Association Between Formalin Fixation Time and Programmed Cell Death Ligand 1 Expression in Patients With Non-Small Cell Lung Cancer

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Abstract. *Background/Aim: The expression of programmed cell death ligand 1 (PD-L1) determined by immunohistochemistry (IHC) may be associated with tissue formalin fixation time in non-small cell lung cancer (NSCLC) samples. We investigated the association between the PD-L1 expression and formalin fixation time, and clarified the optimal duration of fixation for accurate PD-L1 evaluation. Materials and Methods: We collected 55 tumor specimens from resected NSCLC patients. The samples were halved and immediately fixed in 10% buffered formalin for 12-24 h (normal fixation), or 96-120 h (prolonged fixation). Each specimen was stained using two assay systems (22C3 and SP263) for PD-L1. Results: The mean PD-L1 tumor proportion score was not significantly different between normal and prolonged fixation groups for either 22C3 or SP263 (normal fixation: 18.8%; prolonged fixation: 16.3%, p=0.277; normal fixation: 16.2%; prolonged fixation: 17.6%, p=0.560, respectively). Conclusion: Formalin fixation duration for up to 120 h does not affect PD-L1 IHC expression. PD-L1 tumor proportion score of tumor specimens can be evaluated by IHC even if these have been fixed in formalin outside the recommended duration in clinical practice.*

Lung cancer is the leading cause of cancer-related deaths in the world (1). Non-small cell lung cancer (NSCLC) accounts for approximately 80% of all lung cancer cases and most

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NSCLC cases are already advanced, unresectable, and metastatic at initial diagnosis (2). Recently, programmed death 1 (PD-1)/programmed death ligand 1 (PD-L1) axis inhibitors have demonstrated outstanding efficacy in patients with metastatic NSCLC, and have been established as novel standard treatment for NSCLC (3-8).

Tumor proportion score (TPS) for PD-L1, as determined by immunohistochemistry (IHC), is associated with improved treatment efficacy of PD-1 axis inhibitors, such as pembrolizumab (9, 10). Clinical trials have demonstrated the efficacy of pembrolizumab for NSCLC patients with PD-L1 TPS ≥50% or ≥1% (5, 7, 8). Since PD-L1 IHC determines the treatment strategy for NSCLC patients (5, 7-10), an accurate evaluation of PD-L1 expression by IHC is essential.

The evaluation of IHC expression can be affected by technical issues, including formalin fixation time. According to the guidelines established by the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) for HER2 testing, specimens should be fixed in 10% neutral buffered formalin for 6-72 h (11). According to the ATLAS of PD-L1 IHC testing in lung cancer, published by the International Association for the Study of Lung Cancer (IASLC), a formalin fixation time of 6-48 h is recommended for PD-L1 IHC evaluation using the 22C3 assay (12). During PD-L1 evaluation with the SP263 assay, a reasonable formalin fixation time is considered to be 6-72 h (13). Although upper limits for the formalin fixation period have been imposed, the data on which these limits are based, are poorly documented. Therefore, it is important to experimentally verify the appropriate formalin fixation time for PD-L1 evaluation by IHC. In the present study, we investigated the association between formalin fixation time and PD-L1 expression in NSCLC patients, and clarified the optimal duration of fixation for accurate PD-L1 evaluation by IHC.

Materials and Methods

Patients and sample processing. We collected a total of 55 NSCLC samples from resected lungs at Kobe City Medical Center General Hospital from February 2017 to December 2017. We excluded specimens diagnosed with adenocarcinoma *in situ* and minimally invasive adenocarcinoma, because sufficient tumor cells were needed for PD-L1 interpretation. The tumor samples of the resected lung specimens were divided into two parts and both were immediately placed in 10% buffered formalin. One portion of each tumor sample was fixed for 12-24 hours to ensure adequate fixation (normal fixation), which is the duration recommended by established guidelines (12, 13). The other portion was fixed for 96-120 hours (prolonged fixation) (Figure 1). Following fixation, each tumor block was immediately processed for paraffin embedding. The PD-L1 in the resulting paraffin-embedded (FFPE) tumor blocks was then assessed using IHC to establish their TPS.

Patients who reported never having smoked were defined as non-smokers, those who had smoked within 1 year of NSCLC diagnosis were categorized as current smokers, and the remaining patients were considered former smokers. All patients were classified on the basis of clinical stage according to the 7th edition TNM classification (14). All tumor specimens included in the pathological analysis were obtained with informed consent. The study was approved by the Ethics Committee of Kobe City Medical Center General Hospital (Hyogo, Japan). The research was conducted in accordance with the Declaration of Helsinki (15).

Evaluation of tissue IHC. Two assay systems (22C3 and SP263) were used to evaluate the expression of PD-L1 by IHC in NSCLC specimens. Tissue sections (4 μm thick) were cut serially from FFPE tumor blocks and were then routinely deparaffinized and rehydrated. For the 22C3 assay, sections were stained with PD-L1 22C3 mouse monoclonal primary antibodies using the EnVision FLEX visualization system on a Dako Autostainer Link 48 system (Agilent Technologies, Santa Clara, CA) (16, 17). For the SP263 assay, sections were stained with rabbit monoclonal primary antibodies and a matched rabbit immunoglobulin G-negative control with an OptiView DAB IHC Detection Kit on a BenchMark XT automated staining platform (Ventana Medical Systems, Tucson, AZ) (13).

Tumor PD-L1 scores of samples were evaluated by two independent pathologists according to the respective scoring algorithms for these assays. Each slide was randomized and anonymized, and PD-L1 TCs were considered positive when the cell membrane was partially or completely stained, regardless of the staining intensity. Hematoxylin and eosin (H & E) staining was used to orient the IHC slides. The TPS of PD-L1 in TCs was expressed as the percentage of PD-L1-positive TCs in the overall tumor section, and was estimated in increments of 5%, except in the case of 1% positivity. In the case of a disagreement, the slides were re-examined until consensus was reached. We set 1%, 25%, and 50% as the cut-off values, according to the values used in previous prospective clinical trials of PD-1 axis inhibitors.

Statistical analysis. Concordance between normal fixation and prolonged fixation specimens was evaluated using the overall percentage of agreement (OPA) related to the 1%, 25%, and 50% thresholds. A paired *t*-test was used to compare the TPS between specimens with normal fixation and prolonged fixation. A *p*-value of <0.05 was considered to indicate statistical significance. Statistical analyses were performed using JMP 11 software (SAS Institute, Cary, NC, USA).

Results

Patient characteristics. A total of 55 NSCLC patient specimens were collected for the present study, one of which did not contain enough tumor cells. Patient characteristics are summarized in Table I. The median patient age was 70.9 years, and most patients were men (65%), had a smoking history (67%), adenocarcinoma (67%), and stage I NSCLC (74%).

PD-L1 IHC evaluation by 22C3 assay. We compared the TPS of PD-L1 according to the 22C3 assay between the normal and prolonged fixation groups. The mean PD-L1 TPS was not significantly different between normal and prolonged fixation groups (normal fixation: 18.8%; prolonged fixation: 16.3%, *p*=0.277) (Figure 2A). We also assessed the TPS between normal and prolonged fixation groups using the 22C3 assay (Table II). The OPAs at the PD-L1 expression cut-offs of 1%, 25%, and 50% were 85%, 94%, and 91%, respectively. Fourteen samples exhibited discordant category features between the normal and prolonged fixation groups: six belonged to a higher category in the normal fixation group compared to the prolonged one, while eight belonged to a lower category. Of these 14 samples, eight were negative (TPS <1%) in one fixation group, but not in both and exhibited TPS values between 1% and 10% in the positive group. One was discordant because its TPS was 1-24% in one fixation group, but 25-49% in the other. Five samples were strongly positive (TPS ≥50%) in one fixation group, but not in the other, and the TPS values of these five specimens, according to the normal and prolonged fixation group, were 80% and 20%, 75% and 30%, 60% and 25%, 30% and 70%, and 20% and 65%, respectively.

PD-L1 IHC evaluation by SP263 assay. We compared the TPS of PD-L1 using the SP 263 assay between FFPE samples fixed for normal and prolonged periods. The mean PD-L1 TPS was not significantly different between normal and prolonged fixation groups (normal fixation: 16.2%; prolonged fixation: 17.6%, *p*=0.560) (Figure 2B). We also assessed the TPS between normal fixation and prolonged fixation groups using the SP263 assay (Table III). The OPAs at the PD-L1 expression cut-offs of 1%, 25%, and 50% were 80%, 94%, and 93%, respectively. Sixteen samples exhibited discordant category features between the normal and prolonged fixation groups. Of these 16 samples, three belonged to a higher category in the normal fixation group compared to the prolonged one, while 13 belonged to a lower category. In
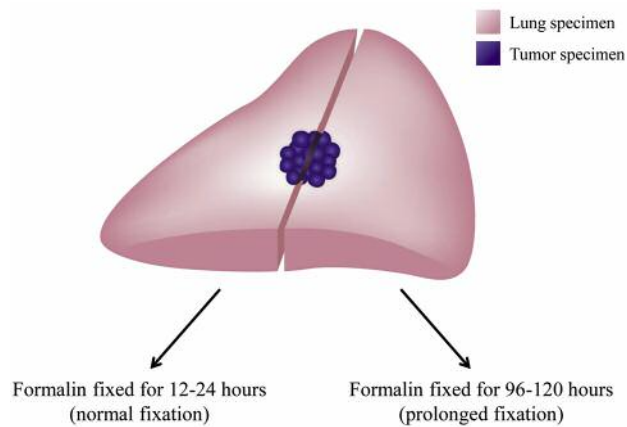
Figure 1. *Schematic of specimen fixation procedure.*

Table I. *Characteristics of 54 non-small cell lung cancer patients recruited to test the effect of formalin fixation duration on programmed cell death ligand 1 immunohistochemistry analysis.*

Characteristics	n (%) (N=54)
Age (years)	
Mean (SD)	70.9 (10.3)
Gender	
Male	35 (65)
Female	19 (35)
Smoking status	
Non-smoker	18 (33)
Current or former	36 (67)
Histology	
Adenocarcinoma	36 (67)
Squamous cell carcinoma	13 (24)
Others	5 (9)
Stage	
I	40 (74)
II	9 (17)
III	4 (7)
IV	1 (2)

SD: Standard deviation.

addition, 11 of these were negative (TPS <1%) in one group, but not in the other. Of these 11 samples, all exhibited TPS values between 1% and 5% in the positive group. One sample was discordant because it had a TPS of 1-24% in one fixation group but 25-49% in the other. Four were strongly positive (TPS ≥50%) in one group but not in the other and in these, the TPS values, according to the normal and prolonged fixation group, were 95% and 25%, 65% and 30%, 20% and 85%, and 10% and 80%, respectively.

PD-L1 IHC evaluation of patients with TPS≥1% by 22C3 assay. We also analyzed 27 tumor samples with a TPS≥1%, according to the 22C3 assay in the sample that had undergone normal fixation. The mean PD-L1 TPS obtained was not significantly different between normal and prolonged fixation groups (normal fixation: 32.6%; prolonged fixation: 32.1%, *p*=0.231) (Figure 2C). The OPAs at the PD-L1 expression cut-offs of 1%, 25%, and 50% were 92%, 89%, and 81%, respectively (Table IV). In these samples, eight exhibited discordant results between normal and prolonged fixation groups, and of these eight samples, six belonged to a higher category in the normal fixation group compared to the prolonged one, and two belonged to a lower category.

PD-L1 IHC evaluation of patients with TPS ≥1% by SP263 assay. We also analyzed 28 tumor samples with TPS ≥1%, according to the SP263 assay in the sample that had undergone normal fixation. The mean PD-L1 TPS according to the SP263 assay was not significantly different between normal and prolonged fixation groups (normal fixation: 31.3%; prolonged fixation: 33.4%, *p*=0.659) (Figure 2D). The OPAs at the PD-L1 expression cut-offs of 1%, 25%, and 50% were 96%, 89%, and 86%, respectively (Table V). In these samples, six were discordant between the normal and

prolonged fixation groups. Of these, three belonged to a higher category in the normal fixation group compared to the prolonged one, and three belonged to a lower category.

Discussion

To the best of our knowledge, this is the first study to investigate the association between the PD-L1 TPS and formalin fixation time. Our results revealed that formalin fixation for up to 120 hours has no apparent effect on PD-L1 IHC expression, according to both 22C3 and SP263 assay, and it is therefore possible to evaluate PD-L1 TPS in tumor samples that have been fixed for extended periods of time up to 120 hours.

In the present study, PD-L1-TPS was not significantly different between specimens with normal and prolonged fixation duration. We observed a similar result in tumor specimens with TPS≥1%. For PD-L1 IHC testing in lung cancer, the previously recommended fixation protocol was with 10% buffered formalin for 6-48 hours (12, 13). However, the relevance of this recommendation was not supported by empirical evidence. Similarly, guidelines for HER2 IHC evaluation suggested a formalin fixation duration of 6-72 h, although previous reports had shown that fixation times between 3 and 168 h do not affect HER2 expression (11, 18-20). Therefore, formalin fixation duration up to 120 h appears to have little to no impact on the expression of routine prognostic markers. Further investigations should be

Table II. *Comparison of programmed cell death ligand 1 tumor proportion score (TPS) at each cut-off level according to the 22C3 assay (N=54).*

TPS	Prolonged fixation			
	<1%	1-24%	25-49%	≥50%
Normal fixation				
<1%	21	6	0	0
1-24%	2	10	0	1
25-49%	0	1	1	1
≥50%	0	1	2	8

Concordant pairs: 0.74 (40/54)
Higher with normal fixation: 0.11 (6/54)
Higher with prolonged fixation: 0.15 (8/54)
Agreement (1%, 25%, 50%): 85%, 94%, 91%

Table V. *Comparison of programmed cell death ligand 1 tumor proportion score (TPS) at each cut-off in patients with a TPS ≥1% according to SP263 assay (N=28).*

TPS	Prolonged fixation			
	<1%	1-24%	25-49%	≥50%
Normal fixation				
<1%	0	0	0	0
1-24%	1	13	1	2
25-49%	0	0	2	0
≥50%	0	0	2	7

Concordant pairs: 0.79 (22/28)
Normal fixation higher: 0.11 (3/28)
Prolonged fixation higher: 0.11 (3/28)
Agreement (1%, 25%, 50%): 96%, 89%, 86%

Table III. *Comparison of programmed cell death ligand 1 tumor proportion score (TPS) at each cut-off level according to the SP263 assay (N=54).*

TPS	Prolonged fixation			
	<1%	1-24%	25-49%	≥50%
Normal fixation				
<1%	16	10	0	0
1-24%	1	13	1	2
25-49%	0	0	2	0
≥50%	0	0	2	7

Concordant pairs: 0.70 (38/54)
Higher with normal fixation: 0.05 (3/54)
Higher with prolonged fixation: 0.24 (13/54)
Agreement (1%, 25%, 50%): 80%, 94%, 93%

Table IV. *Comparison of programmed cell death ligand 1 tumor proportion score (TPS) at each cut-off for patients with a TPS ≥1% according to the 22C3 assay (N=27).*

TPS	Prolonged fixation			
	<1%	1-24%	25-49%	≥50%
Normal fixation				
<1%	0	0	0	0
1-24%	2	10	0	1
25-49%	0	1	1	1
≥50%	0	1	2	8

Concordant pairs: 0.70 (19/27)
Normal fixation higher: 0.22 (6/27)
Prolonged fixation higher: 0.07 (2/27)
Agreement (1%, 25%, 50%): 92%, 89%, 81%

conducted to determine the boundaries of the permissible formalin fixation time for PD-L1 IHC evaluation.

The present study was conducted using two major PD-L1 IHC assay systems, 22C3 and SP263. Currently, PD-L1 IHC 22C3 pharm, using the Dako autostainer, is the only assay that has been approved as a companion diagnostic for pembrolizumab use by the FDA (21). However, the Dako autostainer is not a common laboratory instrument as compared to the Ventana BenchMark platform, which is widely used (22). Therefore, in clinical practice, the utility of the Ventana platforms needs to be demonstrated. While the PD-L1 IHC assays of SP142 and SP263 require the use of Ventana platform, SP142 produced conflicting results, while 28-8, 22C3, and SP263 assays have produced concurring results (23-25). For these reasons, the Conformité Européene has allowed the SP263 assay to be used for PD-L1 IHC assessment to facilitate treatment decisions for PD-1 axis inhibitors (26). We, therefore, chose to assess the effects of fixation time for both the 22C3 and SP263 assays.

In clinical practice, lung biopsy tests or thoracic surgery are often performed on weekdays, while the laboratory that performs PD-L1 IHC evaluation is closed on weekends and holidays. If there is a long weekend, specimens received on Friday will be fixed for a minimum of 72 h, which is beyond the recommended duration of fixation. The present study demonstrated that formalin fixation for up to 120 h has no significant effect on PD-L1 IHC analysis, and therefore, tissue specimens that have been fixed for longer than the recommended duration may be reliable for clinical evaluation of TPS.

In the present study, about 20% of NSCLC tumor samples had ≥50% PD-L1 expression, which is lower than advanced NSCLC tumor samples in several clinical trials (23-30%) (7, 27, 28). However, our study was conducted in patients with
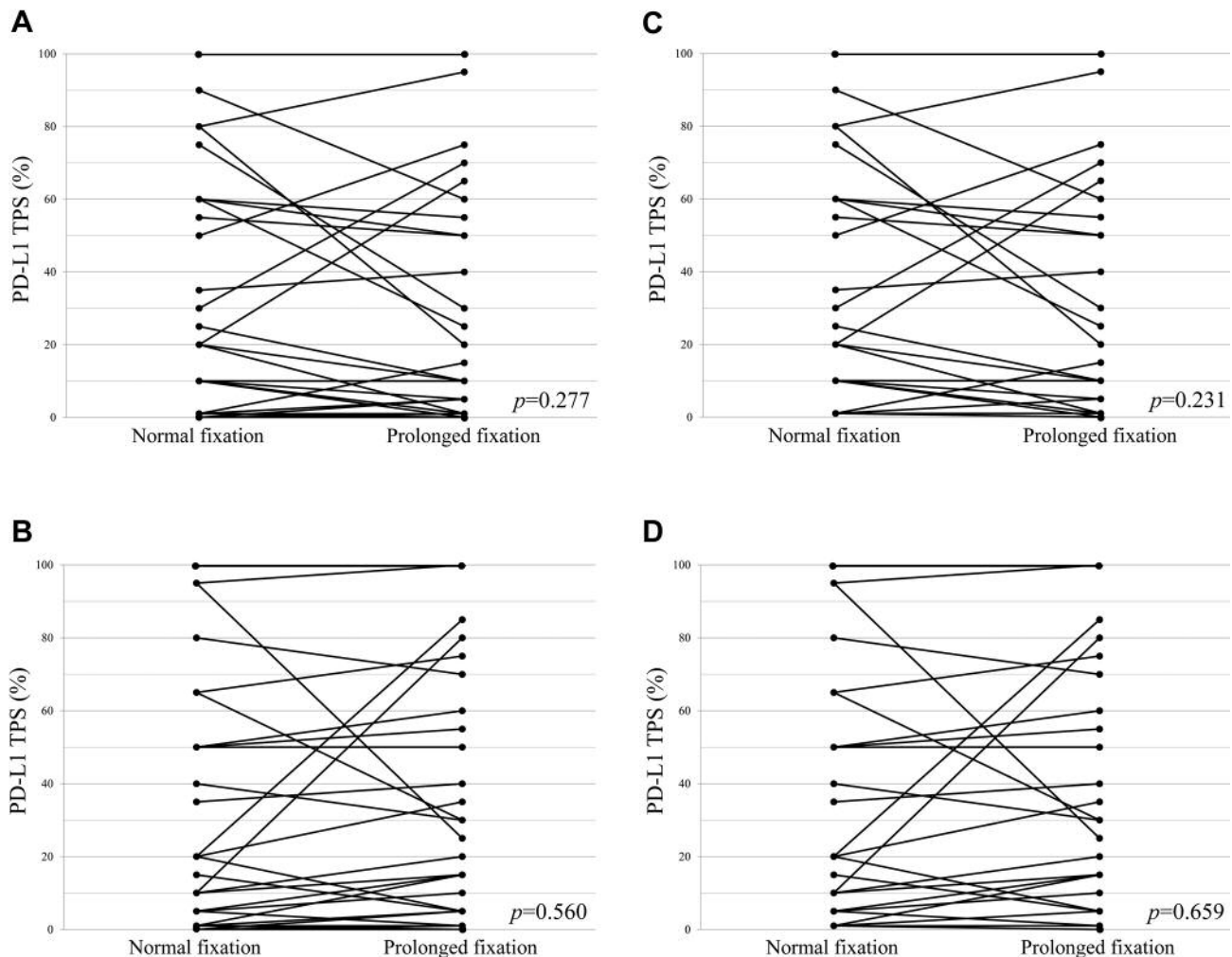
Figure 2. *Comparison of programmed death ligand (PD-L1) tumor proportion score (TPS) values between normal fixation and prolonged fixation in (A) all tumor specimens according to 22C3 assay, (B) all tumor specimens in the SP263 assay (C), tumor specimens with TPS ≥1% according to the 22C3 assay, and (D) tumor specimens with TPS ≥1% according to the SP263 assay.*

an early-stage NSCLC, and in previous studies, 7-26% of the surgically resected NSCLC specimens had ≥50% PD-L1 expression by 22C3 assays (29-31). Therefore, the proportion of samples with ≥50% PD-L1 expression in our cohort is representative of the clinical setting.

Although we found no significant differences in PD-L1 between different fixation treatment groups, our study had several limitations. First, this study had a single-center design with Japanese patients. Second, in advanced NSCLC patients, small biopsy samples are often used for PD-L1 assessment in clinical practice, however, in the present study, we analyzed only surgical specimens with an adequate number of tumor cells. Thus, a larger study cohort with small biopsy samples could validate our findings and establish optimal specimen processing methods for PD-L1 assessment.

In conclusion, we found that formalin fixation duration up to 120 h does not affect PD-L1 IHC expression in NSCLC specimens according to either 22C3 or SP263 assay. We therefore propose that PD-L1 IHC-based TPS can be established in clinical practice using tumor samples that have been formalin fixed for longer than the recommended duration.

Conflicts of Interest

Authors' Contributions

DF designed the study. HK and DF wrote the initial draft of the manuscript, contributed to analysis and interpretation of data and assisted in the preparation of the manuscript. All other authors have contributed to data collection and interpretation, and have critically reviewed the manuscript. All authors approved the final version of the manuscript, and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved.

Acknowledgements

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