmax obtained using FDG PET/CT was related to the CD8/FOXP3 ratio and represented the functionality of the TIME. To the best of our knowledge, this is the first study to consider the relationship between FDG PET/CT and the metabolic activity of the TIME factors in TNBC after adjusting for tumor factors.

TNBC is an aggressive biological subtype associated with poor prognosis and a high risk of early recurrence (31, 32). Predicting pCR after neoadjuvant chemotherapy is essential because pCR is a surrogate marker of prognosis in TNBC (9, 10), a breast cancer subtype that contains the most abundant TILs (3). Previous randomized trials have reported that the abundance of TILs leads to a better prognosis; furthermore, they have indicated the therapeutic effects of neoadjuvant chemotherapy on TNBC due to its high tumor immune cytolytic activity (5-8).

In TIME, TILs include CD8-positive T cells, FOXP3-positive T cells, natural killer cells, dendritic cells, and macrophages. There are various regulatory cell groups in the tumor stroma in addition to lymphocytes, such as bone marrow-derived inhibitory cells, tumor-associated macrophages, cancer-

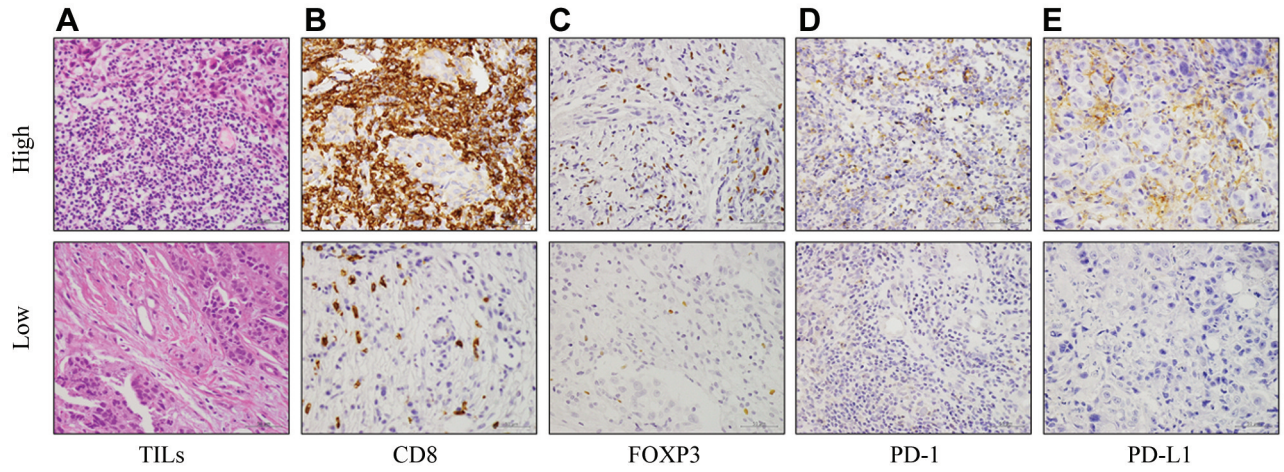

Figure 1. *Representative images of tumor-infiltrating lymphocytes (TILs) (A) and immunohistochemical staining of CD8 (B), forkhead box P3 (FOXP3) (C), programmed cell death-1 (PD-1) (D), and programmed death-ligand 1 (PD-L1) (E).*

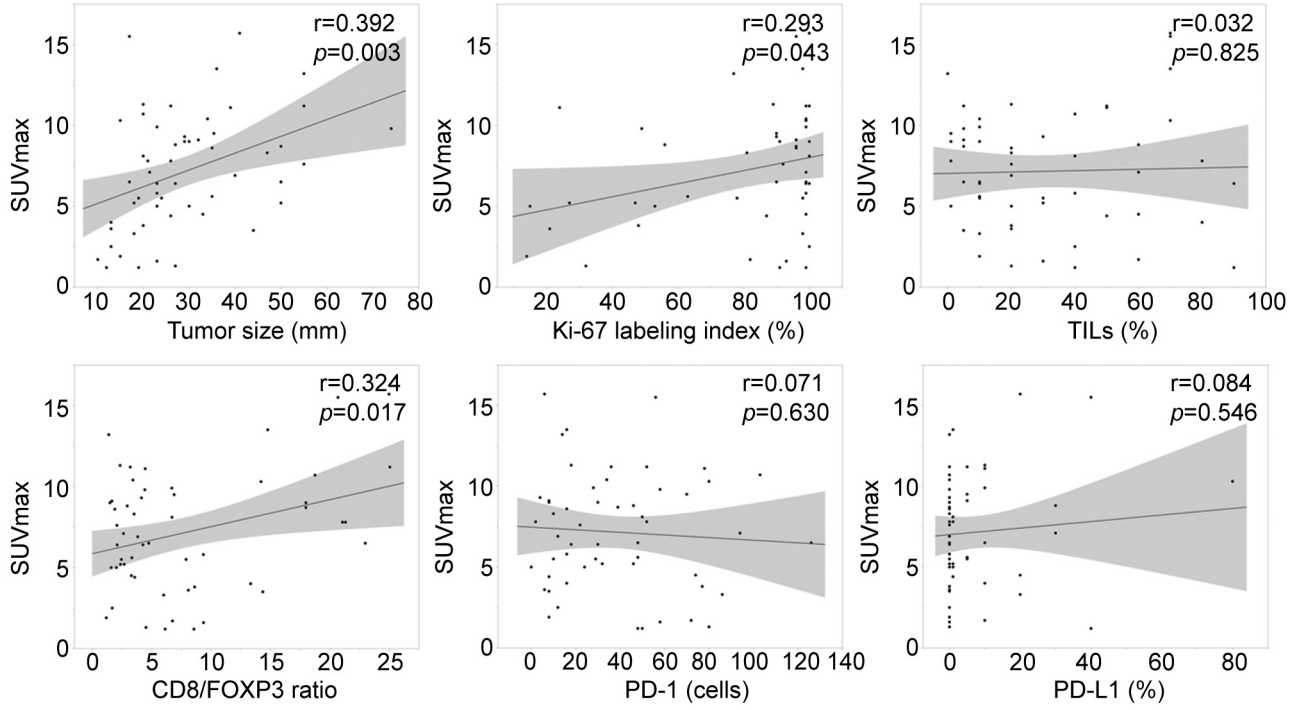

Figure 2. *Correlation of the maximum standardized uptake value (SUVmax) obtained using ^{18}F-fluorodeoxyglucose positron emission tomography/computed tomography with tumor factors and tumor-infiltrating lymphocyte (TIL) subsets. FOXP3: Forkhead box P3; PD-1: programmed cell death-1; PD-L1: programmed death-ligand 1.*

associated fibroblasts, and mesenchymal stem cells (4, 17). In breast cancer, CD8-positive T cells, which are the primary components of TILs, have a critical role in the antitumor immune response. CD8-positive T cells, the so-called CTLs, produce interferon-gamma (IFN-γ) to attack cancer cells (1, 33). Recent studies have reported that CTLs are associated with high pCR rates following neoadjuvant chemotherapy and better survival in TNBC (34). FOXP3-positive T cells, the so-called T-reg cells, act in an immune-suppressive manner against tumors (16). They constitutively express PD-1 and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and suppress the action of effector T cells and dendritic cells (35). Furthermore,

Table II. *Single and multiple linear regression analyses of factors influencing the SUVmax.*

Factors	Single linear regression analysis				Multiple linear regression analysis			
	Regression coefficient	Standard error	t	*p*-Value	Regression coefficient	Standard error	t	*p*-Value
Intercept					0.858	1.82	0.47	0.640
Tumor size (mm)	0.104	0.034	3.07	0.003	0.132	0.034	3.93	<0.001
Ki-67 labeling index (%)	0.040	0.019	2.08	0.043	0.026	0.017	1.51	0.139
CD8/FOXP3 ratio	0.166	0.067	2.47	0.017	0.149	0.072	2.06	0.045
PD-1	–0.008	0.017	–0.49	0.630	–0.029	0.017	–1.76	0.085
PD-L1	0.020	0.033	0.61	0.546	0.059	0.033	1.75	0.087

FOXP3: Forkhead box P3; PD-1: programmed cell death-1; PD-L1: programmed death-ligand 1; SUVmax: maximum standardized uptake value.

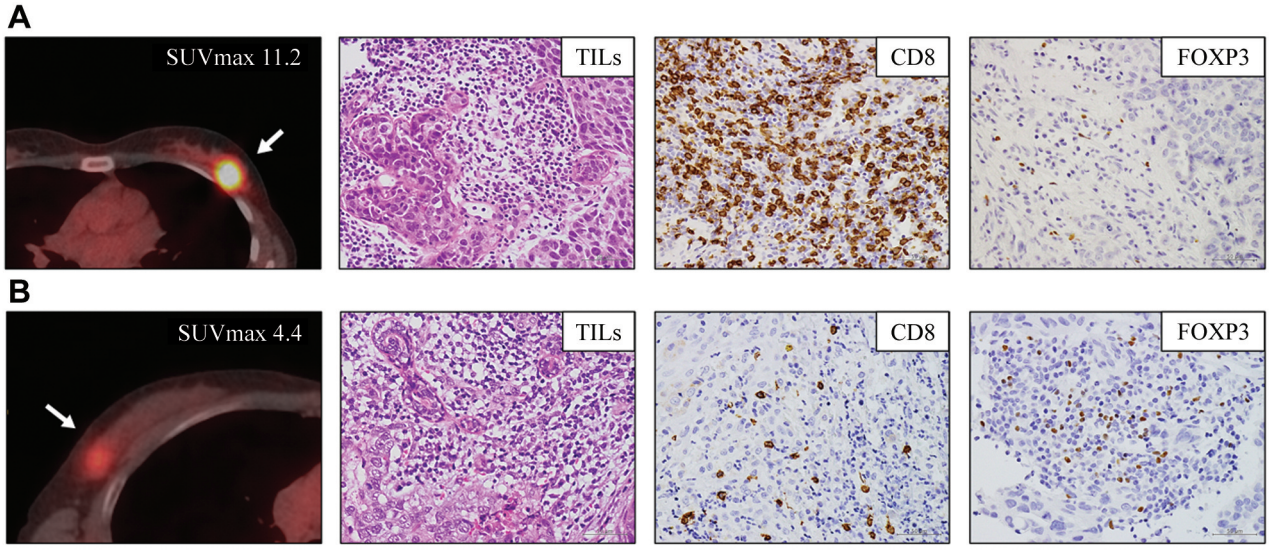

Figure 3. *Representative fluorodeoxyglucose positron emission tomography/computed tomography images, hematoxylin, and eosin staining, and immunohistochemical findings of cases with similar tumor biological features. (A) Infiltrating duct carcinoma not otherwise specified with a tumor size of 26 mm, a nuclear grade of 3, Ki-67 labeling index of 100%, maximum standardized uptake value (SUVmax) of 11.2, 50% tumor-infiltrating lymphocytes (TILs), and CD8/FOXP3 ratio of 25.1. A pathological complete response (pCR) was obtained. (B) Infiltrating duct carcinoma not otherwise specified with a tumor size of 26 mm, nuclear grade of 3, Ki-67 labeling index of 87%, SUVmax of 4.4, 50% TILs, and CD8/FOXP3 ratio of 2.6. A pCR was not obtained. Arrows point to the primary breast tumors.*

T-reg cells produce cytotoxic substances (perforin and granzyme) and suppressive cytokines (interleukin-10 and transforming growth factor-β) that suppress CTLs (36). Since CTLs and T-reg cells have paradoxical effects on tumor microenvironmental immunity, the CD8/FOXP3 ratio is considered a reliable biomarker for predicting neoadjuvant chemotherapy's prognosis and effects. In particular, it has been reported that a high CD8/FOXP3 ratio is related to improved disease-free survival, overall survival, and pCR rates with TNBC (21, 22, 34). Our findings also showed a correlation between the CD8/FOXP3 ratio and pCR rate. Previously, we reported that TIL scoring based on FDG PET/CT was related to pCR (37), and scoring the CD8/FOXP3 ratio might predict a pCR more accurately. PD-L1 is expressed on cancer cells and tumor-infiltrating macrophages by IFN-γ and suppresses VEGF expression and T-cell activity *via* the PD-1/PD-L1 pathway (38). Immune checkpoint inhibitors that block the PD-1/PD-L1 pathway have been demonstrated to improve progression-free survival and the pCR rate following neoadjuvant chemotherapy administered for TNBC (30, 39, 40).

Previous studies have reported that the SUVmax obtained using FDG PET/CT is related to clinicopathological tumor factors, such as the tumor size, nuclear grade, and Ki-67 labeling index (41). However, SUVmax is different for each

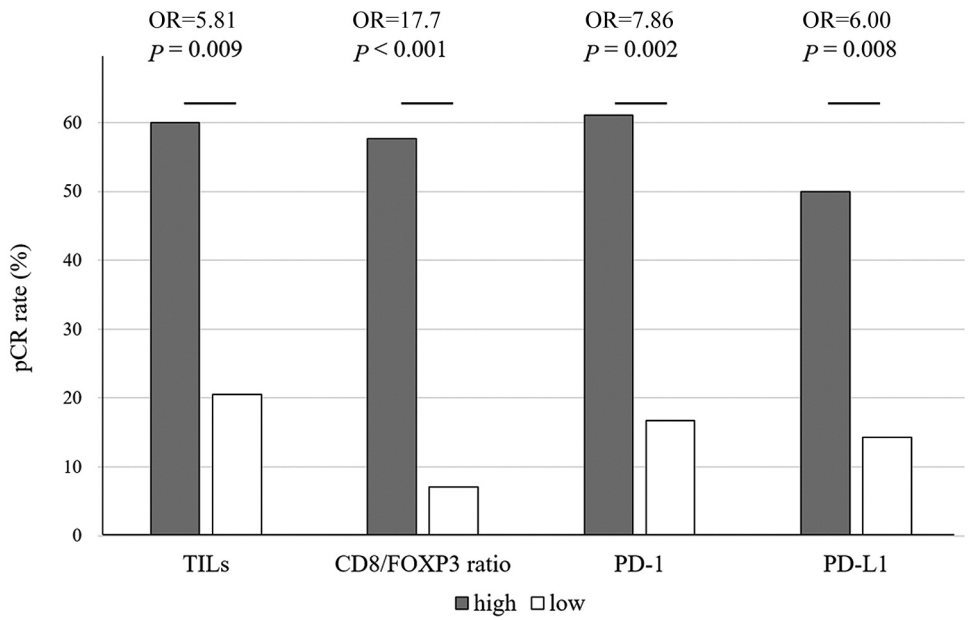

Figure 4. *Relationship between the tumor-infiltrating lymphocyte (TIL) subsets and pathological complete response (pCR) rate. FOXP3: Forkhead box P3; OR: odds ratio; PD-1: programmed cell death-1; PD-L1: programmed death-ligand 1.*

Table III. *Univariate and multivariate logistic regression analyses of factors predicting pCR.*

Factors	Univariate analysis		Multivariate analysis	
	OR (95%CI)	*p*-Value	OR (95%CI)	*p*-Value
T2-4	0.36 (0.11-1.22)	0.100	8.30 (0.42-165.8)	0.166
Ki-67 labeling index_high	2.28 (0.63-8.25)	0.209	0.82 (0.12-5.38)	0.834
CD8/FOXP3 ratio_high	17.7 (3.45-90.9)	<0.001	32.2 (2.26-458.2)	0.010
PD-1_high	7.86 (2.16-28.6)	0.002	8.05 (0.69-94.5)	0.097
PD-L1_high	6.00 (1.62-22.2)	0.007	5.67 (0.78-41.2)	0.086

CI: Confidence interval; FOXP3: forkhead box P3; OR: odds ratio; pCR: pathological complete response; PD-1: programmed cell death-1; PD-L1: programmed death-ligand 1.

tumor, even in those with similar tumor biology, and could be influenced by other factors. Using surgical specimens of early-stage breast cancer, we demonstrated that TILs influenced SUVmax after adjusting for tumor factors (11). Other studies have also reported an association between SUVmax and tumor microenvironment factors (23, 24, 26). However, these analyses did not adjust for tumor factors influencing FDG uptake, and the correlation between SUVmax and TIL subsets remains unclear.

The relationship between SUVmax and TILs is explained by glucose metabolism in the tumor microenvironment. The TIME regulates the tumor metabolism by identifying the interplay of the TIME components depending on the tumor's metabolic activity (42). In the tumor area, tumor and activated immune cells increase glucose metabolism and express glucose transporter 1 (Glut1) (43, 44). Tumor cells and TILs compete for glucose, and the energy balance establishes the tumor microenvironment because of metabolic competition (45, 46). FDG PET/CT is a modality that visualizes the metabolic status of glucose and is expected to detect activated TILs.

This study had some limitations. First, this study involved a small cohort from a single institution and had a retrospective design. Second, TIL subsets were evaluated using biopsy specimens because the patients received neoadjuvant chemotherapy. Moreover, it is difficult to

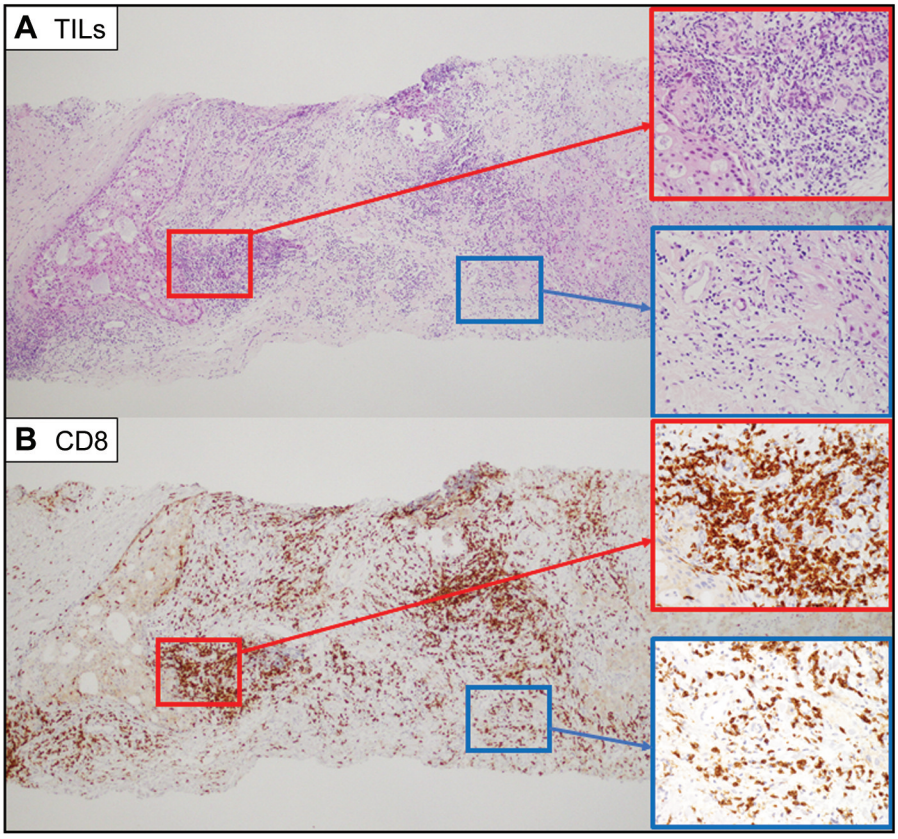

Figure 5. *Representative heterogeneous images of tumor-infiltrating lymphocytes (TILs) (A) and immunohistochemical staining of CD8 (B).*

accurately classify the pattern of TIL invasion due to the heterogeneous TIL expression and distribution in tissues (Figure 5). Thus, the findings of this study may differ from those of evaluations of whole tumors. Third, the evaluation method used for TIME factors is not generalized, and the cut-off values are specific to this study.

In conclusion, the SUVmax obtained using FDG PET/CT reflected the CD8/FOXP3 ratio in early-stage TNBC. Consequently, this study suggests that FDG PET/CT may provide active immunological features in the TIME without additional immunostaining and might help predict the effects of neoadjuvant chemotherapy in TNBC; thus, further research is needed to validate this association.

Conflicts of Interest

The Authors have no conflicts of interest in relation to this study.

Authors' Contributions

Yuri Kimura and Shinsuke Sasada contributed to the study's conception and design. Yuri Kimura, Shinsuke Sasada, Akiko Emi, Norio Masumoto, and Takayuki Kadoya collected clinical data. Yuri Kimura and Koji Arihiro evaluated the pathological findings. Yuri Kimura and Shinsuke Sasada analyzed the data and wrote the manuscript. All Authors commented on previous versions of the manuscript and read and approved the final manuscript.

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