Ann Surg Oncol (2019) 26:1925–1933
https://doi.org/10.1245/s10434-019-07257-3

Annals of
SURGICAL ONCOLOGY
OFFICIAL JOURNAL OF THE SOCIETY OF SURGICAL ONCOLOGY

ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

A Clinicopathological and Prognostic Analysis of PD-L2 Expression in Surgically Resected Primary Lung Squamous Cell Carcinoma

Taichi Matsubara, MD[1], Kazuki Takada, MD, PhD[1], Koichi Azuma, MD, PhD[2], Shinkichi Takamori, MD, PhD[1], Gouji Toyokawa, MD, PhD[3], Akira Haro, MD, PhD[1], Atsushi Osoegawa, MD, PhD[1], Tetsuzo Tagawa, MD, PhD[1], Akihiko Kawahara, PhD[4], Jun Akiba, PhD[4], Isamu Okamoto, MD, PhD[5], Yoichi Nakanishi, MD, PhD[5], Yoshinao Oda, MD, PhD[6], Tomoaki Hoshino, MD, PhD[2], and Yoshihiko Maehara, MD, PhD[1]

[1]Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; [2]Division of Respirology, Neurology, and Rheumatology, Department of Internal Medicine, Kurume University School of Medicine, Fukuoka, Japan; [3]Department of Thoracic Surgery, Clinical Research Institute, National Hospital Organization Kyushu Medical Center, Fukuoka, Japan; [4]Department of Diagnostic Pathology, Kurume University School of Medicine, Fukuoka, Japan; [5]Research Institute for Diseases of the Chest, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; [6]Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

ABSTRACT

Background. Immunotherapy targeting programmed cell death-1 (PD-1) and programmed death-ligand 1 (PD-L1) has shown dramatic therapeutic effects for lung squamous cell carcinoma (SCC), and PD-L1 expression has been shown not only to be a predictive biomarker for response to immunotherapy but also a prognostic factor for lung SCC. However, the clinical significance of programmed death-ligand 2 (PD-L2), another PD-1 ligand, remains unclear. Therefore, we analyzed PD-L2 expression by immunohistochemistry in surgically resected primary lung SCC.

Patients and Methods. PD-L1 and PD-L2 expression on tumor cells were analyzed in 211 primary lung SCC specimens by immunohistochemistry. Additionally, numbers of $CD3^+$, $CD4^+$, and $CD8^+$ tumor-infiltrating lymphocytes were also examined.

Results. The rates of positive PD-L2 expression were 77.3% and 67.3% using 5% and 10% cut-off values, respectively. Low PD-L2 expression on tumor cells was statistically associated with histological type (non-keratinizing/keratinizing) and lymphatic invasion. PD-L2-positive patients had significantly longer postoperative survival time (log-rank test; $p = 0.0170$ at 5% cut-off and $p = 0.0500$ at 10% cut-off). Furthermore, survival analysis according to PD-L1 and PD-L2 expression revealed that PD-L1-positive and PD-L2-negative patients had the most unfavorable prognosis.

Conclusions. PD-L2 protein expression was associated with prognosis in primary lung SCC patients. PD-L2 expression might be a potential biomarker for response to PD-1/PD-L1-targeted immunotherapy, which should be investigated in future studies.

Lung cancer is one of the most common causes of cancer-related deaths worldwide,[1] and non-small cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancers. Adenocarcinoma (AC) and squamous cell carcinoma (SCC) are the two major NSCLC subtypes.[2] Over the past decade, the therapeutic management of NSCLC transitioned from conventional therapy to molecularly targeted therapy, according to the status of oncogenic drivers. In particular, AC patients with

Electronic supplementary material The online version of this article (https://doi.org/10.1245/s10434-019-07257-3) contains supplementary material, which is available to authorized users.

Drs. Taichi Matsubara and Kazuki Takada contributed equally to this work.

First Received: 7 October 2018;
Published Online: 27 February 2019

K. Takada, MD, PhD
e-mail: k_takada@surg2.med.kyushu-u.ac.jp

epidermal growth factor receptor mutations or anaplastic lymphoma kinase translocations showed clinical benefits from tyrosine kinase inhibitors. In contrast to AC, there are no reported therapeutic targets for SCC.

Recently, several clinical studies have shown that immune checkpoint inhibitors targeting programmed cell death-1 (PD-1) or programmed death-ligand 1 (PD-L1) provided favorable outcomes compared with standard chemotherapy.[3–6] In these clinical trials, PD-L1 expression on tumor cells was closely associated with clinical outcomes; however, there is not expected to be an effect of immunotherapy in patients with low PD-L1 expression. Therefore, investigations for other useful biomarkers should be performed.

PD-L1 is generally expressed on a subset of macrophages, and is induced by various inflammatory cytokines.[7,8] The effector phase of T cell function can be attenuated by activation of PD-L1 or programmed death-ligand 2 (PD-L2), which is another PD-1 ligand.[9,10] PD-L2 protein is expressed mainly by dendric cells,[11] but is also expressed on tumor cells.[12] There have been many reports about the clinical significance of PD-L1 expression in NSCLC as it is expected to be a prognostic factor or a predictive biomarker for immunotherapy response.[13–16] However, the clinical significance of PD-L2 expression has not been fully investigated, and the prognostic impact of PD-L2 in lung SCC has not been consistent in previous studies.[17–19] Therefore, in this study, we analyzed associations between PD-L2 expression and clinicopathological characteristics, including PD-L1 expression and the number of tumor-infiltrating lymphocytes (TILs) in surgically resected primary lung SCC samples by immunohistochemistry (IHC). We also investigated the prognostic impact of PD-L2 expression.

PATIENTS AND METHODS

Patients

We retrospectively enrolled patients who were diagnosed with primary lung SCC and underwent complete surgical resection at our department between January 1990 and March 2012. We excluded patients who had a history of SCC of the head and neck or esophagus, or who had received neoadjuvant therapy because of possible metastatic lesions from these tumors because we wanted to ensure endogenous levels of PD-L1, PD-L2, and TILs. Finally, 211 patients were eligible for our study. Pathological stage was defined according to the criteria of the 7th edition of the American Joint Committee on Cancer lung cancer staging system.[20] We investigated the following clinicopathological features: age at surgical resection, sex, smoking status, surgical procedure, histological type, and pathological tumor stage. This study was approved by the Institutional Review Board at Kyushu University (IRB No. 29-261).

Immunohistochemistry for Programmed Death-Ligand 1 (PD-L1), Programmed Death-Ligand 2 (PD-L2), Cluster of Differentiation (CD) 3, CD4, and CD8

For IHC studies, we used formalin-fixed and paraffin-embedded tissue sections, which were cut into 4-μm-thick sections. IHC for PD-L1 was performed as previously described.[16] The PD-L2 IHC protocol was as follows: (1) sections were stained with the B-Bond-III autostainer (Leica Microsystems, Bannockburn, IL, USA); (2) monoclonal antibody incubation was performed following proteinase K (Agilent/Dako, Carpinteria, CA, USA) treatment for 5 min, and then incubating for 30 min; (3) a horse radish peroxidase-polymer was then used as a secondary antibody; and finally (4) the slides were visualized with 3,3′ diaminobenzidine (DAB). This process was automated using the Refine Polymer Detection System (Leica Microsystems). Briefly, IHC staining for TILs was performed as follows: (1) after endogenous peroxidase activity was inhibited with 3% H_2O_2 in methanol for 30 min, sections were pretreated with Target Retrieval Solution (CD3: pH 6.0, CD4 and CD8: pH 9.0; DakoCytomation, Glostrup, Denmark) in a microwave at 100 °C for 20 min, and then incubated with monoclonal antibodies at 4 °C overnight; (2) immunostaining was performed using the Envision Detection System (DakoCytomation); and (3) sections were reacted with DAB, and, finally, counterstained with hematoxylin. The antibodies used are shown in electronic supplementary Table 1.

Immunohistochemical Evaluation

PD-L1 and PD-L2 expression were scored as the proportion of tumor cells showing membrane staining, which was estimated as the percentage of total tumor cells in whole sections. A positive score for PD-L1 expression was defined as $\geq$ 1% according to our previous study.[16] The intensity of PD-L2 expression in the same tumor specimen was evenly distributed throughout. Therefore, we evaluated PD-L2 expression using the proportion score. We then determined the cut-off level of PD-L2 using receiver operating characteristic curve analysis (electronic supplementary Figure 1), and set the cut-off values for PD-L2 positivity at both 5% and 10%. The number of $CD3^-$, $CD4^-$, and $CD8^+$ TILs, which were evaluated within the

borders of the invasive tumor areas,[21] were counted and averaged over three high-power fields for each case, and the median numbers of each molecule were defined as the respective cut-off values.[22,23] All immunohistochemical images were evaluated independently by three investigators (TM, KT, and ST) who were blinded to clinical data. Representative IHC images for each molecule are shown in electronic supplementary Figure 2.

Statistical Analyses

Continuous variables are expressed as means ± standard deviations, and categorical variables are expressed as numerals. We performed statistical evaluations using JMP software version 13 (SAS Institute, Cary, NC, USA). Statistical differences between PD-L2 expression and clinicopathological factors were tested using the Chi square test or Fisher's exact test. Overall survival (OS) was defined as the period between the date of surgery and the date of last follow-up or death; the Kaplan–Meier method with the log-rank test was used to estimate the probability of survival. Univariate and multivariate analyses were used to estimate hazard ratios (HRs) for independent prognostic values via Cox proportional hazards regression models using the backward elimination method. Multivariate analyses of relationships between PD-L2 expression and clinicopathological characteristics were performed by logistic regression analysis using the backward elimination method. p values < 0.05 were regarded as statistically significant.

TABLE 1 PD-L1/PD-L2 expression and tumor-infiltrating lymphocytes in the enrolled primary lung squamous cell carcinoma patients

Molecule	No. of patients (%)
PD-L1, tumor proportion score (%)	
0	98 (46.4)
1–4	36 (17.1)
5–9	11 (5.2)
10–49	28 (13.3)
≥ 50	38 (18.0)
PD-L2, tumor proportion score (%)	
0	0 (0.0)
1–4	48 (22.7)
5–9	21 (10.0)
10–49	49 (23.2)
≥ 50	93 (44.1)
CD3	
Low	98 (46.4)
High	113 (53.6)
CD4	
Low	105 (49.8)
High	106 (50.2)
CD8	
Low	103 (48.8)
High	108 (51.2)

PD-L1 programmed death-ligand 1, *PD-L2* programmed death-ligand 2, *CD3* cluster of differentiation 3, *CD4* cluster of differentiation 4, *CD8* cluster of differentiation 8

RESULTS

Associations Between PD-L2 Expression and Clinicopathological Characteristics of Patients with Surgically Resected Primary Lung Squamous Cell Carcinoma (SCC)

We analyzed PD-L1 and PD-L2 expression on tumor cells, as well as CD3, CD4, and CD8 expression on TILs by IHC in 211 SCC patients. Detailed patient characteristics are shown in electronic supplementary Table 2. The median age of patients was 69 years (range 39–87 years), and over 80% of the cohort were male (82.9%) and heavy smokers (84.8% had ≥ 30 pack years).

The rate of PD-L1 positivity was 53.6% (133 patients), and that for PD-L2 was 77.3% with a 5% cut-off value and 67.3% with a 10% cut-off value (Table 1). At least 1% PD-L2 expression was observed in all tumor specimens. The median CD3, CD4, and CD8 numbers were 34 (range 0–388), 16 (range 0–158), and 8 (range 0–138), respectively, and 113 (53.6%), 106 (50.2%), and 108 (51.2%) patients were assigned to the $CD3^+$, $CD4^+$, and $CD8^+$ high TIL groups, respectively. Table 2 shows associations between PD-L2 expression and clinicopathological factors. PD-L2-negative tumors were significantly correlated with histological type and lymphatic invasion at both cut-off values, and they tended to be correlated with PD-L1 positivity (5%: $p = 0.0708$; 10%: $p = 0.1053$). We further analyzed independent predictive factors of PD-L2 expression. As shown in electronic supplementary Table 3, multivariate logistic analysis for PD-L2 expression showed histological subtype, absence of lymphatic invasion, and presence of vascular invasion were independent predictors of PD-L2 expression. Conversely, a high number of $CD8^+$ TILs and positive PD-L2 expression were independent predictors of PD-L1 expression (electronic supplementary Table 4).

Survival Analysis According to PD-L1 and PD-L2 Expression in Patients with Surgically Resected Primary Lung SCC

Next, we assessed relationships between OS and PD-L2 status at each cut-off value. The median follow-up time

TABLE 2 Associations between PD-L2 expression and clinicopathological factors in primary lung squamous cell carcinoma patients

Factors	*n* (%)	5% cut-off			10% cut-off		
		PD-L2 [*n* (%)]			PD-L2 [*n* (%)]		
		Negative	Positive	*p* value	Negative	Positive	*p* value
Age, years							
< 69	111 (52.6)	28 (58.3)	83 (50.9)	0.4129	39 (56.5)	72 (50.7)	0.4644
≥ 69	100 (47.4)	20 (41.7)	80 (49.1)		30 (43.5)	70 (49.3)	
Sex							
Male	175 (82.9)	37 (77.1)	138 (84.7)	0.2740	54 (78.3)	121 (85.2)	0.2428
Female	36 (17.1)	11 (22.9)	25 (15.3)		15 (21.7)	21 (14.8)	
Smoking status (pack years)							
< 30	32 (15.2)	8 (16.7)	24 (14.7)	0.8191	12 (17.4)	20 (14.1)	0.5439
≥ 30	179 (84.8)	40 (83.3)	139 (85.3)		57 (82.6)	122 (85.9)	
Histological type							
Non-keratinizing	37 (17.5)	16 (33.3)	21 (12.9)	0.0021	23 (33.3)	14 (9.9)	< 0.0001
Keratinizing	174 (82.5)	32 (66.7)	142 (87.1)		46 (66.7)	128 (90.1)	
Pathological stage							
I	114 (54.0)	22 (45.8)	92 (56.4)	0.2487	34 (49.3)	80 (56.3)	0.3780
≥ II	97 (46.0)	26 (54.2)	71 (43.6)		35 (50.7)	62 (43.7)	
Pleural invasion							
Absent	154 (73.0)	34 (70.8)	120 (73.6)	0.7139	51 (73.9)	103 (72.5)	0.8702
Present	57 (27.0)	14 (29.2)	43 (26.4)		18 (26.1)	39 (27.5)	
Lymphatic invasion							
Absent	179 (84.8)	34 (70.8)	145 (89.0)	0.0049	52 (75.4)	127 (89.4)	0.0130
Present	32 (15.2)	14 (29.2)	18 (11.0)		17 (24.6)	15 (10.6)	
Vascular invasion							
Absent	142 (67.3)	35 (72.9)	107 (65.6)	0.3855	50 (72.5)	92 (64.8)	0.2787
Present	69 (32.7)	13 (27.1)	56 (34.4)		19 (27.5)	50 (35.2)	
PD-L1							
Negative	98 (46.4)	28 (58.3)	70 (42.9)	0.0708	38 (55.1)	60 (42.2)	0.1053
Positive	113 (53.6)	20 (41.7)	93 (57.1)		31 (44.9)	82 (57.8)	
CD3							
Low	98 (46.4)	21 (43.7)	77 (47.2)	0.7429	29 (42.0)	69 (48.6)	0.3818
High	113 (53.6)	27 (56.3)	86 (52.8)		40 (58.0)	73 (51.4)	
CD4							
Low	106 (50.2)	22 (45.8)	84 (51.5)	0.5149	33 (47.8)	73 (51.4)	0.6615
High	105 (49.8)	26 (54.2)	79 (48.5)		36 (52.2)	69 (48.6)	
CD8							
Low	103 (48.8)	24 (50.0)	79 (48.5)	0.8709	33 (47.8)	70 (49.3)	0.8839
High	108 (51.2)	24 (50.0)	84 (51.5)		36 (52.2)	72 (50.7)	

PD-L2 programmed death-ligand 2, *PD-L1* programmed death-ligand 1, *CD3* cluster of differentiation 3, *CD4* cluster of differentiation 4, *CD8* cluster of differentiation 8

was 40.2 (range 0.2–192.7) months. As shown in Fig. 1a and c, a statistically significant association between PD-L2 status and OS at both cut-off values was observed (log-rank test; $p = 0.0170$ at 5% cut-off, and $p = 0.0500$ at 10% cut-off). Of note, PD-L2-positive patients had better prognoses compared with those negative for PD-L2 expression. The median OS for PD-L2-positive patients was 88 months at both cut-off values (versus PD-L2-negative patients; 29 months at 5% cut-off, and 42 months at 10% cut-off). Cox proportional hazards regression models showed that advanced stage, the presence of pleural and lymphatic invasions, PD-L1 positivity, and lower PD-L2 expression

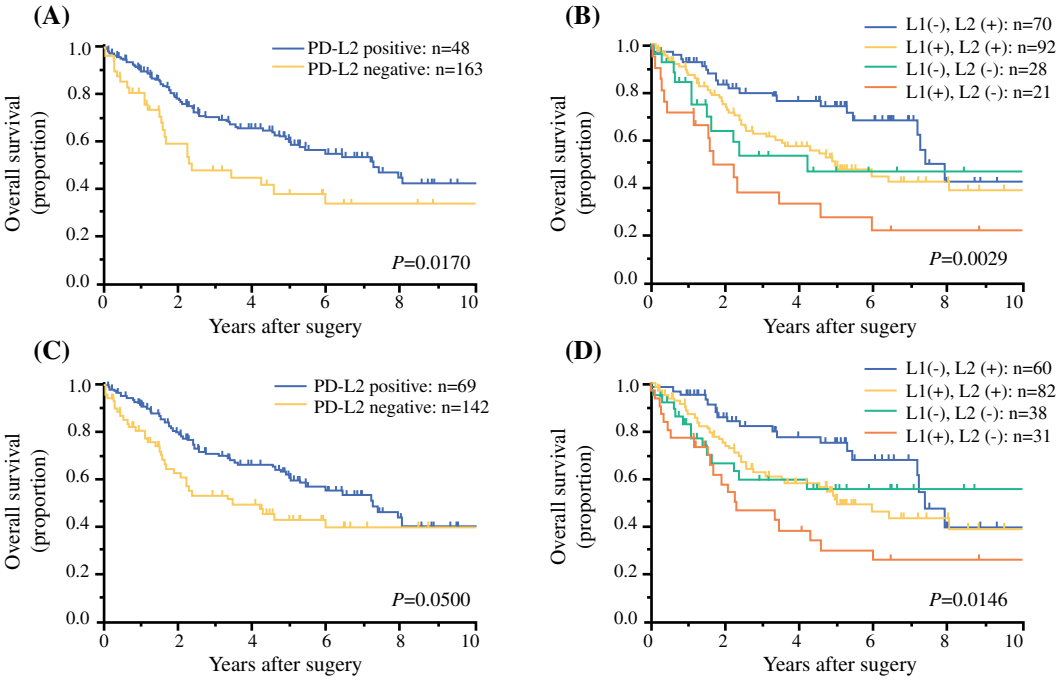

FIG. 1 Kaplan–Meier survival curves according to PD-L1 and PD-L2 expression. Overall survival according to PD-L2 expression determined by the **a** 5% and **c** 10% cut-off values. Overall survival of patients expressing the indicated combinations of PD-L1 and PD-L2. We adopted cut-off values of **b** 5% and **d** 10% for PD-L2 expression. *PD-L1* programmed death-ligand 1, *PD-L2* programmed death-ligand 2

were associated with poorer OS (PD-L1: HR 1.66, $p = 0.0175$; PD-L2: HR 1.74, $p = 0.0243$ at 5% cut-off, HR 1.52, $p = 0.0500$ at 10% cut-off) (Table 3). In multivariate analysis, older age, advanced stage, lymphatic invasion, PD-L1 positivity, low numbers of $CD4^+$ TILs, and PD-L2 negativity were independent poor prognostic factors.

Furthermore, we performed subgroup univariate analyses of OS according to PD-L1 and PD-L2 expression status. PD-L1 and PD-L2 status were significantly associated with OS (PD-L2 cut-off value; 5%: $p = 0.0029$, 10%: $p = 0.0146$) (Fig. 1b and d). PD-L1-positive and PD-L2-negative patients had significantly poorer OS than those who were PD-L1-negative and PD-L2-positive at both cut-off levels (PD-L2 cut-off value; 5%: HR 3.33, 95% confidence interval [CI] 1.69–6.39, $p = 0.0008$; 10%: HR 2.77, 95% CI 1.47–5.25, $p = 0.0019$). Next, we analyzed OS in the subgroup of PD-L1-negative patients. Univariate survival analysis showed that among PD-L1-negative patients, those who were PD-L2-negative had shorter OS compared with those who were PD-L2-positive; however, this was not statistically significant (5% and 10% cut-off: $p = 0.0823$ and $p = 0.1737$, respectively) (Fig. 2a and b). Additionally, we analyzed the independent factors that predict poor prognosis in the PD-L1-negative subgroup. In the multivariate analysis, older age, advanced disease stage, CD4 low, CD8 high, and presence of lymphatic invasion were predictive factors of poor prognosis in SCC patients with negative PD-L1 expression (electronic supplementary Table 5).

DISCUSSION

In the first set of experiments, we evaluated PD-L2 expression on the membrane of tumor cells. When the cut-off value was set at 1%, all SCC specimens were identified as positive. Conversely, PD-L1 positivity was 53.6% at the 1% cut-off level. Both PD-L1 and PD-L2 are ligands of PD-1, and their ligand–receptor interaction induces immune tolerance by suppressing activated immune cells, which can protect tumor cells from T cells.[11,24] Therefore, the mechanisms underlying PD-L1 and PD-L2 expression are expected to overlap; however, in our study, there was a large difference in the expression of these ligands. The expression of these ligands are upregulated by inflammatory cytokines such as interferon-γ11 and are also induced by activating *EGFR* mutations or *ALK* fusions in NSCLC cell lines,[25–27] but these mechanisms are slightly different. First, the affinity of PD-1/PD-L2 is different from that of PD-1/PD-L1. Several reports have demonstrated the binding properties of PD-1 and its ligands using flow cytometric and surface plasmon resonance assays.[28,29] The affinity of PD-L2 for human PD-1 is greater than that of PD-L1 according to the smaller dissociation rate of PD-L2

TABLE 3 Univariate and multivariate analyses of overall survival in primary lung squamous cell carcinoma patients

Factor	(a) 5% cut-off				(b) 10% cut-off			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	HR (95% CI)	*p* value	HR (95% CI)	*p* value	HR (95% CI)	*p* value	HR (95% CI)	*p* value
Age, years								
≥ 69/< 69	1.19 (0.79–1.79)	0.4168	1.54 (1.01–2.36)	0.0438	1.19 (0.79–1.79)	0.4168	1.60 (1.05–2.43)	0.0302
Sex								
Female/male	1.19 (0.68–1.96)	0.5283			1.19 (0.68–1.96)	0.5283		
Smoking status (pack years)								
< 30/≥ 30	1.51 (0.86–2.49)	0.1426			1.51 (0.86–2.49)	0.1426		
Histological type								
Non-keratinizing/ keratinizing	1.25 (0.71–2.06)	0.4170			1.25 (0.71–2.06)	0.4170		
Stage								
≥ II/I	3.74 (2.44–5.82)	<0.0001	3.56 (2.28–5.67)	<0.0001	3.74 (2.44–5.82)	<0.0001	3.67 (2.35–5.83)	<0.0001
Pleural invasion								
Present/absent	1.62 (1.03–2.50)	0.0383			1.62 (1.03–2.50)	0.0383		
Lymphatic invasion								
Present/absent	2.62 (1.56–4.21)	0.0005	1.79 (1.04–2.99)	0.0361	2.62 (1.56–4.21)	0.0005	1.78 (1.03–2.97)	0.0395
Vascular invasion								
Present/absent	1.40 (0.90–2.14)	0.1326			1.40 (0.90–2.14)	0.1326		
PD-L1								
Positive/negative	1.66 (1.09–2.57)	0.0175	1.76 (1.15–2.74)	0.0089	1.66 (1.09–2.57)	0.0175	1.75 (1.14–2.72)	0.0100
PD-L2								
Negative/positive	1.74 (1.08–2.72)	0.0243	1.68 (1.03–2.66)	0.0382	1.52 (1.00–2.31)	0.0500	1.66 (1.07–2.54)	0.0249
CD3								
High/low	1.09 (0.73–1.65)	0.6703			1.09 (0.73–1.65)	0.6703		
CD4								
Low/high	1.55 (1.03–2.37)	0.0365	1.65 (1.07–2.55)	0.0218	1.55 (1.03–2.37)	0.0365	1.69 (1.10–2.63)	0.0164
CD8								
High/low	1.31 (0.87–1.98)	0.1975			1.31 (0.87–1.98)	0.1975		

PD-L1 programmed death-ligand 1, *PD-L2* programmed death-ligand 2, *CD3* cluster of differentiation 3, *CD4* cluster of differentiation 4, *CD8* cluster of differentiation 8, *HR* hazard ratio, *CI* confidence interval

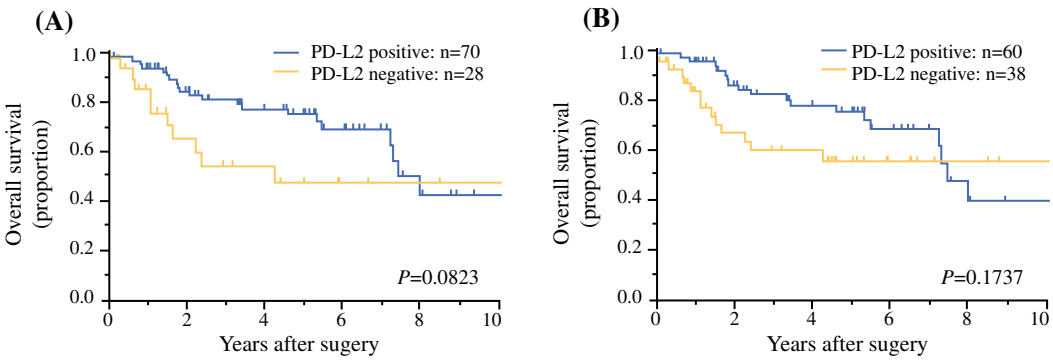

FIG. 2 Kaplan–Meier survival curves according to PD-L2 expression in PD-L1-negative patients. Overall survival according to PD-L2 expression determined by the **a** 5% and **b** 10% cut-off values in PD-L1-negative patients. *PD-L1* programmed death-ligand 1, *PD-L2* programmed death-ligand 2

binding. Second, the differences in expression may derive from the different regulatory cytokine profiles that are involved in PD-L1 and PD-L2 expression. Loke and Allison reported that upregulating PD-L1 depended on toll-like receptor 4 and signal transducers and activator of transcription (STAT) 1, while PD-L2 upregulation depended on interleukin-4α and STAT6.[30,31]

Opposing results were observed regarding the prognostic influences of PD-L1 and PD-L2 expression in this study. Our previous report showed that the postoperative OS of PD-L1-positive patients was shorter than that of PD-L1-negative patients at a cut-off value of 1%.[16] In contrast, PD-L2-negative patients had significantly poorer OS at the 5% and 10% cut-off values, and PD-L1 positivity and PD-L2 negativity were independent predictive factors of poorer OS. Some previous studies have performed survival analyses according to PD-L2 expression in NSCLC, including SCC;[17–19,32] however, there have been no consistent conclusions regarding the prognostic significance of PD-L2 expression in SCC. For example, in cohorts of 48 NSCLC patients (including 23 SCC patients) and 331 patients with resected primary lung SCC, neither PD-L1 nor PD-L2 expression were associated with postoperative survival.[17,18] The lack of a statistically significant association with survival may be due to the small sample size in the latter study.[18] The former study[17] performed IHC for PD-L2 using the same PD-L2 antibody as used in this study, but their IHC evaluation methodology was different from this study. They evaluated the PD-L2 expression by intensity of tumor cells. Currently, the evaluation of PD-L1 expression is generally performed by proportion score, and we thought that it was more practical in that PD-L2 was also evaluated according to proportion score. This was the first study to show that PD-L2-negative SCC patients were significantly associated with poorer survival compared with those with PD-L2 positivity. While complete inhibition of PD-1 signaling through both PD-L1 and PD-L2 is suggested to be more effective in reactivating T cells than inhibiting only the PD-1/PD-L1 interaction,[33] several reports have indicated that the PD-1/PD-L2 interaction does not inhibit T cells or maintain immune homeostasis.[11,34,35]. This hypothesis and the greater affinity of PD-L2 for PD-1 may contribute to the better prognosis of PD-L2-positive patients.

Recently, PD-1 inhibitors, which inhibit PD-1 and PD-L1/PD-L2 interactions, showed great therapeutic effects in advanced NSCLC patients with PD-L1-positive expression compared with standard cytotoxic chemotherapy, regardless of histological subtype.[4,5,36] However, subset analyses of PD-L1-negative patients from the CHECKMATE 017/057 trials demonstrated that the clinical benefit of nivolumab was different between AC and SCC.[3,4] Whereas efficacy endpoints were not achieved in AC patients whose tumors did not express PD-L1, favorable response and survival rates were observed among SCC patients, both PD-L1-positive and PD-L1-negative. These results indicated that there might be a predictive biomarker other than PD-L1 for lung SCC patients. Furthermore, the PD-L1 inhibitor, which inhibits only the PD-1/PD-L1 interaction, improved survival in the PD-L1-negative or undetectable subgroup.[6] These clinical results showed that the anti-PD-1 inhibitor, which inhibits both PD-1/PD-L1 and PD-1/PD-L2 interactions, did not provide benefits, but the anti-PD-L1 inhibitor provided clinical benefit, even in NSCLC patients without detectable PD-L1 expression. We therefore hypothesized that the PD-1/PD-L2 interaction produced these disparate results in PD-L1-negative NSCLC patients. In our cohort, all tumor specimens were positive for PD-L2 protein, even though some tumors did not express PD-L1 protein. Furthermore, we analyzed the significance of PD-L2 expression in the subgroup of PD-L1-negative patients. Increased PD-L2 levels appeared to be associated with prolonged survival; however, this result was not statistically significant. In addition to our results, it has also been reported that the degree of PD-L2 expression in tumors predicts clinical response to anti-PD-1 therapy in a cohort of 172 head and neck SCC patients.[12] From these findings, PD-L2 expression in the subgroup of PD-L1-negative patients could be a biomarker for those who might benefit from this therapy. Conversely, our previous study showed that SCC patients with a PD-L1-negative/PD-L2-positive expression pattern largely had progressive disease on nivolumab treatment.[37] However, the number of SCC cases in the study was very small ($n = 5$); thus, further research is needed to determine the role of PD-L2 on the response to anti-PD-1 drugs. We think that our study will contribute not a little to the important task of predicting the efficacy of immune checkpoint inhibitors. In future, a prospective trial is needed to investigate the effect of immune-checkpoint inhibitors in NSCLC patients with PD-L2 expression, but not PD-L1 expression.

There were some limitations to this present study. First, this was a retrospective single-institution study, thus the potential for bias cannot be excluded. Second, the IHC analyses for PD-L1 and PD-L2 were conducted using only the clones SP142 and 176,611. Positive PD-L1 expression using the SP142 antibody has been reported to be lower than that obtained using other PD-L1 antibodies.[38,39] Further detailed analyses will be possible using these antibodies and/or other cut-off values.

CONCLUSIONS

This study demonstrated that the prognostic significance of PD-L1 and PD-L2 expression was different in primary lung SCC patients. Furthermore, it suggested that PD-L2 expression may predict the clinical benefit from anti-PD-1 therapy for PD-L1-negative patients; these points warrant further investigation.

ACKNOWLEDGEMENT The authors thank James P. Mahaffey, PhD, from Edanz Group (www.edanzediting.com/ac) for editing a draft of this manuscript.

CONFLICTS OF INTEREST The authors declare that they have no conflict of interest.

REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. *CA Cancer J Clin*. 2015;65(1):5–29.
2. Perez-Moreno P, Brambilla E, Thomas R, Soria JC. Squamous cell carcinoma of the lung: molecular subtypes and therapeutic opportunities. *Clin Cancer Res.* 2012;18(9):2443–51.
3. Borghaei H, Paz-Ares L, Horn L, et al. Nivolumab versus docetaxel in advanced nonsquamous non-small-cell lung cancer. *N Engl J Med.* 2015;373(17):1627–39.
4. Brahmer J, Reckamp KL, Baas P, et al. Nivolumab versus docetaxel in advanced squamous-cell non-small-cell lung cancer. *N Engl J Med.* 2015;373(2):123–35.
5. Reck M, Rodriguez-Abreu D, Robinson AG, et al. Pembrolizumab versus chemotherapy for PD-L1-positive non-small-cell lung cancer. *N Engl J Med.* 2016;375(19):1823–33.
6. Rittmeyer A, Barlesi F, Waterkamp D, et al. Atezolizumab versus docetaxel in patients with previously treated non-small-cell lung cancer (OAK): a phase 3, open-label, multicentre randomised controlled trial. *Lancet.* 2017;389(10066):255–65.
7. Dong H, Zhu G, Tamada K, Chen L. B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. *Nat Med.* 1999;5(12):1365–9.
8. Ishida M, Iwai Y, Tanaka Y, et al. Differential expression of PD-L1 and PD-L2, ligands for an inhibitory receptor PD-1, in the cells of lymphohematopoietic tissues. *Immunol Lett.* 2002;84(1):57–62.
9. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer.* 2012;12(4):252–64.
10. Calles A, Liao X, Sholl LM, et al. Expression of PD-1 and its ligands, PD-L1 and PD-L2, in smokers and never smokers with KRAS-mutant lung cancer. *J Thorac Oncol.* 2015;10(12):1726–35.
11. Latchman Y, Wood CR, Chernova T, et al. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat Immunol.* 2001;2(3):261–8.
12. Yearley JH, Gibson C, Yu N, et al. PD-L2 Expression in human tumors: relevance to anti-PD-1 therapy in cancer. *Clin Cancer Res.* 2017;23(12):3158–67.
13. Topalian SL, Hodi FS, Brahmer JR, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med.* 2012;366(26):2443–54.
14. Sacher AG, Gandhi L. Biomarkers for the Clinical Use of PD-1/PD-L1 inhibitors in non-small-cell lung cancer: a review. *JAMA Oncol.* 2016;2(9):1217–22.
15. Takada K, Okamoto T, Shoji F, et al. Clinical significance of PD-L1 protein expression in surgically resected primary lung adenocarcinoma. *J Thorac Oncol.* 2016;11(11):1879–90.
16. Takada K, Okamoto T, Toyokawa G, et al. The expression of PD-L1 protein as a prognostic factor in lung squamous cell carcinoma. *Lung Cancer.* 2017;104:7–15.
17. Kim MY, Koh J, Kim S, Go H, Jeon YK, Chung DH. Clinicopathological analysis of PD-L1 and PD-L2 expression in pulmonary squamous cell carcinoma: Comparison with tumor-infiltrating T cells and the status of oncogenic drivers. *Lung Cancer.* 2015;88(1):24–33.
18. Chen Z, Mei J, Liu L, et al. PD-L1 expression is associated with advanced non-small cell lung cancer. *Oncol Lett.* 2016;12(2):921–7.
19. Arrieta O, Montes-Servin E, Hernandez-Martinez JM, et al. Expression of PD-1/PD-L1 and PD-L2 in peripheral T-cells from non-small cell lung cancer patients. *Oncotarget.* 2017;8(60):101994–2005.
20. Goldstraw P, Crowley J, Chansky K, et al. The IASLC Lung cancer staging project: proposals for the revision of the TNM stage groupings in the forthcoming (seventh) edition of the TNM classification of malignant tumours. *J Thorac Oncol.* 2007;2(8):706–14.
21. Salgado R, Denkert C, Demaria S, et al. The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. *Ann Oncol.* 2015;26(2):259–71.
22. Mansfield AS, Aubry MC, Moser JC, et al. Temporal and spatial discordance of programmed cell death-ligand 1 expression and lymphocyte tumor infiltration between paired primary lesions and brain metastases in lung cancer. *Ann Oncol.* 2016;27(10):1953–8.
23. Tokito T, Azuma K, Kawahara A, et al. Predictive relevance of PD-L1 expression combined with CD8 + TIL density in stage III non-small cell lung cancer patients receiving concurrent chemoradiotherapy. *Eur J Cancer.* 2016;55:7–14.
24. Dong H, Strome SE, Salomao DR, et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med.* 2002;8(8):793–800.
25. Azuma K, Ota K, Kawahara A, et al. Association of PD-L1 overexpression with activating EGFR mutations in surgically resected nonsmall-cell lung cancer. *Ann Oncol.* 2014;25(10):1935–40.
26. Ota K, Azuma K, Kawahara A, et al. Induction of PD-L1 expression by the EML4-ALK oncoprotein and downstream signaling pathways in non-small cell lung Cancer. *Clin Cancer Res.* 2015;21(17):4014–21.
27. Shibahara D, Tanaka K, Iwama E, et al. Intrinsic and Extrinsic regulation of PD-L2 expression in oncogene-driven non-small cell lung cancer. *J Thorac Oncol.* 2018;13(7):926–37.
28. Youngnak P, Kozono Y, Kozono H, et al. Differential binding properties of B7-H1 and B7-DC to programmed death-1. *Biochem Biophys Res Commun.* 2003;307(3):672–7.
29. Cheng X, Veverka V, Radhakrishnan A, et al. Structure and interactions of the human programmed cell death 1 receptor. *J Biol Chem.* 2013;288(17):11771–85.
30. Loke P, Allison JP. PD-L1 and PD-L2 are differentially regulated by Th1 and Th2 cells. *Proc Natl Acad Sci USA.* 2003;100(9):5336–41.
31. Hebenstreit D, Wirnsberger G, Horejs-Hoeck J, Duschl A. Signaling mechanisms, interaction partners, and target genes of STAT6. *Cytokine Growth Factor Rev.* 2006;17(3):173–88.
32. Zhang Y, Wang L, Li Y, et al. Protein expression of programmed death 1 ligand 1 and ligand 2 independently predict poor prognosis in surgically resected lung adenocarcinoma. *OncoTargets Ther.* 2014;7:567–73.

33. Hobo W, Maas F, Adisty N, et al. siRNA silencing of PD-L1 and PD-L2 on dendritic cells augments expansion and function of minor histocompatibility antigen-specific CD8 + T cells. *Blood.* 2010;116(22):4501–11.
34. Brown JA, Dorfman DM, Ma FR, et al. Blockade of programmed death-1 ligands on dendritic cells enhances T cell activation and cytokine production. *J Immunol.* 2003;170(3):1257–66.
35. Akbari O, Stock P, Singh AK, et al. PD-L1 and PD-L2 modulate airway inflammation and iNKT-cell-dependent airway hyperreactivity in opposing directions. *Mucosal Immunol.* 2010;3(1):81–91.
36. Garon EB, Rizvi NA, Hui R, et al. Pembrolizumab for the treatment of non-small-cell lung cancer. *N Engl J Med.* 2015;372(21):2018–28.
37. Takamori S, Takada K, Toyokawa G, et al. PD-L2 expression as a potential predictive biomarker for the response to anti-PD-1 drugs in patients with non-small cell lung cancer. *Anticancer Res.* 2018;38(10):5897–901.
38. Hirsch FR, McElhinny A, Stanforth D, et al. PD-L1 immunohistochemistry assays for lung cancer: results from phase 1 of the blueprint PD-L1 IHC assay comparison project. *J Thorac Oncol.* 2017;12(2):208–22.
39. Rimm DL, Han G, Taube JM, et al. A prospective, multi-institutional, pathologist-based assessment of 4 immunohistochemistry assays for PD-L1 Expression in non-small cell lung cancer. *JAMA Oncol.* 2017;3(8):1051–8.