Expression of programmed death-1 in sentinel lymph nodes of breast cancer

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Background and Objectives: To explore whether lymphocytes in sentinel lymph nodes (SLNs) are highly exposed to tumor neoantigens and thus express high level of programmed death 1 (PD-1), we examined PD-1 expression in SLNs and non-sentinel regional lymph nodes (non-SLNs) in breast cancer.

Methods: We performed PD-1 immunohistochemistry in two cohorts: 40 metastasis-negative SLNs including 10 patients for each subtype (luminal A-like, luminal B-like, HER2, and triple negative breast cancer [TNBC]); and 25 pairs of metastasis-positive SLNs and non-SLNs (10 luminal A-like, 10 luminal B-like, and 5 TNBC).

Results: Among 40 metastasis-negative SLNs, 34 and 6 samples were PD-1 intensity grade 1 (low) and 2 (high), respectively. PD-1 intensity correlated with PD-1-positive lymphocyte numbers (P = 0.005); TNBC had the highest PD-1 lymphocyte numbers among all subtypes. The median PD-1-positive lymphocyte number was higher in SLNs than non-SLNs. In most cases, more lymphocytes in SLNs expressed PD-1 than those in non-SLNs (P < 0.0001).

Conclusions: TNBC had the greatest PD-1 expression among all subtypes, and metastasis-negative SLNs had more PD-1-positive lymphocytes than downstream non-SLNs. These data suggested that lymphocytes in SLNs are activated following exposure to tumor neoantigens and thus tumor specific, and could be utilized as a biomarker platform.

Keywords
breast cancer, PD-1, sentinel lymph node
1 | INTRODUCTION

While research over the last several decades has demonstrated that the immune system suppresses tumorigenesis, the underlying molecular mechanisms have only been recently elucidated. Tumors produce neoantigens, and the neoantigens are presented to T-cells by antigen-presenting cells (APCs), such as dendritic cells, at regional lymph nodes. The T-cells then become activated and specific to the tumor antigens. These activated T-cells move to tumor sites through the bloodstream, and once they specifically recognize and bind to cancer cells, they kill the target cancer cells. In this “cancer-immunity cycle,” both stimulatory and inhibitory signals between APCs or cancer cells and T-cells exist, and immune checkpoint molecules regulate these signals. Cancer cells are believed to escape from elimination by T-cells by directly or indirectly enhancing inhibitory T-cell signals via immune checkpoint molecules. One such molecule is programmed death-1 (PD-1), also known as CD279. PD-1 is expressed on a subset of T-cells and conveys inhibitory signals when stimulated by its ligand, programmed cell death-1 ligand-1 (PD-L1), which is expressed on cancer cells. Targeted drugs against PD-1 or PD-L1 have been recently developed and showed significant clinical efficacy in a variety of malignancies, including melanoma and non-small cell lung, head and neck, renal cell and gastric cancers.

The lack of definitive predictive markers for the efficacy of anti PD-1/PD-L1 therapy is, however, one of the current issues that need to be addressed. In melanoma, PD-1-positive tumor infiltrating lymphocytes (TILs) are tumor-specific and the pre-existence of PD-1-positive CD-8 positive TILs is a predictor of response to the anti-PD-1 antibody. In breast cancer, however, the investigations regarding PD-1 expression in TILs are limited, and only sporadic studies have suggested that the presence of PD-1-positive TILs are associated with higher histologic grade, hormone receptor negativity, triple negative subtypes, and poor prognosis. Therefore, characterization of PD-1 and other immune checkpoint molecules in breast cancer TILs needs to be explored. However, investigating TILs in breast cancer is challenging because the complexity of the breast cancer tumor microenvironment does not allow us to define TILs in an objective manner.

We have hypothesized that lymphocytes in sentinel lymph nodes (SLNs) would be activated in a tumor-specific manner because lymphocytes in SLNs would theoretically be more highly exposed to tumor neoantigens than those in non-sentinel regional lymph nodes (non-SLN). If this hypothesis were true, lymphocytes in SLNs would potentially be ideal biomarkers for individualized anti-immune checkpoint therapies.

To address this hypothesis, we immunohistochemically evaluated PD-1 expression in SLNs of various subtypes of breast cancer compared with non-SLN. To the best of our knowledge, this is the first study that investigates immune checkpoint molecules in SLNs of breast cancer.

2 | MATERIALS AND METHODS

2.1 | Patient selection

We generated a list of breast cancer patients who underwent curative surgery with SLN biopsy (SLNB) at Kobe University Hospital since April 2005. For the first cohort of metastasis-negative SLNB, we selected the 10 newest patients on the patient list for each breast cancer subtype: that is, luminal A-like (estrogen receptor [ER]- or progesterone receptor [PgR]-positive, HER2-negative, and Ki-67 low), luminal B-like (ER- or PgR-positive, HER2-negative, and Ki-67 high), HER2 type (ER- and PgR-negative, and HER2-positive), and triple negative breast cancer (TNBC) (ER-, PgR-, and HER2-negative). For the second cohort of metastasis-positive SLNB that necessitated axillary lymph node dissection (ALND), we attempted to collect a maximum of the 10 newest cases on the patient list for each subtype. We obtained 10, 10, 0, and 5 cases of luminal A-like, luminal B-like, HER2, and TNBC, respectively. Histopathological data used for the patient selection were obtained from the pathology reports. Written informed consent for investigational use of surgical specimens was obtained from each patient. This study was performed in compliance with the Helsinki Declaration and approved by the Institutional Review Board (IRB) in Kobe University.

2.2 | Immunohistochemistry

All tissue samples embedded in paraffin were cut into 4-μm-thick sections and the sections were incubated with a mouse anti-human PD-1 monoclonal antibody (1:40, Clone NAT105, Cell Marque, CA) after deparaffinization and antigen retrieval. For immunostaining, diaminobenzidine (DAB) liquid chromogen (Dako Japan, Kyoto, Japan) was used.

Each case typically had 2-3 SLNs and they were placed on a single slide. A pathologist (T.Y) observed all SLNs (only metastasis-positive SLNs for the cohort of metastasis-positive SLNB) on the slide and determined a representative SLN and the area for cell count under magnification 200×. For the cohort of metastasis-positive SLNB followed by ALND, each case typically had 15-20 dissected non-SLNs and these were placed on 4-5 separate slides, with each slide having 4-5 non-SLNs. We randomly chose one slide for each case: the pathologist observed all non-SLNs on the slide and selected one representative non-SLN and area in the same manner as for SLNs. The pathologist evaluated the PD-1 staining intensity compared with the staining of the germinal center, the internal positive control, under magnification 40×. Staining intensity was categorized into three groups: intensity 0, no staining; intensity 1, positive but weaker than the germinal center; or intensity 2, positive and equivalent to the germinal center (Figure 1). We randomly counted 1000 lymphocytes in the selected area and then recounted PD-1-positive lymphocytes among this group of lymphocytes; from these data, we calculated the ratio of PD-1-positive lymphocytes.

2.3 | Statistical analysis

We conducted Wilcoxon rank-sum test and Kruskal-Wallis test to compare the influence of intensity grade, subtype and metastasis on PD-1 expression. Wilcoxon signed rank-sum test was performed to analyze the difference of PD-1 expression between SLN and non-SLN. In all analyses, significance was set at a P-value of 0.05. Statistical
analyses were performed using the JMP software program, version 11.0 (SAS Institute Inc., Cary, NC).

3 RESULTS

3.1 PD-1 staining in metastasis-negative SLNs and breast cancer subtype

We performed PD-1 staining in 40 metastasis-negative SLNs and categorized 34 samples as PD-1 staining intensity 1 and 6 samples as PD-1 staining intensity 2. Representative staining images of PD-1 staining intensity are shown in Figure 1. No sample was categorized as intensity 0. The median number of PD-1-positive lymphocytes in intensity one group was 35/1000 (range, 6-144/1000) and the median number in intensity two group was 87/1000 (range, 73-234/1000). Even though there were only six intensity two cases, PD-1 intensity was significantly associated with the number of PD-1-positive lymphocytes ($P = 0.005$) (Figure 2).

Among the six intensity two cases, four were TNBC, one was luminal A-like and one was HER2 type, indicating that four of the 10 TNBC cases showed intensity 2 PD-1-positivity (Figure 3A). The median number of PD-1-positive lymphocytes in each breast cancer subtype was as follows: luminal A-like, 25/1000 (range, 6-53/1000); luminal B-like, 24/1000 (range, 10-73/1000); HER2, 50/1000 (range, 14-112/1000), and TNBC, 89/1000 (range, 38-234/1000). There were significant differences between all combinations of subtypes, except for between luminal A-like and B-like, and TNBC had the greatest number of PD-1 lymphocytes among all subtypes (Figure 3B). There was no significant association between the number of PD-1-positive lymphocytes in metastasis-negative SLNs and tumor size or lymphatic invasion (Sup. Figures S1 and S2).

3.2 PD-1 staining in metastasis-positive SLNs and non-SLNs

We next compared PD-1 expression of lymphocytes in 25 metastasis-positive SLNs with their associated non-SLNs dissected after positive SLNB. In metastasis-positive SLNs, PD-1-positive lymphocytes were predominantly distributed in pericancer areas (Figure 4). The median number of PD-1-positive cells in metastasis-positive SLNs was 71/1000 (range, 0-245/1000) and the median number in non-SLNs was 23/1000 (5-90/1000). In the vast majority of cases, more lymphocytes in metastasis-positive SLNs expressed PD-1 than in non-SLNs (median difference, 38/1000, $P < 0.0001$) (Figures 4 and 5A). Among the metastasis-positive SLNs, we categorized 14 samples as PD-1

![FIGURE 1](image1) Immunohistochemical staining of PD-1 in metastasis-negative SLNs. PD-1 intensity was categorized as described in section 2 (under magnification 40×). Representative cases of PD-1 intensity 1 under magnification 40× (A), PD-1 intensity 1 under magnification 200× (B), PD-1 intensity 2 under magnification 40× (C), and PD-1 intensity 2 under magnification 200× (D) are shown.

![FIGURE 2](image2) Distribution of the number of PD-1 positive lymphocytes in metastasis-negative SLNs according to intensity. The intensity of PD-1 immunohistochemical staining was plotted on the x-axis and the number of PD-1-positive lymphocytes (per 1000 cells) was plotted on the y-axis. Each dot represents a single case.
staining intensity 1 and 10 samples as PD-1 staining intensity 2; only one sample was classified as PD-1 staining intensity 0 (Figure 5B). In comparison, in non-SLNs, we regarded only one sample as PD-1 staining intensity 2, and the other 24 samples were categorized as PD-1 staining intensity 1. Furthermore, we examined PD-1 expression in lymphocytes in metastasis-positive SLNs compared with metastasis-negative SLNs. We found that metastasis-positive SLNs had significantly more PD-1-positive lymphocytes than metastasis-negative SLNs (median number of PD-1-positive lymphocytes: 71/1000 vs 37.5/1000, respectively; \( P = 0.047 \) ) (Figure 6).

**FIGURE 3** Ratio of intensity and the distribution of the number of PD-1-positive lymphocytes in metastasis-negative SLNs according to breast cancer subtype. (A) The breast cancer subtypes were plotted on the x-axis and the percent of cases for each intensity was plotted on the y-axis. (B) The breast cancer subtypes were plotted on the x-axis and the number of PD-1 positive lymphocytes (per 1000 cells) was plotted on the y-axis. Each dot represents a single case.

**FIGURE 4** Immunohistochemical staining of PD-1 in metastasis-positive SLN and its downstream non-SLN. Representative case of metastasis-positive SLN under magnification 40× (A), metastasis-positive SLN under magnification 200× (B), non-SLN under magnification 40× (C), and non-SLN under magnification 200× (D) are shown.

### DISCUSSION

In this study, we investigated the expression of PD-1 in SLNs of breast cancer. We found that almost all SLNs regardless of the presence or absence of metastasis showed expression of PD-1, and the TNBC subtype showed the greatest number of PD-1-positive lymphocytes and the highest PD-1 intensity. We also found that metastasis-positive SLNs had more PD-1-positive lymphocytes than their associated downstream non-SLNs.

To the best of our knowledge, this is the first study to evaluate expression of PD-1 in SLNs of breast cancer. Even in melanoma, in
which both SLNB and anti-PD-1/PD-L1 therapy are adopted as standard of care, little information on the expression of immune checkpoint molecules in SLNs is available. Kakavand et al investigated PD-1 expression in metastasis-positive SLNs and found that the presence of peri-tumoral PD-1-positive lymphocytes in SLNs was associated with poor survival. However, the authors did not refer to the frequency of PD-1 expression in melanoma SLNs. Therefore, more collective data are required to define the roles of expression of immune checkpoint molecules in SLNs as prognostic and/or predictive biomarkers for both melanoma and breast cancer.

Our finding that TNBC had the greatest numbers of PD-1-positive lymphocytes in SLNs is consistent with previous studies suggesting that TNBC may be more immunogenic than the other breast cancer subtypes. A gene expression profiling study demonstrated an association between the expression of immunomodulatory genes and better clinical outcomes in TNBC. Several studies reported that TNBC has more PD-1-positive TILs than other subtypes. Furthermore, a phase Ib study of pembrolizumab, an anti-PD-1 antibody, in TNBC showed promising activity of the drug. Our finding supports the strategy of developing immune checkpoint inhibitors against TNBC.

Another finding of our study is that the vast majority of metastasis-positive SLNs had more PD-1-positive lymphocytes than non-SLNs. This result led us to speculate that this may be because SLNs may be exposed to tumor neoantigens more highly than the other downstream non-SLNs. While no previous study has thoroughly analyzed PD-1 expression from the perspective of distance from the source of tumor neoantigens, Gros et al compared PD-1 expression in melanoma TILs with that of peripheral blood mononuclear cells (PBMCs). The authors showed that TILs had more PD-1-positive T cells than PBMCs, indicating that the closer that lymphocytes are to the source of tumor neoantigens, the more remarkably the lymphocytes express PD-1. The authors also showed that CD8-positive/PD-1-positive lymphocytes are tumor specific. These findings support the hypothesis that PD-1-positive lymphocytes in SLNs are activated tumor specifically, even though further studies are required to conclusively demonstrate this point. On the other hand, previous studies suggested that lymphocytes in SLNs of breast cancer and melanoma are immunosuppressed even before SLN metastasis occurs. Interestingly, Cochran et al showed that the immunosuppression is more prominent in SLNs than in downstream non-SLNs. These findings indicate that the primary tumor could downregulate local immune defense to enable lymph node metastasis. Taken together, because antigen binding to T cell receptor on lymphocyte is known to result in lymphocyte activation and upregulation of PD-1 expression, lymphocytes in SLNs may eventually be immunosuppressed through PD-1 signaling pathway albeit transient activation.

If our hypothesis is proven to be true, this suggests that SLNs may be an ideal source of biomarkers for individualized anti-immune checkpoints therapies. While TILs are indicated to provide prognostic or predictive biomarkers, lymphocytes in SLNs are much easier to collect and analyze than TILs. In particular, the applicability to flow cytometry, which will enable more comprehensive analysis of lymphocyte surface

**FIGURE 5** Distribution of the number of PD-1 positive lymphocytes and the ratio of each intensity in metastasis-positive SLNs and non-SLNs. (A) The number of PD-1 positive lymphocytes (per 1000 cells) was plotted on the y-axis in SLNs and non-SLNs (on the x-axis). Each dot represents a single case. Pairs of SLN and non-SLN in each case are connected with a line. (B) The ratio of each intensity was plotted on the y-axis in SLNs and non-SLNs (on the x-axis).

**FIGURE 6** Distribution of the number of PD-1-positive lymphocytes in metastasis-negative and metastasis-positive SLNs. The number of PD-1-positive lymphocytes (per 1000 cells), plotted on the y-axis, in metastasis-negative and metastasis-positive SLNs, plotted on the x-axis. Each dot represents a single case.
proteins, is attractive, because numerous immune checkpoint molecules other than PD-1 are believed to be involved in anti-tumor immunity and thus to be therapeutic targets. In fact, Gros et al showed that PD-1-positive TILs tend to co-express other immune checkpoint molecules, such as CTLA-4, CD137, TIM-3, and LAG-3, in melanoma.\(^\text{10}\)

There are some limitations of our study. First, the number of samples is small. An extended larger investigation is necessary for validation of our findings. Second, immunohistochemistry is not very quantitative, and standardized methodology should be established for the generalization of our findings. Further, validation with more quantitative methods such as flow cytometry and RT-PCR are necessary, whereas sacrificing fresh SLNs for such investigational purposes is challenging because these lymph nodes provide critical information in the clinic. Third, we have not evaluated checkpoint molecules other than PD-1. Considering that many other checkpoint molecules are currently regarded as potential therapeutic targets, they should be evaluated to understand the tumor immunity landscape.

In conclusion, our current findings of the highest PD-1 expression in TNBC and more PD-1-positive lymphocytes in metastasis-positive SLNs than in downstream non-SLNs suggest that lymphocytes in SLNs are activated following high exposure to tumor neoantigens and thus are tumor-specific, and could be utilized as a biomarker platform.

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REFERENCES


SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.
SYNOPSIS
To explore the hypothesis that lymphocytes in sentinel lymph nodes (SLNs) are highly exposed to tumor neoantigen and express high level of programmed death 1 (PD-1), we immunohistochemically evaluated expression of PD-1 in SLNs of each breast cancer subtype and non-sentinel regional lymph nodes (non-SLNs) in breast cancer. Triple negative breast cancer (TNBC) had the greatest PD-1 expression in lymphocytes in SLNs among the breast cancer subtypes, and metastasis-positive SLNs had more PD-1-positive lymphocytes than non-SLNs. These results suggest that lymphocytes in SLNs are tumor-specifically activated.