Showa Univ J Med Sci 34(2), 64−77, June 2022

Original Paper

The PD-L1/22C3 assay for primary lung cancer is feasible for daily clinical practice irrespective of the diagnostic procedure

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Received: 21 September 2021 / Accepted: 19 November 2021

Abstract

The programmed death ligand 1 immunohistochemistry 22C3 pharm DX assay (PD-L1/22C3) is commonly used for assessing PD-L1 expression in non-small-cell lung cancer. Although various sample types have been used for the PD-L1 assay, the feasibility of the PD-L1/22C3 assay in clinical practice remains undefined. At Showa University Hospital, 270 patients diagnosed with primary lung cancer and 271 pathological specimens were assessed. The overall failure rate of the PD-L1/22C3 assay, tumor proportion score (TPS) distribution, and clinical characteristics were retrospectively reviewed. Efficacy, including objective response rate, progression-free-survival, and overall survival, following pembrolizumab monotherapy for patients with high PD-L1 expression and anti-PD-1/PD-L1 treatment for previously treated patients were also retrospectively analyzed. The overall failure rate for the PD-L1/22C3 assay was 3.0%. PD-L1 expression classified by TPS＜1%, 1−49%, and ≥ 50% was 31%, 33%, and 33%, respectively. Thirty-one patients with high PD-L1 expression (TPS ≥ 50%) received first-line pembrolizumab monotherapy, which exhibited high efficacy and outcome, irrespective of the diagnostic procedure. In 65 patients, anti-PD-1/PD-L1 monotherapy used as second- or further-line treatment showed moderate efficacy, irrespective of the diagnostic procedure and the period between tumor acquisition and PD-L1 assay. However, PD-L1 positivity did not affect clinical outcome. The PD-L1/22C3 assay is feasible in a clinical setting because of its low failure rate and it is a good predictor of pembrolizumab efficacy. For previously treated patients, prediction of the effectiveness of anti-PD-1/PD-L1 treatment based on PD-L1 expression should be considered.

Key words: programmed death ligand 1, immunohistochemistry, 22C3, pembrolizumab, diagnostic procedure

Introduction

Non-small-cell lung cancer (NSCLC) is a leading cause of death globally. Platinum-based chemotherapy is the standard treatment for advanced NSCLC. Numerous oral kinase inhibitors targeting genetic abnormalities, such as *EGFR* mutations, *ALK*, and *ROS-1* rearrangements, have also been introduced into treatment regimens. These drugs produce enhanced tumor responses and significantly prolong survival[1−3]. Despite their success, the emergence of drug resistance is inevitable and the overall survival benefits are limited. Therefore, new approaches to effectively treat NSCLC are needed. Immune

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checkpoint inhibition with anti-programmed cell death-1 (PD-1)/programmed cell death ligand-1 (PD-L1) antibodies have demonstrated significant antitumor effects and survival benefits in NSCLC patients, with a substantial population achieving a long-term response[4, 5]. Thus, anti-PD-1/PD-L1 treatment has become standard treatment for advanced NSCLC. Four drugs, including the anti-PD-1 antibodies, nivolumab and pembrolizumab, and the anti-PD-L1 antibodies, atezolizumab and durvalumab, have been approved for NSCLC treatment in various clinical settings. PD-L1 expressed on tumor cells as determined by immunohistochemistry (IHC) is a reliable biomarker to predict response to these drugs. Several antibody clones, such as 28-8, 22C3, and SP-142, have been used to evaluate PD-L1 expression in tumor specimens[6]. The PD-L1 assay using the 22C3 clone (PD-L1/22C3) is an approved companion diagnostic for pembrolizumab efficacy[7]. Among patients exhibiting PD-L1 overexpression on more than 50% of their tumor cells as assessed by PD-L1/22C3, pembrolizumab showed a significantly higher progression-free survival compared with platinum-based chemotherapy in the first-line setting[8, 9]. This assay has been used in clinical practice because of its high concordance with other clones including 28-8 and SP142[10, 11]. Therefore, PD-L1/22C3 has become the most popular PD-L1 assay in clinical practice. In early landmark clinical trials, tumor samples used to evaluate PD-L1 expression were limited to core-needle biopsies (CNB) or excisional biopsy, because accurate PD-L1 measurement requires an adequate number of tumor cells (at least 100 cells)[7]. It is also believed that the quality of tumor staining deteriorates naturally; therefore, fresh specimens obtained within six months are recommended[7, 8]. In real-world settings, various sample types, such as transbronchial biopsy (TBB), endobronchial ultrasound needle aspiration (EBUS-TBNA), and CNB have been used, and the time between obtaining the samples and assessing PD-L1 expression varies. Since the feasibility of measuring PD-L1 in clinical practice has not been fully evaluated, it remains a major concern for physicians. Therefore, we retrospectively evaluated the feasibility of the PD-L1/22C3 assay in clinical practice.

Materials and methods

Study subjects and data collection

Patients diagnosed with primary lung cancer at the Showa University Hospital between March 2017 and December 2019 were selected and the associated pathological specimens that were assessed for PD-L1 expression using the PD-L1 IHC 22C3 pharm DX assay were analyzed.

Demographic characteristics, including age, sex, smoking status, histology, tumor stage at the time of the PD-L1 assay (based on the International Association for the Study of Lung Cancer 8th edition), and gene alteration profiles were collected from medical records. Diagnostic procedures to obtain pathological specimens, PD-L1 expression, and the time between tumor collection and PD-L1 assay were also obtained from the medical records. For the patients receiving anti-PD-1/PD-L1 treatment, clinical efficacy, including objective response rate (ORR), progression-free survival (PFS), and overall survival (OS), was retrospectively reviewed.

The study was conducted in accordance with the Declaration of Helsinki and the Ethics Committee of Showa University approved the protocol (approval number 3111). Written informed consent from the patients was waived. Eligible patients were given the opportunity to opt out from the study.

Efficacy assessment and statistics

The ORR was assessed using the physician-based Response Evaluation Criteria in Solid Tumors version 1.1. The correlation between ORR and the selected variable was analyzed. Categorical and continuous variables were analyzed using Fisher's exact test and Wilcoxon rank-sum test, respectively. PFS was defined as the date from the start of anti-PD-1/PD-L1 treatment to confirmation of disease progression as determined by the treating physician or death. The OS was defined as the date from the start of anti-PD-1/PD-L1 treatment to death, regardless of the cause of death. PFS and OS were estimated by the Kaplan-Meyer method. The risk ratios of selected factors for PFS and OS were analyzed using a Cox regression model. A *P*-value less than 0.05 was considered statistically significant. The analyses were conducted using JMP pro version 15 (SAS institute CA USA).

Results

Study subjects and baseline characteristics

A total of 270 patients with 271 specimens were enrolled and reviewed (a TBB specimen and surgical specimen were obtained from one patient). The characteristics of the patients are listed in Table 1. The median age was 70 years and the majority were males. The majority (78.9%) of the patients were

Table 1. Patient characteristics (N = 270)

Characteristic	No. of Patients (%)
Age, years	
Median (range)	70 (34-89)
Sex	
Male	193 (71.5%)
Female	77 (28.5%)
Smoking Status	
Current	82 (30.4%)
Former	131 (48.5%)
Never	54 (20.0%)
Unknown	3 (1.1%)
ECOG performance status	
0-1	197 (73.0%)
2 ≤	73 (27.0%)
Histology	
Adeno	190 (70.4%)
Squamous	55 (20.4%)
Adenosquamous	2 (0.7%)
NSCLC (NOS)	4 (1.5%)
Undifferentiated	9 (3.3%)
Neuroendocrine	5 (1.9%)
SCLC	2 (0.7%)
Pleomorphic	1 (0.4%)
Large cell	1 (0.4%)
Sarcomatoid	1 (0.4%)
Driver gene alterations	
EGFR mutations	
Positive	51 (18.9%)
Negative	202 (74.8%)
Unknown	17 (6.3%)
ALK rearrangements	
Positive	6 (2.2%)
Negative	188 (69.6%)
Unknown	76 (28.1%)
ROS-1 rearrangements	
Positive	6 (2.2%)
Negative	129 (47.8%)
Unknown	135 (50.0%)
Tumor stage	
ⅠA-ⅡB	19 (7.0%)
ⅢA-ⅢC	68 (25.2%)
ⅣA	75 (27.8%)
ⅣB	106 (39.3%)
Unknown	2 (0.7%)

Abbreviations
ECOG : Eastern Cooperative Onclogy Group
NSCLC : Non-small-cell lung cancer
NOS : Non other specified
SCLC : Small-cell lung cancer

Table 2. Diagnostic procedures

Diagnostic Procedures	No. of Patients (%)	
Endoscopic biopsy	123	45.4%
TBB	98 (79.7%)	
EBUS-TBNA	16 (13.0%)	
EUS-FNA	8 (6.5%)	
Esophageal biopsy	1 (0.8%)	
Resection or excisional biopsy	76	28.0%
Lung	54 (71.1%)	
Pleura	4 (5.3%)	
Subclavian LN	6 (7.9%)	
Mediastinal LN	4 (5.3%)	
Axillary LN	2 (2.6%)	
Inguinal LN	1 (1.3%)	
Brain	5 (6.6%)	
Core needle biopsy (N = 71)	71	26.2%
Lung or Pleura	50 (70.4%)	
Subclavian LN	9 (12.7%)	
Bone or soft tissues	6 (8.5%)	
Liver	5 (7.0%)	
Adrenal gland	1 (1.4%)	
Others (N = 1)	1	0.4%
Pleural effusion	1 (100%)	

Abbreviations
TBB : Transbronchial biopsy
EBUS-TBNA : Endobronchial ultrasound needle aspiration
EUS-FNA : Endoscopic ultrasound fine-needle aspiration
LN : Lymph node

smokers and 70.4% had adenocarcinoma. Although seven patients (2.6%) were finally diagnosed with neuroendocrine or small cell carcinoma, which was not indicated for PD-L1 assay, the initial diagnosis was undifferentiated carcinoma. Driver gene alterations were observed in 63 patients. The most common gene alteration was *EGFR* mutation, which was observed in 51 cases (18.9%), followed by *ALK* rearrangements in six patients (2.2%), and *ROS-1* rearrangements in six cases (2.2%). Although 19 cases (7%) were in the early stage of disease, most patients were at an advanced stage.

Diagnostic procedures and site of collection of tumor specimens

Among the 270 patients, 271 specimens were analyzed for PD-L1 expression. In total, 123 (45.4%), 76 (28.0%), and 71 (26.2%) specimens were obtained by endoscopic, resection or excisional,

or CNB, respectively (Table 2). In the case of endoscopic biopsy, 79.7% of the specimens were obtained by TBB, whereas 13.0% and 7.3% were obtained by EBUS-TBNA and gastrointestinal endoscopy, respectively. Most resection or excisional biopsies were collected from the intrathoracic region, whereas five cases (6.6%) were from distant brain metastases. For CNB, most target sites were intrathoracic regions, such as the lung and pleura, whereas distant metastases, including soft tissues, liver, and adrenal gland, accounted for 16.9% of the cases.

PD-L1 expression and period between tumor collection and PD-L1 assay

PD-L1 expression was not evaluated in 3.0% (8 out of 271) of the cases. One of these cases was a malignant pleural effusion, two cases were conventional TBB, two cases were TBB samples obtained by the radial endobronchial ultrasound guide sheath (r-EBUS-GS) method, two were obtained by EBUS-TBNA, and the remaining were metastatic adrenal glands obtained by core-needle biopsy (Table 3A). In the total population, the proportions of PD-L1 expression classified by tumor proportion score (TPS) <1%, 1–49%, and ≥ 50% were 31% (84 of 271), 33% (89 of 271), and 33% (90 of 271), respectively (Figure 1A). Moreover, in the population with unknown driver mutations or without driver mutations (N=208), the proportions of PD-L1 expression classified by TPS were lower compared with those of the total population and were 28.4% (59 of 208), 32.2% (67 of 208), and 36.1% (90 of 208) for TPS <1%, 1–49%, and ≥ 50%, respectively. Among the 21 distant metastatic cases, the proportion of PD-L1 expression was 33.3% (7 of 21) for TPS <1%, 28.6% (6 of 21) for TPS 1–49%, 28.6% (7 of 21) for TPS ≥ 50%, and 4.8% (1 of 21) for non-evaluable cases. Similarly, among the 250 intrathoracic cases, the proportion of PD-L1 expression was 30.8% (77 of 250) for TPS <1%, 33.6% (84 of 250) for TPS 1–49%, 32.8% (82 of 250) for TPS ≥ 50%, and 2.8% (7 of 250) for the non-evaluable cases.

The period between tumor acquisition and PD-L1 assay is shown in Figure 1B. More than half of the samples were acquired within 30 days, 10% were acquired between 30 and 90 days, and 6% were acquired between 90 days and 6 months. In brief, about one-fourth of the cases were obtained after more than 6 months. However, the proportion of archival specimens stored over 6 months decreased annually, being 36.6% (41/112) for 2017, 26.0% (20/77) for 2018, and 13.4% (11/84) for 2019. PD-L1 expression and the period profile according to diagnostic procedure are summarized in Table 3B.

Efficacy analysis and its association with patient factors

Of the 270 patients, 31 exhibited high tumor PD-L1 expression (TPS ≥ 50%) that also had adequate organ function. These patients, who were candidates for systemic immunotherapy as determined by the treating physicians, received first-line pembrolizumab monotherapy as standard first-line treatment. Patient demographics are summarized in Table 4A. For the patients who received first-line pembrolizumab monotherapy, the ORR and disease control rate (DCR) were 53.1% and 87.5%, respectively. Of the 270 patients, 65 who had adequate organ function and were candidates for systemic immunotherapy, received anti-PD-1/PD-L1 monotherapy (nivolumab, pembrolizumab, atezolizumab) as second-line or further-line treatment. The use of anti-PD-1/PD-L1 agents depended on the selection of the physician. Patient demographics including PD-L1 expression profiles are shown in Table 4B. For second-line or further-line anti-PD-1/PD-L1 treatment (n=65), 51 patients (78.5%) were treated with nivolumab, nine patients (13.8%) with pembrolizumab, and five (7.7%) with atezolizumab. Of the 65 patients who received anti-PD-1/PD-L1 monotherapy as a second- or further-line therapy, the ORR and DCR were 21.5% and 61.5%, respectively. The response profiles are summarized in Table 4C. Next, we analyzed the association of tumor response with patient factors and other factors, including diagnostic procedures and the time between tumor collection and PD-L1 measurement. The responders and non-responders were defined as patients who achieved a CR or PR, and those who did not, respectively. No significant differences were observed between the responding and non-responding groups (Table 5A, 5B).

Survival outcomes of patients receiving anti-PD-1/PD-L1 treatment and its association with patient factors

For patients with high PD-L1 expression (TPS ≥ 50%) who received pembrolizumab monotherapy, the median PFS and median OS were 14.3 months (95% C.I. 7.4–30.1) and 34.3 months (95% C.I. 23.9-not estimated), respectively (Figure 2A, 2B).

A subgroup analysis using a Cox regression model was performed to identify patient factors that affected survival. Poor performance status (≥ 2) was significantly associated with a worse PFS, but not OS. Other factors including age, sex, tumor

Table 3A. Case profile for failure of the programmed cell death ligand-1 (PD-L1) assay

Case No.	Age	Gender	Histology	Tumor stage	PS	Period of PD-L1 assay (days)	Period of PD-L1 assay (months)	Biopsy sample	Diagnostic procedure	Number of biopsy samples	Reason for failure
1	59	Male	Adeno	IVA	1	439	>6 months	Malignant effusion	Thoracentesis	N/A	not indicated
2	73	Female	Adeno	IVB	1	12	≤ 6 months	Lung (primary lesion)	Conventional TBB	3	tumor cell <100
3	64	Female	Adeno	IVB	1	13	≤ 6 months	Lymhonode	EBUS-TBNA	3	tumor cell <100
4	59	Male	Adeno	IVB	1	1188	>6 months	Adrenal gland	Image guided CNB	6	tumor cell <100
5	77	Male	Adeno	IVB	1	13	≤ 6 months	Lung (primary lesion)	r-EBUS-GS	4	tumor cell <100
6	85	Female	Adeno	IVB	1	13	≤ 6 months	Lung (primary lesion)	Conventional TBB	5	tumor cell <100
7	73	Female	Squamous	IVA	1	5	≤ 6 months	Lung (primary lesion)	r-EBUS-GS	4	tumor cell <100
8	78	Male	Adeno	IVB	3	8	≤ 6 months	Lymhonode	EBUS-TBNA	2	tumor cell <100

Abbreviations, TBB : Transbronchial biopsy EBUS-TBNA : Endobronchial ultrasound needle aspiration CNB : Core needle biopsy r-EBUS-GS : Radial endobronchial ultrasound guide sheath

Table 3B. The PD-L1 expression and the period profile according to the diagnostic procedure

	Endoscopic biopsy N = 123 (%)	Resection or excisional biopsy N = 76 (%)	Core needle biopsy N = 71 (%)
Period between tumor obtainig and PD-L1 testing			
≤ 6 months	96 (78.0%)	40 (52.6%)	63 (88.7%)
>6 months	27 (22.0%)	36 (47.4%)	8 (11.3%)
Tumor Proportion Score			
<1%	39 (31.7%)	29 (38.2%)	16 (22.5%)
1–49%	38 (30.9%)	24 (31.6%)	27 (38.0%)
50%≤	40 (32.5%)	23 (30.3%)	27 (38.0%)
Not evaluable	6 (4.9%)	0 (0%)	1 (1.4%)

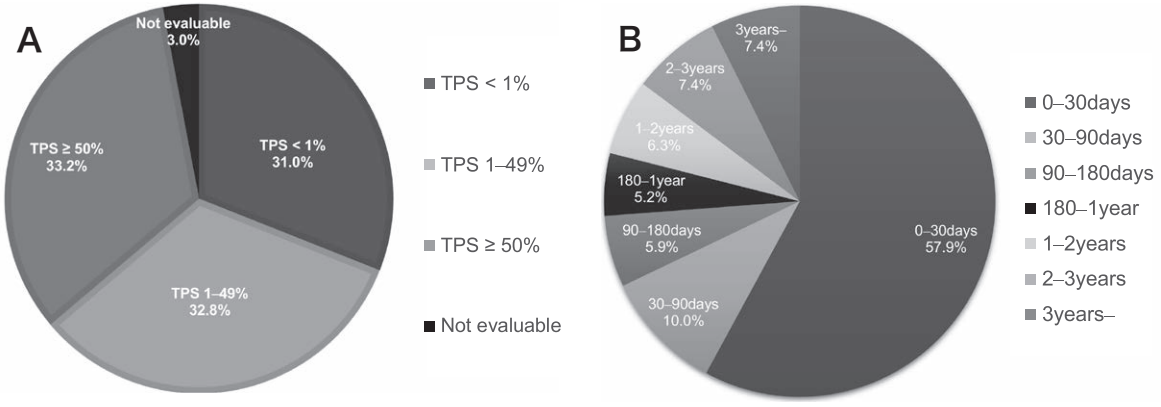

Fig. 1.
A : Programmed cell death ligand-1 (PD-L1) expression classified by tumor proportion score (TPS)
B : The period between tumor sample acquisition and PD-L1 assay

Table 4A. Patient characteristics (N=31) 1st line pembrolizumab

Characteristics	No. of Patients (%)
Age (median, years, range)	69 (40-83)
Sex	
Female	6 (19.4%)
Male	25 (80.6%)
Histology	
Adeno	19 (61.3%)
Squamous	11 (35.5%)
Adenosquamous	1 (3.2%)
Smoking Status	
Current	14 (45.2%)
Former	14 (45.2%)
Never	3 (9.7%)
Tumor stage	
Ⅲ	8 (25.8%)
Ⅳ	23 (74.2%)
PS	
0-1	22 (71.0%)
2≤	9 (29.0%)
Driver mutation	
Negative	30 (96.8%)
Positive*	1 (3.2%)
Diagnostic procedures	
Resection	7 (22.6%)
CNB	9 (29.0%)
Endoscopic	15 (48.4%)
Period between tumor obtaining and PD-L1 assay	
≤6 months	28 (90.3%)
>6 months	3 (9.7%)

**ROS-1* rearrangement case
Abbreviations
PS : Performance status CNB : Core needle biopsy
NOS : Non other specified TPS : Tumor proportion score

Table 4B. Patient characteristics (N=65) anti-PD-1/PD-L1 treatment for previously treated patients

Characteristics	No. of Patients (%)
Age (median, years, range)	68 (43-86)
Sex	
Female	17 (26.2%)
Male	48 (73.8%)
Histology	
Adeno	49 (75.4%)
Squamous	11 (16.9%)
NSCLC (NOS)	1 (1.5%)
Pleomorphic	1 (1.5%)
Undifferentiated	3 (4.6%)
Smoking Status	
Current	13 (20.0%)
Former	40 (61.5%)
Never	12 (18.5%)
Tumor stage	
Ⅱ-Ⅲ	15 (23.1%)
Ⅳ	50 (76.9%)
PS	
0-1	57 (87.7%)
2≤	8 (12.3%)
PD-L1 expression	
TPS ≥ 50%	14 (21.5%)
TPS 1-49%	22 (33.8%)
TPS < 1%	26 (40.0%)
Unknown	3 (4.6%)
Driver mutation	
Negative	58 (89.2%)
Positive	7 (10.8%)
Diagnostic procedures	
Resection	16 (24.6%)
CNB	18 (27.7%)
Endoscopic	30 (46.2%)
Others	1 (1.5%)
Period between tumor obtaining and PD-L1 assay	
≤6 months	31 (47.7%)
>6 months	34 (52.3%)

Table 4C. Response profile of anti-PD-1/PD-L1 treatment

	First-line pembrolizumab monotherapy for patients with TPS ≥ 50% (N = 31)			anti-PD-1/PD-L1 treatment for previously treated patients (N = 65)		
Response Outcomes	N	%	95% C.I.	N	%	95% C.I.
Complete Response (CR)	0	0		0	0	
Partial Response (PR)	16	51.6%	34.0-69.2	14	21.5%	11.5-31.5
Stable Disease (SD)	11	35.5%	18.6-52.3	26	40.0%	28.1-51.9
Progressive Disease (PD)	4	12.9%	1.10-24.7	23	35.4%	23.7-47.0
Not evaluable (NE)				2	3.1%	
Objective Response Rate (ORR)		51.6%	34.0-69.2		21.5%	11.5-31.5
Disease Controle Rate (DCR)		87.1%	75.3-98.9		61.5%	49.7-73.4

Abbreviations, TPS : Tumor proportion score C.I. : Confidential Interval

Table 5A. Correlation between patients' characteristics and tumor response of first-line pembrolizumab

		N (%)		
		Responders (N = 16)	Non-Responders (N = 15)	*P* value
Age (median, years)		67	70	0.056
Sex	Female	2 (33.3%)	4 (66.7%)	0.39
	Male	14 (56.0%)	11 (44.0%)	
Histology	Squamous	7 (58.3%)	5 (41.7%)	0.72
	Non-Squamous	9 (47.4%)	10 (52.6%)	
Smoking Status	Current / Former	15 (53.6%)	13 (46.4%)	0.60
	Never	1 (33.3%)	2 (66.7%)	
Tumor stage	Ⅲ	5 (62.5%)	3 (37.5%)	0.59
	Ⅳ	11 (47.8%)	12 (52.2%)	
PS	0–1	13 (59.1%)	9 (40.9%)	0.25
	2 ≤	3 (33.3%)	6 (66.7%)	
Diagnostic procedures	Resection / CNB	9 (56.4%)	7 (43.6%)	0.72
	Endoscopic / Others	4 (46.7%)	6 (53.3%)	
Period between tumor collection and PD-L1 assay	≤ 6 months	15 (53.6%)	13 (46.4%)	0.60
	>6 months	1 (33.3%)	2 (66.7%)	

Abbreviations PS : Performance status CNB : Core needle biopsy

Table 5B. Correlation between patient characteristics and tumor response of anti-PD-1/PD-L1 treatment for a previously treated population

		N (%)		
		Responders (N = 14)	Non-Responders (N = 51)	*P* value
Age (median, years)		68	68	0.71
Sex	Female	3 (17.6%)	14 (82.4%)	0.74
	Male	11 (22.9%)	37 (77.1%)	
Histology	Squamous	3 (27.3%)	8 (72.7%)	0.69
	Non-Squamous	11 (20.4%)	43 (79.6%)	
Smoking status	Current / Former	12 (22.6%)	41 (77.4%)	1.00
	Never	2 (16.7%)	10 (83.3%)	
stage	Ⅱ-Ⅲ	6 (40.0%)	9 (60.0%)	0.72
	Ⅳ	8 (16.0%)	42 (84.0%)	
PS	0–1	14 (24.6%)	43 (75.4%)	0.18
	2 ≤	0 (0.0%)	8 (100%)	
PD-L1 expression (TPS ≥ 1%)	Negative / Unknown	6 (17.4%)	23 (82.6%)	1.00
	Positive	8 (28.6%)	28 (71.4%)	
Driver mutations	Negative	13 (22.4%)	45 (77.6%)	1.00
	Positive	1 (14.3%)	6 (85.7%)	
Diagnostic procedures	Resection / CNB	8 (23.5%)	26 (76.5%)	0.77
	Endoscopic / Others	6 (19.4%)	25 (80.6%)	
Period between tumor collection and PD-L1 assay	≤ 6 months	7 (26.9%)	24 (73.1%)	1.00
	>6 months	7 (21.2%)	27 (78.8%)	

Abbreviations PS : Performance status CNB : Core needle biopsy

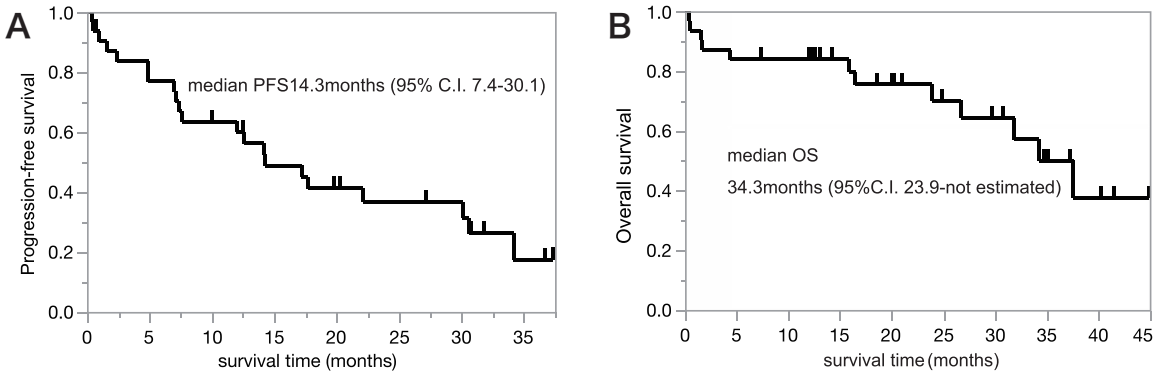

Fig. 2. Survival curves for patients with high PD-L1 expression (TPS≧50%) who received pembrolizumab monotherapy

histology, smoking status, tumor stage, and diagnostic procedures, were not significantly associated with PFS and OS according to univariate analysis. The time between tumor acquisition and PD-L1 assay was not significantly associated with PFS. However, the Cox regression method was not applied to evaluate the association of this factor with OS because of a lack of mortality events in the samples stored for ≥ 6 months (Table 6A, 6B). In the second-line or the further-line anti-PD-1/PD-L1 treatment groups, the median PFS and median OS were 7.2 months (95% C.I. 3.9–9.8) and 20.9 months (95% C.I. 13.7–30.4), respectively (Figure 3A, 3B). Furthermore, another subgroup analysis using the Cox regression model was used to identify significant patient factors that affected survival. However, none of the factors, including PD-L1 positivity, driver mutational status, diagnostic procedures, or the time between tumor acquisition and PD-L1 assay, were significant as determined by univariate analysis (Table 7A, 7B).

Discussion

In the present study, we showed that the feasibility of the PD-L1/22C3 assay in a daily clinical setting was high, irrespective of the diagnostic procedure and sample storage time. The overall failure rate of the PD-L1/22C3 assay was low, and diagnostic procedures and tumor sample storage time did not significantly affect the efficacy of anti-PD-1/PD-L1 treatment. However, PD-L1 positivity was not associated with the efficacy of anti-PD-1/PD-L1 treatment in previously treated patients.

Our analysis included various clinical samples, such as endoscopic biopsies, CNB, and resected samples. The overall failure rate for the PD-L1 assay was 3.0%. A global retrospective study showed that PD-L1 expression could not be assessed in 6% (170 out of a cohort of 2613) of the cases[12]; however, the detailed biopsy procedures were not described. Additionally, a prospective observational investigation of PD-L1 expression in small biopsy samples obtained at a Japanese institution revealed that the PD-L1 undetermined rate was 2.6%[13]. The failure rate for the PD-L1 assay in our study was comparable to that of previous studies. It is essential to assess the causes of assay failure to determine its feasibility. At our institution, six of eight cases in which the PD-L1 assay failed were endoscopic cases and the overall failure rate was 4.6%. Thus, in the future, it is important to optimize the endoscopic procedure to improve the PD-L1 assay, considering that approximately 50% of the cases for the PD-L1 assay in our study were endoscopic biopsy samples.

TBB with r-EBUS-GS is a well-established biopsy method and an efficient and safe diagnostic procedure, which is only limited by sample size. A previous report showed that biopsy samples obtained by the r-EBUS-GS method with thin bronchoscopy were smaller and had fewer tumor cells compared with those obtained by normal bronchoscopy and core-needle biopsy methods, with a failure rate of 3.6%[13]. At our institution, the PD-L1 assay failed in two samples obtained with r-EBUS-GS using thin bronchoscopy because of the small number of tumor cells. Another report showed that additional conventional TBB following r-EBUS-GS biopsy was effective at improving the diagnostic result[14]. Percutaneous transthoracic biopsy has a high diagnostic accuracy for peripheral lung lesions, although the complications are relatively high[15]. Alternative methods, such as second bronchoscopy or percutaneous transthoracic biopsy, may be considered to obtain adequate tumor tissue. Nonetheless, in

Table 6A. Correlation progression-free survival (PFS) with patient factors

Factors	Variables	Univariate Analysis		
		Risk Ratio	95% C.I.	*P* value
Age	＜70	1		
	≥ 70	0.74	0.31–1.77	0.50
Sex	Male	1		
	Female	1.23	0.45–3.39	0.69
Histology	Non-Squamous	1		
	Squamous	0.83	0.34–2.01	0.83
Smoking	Never	1		
	Current / Former	0.66	0.19–2.27	0.51
Stage	Ⅲ	1		
	Ⅳ	2.25	0.74–6.82	0.15
PS	0–1	1		
	2 ≤	2.66	1.02–6.97	0.047＜0.05
Diagnostic Procedure	Resection / CNB	1		
	Endoscopic / Others	1.77	0.74–4.23	0.20
Period between tumor collection and PD-L1 assay	≤ 6 months	1		
	＞6 months	0.56	0.13–2.44	0.44

Table 6B. Correlation overall survival (OS) with patient factors

Factors	Variables	Univariate Analysis		
		Risk Ratio	95% C.I.	*P* value
Age	＜70	1		
	≥ 70	0.97	0.30–3.12	0.96
Sex	Male	1		
	Female	0.60	0.13–2.82	0.52
Histology	Non-Squamous	1		
	Squamous	0.51	0.14–1.89	0.31
Smoking	Never	1		
	Current / Former	2.50	0.30–20.54	0.39
Stage	Ⅲ	1		
	Ⅳ	5.21	0.67–40.59	0.11
PS	0–1	1		
	2 ≤	2.54	0.56–11.55	0.23
Diagnostic Procedure	Resection / CNB	1		
	Endoscopic / Others	2.98	0.84–10.62	0.09
Period between tumor collection and PD-L1 assay	≤ 6 months			
	＞6 months		Not calculated	

Abbreviations PS : Performance status CNB : Core needle biopsy C.I. : Confidential Interval

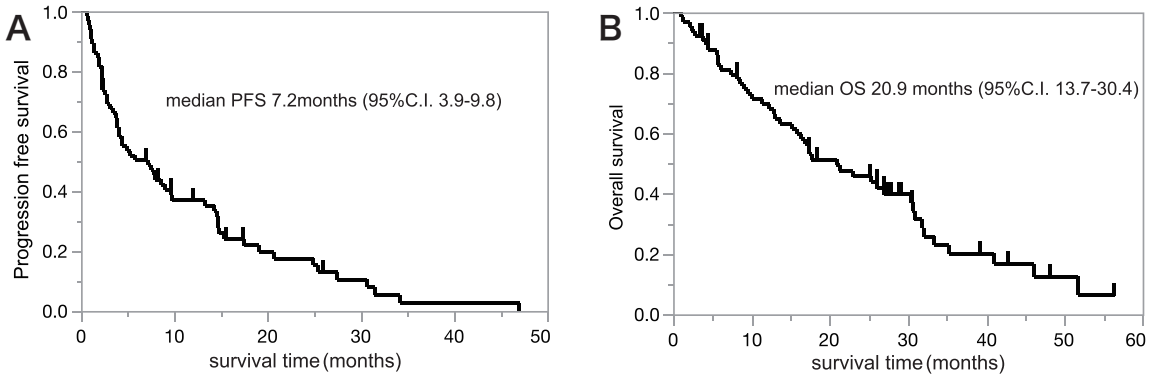

Fig. 3. Survival curves following anti-PD-1/PD-L1 treatment for previously treated patients

the other two failed cases in which samples were obtained by EBUS-TBNA, biopsy samples had a few tumor cells with blood clots or necrotic tissue. An expert panel report for EBUS-TBNA recommends using a rapid on-site evaluation (ROSE) to improve diagnostic accuracy and suggests a minimum of three separate passes per sampling site if ROSE is not available[16], although ROSE has not been used at our institution. Since early passes may obtain more tumor cells compared with late passes, it is essential to obtain several lymph nodes with TBNA for adequate tumor specimens. Overall, the failure of the PD-L1 assay is rare and usually caused by technical issues, which can be managed and minimized.

In the present study, the PD-L1 positivity rate was 66% and the proportions of PD-L1 expression classified by TPS <1%, 1–49%, and ≥50% were 31%, 33%, and 33%, respectively. A global cohort study revealed that the proportions of PD-L1 expression classified by TPS <1%, 1–49%, and ≥50% in the Asia-Pacific region were 47%, 53%, and 22%, respectively[6]. Of these, 72% of the samples were biopsy samples, whereas 26% were resected samples. Additionally, another Japanese prospective study of small biopsies showed that the proportions of PD-L1 expression classified by TPS <1%, 1–49%, and ≥ 50% were 34.6%, 31.4%, and 31.4%, respectively[13]. The KEYNOTE-001 study showed that PD-L1 positivity was 60.8% and the PD-L1 expression rates classified by TPS <1%, 1–49%, and ≥ 50% were 39.2%, 37.6%, and 23.2%, respectively.[7] The PD-L1 positivity and PD-L1 distribution were also consistent with those of previous studies, suggesting that the PD-L1 assay results in our study reflects the situation of real-world clinical practice.

Based on PD-L1 expression evaluated using samples obtained during daily practice, patients with NSCLC were treated with anti-PD-1/PD-L1 antibodies. In the present study, the treatment efficacy in the high PD-L1 expression (TPS ≥ 50%) group was high, regardless of clinical characteristics including diagnostic procedure. These outcomes were considered relatively high compared with those of the landmark clinical trials or real-world studies that assessed the efficacy of pembrolizumab monotherapy for NSCLC with high PD-L1 expression[8, 9, 17]. Several studies have shown that patients with a very high PD-L1 expression (TPS ≥ 90%) may exhibit a higher tumor response and longer survival compared with those in the 50%–89% range[18, 19]. The high efficacy of pembrolizumab monotherapy in our TPS ≥ 50% group may be attributed to the underestimation of very high PD-L1 expression (TPS ≥ 90%). High PD-L1 expression assessed using small samples can be overestimated because of the heterogeneity of PD-L1 expression in primary lung tumors[20–22]. In the KEYNOTE-042 study, tumor treatment efficacy was evaluated at different cut-off points (i.e., 50%, 20%, and 1%). The ORRs of the patient groups with TPS ≥ 50%, TPS ≥ 20%, and TPS ≥ 1% were 39%, 33%, and 27%, respectively. Substantial objective tumor responses were observed across the cut-off lines. Even if PD-L1 expression deteriorates in archival specimens, a substantial response is expected in patients with PD-L1 expression ≥ 50%. Collectively, a substantial efficacy of first-line pembrolizumab monotherapy for high PD-L1 expressing patients may be expected, even if the specimens are small biopsy or archival samples. Our study population receiving first-line pembrolizumab monotherapy with high PD-L1 expression proved that the PD-L1/22C3 assay is useful for predicting the efficacy of first-line pembrolizumab monotherapy.

In our study populations that received anti-PD-1/PD-L1 treatment for previously treated NSCLC, the ORR was 21.5% and the median PFS and median

Table 7A. Correlation of PFS with patient factors

Factors	Variables	Univariate Analysis		
		Risk Ratio	95% C.I.	*P* value
Age	<70	1		
	≥ 70	0.99	0.57–1.72	0.98
Sex	Male	1		
	Female	0.87	0.47–1.64	0.67
Histology	Non-Squamous	1		
	Squamous	1.44	0.74–2.81	0.28
Smoking status	Never	1		
	Current / Former	0.89	0.45–1.78	0.75
Tumor stage	Ⅱ–Ⅲ	1		
	Ⅳ	1.30	0.70–2.43	0.41
PS	0–1	1		
	2 ≤	1.79	0.84–3.83	0.13
Driver mutations	Negative	1		
	Positive	1.17	0.46–2.96	0.75
PD-L1 expression	Positive	1		
	Negative / Unknown	1.60	0.93–2.74	0.090
Diagnostic Procedure	Resection / CNB	1		
	Endoscopic / Others	0.69	0.40–1.18	0.17
Period between tumor collection and PD-L1 assay	≤ 6 months	1		
	>6 months	0.84	0.50–1.43	0.53

Table 7B. Correlation of OS with patient factors

Factors	Variables	Univariate Analysis		
		Risk Ratio	95% C.I.	*P* value
Age	<70	1		
	≥ 70	0.91	0.50–1.67	0.77
Sex	Male	1		
	Female	0.54	0.26–1.14	0.11
Histology	Non-Squamous	1		
	Squamous	1.52	0.75–3.09	0.24
Smoking status	Never	1		
	Current / Former	1.19	0.55–2.57	0.67
Tumor stage	Ⅱ–Ⅲ	1		
	Ⅳ	0.98	0.49–1.94	0.95
PS	0–1	1		
	2 ≤	1.99	0.88–4.51	0.10
Driver mutations	Negative	1		
	Positive	0.80	0.28–2.28	0.67
PD-L1 expression	Positive	1		
	Negative / Unknown	0.90	0.50–1.63	0.73
Diagnostic Procedure	Resection / CNB	1		
	Endoscopic / Others	0.61	0.34–1.09	0.10
Period between tumor collection and PD-L1 assay	≤ 6 months	1		
	>6 months	0.78	0.42–1.42	0.41

Abbreviations PS : Performance status CNB : Core needle biopsy C.I. : Confidential Interval

OS were 7.2 and 20.9 months, respectively. These outcomes were comparable to those of several clinical trials of anti-PD-1/PD-L1 monotherapy for previously treated NSCLC[4, 5, 23, 24]. Similar to pembrolizumab monotherapy for high PD-L1 expression, the diagnostic procedure and the time between tumor acquisition and PD-L1 assay did not affect efficacies in these studies. Moreover, PD-L1 positivity did not affect clinical outcomes in our study population.

Pivotal studies have shown that PD-L1 expression is a predictive marker for a better tumor response and longer survival. In the nivolumab clinical study, PD-L1-positive patients had favorable outcomes, including ORR, compared with PD-L1-negative patients[5]. For pembrolizumab monotherapy in previously treated NSCLC patients, the TPS ≥ 50% population exhibited a tumor response of approximately 30% and a longer survival compared with the TPS 1–49% population[23]. In our study population, NSCLC patients with high PD-L1 expression (TPS ≧ 50%) had a lower tumor response and shorter survival outcomes compared with the other PD-L1 expression or unknown groups (Table 8). This result is inconsistent with previous reports and should have a negative impact on the PD-L1 assessment for predicting anti-PD-1/PD-L1 treatment efficacies in a previously treated patient population. Several previous studies showed that PD-L1 expression was correlated to tumor aggressiveness and poor prognosis[25, 26]. Therefore, PD-L1 overexpression is a predictive marker of tumor response to anti-PD-1/PD-L1 treatment and reflects tumor aggressiveness and poor prognosis. In our study, 42.8% (6 of 14) of the patients with PD-L1 overexpression experienced primary progressive disease (PD) during anti-PD-1/PD-L1 treatment as a second or further-line treatment. Furthermore, the proportion of primary PD was higher compared with that of the TPS 1–49% group (22.7%: 5/22) and negative or unknown groups (28%: 7/25), which resulted in poor survival outcomes. Because of the small population and retrospective nature of our study, tumor aggressiveness may be more prominent than tumor responsiveness to treatment. Other factors, except PD-L1 expression, may exist to explain the primary resistance to anti-PD-1/PD-L1 treatment. Tumors with genetic alterations are reportedly resistant to PD-1 inhibition. *EGFR* mutations or *ALK* alterations confer resistance to PD-1/PD-L1 treatment[27]. However, only seven out of 65 cases carried driver mutations and two harbored *EGFR* mutations in PD-L1-overexpressing tumors among patients who received anti-PD-1/PD-L1 treatment as second-line or after treatment. Other mutations, such as *JAK1/2* in melanoma[28] and *SKT11/LKB1* alteration in *K-ras* mutant lung adenocarcinoma, are associated with primary resistance to PD-1 inhibitors[29]; however, these mutations were not assessed in the present study. We believe that apart from PD-L1 expression, there are other factors that determine response to PD-1/PD-L1 inhibitors, which should be the subject of future investigations.

This study had several limitations. First, the spatial and temporal discordance in PD-L1 expression were not assessed. Intertumoral PD-L1 expression heterogeneity and its discordance between small

Table 8. Efficacy profile classified by tumor proportion score (TPS)

Factors	Variables	TPS ≥ 50% N = 14	TPS1–49% N = 22	TPS<1% N = 26	TPS unknown N = 3
Diagnostic procedures	Resection / CNB	8 (57.1%)	8 (36.4%)	17 (65.4%)	1 (33.3%)
	Endoscopic / Others	6 (42.9%)	14 (63.6%)	9 (34.6%)	2 (66.7%)
Period between tumor collection and PD-L1 assay	≤ 6 months	5 (35.7%)	12 (54.5%)	13 (50.0%)	1 (33.3%)
	>6 months	9 (64.3%)	10 (45.5%)	13 (50.0%)	2 (66.7%)
Tumor response	Responder	2 (14.3%)	6 (27.3%)	5 (19.2%)	1 (33.3%)
	Non-responder	12 (85.7%)	16 (72.7%)	21 (80.8%)	2 (66.7%)
Survival	median PFS months (95% C.I.)	3.4 (1.4–19.1)	9.0 (3.9–27.5)	5.3 (2.6–9.7)	
	median OS months (95% C.I.)	13.0 (5.6–30.4)	30.9 (12.3–35.3)	20.9 (15.2–32.0)	

Abbreviations, TPS : Tumor proportion score CNB : Core needle biopsy PFS : Progression-free survival OS : Overall survival C.I. : Confidential Interval

biopsy and paired resected specimens, spatial discordance between primary and metastatic sites, and temporal dynamic changes in PD-L1 expression in tumors have also been investigated[30]. Thus, only one-point evaluation using a small biopsy or archival sample may not reflect true PD-L1 expression at the start of anti-PD-1/PD-L1 treatment. Second, our study was retrospective and conducted in a small population at a single institution, thus efficacy analyses were not adequate. Third, other PD-L1 antibodies, such as 28-8 and SP142, or other predictive markers, such as tumor mutational burden, were not evaluated.

In conclusion, we believe that our study presents credible evidence that the PD-L1/22C3 assay is feasible in daily clinical practice and should be further validated, although a few cases with biopsy specimens obtained via endoscopic procedure may fail the assessment. The efficacy of first-line pembrolizumab monotherapy for PD-L1-overexpressing NSCLC can be predicted using routine clinical samples, irrespective of the diagnostic procedure. For previously treated patients, the prediction of the efficacy of anti-PD-1/PD-L1 treatment based on PD-L1 expression should be considered on an individual basis. Further investigations related to an accurate PD-L1 assessment to reflect the actual PD-L1 status are warranted.

Conflict of interest disclosure

The authors declare no conflicts of interest associated with this article.

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