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Effect of Tissue Sample Type on The Evaluation of PD-L1 (SP142) Expression in Breast Cancer

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Abstract

Background/Aim: PD-L1 expression is a key biomarker for immune checkpoint inhibitor therapy in breast cancer treatment. However, many factors affect PD-L1 assessment. This study evaluated how specimen-related factors affect PD-L1 expression in breast cancer and provides a guide for optimal specimen selection.

Patients and Methods: We retrospectively analyzed 30 consecutive breast cancer specimens submitted for PD-L1 testing at the Osaka University Hospital between November 2019 and November 2021. PD-L1 expression was evaluated using the VENTANA SP142 immunohistochemistry assay. Expression in tumor-infiltrating immune cells (IC) was quantified as the percentage of PD-L1-positive IC within the tumor area, and positivity was defined as IC $\geq 1\%$.

Results: The overall PD-L1 positivity rate was 47%. PD-L1 expression was significantly higher in surgical specimens compared with biopsy samples (65% vs. 23%, $p=0.032$) and in primary tumors compared with metastatic/recurrent sites (58% vs. 0%, $p=0.018$). All specimens from patients with Stage IV or recurrent disease were PD-L1-negative, compared with 74% positivity at earlier stages ($p<0.001$). Although not statistically significant, specimens from patients who had received chemotherapy within 40 days showed lower PD-L1 positivity than chemotherapy-naïve specimens (14% vs. 57%, $p=0.086$). In advanced disease (Stage IV/recurrent), 82% of specimens were from biopsies and 55% from metastatic sites, potentially explaining the lower PD-L1 positivity.

Conclusion: For optimal PD-L1 assessment in breast cancer, surgical specimens from primary tumors without prior therapy are preferable due to larger evaluable tumor areas. For patients requiring neoadjuvant chemotherapy or with *de novo* Stage IV disease, multiple biopsies of primary tumors using thick needles before treatment, with attention being paid to sampling tumor margins to account for potential immune-excluded phenotypes, are recommended.

Keywords: PD-L1, SP142, breast cancer, neoadjuvant chemotherapy, primary tumor.

Introduction

PD-L1 expression serves as a key biomarker for immune checkpoint inhibitor therapy in patients with breast cancer

and other cancer types. Clinical trials have demonstrated the efficacy of PD-L1 inhibitors in the treatment of metastatic/recurrent triple-negative breast cancer (mTNBC). The IMpassion130 trial showed improved



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progression-free survival with atezolizumab plus chemotherapy in patients with tumor-infiltrating immune cells (IC) PD-L1 expression $\geq 1\%$ (1, 2). Similarly, the KEYNOTE-355 trial demonstrated a significant overall survival benefit with pembrolizumab plus chemotherapy in PD-L1-positive (Combined Positive Score ≥ 10) patients (3). Given the benefit of treatment with immune checkpoint inhibitors, these drugs should be provided to every patient with mTNBC expressing PD-L1. Two monoclonal antibody clones (SP142 and 22C3) are widely used to assess PD-L1 in TNBC. Because they target different epitopes and use distinct scoring criteria, the assays are not analytically equivalent (4).

Several factors affect the expression of PD-L1 protein in IC and tumor cells. For example, PD-L1 positivity rates using the SP142 assay vary between metastatic/recurrent TNBC (41%) and non-metastatic TNBC (46-56%) (5, 6). Expression levels also differ between primary and metastatic sites (44.0% vs. 35.6%) (7, 8). Notably, one-third of PD-L1-negative cases in pre-operative specimens converted to positive in post-operative samples (9). Changes in PD-L1 expression have also been observed in post-neoadjuvant chemotherapy (NAC) specimens compared with pre-NAC samples (10, 11).

These findings indicate that specimen type, collection method, and disease stage may influence PD-L1 assessment. While PD-L1-positive patients show improved survival outcomes regardless of specimen characteristics (7, 8), careful specimen selection is crucial to avoid underestimating PD-L1 expression. This is particularly important in Japan, where insurance coverage limits PD-L1 testing to once per patient. Here, we retrospectively analyzed 30 cases to evaluate how specimen selection affects the results of PD-L1 testing. Based on our findings, a guide to specimen selection for PD-L1 (SP142) testing is provided.

Patients and Methods

Patient selection. The study included 30 consecutive breast cancer specimens submitted for PD-L1 testing from Osaka University Hospital between November 2019 and November

2021. The study conforms to the principles of the Declaration of Helsinki and was approved by the Institutional Review Board of Osaka University Hospital (approval no. 21227).

Specimen collection and processing. Core needle biopsies were performed using 8G, 10G, or 14G needles, with immediate fixation in 10% neutral buffered formalin (NBF). For metastatic lesions and surgical specimens, resected tissue was promptly fixed in 10% NBF. All specimens were processed into formalin-fixed paraffin-embedded blocks for analysis.

Immunohistochemistry. PD-L1 expression was evaluated using the VENTANA SP142 PD-L1 immunohistochemistry assay (Ventana Medical Systems, Oro Valley, AZ, USA). Detection was performed using an anti-PD-L1 (clone SP142) rabbit monoclonal antibody with the Ventana OptiView DAB Universal Kit and VENTANA amplification reagent. Tonsil tissue fixed in 10% NBF served as control, showing positive staining in germinal center lymphocytes and macrophages, scattered positive IC in interfollicular regions, diffuse positive staining in reticular crypt epithelial cells, and negative staining in superficial squamous epithelial cells. A control rabbit monoclonal antibody was used as a negative control when evaluating PD-L1-staining. PD-L1 expression in tumor-infiltrating IC was quantified as the percentage of PD-L1-positive IC in the tumor area, regardless of staining intensity. Results were categorized as IC0 (<1%), IC1 ($\geq 1\%$ to <5%), IC2 ($\geq 5\%$ to <10%), and IC3 ($\geq 10\%$). Immunohistochemical analysis and quantification of PD-L1-positive immune cells (ICs) were performed in a College of American Pathologists-accredited central laboratory (SRL Inc., Akiruno, Japan). Four tissue sections per sample were analyzed without repetition. Exceptionally, two cases required restaining because of poor quality, but the results remained consistent. Based on the IMpassion130 trial results, PD-L1 positivity was defined as IC $\geq 1\%$ (1, 12, 13).

Statistical analysis. Categorical comparisons were performed using chi-square tests with JMP Pro 16

software (JMP Statistical Discovery, Cary, NC, USA). Statistical significance was set at $p < 0.05$.

Results

Patient characteristics. All patients were females. Of the 30 specimens, 17 were surgical, and 13 were biopsy specimens. The biopsy methods included vacuum-assisted biopsy (n=7), core needle biopsy (n=4), CT-guided lung biopsy (n=1), and skin biopsy (n=1). Twenty-four specimens were from primary tumors, and six were from metastatic sites. Histologically, 27 cases were invasive ductal carcinoma, and three were not. Twenty-four out of 26 cases showed Ki-67 positivity >20%. Disease staging at the time of specimen collection was as follows: Stage I (n=4), Stage II (n=12), Stage III (n=3), and Stage IV, including *de novo* and distant recurrence (n=11). Seven of the 30 cases had received chemotherapy within the 40 days before specimen collection. The chemotherapy regimens included weekly paclitaxel followed by fluorouracil plus epirubicin plus cyclophosphamide (n=4), docetaxel plus cyclophosphamide (n=1), epirubicin plus cyclophosphamide (n=1), and dose-dense doxorubicin plus cyclophosphamide followed by dose-dense paclitaxel (n=1).

PD-L1 expression analysis. The overall distribution of PD-L1 (clone SP142) expression was classified: IC0 (<1%), 16 cases; IC1 (1%-4%), 5 cases; IC2 (5%-9%), 3 cases; and IC3 (>10%), 6 cases, a total positivity rate of 47%. This was comparable to the IMpassion130 trial's positivity rate (40.8%). Representative immunohistochemistry images of PD-L1 expression with IC scores are shown in Figure 1.

Correlation of PD-L1 status with clinical and specimen collection-associated factors. Based on baseline patient characteristics, PD-L1 expression was significantly associated with clinical stages (Stages I, II, and III vs. Stage IV and recurrence; Table I). Notably, all specimens derived from patients with Stage IV or recurrent disease were negative for PD-L1.

The influence of sample conditions on PD-L1 status was explored (Table II). PD-L1 positivity was significantly higher in surgical specimens than in biopsy samples and in primary tumors than in metastatic or recurrent tumors, suggesting that smaller evaluable areas in biopsy specimens than in surgical specimens affect the assessment of PD-L1 positivity (Figure 2A-D). Additionally, PD-L1 positivity was relatively higher in tumors obtained from patients not receiving drug treatment within 40 days than in tumors from patients receiving drug treatment within 40 days of specimen collection; however, the difference was not statistically significant. As NAC often markedly reduces the areas of tumor nests, evaluable areas containing IC may also be reduced (Figure 2E, F).

Finally, an association of clinical stages with sample conditions was explored to elucidate the reason for the low PD-L1 positivity in Stage IV and recurrent tumors (Table III). In Stage IV and recurrent tumors, 9 out of 11 specimens (82%) were from biopsies, and 6 out of 11 (55%) of these specimens were obtained from metastatic or recurrent tumors, suggesting that biopsy specimens are suboptimal for examining PD-L1 expression in patients with advanced breast cancer.

Discussion

Our study found a statistically significant higher rate of PD-L1 positivity in surgical specimens than in biopsy specimens as shown in Table II. Previous studies reported that 45% of cases that were ultimately PD-L1-positive in surgical specimens were initially negative in biopsy samples (14). This discrepancy may be attributed to tumor heterogeneity. While heterogeneity is reported to be more frequent in tumor-infiltrating IC than in tumor cells, the SP142 assay's IC >1% criterion for atezolizumab eligibility in TNBC may be particularly susceptible to tumor heterogeneity effects (15).

In immune-excluded phenotype cancers, immune responses are present at tumor margins but scarce in the tumor center (an immune desert state), potentially

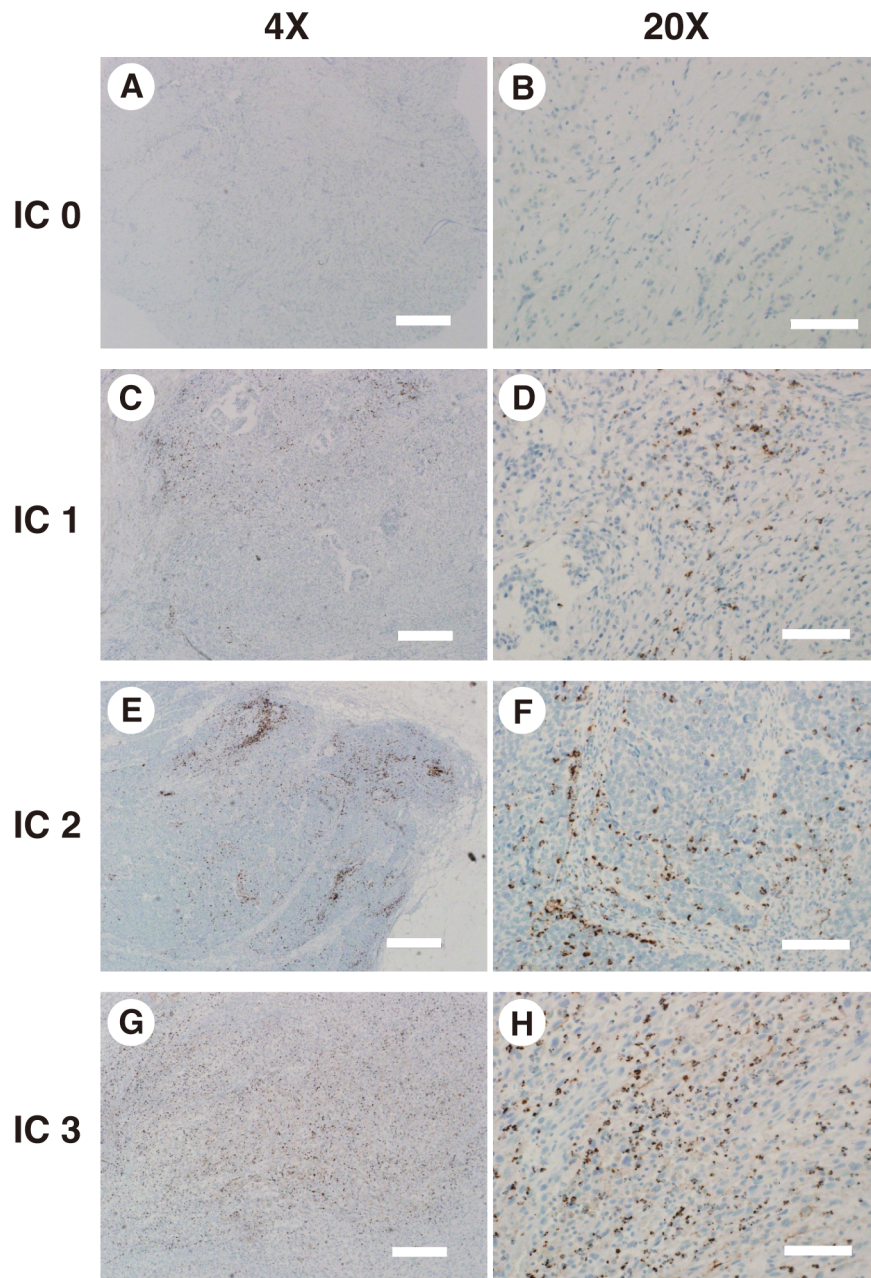


Figure 1. Representative microscopic images of immunohistochemical staining for PD-L1 (SP142) with the assigned immune-cells (IC) scores, representing ratios of PD-L1-positive tumor-infiltrating IC; IC 0 (<1% PD-L1-positive; A, B), IC 1 ($\geq 1\%$ and <5% PD-L1-positive; C, D), IC 2 ($\geq 5\%$ and <10% PD-L1-positive; E, F), IC 3 ($\geq 10\%$ PD-L1-positive; G, H). Scale bars: 0.4 mm (4 \times) and 0.1 mm (20 \times).

complicating the assessment of PD-L1-positive immune cells (15). Therefore, it is recommended that biopsies target tumor margins, and, when feasible, repeat biopsies

are considered for negative cases to limit the potential for false negatives. To address intratumoral heterogeneity, efforts to develop and validate serum PD-L1 quantification

Table I. Association of PD-L1 status with baseline patient characteristics.

Factor	PD-L1 status		p-Value (Fisher)
	Positive n (%)	Negative n (%)	
Age			
≤50 years	4 (13)	6 (20)	0.708
>50 years	10 (33)	10 (33)	
Clinical Stage			
I, II, and III	14 (47)	5 (17)	<0.001
IV (<i>de novo</i>) and recurrence	0 (0)	11 (37)	
Tumor type			
Invasive ductal carcinoma	12 (40)	15 (50)	0.586
Others	2 (7)	1 (3)	
Histological grade			
1 and 2	2 (7)	4 (13)	0.341
3	11 (37)	6 (20)	
Unknown	1 (3)	6 (20)	
Ki-67 index			
≤20%	0 (0)	2 (7)	0.203
>20%	14 (47)	10 (33)	
Unknown	0 (0)	4 (13)	
Subtype			
HR-negative/HER2-negative	13 (43)	16 (53)	0.466
HR-negative/HER2-positive	1 (3)	0 (0)	

HR, Hormone receptor.

as a predictive biomarker for immune checkpoint inhibitors are underway (16).

We found higher PD-L1 positivity rates in Stages I-III compared with Stage IV (*de novo* and recurrent) disease (Table I). This difference may be partially explained by the higher proportion of surgical specimens evaluated in Stages I-III (15/19; 79%) compared with Stage IV (2/11; 18%) shown in Table III.

Specimens from patients who received prior chemotherapy tended towards lower PD-L1 positivity rates, although this was not statistically significant (Table II). While previous studies have reported varying effects of chemotherapy on PD-L1 expression – including decreased expression or unchanged positivity rates (11, 17) – our findings may indicate reduced PD-L1 positivity post-chemotherapy. As indicated above, the primary cause of chemotherapy-induced changes in PD-L1 expression may be the reduction/extinction of tumor nests observed in post-chemotherapy specimens.

Table II. Association of PD-L1 status with sample conditions.

Factor	PD-L1 status		p-Value (Fisher)
	Positive n (%)	Negative n (%)	
Specimen type			
Surgical specimen	11 (37)	6 (20)	0.032
Biopsy specimen	3 (10)	10 (33)	
Site of tumor sample			
Primary tumor	14 (47)	10 (33)	0.018
Metastatic/recurrent tumor	0 (0)	6 (20)	
Prior therapy within 40 days			
Yes	1 (3)	6 (20)	0.086
No	13 (43)	10 (33)	
Time from sampling to testing			
≤180 days	7 (23)	9 (30)	1.0
>180 days	7 (23)	7 (23)	

Table III. Association of clinical stages with sample conditions.

Factor	Clinical stage		p-Value (Fisher)
	I, II, III n (%)	IV n (%)	
Specimen type			
Surgical specimen	15 (50)	2 (7)	0.002
Biopsy specimen	4 (13)	9 (30)	
Site of tumor sample			
Primary tumor	19 (63)	5 (17)	<0.001
Metastatic/recurrent tumor	0 (0)	6 (20)	

Another proposed cause involves alterations in the tumor microenvironment (18).

One potential explanation for the low PD-L1 positivity in stage IV TNBC is sampling site. In our cohort, all specimens obtained from metastatic lesions (exclusively from patients with stage IV TNBC) were PD-L1 negative (Table II and Table III). A similar finding was reported by Han *et al.* (19). As previously described, metastatic tumors are typically more aggressive and exhibit a more immunosuppressive microenvironment than primary tumors (20). Accordingly, when both are available, PD-L1 assessment with the SP142 assay should preferentially be performed on primary tumor tissue rather than metastatic lesions.

Our study had a small sample size and lacked paired comparisons (pre/post-surgery, pre/post-chemotherapy,

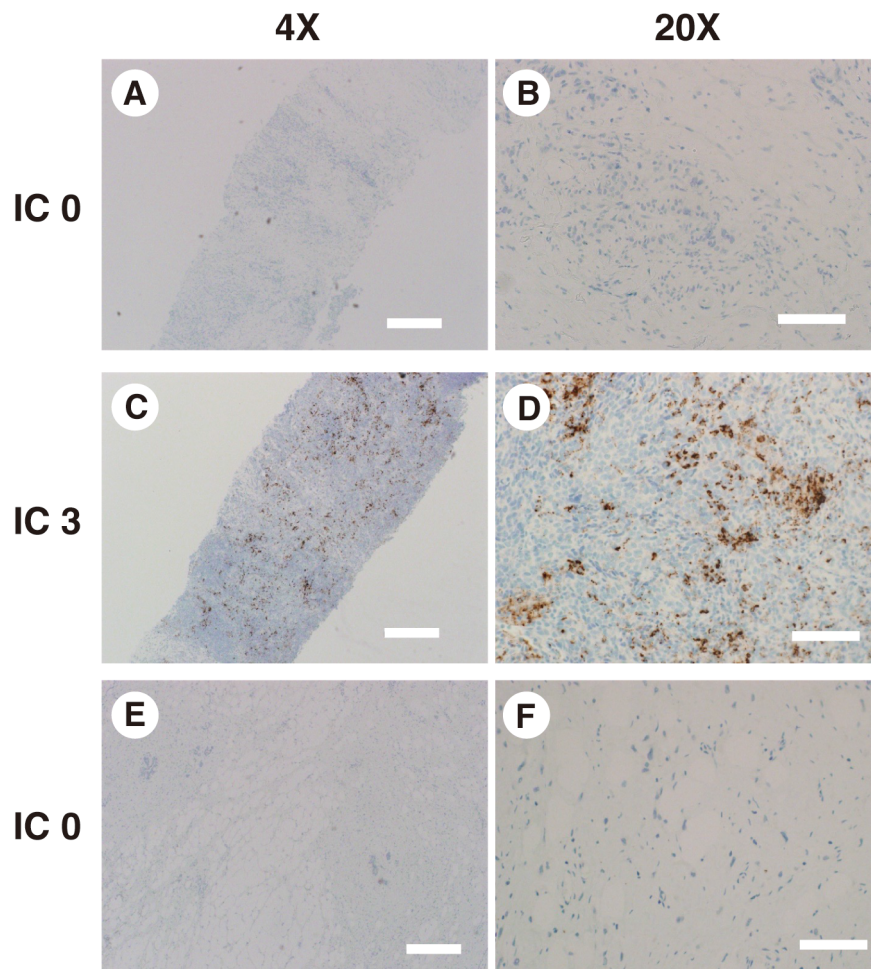


Figure 2. Representative microscopic images of immunohistochemical staining for PD-L1 (SP142) with the assigned immune-cells (IC) scores, representing ratios of PD-L1-positive tumor-infiltrating IC; IC 0 (<1% PD-L1-positive; A, B, E, F), IC 3 ($\geq 10\%$ PD-L1-positive; C, D). (A-D) PD-L1 expression in biopsy specimens. (E, F) PD-L1 expression in a surgical specimen collected after neoadjuvant chemotherapy. Scale bars: 0.4 mm (4 \times) and 0.1 mm (20 \times).

primary/metastatic sites), and while we analyzed PD-L1-positivity rates, we did not evaluate clinical outcomes in patients treated with atezolizumab based on PD-L1 status; therefore, further studies are warranted. Recent studies investigating changes in the tumor immune micro-environment (PD-L1 and tumor-infiltrating lymphocytes) as predictive markers for chemotherapy and immunotherapy response highlight the importance of appropriate tissue selection for PD-L1 testing (21, 22).

In conclusion, the most appropriate specimens for investigating PD-L1 expression in patients with breast

cancer are surgical specimens obtained from primary tumors without prior therapy because the evaluable tumor areas are large. For patients planning NAC and for patients with *de novo* Stage IV cancer, multiple biopsies of primary tumors using thick biopsy needles before treatment are recommended.

Conclusion

Specimen selection for PD-L1 testing should prioritize sampling the tumor margins during biopsy and, when

possible, use surgical specimens that include tumor margins and large tissue samples. Although the impact of chemotherapy on PD-L1 expression requires further investigation, chemotherapy-naïve tumor specimens may be more appropriate than those post-chemotherapy.

Conflicts of Interest

The Authors declare the following conflicts of interest: MT and MS have received honoraria from MSD K.K. and Chugai Pharmaceutical Co., Ltd., and NM and KS have received honoraria from Nippon Kayaku Co., Ltd. for lectures.

Authors' Contributions

Conceptualization, MS; Data curation, TU; Formal analysis, TU and MS; Funding acquisition, N/A; Investigation, TU and MS; Methodology, TU; Project administration, MS; Resources, MS, KA, NM, MT, TY, YS, TM, TT, and KS; Software, N/A; Supervision, KS; Validation, TU and MS; Visualization, TU; Roles/Writing – original draft, TU and MS; Writing – review and editing, all Authors.

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Artificial Intelligence (AI) Disclosure

No artificial intelligence (AI) tools, including large language models or machine learning software, were used in the preparation, analysis, or presentation of this manuscript.

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