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PD-L1 expression on tumor-infiltrating immune cells is highly associated with M2 TAM and aggressive malignant potential in patients with resected non-small cell lung cancer

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ABSTRACT

Objectives: PD-L1 expression on tumor cells (TCs) and tumor-infiltrating immune cells (ICs) plays important roles in regulating the antitumor T cell response. However, the mechanistic and clinical significance of the effect of PD-L1 on TCs versus ICs remains unclear. On the other hand, tumor-associated macrophages (TAMs), M2 macrophages in particular, can promote tumor progression.

Methods: We evaluated PD-L1 expression on TCs and ICs using Ventana SP263 assay and the stromal M2 TAM distribution using CD163 staining in 160 consecutive patients with resected non-small cell lung cancer (NSCLC).

Results: PD-L1 expression on TCs and ICs was significantly higher in stromal M2 TAM-high group than in stromal M2 TAM-low group ($p < 0.001$ and $p < 0.001$, respectively). Regarding the clinical significance of PD-L1, PD-L1 expression on TCs was significantly associated with histology ($p = 0.001$), tumor differentiation ($p < 0.001$) and nodal status ($p = 0.029$). Furthermore, PD-L1 expression on ICs was significantly associated with histology ($p < 0.001$), tumor differentiation ($p < 0.001$), tumor status ($p = 0.024$), nodal status ($p = 0.016$), and pathologic stage ($p = 0.004$). The disease-free survival rate was significantly lower in patients with PD-L1-positive TC than in those with PD-L1-negative TC ($p = 0.023$), as well as in patients with PD-L1-positive IC than in those with PD-L1-negative IC ($p < 0.001$). Furthermore, the overall survival rate was significantly lower in patients with PD-L1-positive IC than in those with PD-L1-negative IC ($p = 0.023$).

Conclusions: During tumor progression in NSCLC, the presence of M2 TAMs might affect PD-L1 expression both on TCs and ICs. In patients with NSCLC, PD-L1 expression both on TCs and ICs was associated with malignant behaviors, which was more in case of ICs.

1. Introduction

Non-small cell lung cancer (NSCLC), accounting for approximately 85% of all cases of lung cancer, remains to be the leading cause of cancer-related mortality worldwide despite the availability of advanced cytotoxic chemotherapies. Molecular-targeted therapies, such as EGFR–tyrosine kinase inhibitors [1], are also ineffective for cancers without mutations of the target genes. Therefore, it is important to further elucidate tumor biology of NSCLC to develop new treatment strategies for the patients. Recent studies have reported agents that target the programmed death-ligand 1 (PD-L1)/programmed death-1 (PD-1) axis, which are immune-check point inhibitors, and this has been used as a standard treatment for patients with metastatic NSCLC [2–4]. Pembrolizumab (anti-PD-1 antibody) has been approved as monotherapy in patients with tumors that have highly upregulated expression of PD-L1 on tumor cells (≥50% of tumor proportion score, TPS) [3]. This finding made PD-L1 testing a mandatory diagnostic test during treatment planning in patients with NSCLC. In case of the second-line setting and beyond, atezolizumab (anti-PD-L1 antibody) has been approved as monotherapy in PD-L1-unselected NSCLC patients [4]. Furthermore, durable clinical responses to atezolizumab were observed not only in patients with tumors with high PD-L1 expression on their tumor cells (TCs), but also in patients with tumors that expressed high levels of PD-L1 on their tumor-infiltrating immune cells (ICs) [5].

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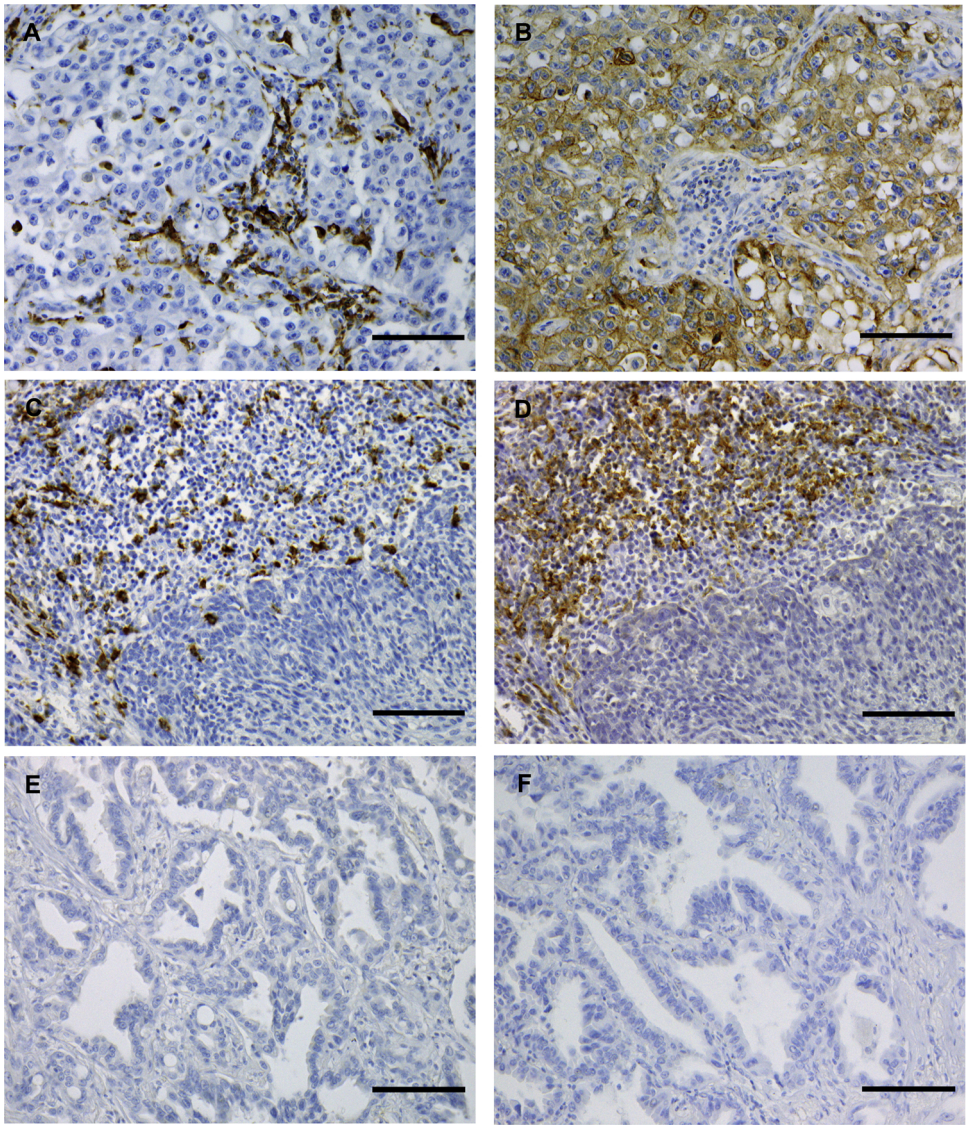

Fig. 1. Immunostaining of lung cancers. A carcinoma with high density of CD163-positive TAM in tumor stroma (A), and positive expression of PD-L1 only on tumor cells (B). A carcinoma with high density of CD163-positive TAM in tumor stroma (C), and positive expression of PD-L1 only on tumor-infiltrating immune cells (D). A carcinoma with low density of CD163-positive TAM in tumor stroma (E), and a negative expression of PD-L1 on tumor cells and tumor-infiltrating immune cells (F). Bar, 100 μm.

Although these observations have suggested that PD-L1 expression on TCs and ICs plays important roles in regulating the anti-tumor T cell response, the mechanistic and clinical significance of PD-L1 on TCs versus ICs is still unclear.

On the other hand, infiltrating macrophages in tumors, known as tumor-associated macrophages (TAMs), are considered to be key components of tumor microenvironment that influences tumor progression [6,7]. Based on a physiological or pathological situation, macrophages can be polarized into different phenotypes. In general, M1 macrophages act in tumor-inhibiting manner, and M2 macrophages act in tumor-promoting manner [8,9]. Recent clinical studies have reported that TAMs expressing CD163, a M2 macrophage marker, were associated with PD-L1 expression on TCs in several human cancers [10–12]. In addition, macrophages are distributed in various tissue compartments in lung cancer, such as tumor stroma and tumor islets. The M2 TAMs have been reported to be chiefly located in tumor stroma, while M1 TAMs are mainly found in tumor islets [8,13].

Taking all these previous findings into consideration, in order to elucidate the mechanical and clinical significance of PD-L1 expression on TCs versus ICs in NSCLC, we performed a clinical study to investigate the PD-L1 expression on TCs and ICs in relation to the stromal M2 TAMs.

2. Materials and methods

2.1. Patients

Consecutive 160 NSCLC patients who underwent surgery at the Department of Thoracic Surgery, Kitano Hospital from November 2011 to October 2014 were studied. This study was approved by the Institute's Ethics Committee (P181200300), and written informed consent was obtained from each patient. Pathological staging was determined using the 8th tumor node metastasis (TNM) classification system [14]. The histological type and the grade of differentiation of the tumors were determined according to the classification system developed by the World Health Organization [15]. The patients' medical records and histopathological diagnoses were fully documented. The patient records included follow-up data till August 2018. The overall median follow-up period was 42.8 months.

Table 1
Distributions of PD-L1 expression on tumor cells among NSCLC patients according to clinicopathological characteristics.

Characteristics	n	< 1%	1%–49%	≥50%	mean ± SD (%)	p value
Smoking						
Non-smoker	85	49	22	14	16.7 ± 28.1	0.880
Smoker	75	44	15	16	16.1 ± 27.3	
Gender						
Male	88	55	15	18	16.7 ± 28.8	0.890
Female	72	38	22	12	16.1 ± 26.4	
Tumor status						
T0	8	6	1	1	11.9 ± 28.0	0.348
T1	72	50	11	11	13.4 ± 25.8	
T2, T3, T4	80	37	25	18	19.6 ± 29.2	
Nodal status						
N0	123	76	28	19	13.8 ± 25.3	0.029
N1, N2, N3	37	17	9	11	25.1 ± 33.1	
Pathological stage						
0	7	6	1	0	2.1 ± 5.7	0.127
I	100	62	22	16	14.6 ± 26.5	
II	24	12	7	5	16.7 ± 26.1	
III	29	13	7	9	25.9 ± 33.6	
Differentiation						
Well	33	25	7	1	6.2 ± 17.6	< 0.001
Moderately	93	55	24	14	13.8 ± 24.9	
Poorly	34	13	6	15	33.5 ± 35.2	
Histology						
Adenocarcinoma	128	77	35	16	13.0 ± 24.8	0.001
Squamous cell carcinoma	25	14	2	9	25.8 ± 33.7	
Large cell carcinoma	7	2	0	5	45.7 ± 33.1	
Total number of patients	160	93	37	30	16.4 ± 27.6	

SD, standard deviation.

2.2. Immunohistochemistry

We performed immunohistochemical studies to evaluate the PD-L1 expression on TCs and ICs using the Ventana SP263 assay [16], and examined the M2 TAM distribution of the tumor stroma by CD163 staining, using the Ventana BenchMark GX system (Roche/Ventana Medical Systems, Tucson, USA), according to the recommended protocol. The following antibodies were used: a rabbit monoclonal anti-human PD-L1 antibody (clone SP263, Roche/Ventana), and a mouse monoclonal anti-human CD163 antibody (clone 760–4437, Roche/Ventana). Formalin-fixed paraffin-embedded tissue was cut into 4 μm sections and mounted on poly-L-lysine-coated slides. The sections were deparaffinised and rehydrated. Antigen retrieval was performed with Cell Conditioner 1 for 64 min against SP263, and for 32 min against CD163. The sections were then incubated with the specific primary antibody for 16 min against both SP263 and CD163. Subsequently, the sections were treated with the OptiView HQ Linker for 8 min and the OptiView HRP Multimer for 8 min. Finally, counterstaining was performed with Mayer's haematoxylin and Scott's tap water bluing reagent.

The evaluation of the stained tissue sections was performed by two investigators (Sumitomo and Hirai) who had no knowledge of patients' clinical status. Cases with discrepancies were jointly re-evaluated until a consensus was reached. PD-L1 expression was calculated as the percentage of membrane staining on TCs or ICs in the overall area of the tumor, regardless of intensity. Regarding PD-L1 expression on TCs, cases were classified by two different cut-off values, 1% and 50%, based on the previously described method showing the association of this cut-off with anti-PD-1 therapeutic response [3]. On the other hand, regarding PD-L1 expression on ICs, cases were classified by two different cut-off values, 1% and 10%, as reported previously [17].

For CD163-staining, five most representative high-power fields (400x; 0.0625 mm^2) of the tumor stroma were selected (Fig. 1). Tumor stroma was defined as the area where tumor stromal cells accounted for more than 70% of the total cells [18]. The number of CD163-positive cells in each area was counted, and the average number of fields in each area was calculated. Finally, the CD163-positive macrophage density in the tumor stroma (stromal M2 TAM density) was defined as the cell number per mm^2. As previous clinical studies reported that the level of C-reactive protein (CRP), a marker of inflammatory response, was related to cancer risk and prognosis [19,20], receiver operating characteristic curve analysis was performed to determine the optimal cut-off value of the stromal M2 TAM density with maximal sensitivity and specificity for distinguish < 1 mg/l and ≥1 mg/l of CRP [19,20]. Consequently, the sample was classified as stromal M2 TAM-high when the stromal M2 TAM density was > 380. The CRP level was significantly higher in stromal M2 TAM-high group than in stromal M2 TAM-low group (4.41 ± 7.88 mg/l vs 2.29 ± 3.52 mg/l, p = 0.025).

2.3. Statistical analysis

Because the distributions of three values, including the percentage of PD-L1-positive TCs (p = 0.326), the percentage of PD-L1-positive ICs (p = 0.212), and the stromal M2 TAM density (p = 0.165), showed normal distributions (Kolmogorov-Smirnov analysis), the statistical significances regarding these values were assessed by the *t*-test, ANOVA with Bonferroni/Dunn test or Pearson's correlation coefficient. Categorical variables were compared using χ^2 test.

Disease-free survival (DFS) was defined as the time from the treatment initiation (surgical resection, chemotherapy or radiation) to the date of disease recurrence or death due to any cause. Overall survival (OS) was defined as the time from the treatment initiation to the date of death due to any cause. The Kaplan-Meier method was used to estimate the probability of DFS and OS as a function of time, and the differences in the survivals of subgroups of patients were compared by using Mantel's log-rank test. The Cox regression model was used to evaluate the effects of survival. Statistical analyses were performed with SPSS 23.0 for Windows (IBM Corp., Armonk, NY, USA). All p values were based on the two-sided statistical analysis, and a p value < 0.05 was considered as statistically significant.

Table 2
Distributions of PD-L1 expression on tumor-infiltrating immune cells among NSCLC patients according to clinicopathological characteristics.

Characteristics	n	< 1%	1% - 9%	≥10%	mean ± SD (%)	p value
Smoking						
Non-smoker	85	23	32	30	10.6 ± 12.7	0.688
Smoker	75	17	30	28	9.8 ± 10.6	
Gender						
Male	88	20	34	34	10.5 ± 11.8	0.703
Female	72	20	28	24	9.8 ± 11.7	
Tumor status						
T0	8	4	3	1	5.8 ± 10.2	0.024
T1	72	17	37	18	7.9 ± 9.4	
T2, T3, T4	80	19	22	39	12.7 ± 13.3	
Nodal status						
N0	123	35	50	38	9.0 ± 10.8	0.016
N1, N2, N3	37	5	12	20	14.3 ± 13.8	
Pathological stage						
0	7	4	3	0	2.3 ± 3.2	0.004
I	100	30	42	28	9.4 ± 10.8	
II	24	2	10	12	13.6 ± 10.8	
III	29	4	7	18	15.4 ± 14.4	
Differentiation						
Well	33	10	17	6	6.1 ± 8.7	< 0.001
Moderately	93	27	38	28	9.3 ± 11.6	
Poorly	34	3	7	24	16.7 ± 12.4	
Histology						
Adenocarcinoma	128	35	55	38	8.6 ± 10.0	< 0.001
Squamous cell carcinoma	25	4	6	15	18.1 ± 17.1	
Large cell carcinoma	7	1	1	5	11.0 ± 6.7	
Total number of patients	160	40	62	58	10.2 ± 11.8	

SD, standard deviation.

3. Results

3.1. Expression of PD-L1 on TCs and ICs among 160 resected NSCLCs

The percentages of PD-L1-positive TCs varied greatly among the 160 tumor tissues studied (mean, 16.4 ± 27.6%; Fig. 1 and Table 1), and they were < 1% in 93 (58.1%) tumors, 1%–49% in 37 (23.1%) tumors, and ≥50% in 30 (18.8%) tumors.

The percentages of PD-L1-positive ICs also varied greatly among the 160 tumor tissues (mean, 10.2 ± 11.8%; Fig. 1 and Table 2), and they were < 1% in 40 (25.0%) tumors, 1%–9% in 62 (38.8%) tumors, and ≥10% in 58 (36.3%) tumors.

3.2. PD-L1 expression on TCs and ICs in relation to the stromal M2 TAM density

The stromal M2 TAM density varied greatly among the 160 tumor tissues (mean, 407.0 ± 389.2; Fig. 1). Sixty-seven tumors (41.9%) were categorized in the stromal M2 TAM-high group, and 93 tumors (58.1%) were in the stromal M2 TAM-low group.

Regarding the PD-L1 expression on TCs, the stromal M2 TAM density significantly correlated with the percentage of PD-L1-positive TCs ($r = 0.385$, $p < 0.001$). The percentage of PD-L1-positive TCs was significantly higher in stromal M2 TAM-high group than in stromal M2 TAM-low group (26.5 ± 32.3% vs 9.2 ± 21.1%, $p < 0.001$) (Fig. 2A). With respect to tumor histology, among 128 patients with lung adenocarcinoma, the stromal M2 TAM density significantly correlated with the percentage of PD-L1-positive TCs ($r = 0.388$, $p < 0.001$). The percentage of PD-L1-positive TCs was also significantly higher in stromal M2 TAM-high group than in stromal M2 TAM-low group (22.5 ± 31.5% vs 8.1 ± 19.0%, $p = 0.002$) (Fig. 2B).

On the other hand, regarding the PD-L1 expression on ICs, the stromal M2 TAM density also significantly correlated with the percentage of PD-L1-positive ICs ($r = 0.349$, $p < 0.001$). The percentage of PD-L1-positive ICs was significantly higher in stromal M2 TAM-high group than in stromal M2 TAM-low group (14.8 ± 13.6% vs 6.9 ± 8.9%, $p < 0.001$) (Fig. 2C). With respect to tumor histology, among patients with lung adenocarcinoma, the stromal M2 TAM density significantly correlated with the percentage of PD-L1-positive ICs ($r = 0.360$, $p < 0.001$). The percentage of PD-L1-positive ICs was significantly higher in stromal M2 TAM-high group than in stromal M2 TAM-low group (13.4 ± 12.0% vs 6.2 ± 7.9%, $p < 0.001$) (Fig. 2D).

3.3. Clinical significance of PD-L1 expression on TCs among resected NSCLC

Distributions of the PD-L1 expression on TCs according to clinicopathological characteristics are shown in Table 1. With respect to tumor histology, PD-L1 expression on TCs was significantly higher in squamous cell carcinomas than in adenocarcinomas ($p = 0.001$). In addition, PD-L1 expression on TCs was also significantly associated with tumor differentiation ($p < 0.001$). The PD-L1 expression on TCs was significantly higher in poorly differentiated tumors than in moderately and well differentiated tumors ($p < 0.001$ and $p < 0.001$, respectively). With respect to nodal status, the PD-L1 expression on TCs was significantly higher in node-positive tumors than in node-negative tumors ($p = 0.029$).

Among patients with lung adenocarcinoma, PD-L1 expression on TCs was also significantly associated with tumor differentiation ($p = 0.001$) (Supplemental Table 1). The PD-L1 expression on TCs was significantly higher in poorly differentiated tumors than in moderately and well differentiated tumors ($p = 0.002$ and $p < 0.001$, respectively). In addition, the PD-L1 expression on TCs was significantly higher in node-positive tumors than in node-negative tumors ($p = 0.032$).

3.4. Clinical significance of PD-L1 expression on ICs among resected NSCLC

Distributions of the PD-L1 expression on ICs according to clinicopathological characteristics are shown in Table 2. With respect to tumor histology, PD-L1 expression on ICs was also significantly higher in squamous cell carcinomas than in adenocarcinomas ($p < 0.001$). In addition, the PD-L1 expression on ICs was also significantly associated with tumor differentiation ($p < 0.001$). The PD-L1 expression on ICs

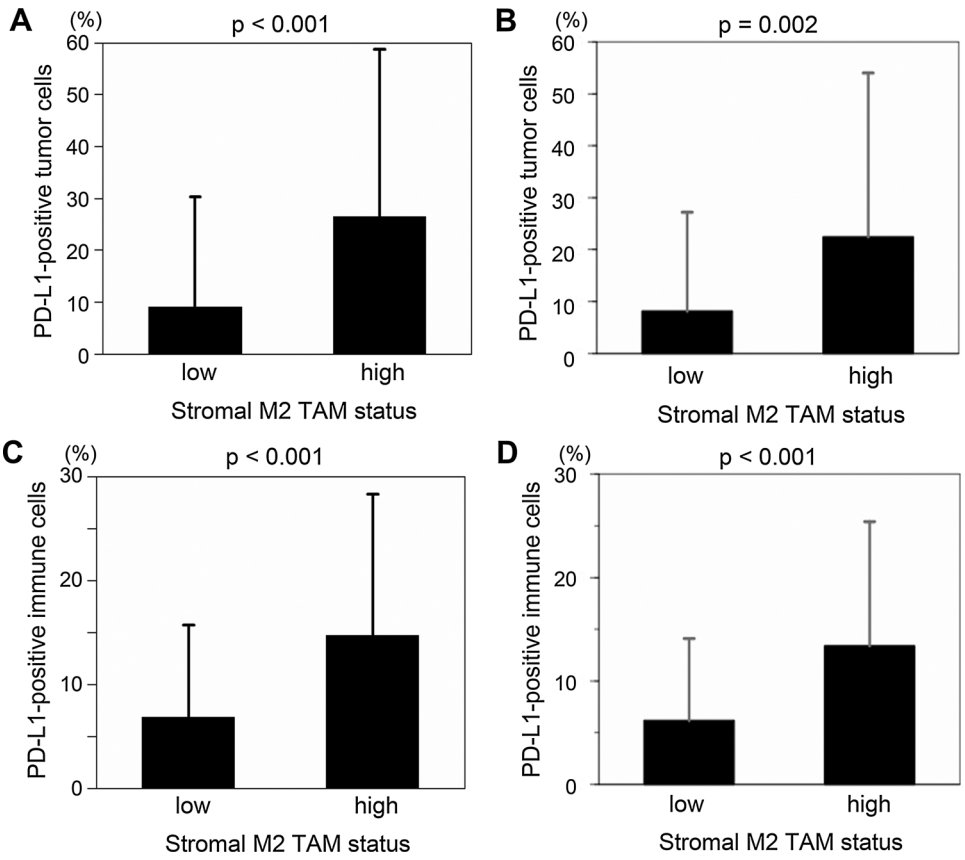

Fig. 2. (A) The relationship between the stromal M2 TAM density and the PD-L1 expression on tumor cells among 160 NSCLCs. (B) The relationship between the stromal M2 TAM density and the PD-L1 expression on tumor cells among 128 lung adenocarcinomas. (C) The relationship between the stromal M2 TAM density and the PD-L1 expression on tumor-infiltrating immune cells among 160 NSCLCs. (D) The relationship between the stromal M2 TAM density and the PD-L1 expression on tumor-infiltrating immune cells among 128 lung adenocarcinomas.

was significantly higher in poorly differentiated tumors than in moderately and well differentiated tumors ($p = 0.001$ and $p < 0.001$, respectively). Furthermore, the PD-L1 expression on ICs was significantly associated with tumor status ($p = 0.024$). With respect to nodal status, the PD-L1 expression on ICs was significantly higher in node-positive tumors than in node-negative tumors ($p = 0.016$). Additionally, the PD-L1 expression on ICs was significantly associated with pathologic stage ($p = 0.004$). The PD-L1 expression on ICs was significantly higher in advanced stage than in early stage (stage II vs stage I, $p = 0.048$; stage III vs stage I, $p = 0.004$).

Among patients with lung adenocarcinoma, PD-L1 expression on ICs was also significantly associated with tumor differentiation ($p < 0.001$) (Supplemental Table 2). The PD-L1 expression on ICs was significantly higher in poorly differentiated tumors than in moderately and well differentiated tumors ($p < 0.001$ and $p < 0.001$, respectively). Furthermore, the PD-L1 expression on ICs was significantly associated with tumor status ($p = 0.023$). Additionally, the PD-L1 expression on ICs was significantly associated with pathologic stage ($p = 0.016$). The PD-L1 expression on ICs was significantly higher in advanced stage than in early stage (stage II vs stage I, $p = 0.022$; stage III vs stage I, $p = 0.026$).

3.5. DFS of patients with resected NSCLC in relation to PD-L1 expression on TCs and ICs

With respect to the PD-L1 expression on TCs, the DFS was almost the same between patients with 1%–49% of positive PD-L1 expression on TCs and patients with ≥50% of positive PD-L1 expression on TCs (Supplemental Fig. 1A). Therefore, cases were classified as PD-L1-positive TC when the percentage of PD-L1-positive TCs was ≥1%, and cases were classified as PD-L1-negative TC when the percentage of PD-L1-positive TCs was < 1%. The DFS rate was significantly lower in patients with PD-L1-positive TC than in those with PD-L1-negative TC (56.1% vs 72.0% in 5-year DFS, $p = 0.023$) (Fig. 3A). In particular, among early stages (0 and I), the DFS rate was significantly lower in patients with PD-L1-positive TC than in those with PD-L1-negative TC (65.1% vs 83.7% in 5-year DFS, $p = 0.019$) (Fig. 3B). However, among advanced stages (II and III), there was no difference in the DFS between these groups (Fig. 3C).

Among patients with lung adenocarcinoma, there was no significant difference in the DFS between patients with PD-L1-positive TC and those with PD-L1-negative TC ($p = 0.050$) (Supplemental Fig. 2A). However, among early stages (0 and I), the DFS rate was significantly lower in patients with PD-L1-positive TC than in those with PD-L1-negative TC (65.1% vs 86.8% in 5-year DFS, $p = 0.015$) (Supplemental Fig. 2B). In contrast, among advanced stages (II and III), there was no difference in the DFS between these groups (Supplemental Fig. 2C).

With respect to the PD-L1 expression on ICs, the DFS was almost the same between patients with < 1% of positive PD-L1 expression on ICs and patients with 1%–9% of positive PD-L1 expression on ICs (Supplemental Fig. 1B). Therefore, cases were classified as PD-L1-positive IC when the percentage of PD-L1-positive ICs was ≥10%, and cases were classified as PD-L1-negative IC when the percentage of PD-L1-positive ICs was < 10%. The DFS rate was also significantly lower in patients with PD-L1-positive IC than in those with PD-L1-negative IC (51.6% vs 72.3% in 5-year DFS, $p < 0.001$) (Fig. 3D). Especially, among early stages (0 and I), the DFS rate was significantly lower in patients with PD-L1-positive IC than in those with PD-L1-negative IC (65.6% vs 79.8% in 5-year DFS, $p = 0.011$) (Fig. 3E). However, among advanced stages (II and III), there was no difference in the DFS between these groups (Fig. 3F).

Among patients with lung adenocarcinoma, the DFS rate was also significantly lower in patients with PD-L1-positive IC than in those with PD-L1-negative IC (56.6% vs 72.7% in 5-year DFS, $p = 0.012$) (Supplemental Fig. 2D). Especially, among early stages (0 and I), the DFS rate was significantly lower in patients with PD-L1-positive IC than

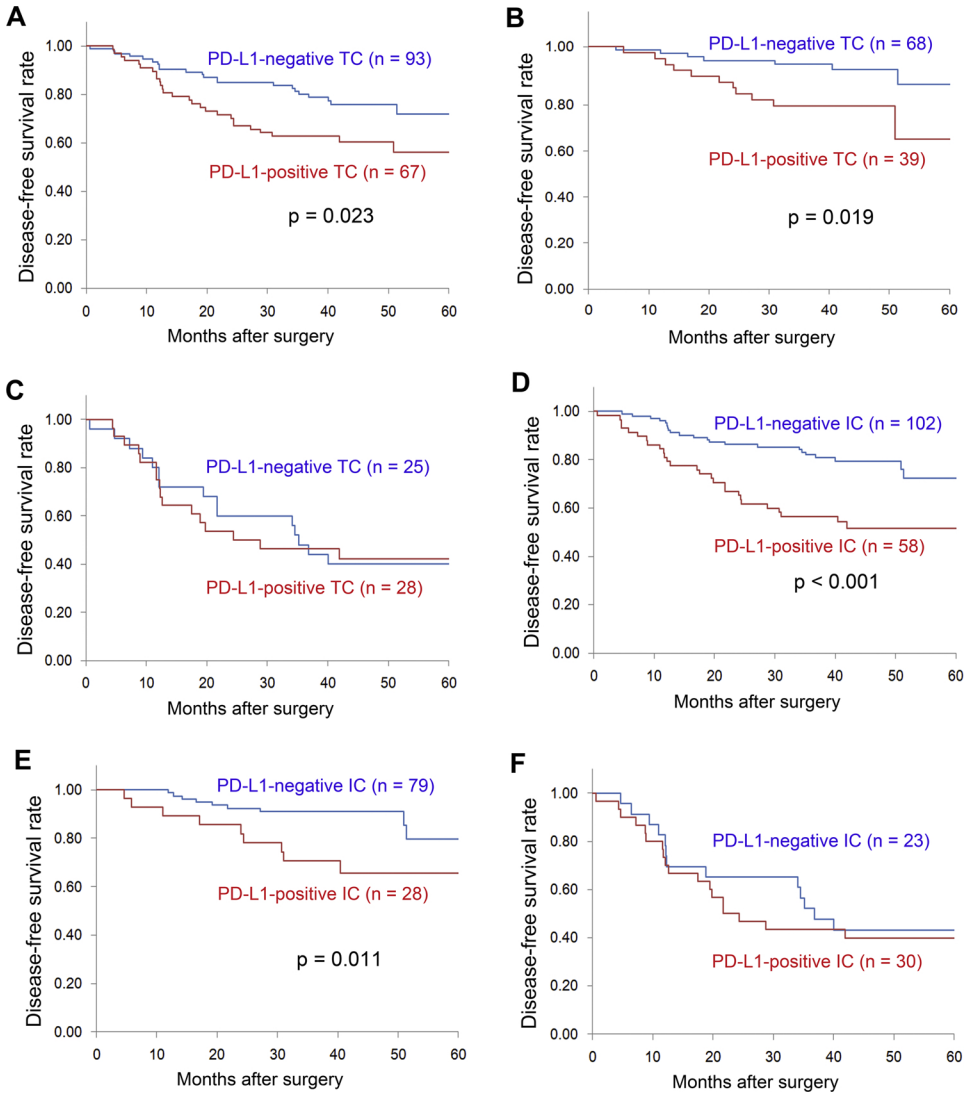

Fig. 3. Disease-free survival: (A) among 160 NSCLC patients in relation to PD-L1 expression on TCs; (B) among 107 patients with early stage NSCLC in relation to PD-L1 expression on TCs; (C) among 53 patients with advanced stage NSCLC in relation to PD-L1 expression on TCs; (D) among 160 NSCLC patients in relation to PD-L1 expression on ICs; (E) among 107 patients with early stage NSCLC in relation to PD-L1 expression on ICs; (F) among 53 patients with advanced stage NSCLC in relation to PD-L1 expression on ICs. TC, tumor cell; IC, tumor-infiltrating immune cell.

in those with PD-L1-negative IC (65.2% vs 82.4% in 5-year DFS, p = 0.009) (Supplemental Fig. 2E). However, among advanced stages (II and III), there was no difference in the DFS between these groups (Supplemental Fig. 2F).

Uni-variate analyses using the Cox regression model also demonstrated that both the PD-L1 expression on TCs (HR = 1.903 [95% CI: 1.084–3.344]; p = 0.025) and ICs (HR = 2.603 [95% CI: 1.481–4.573]; p < 0.001) were significant factors for predicting DFS of patients with resected NSCLC.

3.6. OS of patients with resected NSCLC in relation to PD-L1 expression on TCs and ICs

PD-L1 expression on TCs was not associated with the OS (Fig. 4A). On the other hand, the OS rate was significantly lower in patients with PD-L1-positive IC than in those with PD-L1-negative IC (71.1% vs 86.9% in 5-year OS, p = 0.023) (Fig. 4D). However, among patients with lung adenocarcinoma, there was no significant difference in the OS between these groups (data not shown). Uni-variate analysis using the Cox regression model also demonstrated that the PD-L1 expression on ICs (HR = 2.428 [95% CI: 1.102–5.349]; p = 0.029) was a significant factor for predicting OS of patients with resected NSCLC.

4. Discussion

In this clinical study, we evaluated the PD-L1 expression on TCs and ICs and the distribution of M2 TAM by immunohistochemistry. As PD-L1 testing is considered to be a mandatory diagnostic test for the treatment planning against NSCLC patients, there are several immunohistochemichal assays for analysing PD-L1 expression, such as Agilent 28-8 pharmDx against nivolumab, Agilent 22C3 pharmDx against pembrolizumab, Ventana SP142 assay against atezolizumab, and Ventana SP263 assay against durvalumab. Several studies have reported highly comparable staining by the 28-8, 22C3 and SP263 assays [16,21,22], and the SP263 assay exhibited greatest sensitivity [16,23]. In contrast, SP142 assay was reported to show less sensitivity [16,24]. Therefore, we used Ventana SP263 assay to evaluate the PD-L1 expression in the present study.

First, the present study clearly demonstrated that stromal M2 TAM density was associated with PD-L1 expression both on TCs and ICs. Based on the physiological or pathological situation, macrophage can be polarized into various phenotypes with different biological

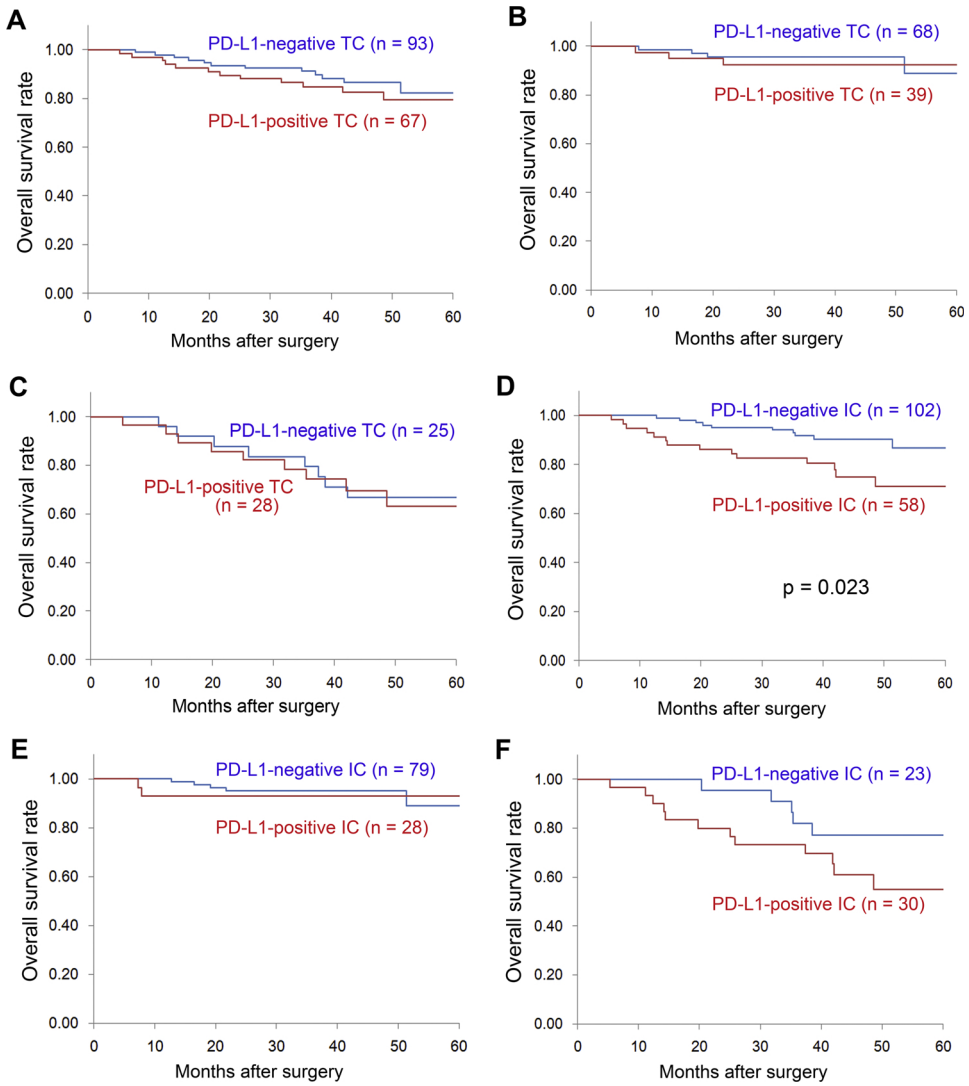

Fig. 4. Overall survival: (A) among 160 NSCLC patients in relation to PD-L1 expression on TCs; (B) among 107 patients with early stage NSCLC in relation to PD-L1 expression on TCs; (C) among 53 patients with advanced stage NSCLC in relation to PD-L1 expression on TCs; (D) among 160 NSCLC patients in relation to PD-L1 expression on ICs; (E) among 107 patients with early stage NSCLC in relation to PD-L1 expression on ICs; (F) among 53 patients with advanced stage NSCLC in relation to PD-L1 expression on ICs. TC, tumor cell; IC, tumor-infiltrating immune cell.

properties, such as tumor-inhibiting M1 macrophages and tumor-promoting M2 macrophages [9,18]. TH2-derived cytokines, such as IL4, IL10 and IL13, or TGFβ could induce M2 activation of macrophages [18]. During tumor progression, these signals originating from tumor cells and stromal cells could induce the production of M2 TAMs in the tumor microenvironment, which can promote tumor cell proliferation [25]. Experimental studies have reported that tumor cells can induce M2 macrophages with increased expression of PD-L1 [26,27]. PD-L1 expression is generally considered to be induced at the transcriptional level after exposure to IFN-γ released by T effector cell [28–30]. In fact, it was revealed that PD-L1 induced by IFN-γ from TAMs promoted the progression of lung cancer [31]. In addition, recent studies have shown that other signals derived from macrophage, such as TNF-α, VEGF and CXCL8, could induce PD-L1 expression [32–34]. Consequently, clinical studies reported that the M2 TAM was associated with PD-L1 expression on TCs in human cancer [10–12]. Another study in squamous cell lung carcinoma also reported that PD-L1 expression on tumor-infiltrating ICs was correlated with the T effector cells and IFN-γ signature [35]. Furthemore, a clinical study in colorectal cancers revealed that PD-L1 was expressed on both TCs and M2 TAMs at the invasive front [36].

Next, the present study revealed that the PD-L1 expression on TCs was associated with tumor histology, tumor differentiation, lymph node metastasis, and DFS among patients with resected NSCLCs. PD-L1 expression on TCs was higher in squamous cell carcinomas than in adenocarcinomas, as reported previously [37]. A previous study also reported that the lung adenosquamous carcinoma had heterogeneous PD-L1 expression that was higher in squamous component than in adenomatous component [38]. PD-L1 expression on TCs was also associated with tumor differentiation and it was higher in poorly differentiated tumors. This was consistent with a previous study in human cancers, including lung cancer and colorectal cancer [36,39–41]. In addition, PD-L1 expression on TCs was reported to be associated with lymphatic invasion and vascular invasion [10,36,39]. Subsequently, high expression of PD-L1 on TCs was also associated with poor prognosis, as reported previously [39,40,42,43]. Thus, along with these clinical findings, our results revealed that high expression of PD-L1 on TCs is associated with aggressive malignant behaviour.

Furthermore, the present study found that the PD-L1 expression on ICs was associated with more aggressive malignant potentials in NSCLC. The PD-L1 expression on ICs was associated with tumor histology, tumor differentiation, tumor status, lymph node metastasis, and pathologic stage. Consequently, high expression of PD-L1 on ICs was a

poor prognostic factor both in DFS and in OS among patients with resectable NSCLCs. A recent study has found that ICs play a critical role in regulating T cell responses independent of PD-L1 expression on TCs, and that PD-L1 expression on ICs was an important indicator of active immune suppression in tumors [5]. A previous study in colorectal cancers also reported the PD-L1 expression on ICs to correlated with poor differentiation, lymphatic invasion and vascular invasion [36]. To our knowledge, the present study was the first report clearly demonstrating the clinical significance of the PD-L1 expression on ICs in NSCLC. In fact, a recent study also found that durable clinical responses to atezolizumab (anti-PD-L1) were observed in patients with tumors expressing high PD-L1 expression on ICs alone [5].

Although the prognostic significance of PD-L1 expression on TCs and ICs was found only on univariate analysis using the Cox regression model in the present study, the statistical analyses regarding prognosis did not reach statistical significance on multivariate analysis using the Cox regression model. These results may be partly due to the relatively small number of patients, and further clinical studies using a higher number of patients are required.

In conclusions, during tumor progression in NSCLC, the tumor-promoting M2 TAM might affect PD-L1 expression both on TCs and ICs. PD-L1 expression on TCs was associated with aggressive malignant behaviors. Furthermore, PD-L1 expression on ICs was associated with more aggressive malignant potentials in NSCLC. Further investigations are necessary to develop new treatment strategy against NSCLC. For example, recent experimental studies found that anti-PD-1/PD-L1 therapy redirects macrophages from a tumor-promoting M2 to a tumor-inhibiting M1 phenotype [44,45].

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Authors' contributions

RS and CH developed the study. RS, TH, MF, and CH designed and performed experiments. RS, HM, and YO collected the data. RS and CH analyzed and interpreted of data and wrote the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

None.

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Not applicable.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.lungcan.2019.08.023.

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