Original Study

Prognostic Impact of Programmed Death-ligand 1 and Surrounding Immune Status on Stage I Lung Cancer

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Abstract

This study aimed to elucidate the prognostic impact of the programmed cell death protein 1/programmed death-ligand 1 (PD-L1) pathway on the surrounding immune microenvironment. PD-L1 positivity was associated with poor prognosis when there were few concurrent intratumoral CD8 cells; it was not associated with prognosis when an adequate number of concurrent intratumoral CD8 cells existed. Hence, our study suggests that the prognostic impact of PD-L1 expression was distinct to the intratumoral CD8 status.

Background: The programmed death 1/programmed death-ligand 1 (PD-L1) pathway reportedly is as an important factor determining effects of immunotherapy; however, its prognostic impact is controversial, and its association with the surrounding immune microenvironment has not yet been elucidated. **Patients and Methods:** We retrospectively analyzed 126 patients with pathologic stage I non–small-cell lung cancer. Patients with lepidic-dominant adenocarcinoma were excluded. PD-L1 expression was evaluated with immunohistochemistry correlated with clinicopathologic features and surrounding immune microenvironment status, including CD4, CD8, regulatory T cells, and human leukocyte antigen class I. Factors affecting prognosis were assessed by Kaplan-Meier and Cox regression analyses. **Results:** Twenty-three (18.3%) patients were positive for PD-L1 expression. No significant correlation was observed between PD-L1 expression and the surrounding immune microenvironment status. The PD-L1–positive group had a worse prognosis than the PD-L1–negative group (5-year recurrence-free survival rates, 63.4% vs. 81.0%; $P = .061$). Among surrounding immune cells, intratumoral CD8 status had the strongest impact on prognosis ($P = .12$). In the intratumoral CD8–high group, PD-L1 expression demonstrated no significant prognostic impact, whereas in the intratumoral CD8–low group, patients positive for PD-L1 demonstrated a significantly worse prognosis than those negative for PD-L1 (5-year recurrence-free survival rates, 41.7% vs. 78.6%; $P = .034$). Multivariable Cox regression analysis revealed that 'PD-L1–positive and intratumoral CD8–low' status was an independent prognostic factor (hazard ratio, 3.80; 95% confidence interval, 1.22-10.5; $P = .023$). **Conclusions:** The prognostic impact of the PD-1/PD-L1 pathway may be distinct according to concurrent intratumoral CD8 status.

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Introduction

Immune checkpoint inhibition against programmed death 1 (PD-1) and programmed death-ligand 1 (PD-L1) has demonstrated much benefit in the survival of patients with lung cancer and has exhibited favorable results compared with conventional standard therapy.[1,2] The PD-1/PD-L1 pathway has attracted attention as an important factor determining immunotherapy effects. However, the prognostic impact of PD-L1 expression itself remains controversial; the presence of PD-L1 positivity in cohorts of patients is reportedly related from a poor prognosis to better locoregional control and

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prognosis.[3,4] Briefly, tumor cells expressing PD-L1 may survive immune regulation by binding to PD-1 on CD8+ cytotoxic T cells (CTLs), thereby downregulating CTL function, which otherwise would attack and kill the tumor cells.[1] Thus, the PD-1/PD-L1 pathway shows effects by interacting with the surrounding immune microenvironment. Moreover, cancer immunity itself comprised a great variety of immune cells. So far, few studies have evaluated the relationship between the PD-1/PD-L1 pathway and the surrounding immune microenvironment.

Tumor-preventing and tumor-promoting lymphocytes play prognostic roles in several tumors, including non−small-cell lung cancer (NSCLC).[5-10] Among the different cell subsets of tumor-infiltrating lymphocytes (TILs), the most promising biomarkers in cancer immunity are CD8+ cytotoxic T lymphocytes, which are pivotal components of the cell-mediated antitumor immune responses and crucial in suppressing cancer development and controlling disease progression.[5] In NSCLC, together with other malignancies, many studies have evaluated the clinical value of CD8+ cells, demonstrating a strong independent positive prognostic effect.[6-8] Conversely, regulatory T cells (Tregs) are a subtype of CD4+T cells with immunosuppressive properties; the most reliable phenotypic marker for Tregs is FOXP3. Presence of FOXP3+ lymphocytes is associated with poor prognosis or better locoregional control and prognosis.[9,10] TILs, including CD4+, CD8+, and FOXP3+ cells, may affect prognosis and the PD-1/PD-L1 pathway.

Additionally, human leukocyte antigen (HLA) class I molecules play critical roles in the steps of cancer immunity, recognition, and binding tumor cells by CTLs, and tumor cells with reduced expression of HLA class I molecules may evade recognition by CTLs to survive.[11-13] PD-L1 expression also plays an important role in immune evasion at the final step by inhibiting activated CTLs. Accordingly, the effect of the PD-1/PD-L1 pathway may depend on HLA class I expression status.

We hypothesized that the prognostic impact of the PD-1/PD-L1 pathway is affected by other biological factors' statuses involved in cancer immunity, such as CD4, CD8, Tregs, and HLA class I molecules, which also play critical roles in cancer immunity. We evaluated the prognostic impact of the PD-1/PD-L1 pathway and surrounding immune microenvironment and their relationship in patients with pathologic stage I lung cancers by immunohistochemistry.

Patients and Methods

Study Population

The Institutional Review Board of the Hiroshima University Hospital approved this study. The Institutional Review Board ethics approval number is E901, and the recognition date is September 14, 2017. Among 391 patients with NSCLC who underwent surgery at our institution between April 2013 and December 2015, we studied 126 with pathologic stage I NSCLC who underwent complete resection without induction therapy (see Supplemental Figure 1 in the online version). Formalin-fixed, paraffin-embedded samples obtained from tumor resections in these patients were used, retrieved from the registry of the Department of Anatomical Pathology, Hiroshima University. Patients with lepidic-dominant adenocarcinoma were excluded. Lepidic-dominant adenocarcinoma was defined as adenocarcinoma that pathologically had more lepidic than invasive components (acinar, papillary, solid, micropapillary) by $>$ 50% to 100% (50% $<$ lepidic components $\leq$ 100%). Staging was determined as per Tumor, Nodes, and Metastasis (TNM) Classification of Malignant Tumours, 7th edition.[14] All patients were examined at 3- to 6-month intervals for 5 years and at 1-year intervals thereafter until death or date of last follow-up. Evaluations included physical examinations, chest radiography or computed tomography, positron emission tomography with 18-fluorodeoxyglucose, and tumor marker detection.

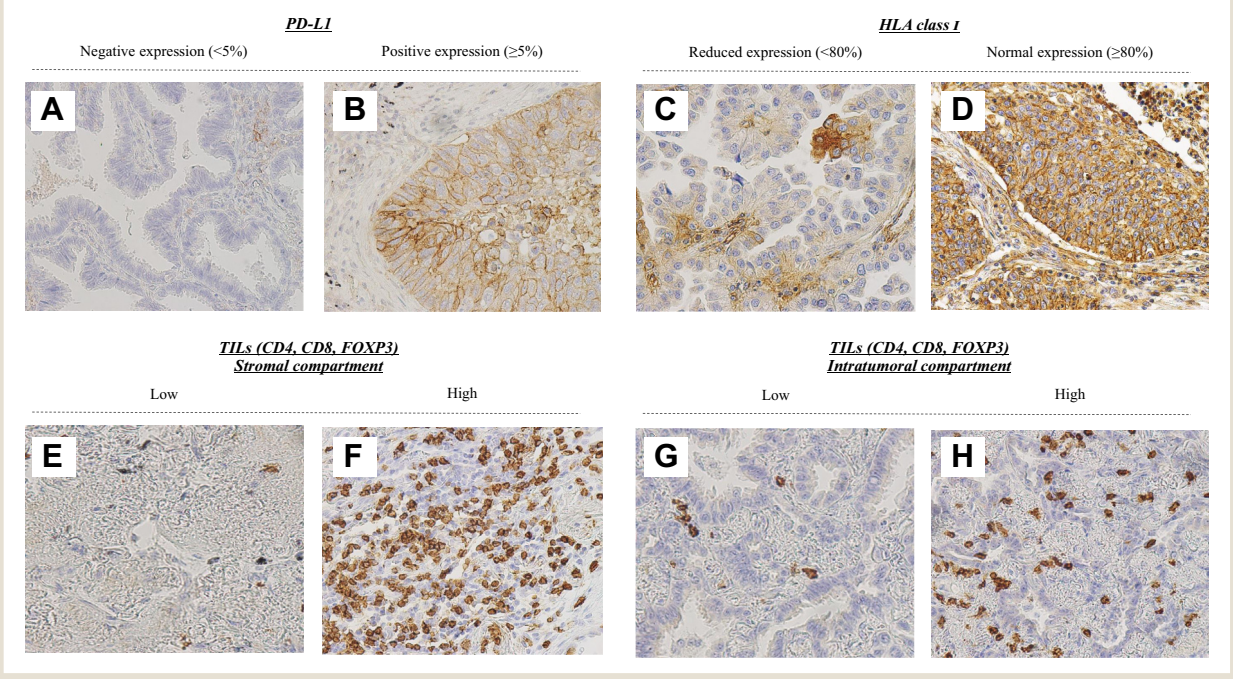
Immunohistochemistry

Formalin-fixed, paraffin-embedded tumor tissue was sectioned at 4 μm, and sections were pasted on coated glass slides for immunohistochemistry. PD-L1 immunohistochemistry was performed using 28-8 antibody (clone, 28-8; Abcam, Cambridge, United Kingdom) for tumor cell membrane staining, as per manufacturer's recommendations. Slides were stained with Dako Autostainer Link 48 (Dako, Santa Clara, CA). Antigen retrieval was performed in Target Retrieval Solution Low pH. The primary antibody of PD-L1 was diluted at 1:400 and detected with Rabbit (LINKER) and EnVision FLEX/HRP. Each slide was examined without any knowledge of the clinical data. Each tumor cell was judged as positive staining for PD-L1 when the membrane was stained at any intensity. PD-L1 expression status was subdivided into positive and negative groups by the percentage of tumor cells expressing PD-L1 at the cutoff value of 5%, as in previous studies.[3,4] HLA class I expression was evaluated in a previous study using an anti-HLA class I antibody (EMR8-5; Cosmo Bio, Tokyo, Japan).[12,13] According to the percentage of tumor cells expressing HLA class I molecules, each patient was classified as showing 'normal' or 'reduced' expression at the cutoff value of 80%, as in previous studies.[12,13] For assessment of TILs (CD4; SP35; Roche Diagnostics, Florham Park, NJ), CD8 (SP53; Roche Diagnostics), FOXP3 (D2W8E; Cell Signaling Technology, Danvers, MA), we referred to the recommendations by the International TILs Working Group 2014.[15] High-power fields (magnification ×400) were randomly analyzed in 10 stromal or intratumoral compartments per sample; the absolute number of TILs was determined, and the median was obtained in each case. According to the median of positive cells infiltrating stromal and intratumoral compartments, these 3 markers were subdivided into 'high' and 'low' groups, such as stromal CD4-low group, intratumoral CD8-high group, and so forth (Figure 1).

Statistical Analysis

Summarized data are presented as number or median and interquartile range. Differences in various variables between PD-L1−positive and −negative groups were evaluated using the Fisher exact test for categorical variables and the Mann-Whitney *U* test for continuous variables. Moreover, we investigated the significant factors for PD-L1 positivity using logistic regression analyses. Recurrence-free survival (RFS) was the interval between day of operation and date of death or proven detection of recurrence or metastases. Survival data were estimated by the Kaplan-Meier method and compared using the log-rank test. Cox regression analyses for RFS

Figure 1 Representative Immunohistochemical Staining of PD-L1, HLA Class I, TILs (CD4, CD8, FOXP3) in Stromal and Intratumoral Compartments. Original Magnification ×400. A, Negative Expression (< 5%) of PD-L1. B, Positive Expression (≥ 5%) of PD-L1. C, Reduced Expression (< 80%) of HLA Class I. D, Normal Expression (≥ 80%) of HLA Class I. E, Low Infiltration of TILs in Stromal Compartment. F, High Infiltration of TILs in Stromal Compartment. G, Low Infiltration of TILs in Intratumoral Compartment. H, High Infiltration of TILs in Intratumoral Compartment. TILs Are Subdivided to High and Low Groups According to Median Positive Cells Infiltrating



Abbreviations: HLA = human leukocyte antigen; PD-L1 = programmed death-ligand 1; TILs = tumor-infiltrating lymphocytes.

were used to identify the significant prognostic factors in stage I NSCLC; $P < .05$ was considered significant. All data were analyzed statistically using Jmp 12.0 (SAS Institute, Inc, Cary, NC).

Results

Immunohistochemical Results

Figure 1 shows a representative immunohistochemical staining of PD-L1, HLA class I, and TILs (CD4/CD8/FOXP3) in stromal and intratumoral compartments. Among 126 total patients, 23 (18.3%) were positive and 103 (81.7%) were negative for PD-L1 expression. In this cohort, HLA class I expression was 'normal' in 52 (41.3%) patients and 'reduced' in 74 (58.7%) patients. The number of positive cells per high power field in the stromal compartment ranged from 3 to 55 (median, 20.0; mean, 22.9) for CD4, 4 to 70 (median, 25.0; mean, 26.2) for CD8, and 0 to 10 (median, 5.0; mean, 5.6) for FOXP3. Conversely, in the intratumoral compartment, numbers ranged from 0 to 15 (median, 4.0; mean, 5.9), 0 to 28 (median, 13.0; mean, 11.2), and 0 to 5 (median, 2; mean, 2.4), respectively.

PD-L1 Expression and Clinicopathologic, Immunohistochemical Results

Table 1 shows characteristics of patients in the PD-L1−positive (median age, 68.0 years; interquartile range, 63.0-74.0 years; 18 [78.3%] males and 5 [21.7%] females) and PD-L1−negative groups. The Fisher exact test and the Mann-Whitney U test demonstrated that positive PD-L1 expression was significantly associated with non-adenocarcinomas ($P = .023$) and having vascular invasion ($P = .0042$). Eighty-seven adenocarcinomas were divided into 4 groups according to subtype as follows: papillary (n = 79), acinar (n = 3), micropapillary (n = 3), and solid (n = 2) dominant type. We investigated the significant factors for PD-L1 positivity using logistic regression analyses and demonstrated that nonadenocarcinomas ($P = .018$) and vascular invasion ($P = .0024$) were significant factors associated with PD-L1 positivity (see Supplemental Table 1 in the online version). No significant correlation was observed between PD-L1 expression and the surrounding immune microenvironment status, such as CD4, CD8, FOXP3, and HLA class I expression in the Fisher exact test (Table 1) and in logistic regression analyses (see Supplemental Table 1 in the online version). Similar results were obtained when we analyzed the characteristics of patients in the PD-L1-positive and intratumoral CD8-low and other groups (see Supplemental Table 2 in the online version).

Prognostic Values of PD-L1, HLA Class I, and TILs (CD4, CD8, FOXP3)

The median follow-up for all patients was 47.5 months. In log-rank analysis, the 5-year RFS rate of patients positive for PD-L1 was worse than that of patients negative for PD-L1 (63.4% vs.

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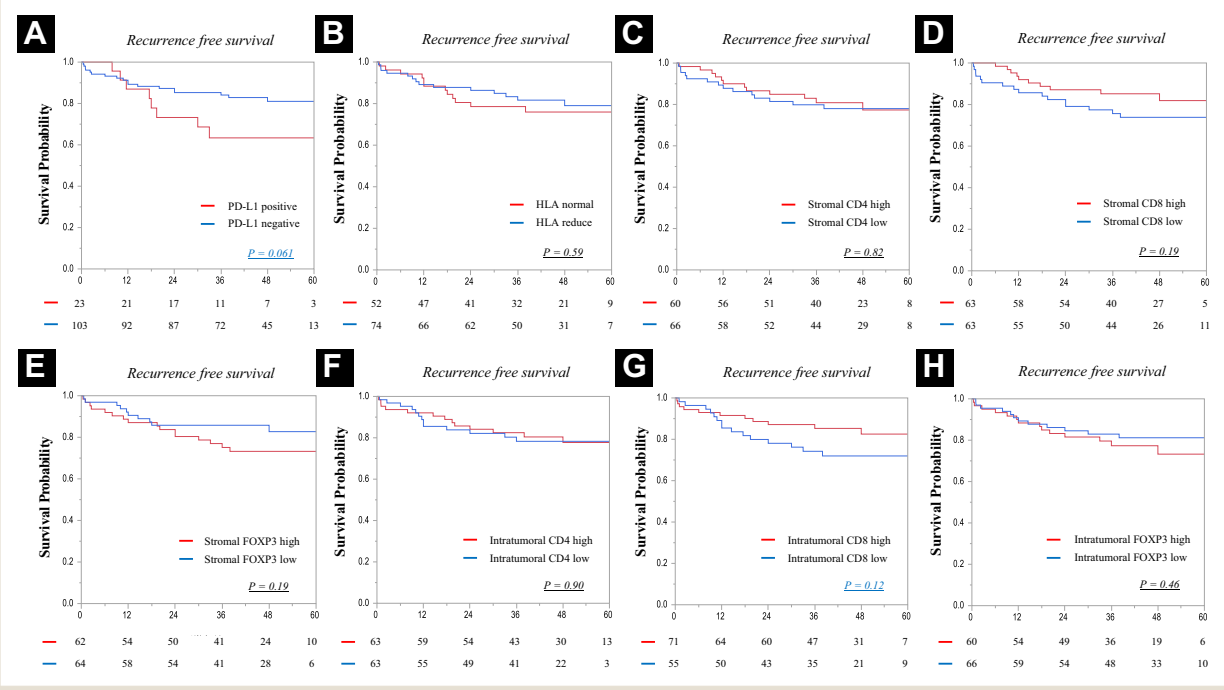
Table 1 Patient and Tumor Characteristics of PD-L1–positive and –negative Patients

Variables	PD-L1–positive N = 23	PD-L1–negative N = 103	P Value
Age, y	68.0 (63.0-74.0)	71.0 (62.0-74.0)	.59
Gender			.10
Male	18 (78.3)	61 (59.2)	
Female	5 (21.7)	42 (40.8)	
Smoking			.088
Yes	19 (82.6)	64 (62.1)	
Never	4 (17.4)	39 (37.9)	
Surgical procedure			.15
Lobectomy	18 (78.3)	62 (60.2)	
Sublobar resection	5 (21.7)	41 (39.8)	
Adjuvant chemotherapy	8 (34.8)	28 (27.2)	.46
Histology			.023
Adenocarcinoma	11 (47.8)	76 (73.8)	
Non-adenocarcinoma	12 (52.2)	27 (26.2)	
Visceral pleural invasion	6 (26.1)	16 (15.5)	.23
Lymphatic invasion	7 (30.4)	16 (15.5)	.13
Vascular invasion	13 (56.5)	24 (23.3)	.0042
Stromal CD4			.17
High	14 (60.9)	46 (44.7)	
Low	9 (39.1)	57 (55.3)	
Intratumoral CD4			1.00
High	12 (52.2)	51 (49.5)	
Low	11 (47.8)	52 (50.5)	
Stromal CD8			.65
High	10 (43.5)	53 (51.5)	
Low	13 (56.5)	50 (48.5)	
Intratumoral CD8			.49
High	11 (47.8)	60 (58.3)	
Low	12 (52.2)	43 (41.7)	
Stromal FOXP3			.82
High	12 (52.2)	50 (48.5)	
Low	11 (47.8)	53 (51.5)	
Intratumoral FOXP3			.37
High	13 (56.5)	47 (45.6)	
Low	10 (43.5)	56 (54.4)	
HLA class I			.25
Normal	12 (52.2)	40 (38.8)	
Reduced	11 (47.8)	63 (61.2)	

Values are median (interquartile range) or n (%).
Abbreviations: HLA = human leukocyte antigen; PD-L1 = programmed death-ligand 1.

81.0%; $P = .061$) (Figure 2). We performed subgroup analyses according to subtype, malignant grade of the adenocarcinoma (see Supplemental Figure 2 in the online version), and different cutoff values (see Supplemental Figure 3 in the online version). Regarding the surrounding immune cells markers, low presence of CD8 cells in the intratumoral compartment, according to the median positive cells infiltrating, was associated with poorer RFS (71.9% vs. 82.5%; $P = .12$), whereas other markers, including HLA class I ($P = .59$), stromal CD4 ($P = .82$), CD8 ($P = .19$), FOXP3 ($P = .19$), intratumoral CD4 ($P = .90$), and FOXP3 ($P = .46$) did not have a strong impact on prognosis (Figure 2). We performed analyses for comparison between PD-L1–positive and –negative tumors according to the surrounding immune status and found a correlation between the prognostic impact of PD-L1 expression and intratumoral CD8 status, whereas other immune status did not have a strong effect on the prognostic impact of PD-L1 expression

Figure 2 RFS Curves for Patients Positive (*red line*) and Negative (*blue line*) for PD-L1 (A), Normal (*red line*) and Reduced (*blue line*) HLA Class I Groups (B), and TILs High (*red lines*) and Low (*blue lines*) Groups in Stromal (C-E) and Intratumoral (F-H) Compartments. The X-axis Shows the Survival Time in Months, and the Y-axis Shows the Survival Probability. The Number of Patients at Risk is Listed at the Bottom of the Figure. A, PD-L1. B, HLA Class I. C, Stromal CD4. D, Stromal CD8. E, Stromal FOXP3. F, Intratumoral CD4. G, Intratumoral CD8. H, Intratumoral FOXP3



Abbreviations: HLA = human leukocyte antigen; PD-L1 = programmed death-ligand 1; RFS = recurrence-free survival; TILs = tumor-infiltrating lymphocytes.

(Figure 3, Supplemental Figure 4 [in the online version]). In subanalysis of the intratumoral CD8-high group, PD-L1 expression demonstrated no significant difference in prognosis, whereas in the intratumoral CD8-low group, patients positive for PD-L1 demonstrated significantly worse prognosis than those negative for PD-L1 (5-year RFS rates, 41.7% vs. 78.6%; $P = .034$) (Figure 4).

We investigated the prognostic factors in patients with pathologic stage I NSCLC using Cox regression analyses. Univariable Cox regression analyses revealed that smoking history, non-adenocarcinomas, pleural invasion, vascular invasion, and PD-L1−positive and intratumoral CD8-low status were independent prognostic factors. Multivariable Cox regression analyses revealed that immune status of 'PD-L1−positive and intratumoral CD8-low' was an independent prognostic factor (hazard ratio, 3.80; 95% confidence interval, 1.22-10.5; $P = .023$) (Tables 2 and 3).

Discussion

Currently, PD-L1 expression has attracted increasing attention because many studies demonstrated that positive PD-L1 expression correlated with favorable clinical benefits achieved with anti−PD-1/PD-L1 antibodies.[1,2] However, the prognostic impact of PD-L1 expression itself remains controversial, and the presence of PD-L1 positivity in patient cohorts is reportedly related from poor prognosis to a better locoregional control and prognosis.[3,4] Cancer immunity comprises a series of steps[1,16]: (1) release of neoantigens from tumor cells; (2) capture and processing of neoantigens by antigen presenting cells (APCs); (3) activation of CD8+ CTLs through presentation of processed neoantigens by APCs; (4) trafficking and infiltrating of CD8+ CTLs to tumor cells; (5) recognition and binding to tumor cells by CD8+ CTLs; and (6) killing of target tumor cells by CD8+ CTLs. The PD-1/PD-L1 pathway acts on step 6; namely, PD-L1 expressed on tumor cells bind to PD-1 on CD8+ CTLs, thereby downregulating its function, which otherwise would attack and kill the tumor cells.[1] Considering the mechanism of cancer immunity and the PD-1/PD-L1 pathway, because the PD-1/PD-L1 pathway shows its effect by interaction with the surrounding immune microenvironment, we hypothesised that the prognostic impact of PD-L1 expression is influenced by other factors associated with cancer immunity, such as CD4, CD8, Tregs, and HLA class I molecules; thus, the prognostic impact of PD-L1 has diversity. We planned this translational study to verify this hypothesis.

Among TILs, CD8+ cells form the effector arm of adaptive immunity with cytotoxic activity and are considered to have strong tumor preventive effects. Ruffini et al[6] observed that CD8+ cells were associated with prolonged survival in 1290 patients with stage I to IIIA NSCLC. Approximately simultaneously, Al-Shibli et al[7] observed that high CD8+ lymphocyte infiltration in stromal and intratumoral areas was associated with better survival in 335 patients with resected stage I to IIIA NSCLC. It has been demonstrated that

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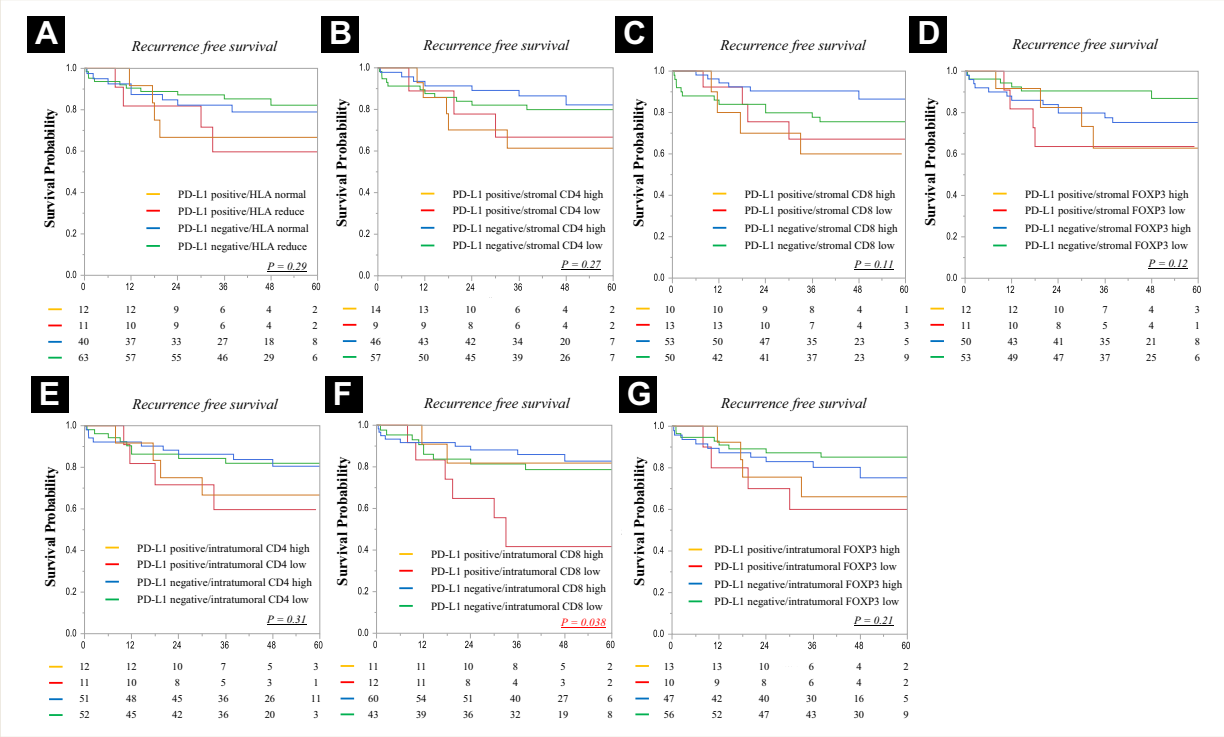
Figure 3 RFS Curves for Patients Positive (*yellow* and *red lines*) and Negative (*blue* and *green lines*) for PD-L1 Stratified by the Surrounding Immune Status. The X-axis Shows the Survival Time in Months, and the Y-axis Shows the Survival Probability. The Number of Patients at Risk is Listed at the Bottom of the Figure. A, HLA Class I. B, Stromal CD4. C, Stromal CD8. D, Stromal FOXP3. E, Intratumoral CD4. F, Intratumoral CD8. G, Intratumoral FOXP3



Abbreviations: HLA = human leukocyte antigen; PD-L1 = programmed death-ligand 1; RFS = recurrence-free survival.

CD8+ cell density, owing to its significant independent prognostic impact, might be a good candidate marker for establishing a TNM immunoscore in resected NSCLC.[8] In agreement with previous studies, we observed that presence of CD8+ cells, especially those existing in the tumor compartment, is highly associated with prognosis.

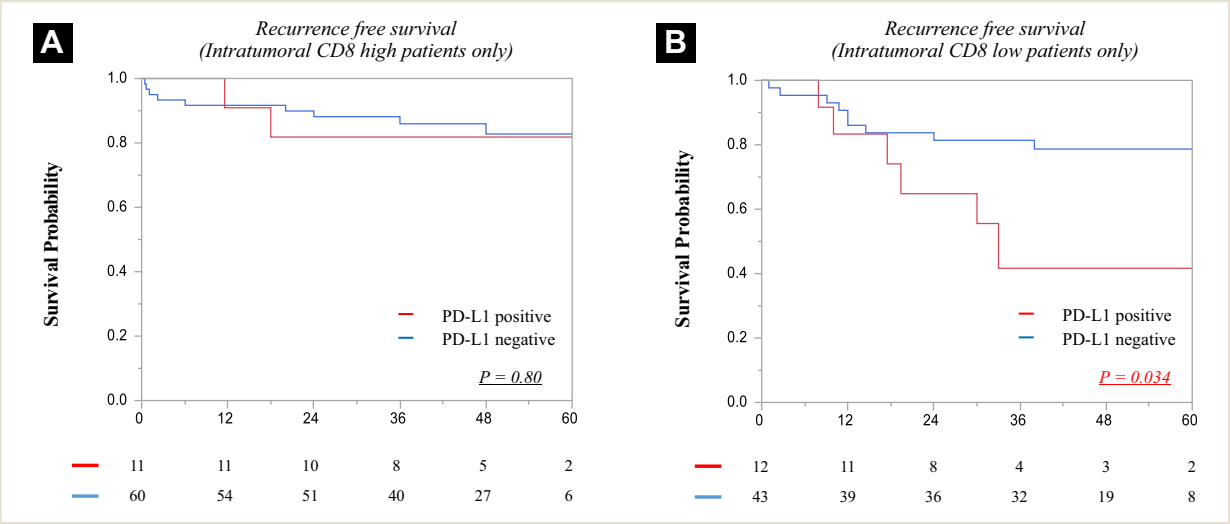
Conversely, presence of CD4+ and FOXP3+ cells has yielded contradictory results regarding prognosis, has been associated with poor and improved prognosis, or had no impact on prognosis.[9,10,17] This discrepancy may result from CD4+ and FOXP3 TILs forming a heterogeneous population of cells with different phenotypes and even opposing actions in the tumor microenvironment.[5,17] We observed that the presence of CD4+ and FOXP3+ cells in the stromal and intratumoral compartment is not strongly associated with prognosis; we speculated this is partly because TILs having opposing effect on prognosis were cancelled out in CD4 and FOXP3 cells.

HLA class I molecules play critical roles in the step of recognition and binding by CTLs (above step 5)[11]; PD-L1 plays an important role in step 6. We hypothesized that the prognostic impact of PD-L1 expression is influenced by concurrent HLA class I molecule status. Consequently, apparent correlation between the PD-1/PD-L1 pathway and HLA class I was not observed. Reportedly, the prognostic impact of PD-L1 expression was distinct according to HLA class I expression status in resected adenocarcinomas of the lung.[12] Correlation between the PD-1/PD-L1 pathway and HLA class I may differ depending on histology. Further investigations in HLA class I are needed.

The results, which demonstrate a significant association of PD-L1 positivity with non-adenocarcinoma histology and vascular invasion, are consistent with the results of previous studies.[12,18,19] Although it remains unclear whether PD-L1 expression depends on histology, contributes to the acquisition of pathologically invasive features, or whether more invasive lung cancers express a greater level of PD-L1 protein, one explanation might address this issue: some factor, such as interferon gamma, may induce PD-L1 expression, which leads to immune escape of tumor cells from T cells during the process by which lung cancers acquire invasive characteristics. Supporting this speculation, a recent report showed that the MYC oncogene regulates PD-L1 expression through transcriptional modulation in various types of cancer, including lung cancer.[20] Given this evidence, MYC might play an essential role in the acquisition of invasive features in lung cancer through the positive regulation of PD-L1 expression, and this factor might show different effects in different histologic types, which needs to be elucidated in future preclinical and clinical studies.

Mentioned above, among the immune microenvironment, CD8 cells are strong, promising biomarkers as pivotal components of

Figure 4 RFS Curves for Patients Positive (*red line*) and Negative (*blue line*) for PD-L1 Stratified by Intratumoral CD8 Expression Status. The X-axis Shows the Survival Time in Months, and the Y-axis Shows the Survival Probability. The Number of Patients at Risk is Listed at the Bottom of the Figure. A, Analysis for Intratumoral CD8-High Patients. B, Analysis for Intratumoral CD8-low Patients



Abbreviations: PD-L1 = programmed death-ligand 1; RFS = recurrence-free survival.

cell-mediated antitumor immune responses; we validated this finding in this study. The PD-1/PD-L1 pathway shows its effect by interacting with the surrounding immune microenvironment, especially by suppressing cytotoxic CD8 cells. Accordingly, the effect of PD-1/PD-L1 may differ depending on concurrent CD8. Tumors with adequate CD8 cells may originally have 'strong attack power by cancer immunity,' and be immune-attacked regardless of suppression by the PD-1/PD-L1 pathway. Tumors with adequate CD8 cells may have good prognosis, and the prognostic impact of PD-L1 status may be lacking. Conversely, tumors with a few CD8 cells may originally have 'weak attack power by cancer immunity,' and tumor cells may evade immune attack if suppression by the PD-1/PD-L1 pathway is added up. Therefore, tumors with a few CD8 cells may have poor prognosis especially when PD-L1 was positive; thus, the prognostic impact of PD-L1 status may be enhanced in tumors with a few CD8 cells. In our study, PD-L1 positivity was crucial in predicting poor survival in all patients; however, its statistical significance was marginal. When we subdivided patients into 2 groups according to intratumoral CD8, the prognostic impact of PD-L1 was not evident in tumors with adequate CD8 cells, whereas it was enhanced in tumors with few intratumoral CD8 cells. These results may indicate that the prognostic impact of the PD-1/PD-L1 pathway depends on concurrent CD8 status.

Study limitations include this being a single-institute, retrospective review. The respective smaller patient groups might have introduced bias. With reference to several previous reports,[12,21] we decided to perform our study with 126 patients with NSCLC. Greater data may remedy this problem. We performed survival analysis only for RFS because, among the 126 patients with resected NSCLC, only 12 (9.5%) died during follow-up; this number is too small for statistical analysis for overall survival; 26 (20.6%) patients relapsed or died; we analyzed RFS only. Patients with lepidic-dominant adenocarcinoma were excluded because they reportedly have good prognosis and few recurrences[22,23]; few have been reported with PD-L1 positivity.[18,24,25] Thus, we aimed to investigate whether the relationship between the PD-1/PD-L1 pathway and the surrounding immune microenvironment affected prognosis more clearly. Chan et al assessed the difference using antibodies.[19] They assessed PD-L1 expression in 713 consecutive NSCLCs using several commercially available PD-L1 immunohistochemical assays, 3 (28-8, 22C3, SP263) of which showed good analytical performance and a high agreement with each other. In addition, the Blueprint PD-L1 IHC Assay Comparison Project revealed that these 3 (28-8, 22C3, SP263) assays were also closely aligned in terms of tumor cell staining.[26] Among them, 28-8 was assessed for its mutual relationship with other antibodies in these studies. From these results, we selected the 28-8 antibody to use in our study. Second, regarding the cutoff value, we decided to adopt 5% as the cutoff for our main result. Hirai et al[12] have previously summarized the reported studies on PD-L1 expression in NSCLC. Among them, a cutoff value of "5%" was the most frequently used. It was speculated that a cutoff value of "5%" was adopted to counteract the possibility of false positivity owing to variations in staining (If a cutoff value of "1%" is adopted, only a few stains might be judged to be positive according to staining variation, whereas if a cutoff value of "5%" is adopted, only samples that are stained to some extent are diagnosed as positive). After a thorough consultation with our co-author pathologists, we decided on the cutoff value of 5%. Other antibodies, such as 22C3, SP142, and SP263, and cutoff values should be investigated in future studies.

Table 2 Univariable Cox Regression Analysis for Recurrence-free Survival in Patients With Stage I Lung Cancer

Variables	Univariable		
	Hazard Ratio	95% Confidence Interval	*P* Value
Age: ≥ 68.5 y[a] (vs. < 68.5 y)	1.48	0.68-3.29	.32
Gender: male (vs. female)	2.22	0.95-6.09	.067
Smoking: yes (vs. never)	3.27	1.25-11.2	.014
Surgical procedure: lobectomy (vs. sublobar resection)	1.05	0.48-2.47	.90
Adjuvant chemotherapy: yes (vs. no)	1.04	0.43-2.32	.92
Histology: nonadenocarcinoma (vs. adenocarcinoma)	2.40	1.08-5.23	.031
Visceral pleural invasion: positive (vs. negative)	3.65	1.60-7.96	.0029
Lymphatic invasion: positive (vs. negative)	2.38	0.97-5.31	.057
Vascular invasion: positive (vs. negative)	3.06	1.40-6.68	.0055
PD-L1: positive (vs. negative)	2.18	0.89-4.85	.085
Stromal CD4: high (vs. low)	0.91	0.41-1.98	.82
Intratumoral CD4: high (vs. low)	0.95	0.43-2.08	.90
Stromal CD8: high (vs. low)	0.59	0.26-1.29	.19
Intratumoral CD8: high (vs. low)	0.55	0.25-1.19	.13
Stromal FOXP3: high (vs. low)	1.69	0.77-3.85	.19
Intratumoral FOXP3: high (vs. low)	1.33	0.61-2.93	.47
HLA class I: normal (vs. reduced)	1.23	0.56-2.67	.59
PD-L1—positive & intratumoral CD8 low (vs. other)	3.49	1.27-8.25	.018

Abbreviations: HLA = human leukocyte antigen; PD-L1 = programmed death-ligand 1.
[a]Continuous variables were dichotomized and converted to categorical variables using the mean values.

Conclusion

Our study suggested that prognostic impact of PD-L1 expression was distinct according to intratumoral CD8 status in resected lung cancer. PD-L1 positivity on tumor cells was associated with poor prognosis when concurrent intratumoral CD8 were few, whereas it was not associated with prognosis when an adequate number of concurrent intratumoral CD8 cells existed. To better elucidate the clinical significance of the PD-1/PD-L1 pathway, it may be important to evaluate its correlation with the surrounding immune microenvironment, especially intratumoral CD8. Further

Table 3 Multivariable Cox Regression Analysis for Recurrence-free Survival in Patients With Stage I Lung Cancer

Variables	Multivariable		
	Hazard Ratio	95% Confidence Interval	*P* Value
Age: ≥ 68.5 y[a] (vs. < 68.5 y)	1.53	0.62-3.86	.34
Gender: male (vs. female)	0.68	0.21-2.55	.54
Smoking: yes (vs. never)	3.06	0.71-14.2	.13
Surgical procedure: lobectomy (vs. sublobar resection)	0.80	0.33-2.01	.63
Adjuvant chemotherapy: yes (vs. no)	0.94	0.32-2.58	.91
Histology: nonadenocarcinoma (vs. adenocarcinoma)	1.49	0.57-4.07	.42
Visceral pleural invasion: positive (vs. negative)	2.26	0.86-5.89	.097
Lymphatic invasion: positive (vs. negative)	1.69	0.65-4.07	.27
Vascular invasion: positive (vs. negative)	1.29	0.50-3.28	.59
Immune status: PD-L1 positive & intratumoral CD8 low (vs. other)	3.80	1.22-10.5	.023

Abbreviation: PD-L1 = programmed death-ligand 1.
[a]Continuous variables were dichotomized and converted to categorical variables using the mean values.

investigations on the correlation between the PD-1/PD-L1 pathway and the surrounding immune microenvironment, especially CD8 in lung cancer, are needed.

Clinical Practice Points

- Many studies demonstrated that positive PD-L1 expression correlated with favorable clinical benefits achieved with anti−PD-1/PD-L1 antibodies; however, the prognostic impact of PD-L1 expression itself remains controversial, and the presence of PD-L1 positivity in patient cohorts is reportedly related from poor prognosis to a better locoregional control and prognosis.
- The present study analyzed the prognostic impact of the PD-1/PD-L1 pathway and surrounding immune microenvironment such as CD 4, CD 8, Tregs, and HLA class I molecules, and the relationship between them. We showed that positive PD-L1 expression status on tumor cells was a potential factor to predict a poor prognosis, which may depend on concurrent CD 8 status, especially existing in the intratumoral compartment in lung cancer. PD-L1 positivity was found to be correlated with a poor prognosis when concurrent intratumoral CD 8 were few in number, whereas PD-L1 expression status provided no prognostic impact when concurrent intratumoral CD 8 adequately existed.
- Our study suggests that the prognostic impact of the PD-1/PD-L1 pathway may be distinct according to concurrent intratumoral CD8 status. To better elucidate the clinical significance of the PD-1/PD-L1 pathway, it may be important to evaluate correlation with the surrounding immune microenvironment, especially intratumoral CD8.

Disclosure

The authors have stated that they have no conflicts of interest.

Supplemental Data

Supplemental figures and tables accompanying this article can be found in the online version at https://doi.org/10.1016/j.cllc.2020.01.013.

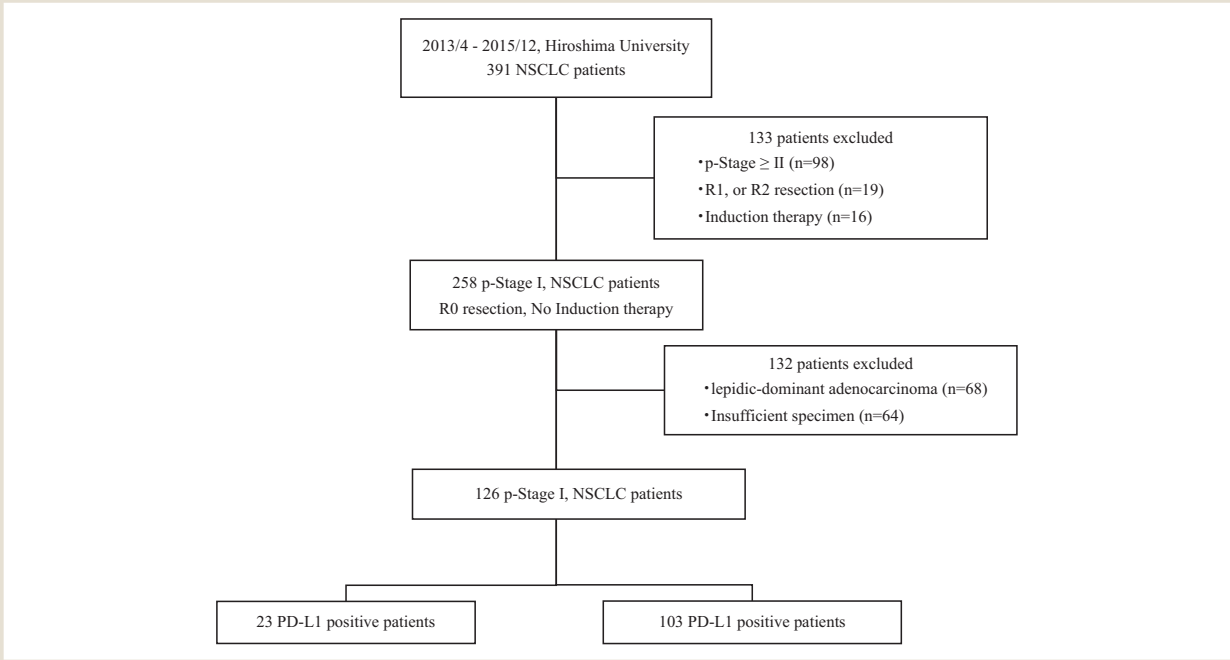
References

1. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer* 2012; 12:252-64.
2. Reck M, Rodríguez-Abreu D, Robinson AG, et al, KEYNOTE-024 Investigators. Pembrolizumab versus chemotherapy for PD-L1-positive nonsmall-cell lung cancer. *N Engl J Med* 2016; 375:1823-33.
3. 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; 11:1879-90.
4. Ohue Y, Kurose K, Nozawa R, et al. Survival of lung adenocarcinoma patients predicted from expression of PD-L1, galectin-9, and XAGE1 (GAGED2a) on tumor cells and tumor-infiltrating T cells. *Cancer Immunol Res* 2016; 4:1049-60.
5. Fridman WH, Pagès F, Sautès-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer* 2012; 12:298-306.
6. Ruffini E, Asioli S, Filosso PL, et al. Clinical significance of tumorinfiltrating lymphocytes in lung neoplasms. *Ann Thorac Surg* 2009; 87:365-71 [discussion 371-2].
7. Al-Shibli KI, Donnem T, Al-Saad S, Persson M, Bremnes RM, Busund LT. Prognostic effect of epithelial and stromal lymphocyte infiltration in non-small cell lung cancer. *Clin Cancer Res* 2008; 14:5220-7.
8. Donnem T, Hald SM, Paulsen EE, et al. Stromal CD8+ T-cell density—a promising supplement to TNM staging in non-small cell lung cancer. *Clin Cancer Res* 2015; 21:2635-43.
9. Bates GJ, Fox SB, Han C, et al. Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. *J Clin Oncol* 2006; 24:5373-80.
10. Salama P, Phillips M, Grieu F, et al. Tumor-infiltrating FOXP3+ T regulatory cells show strong prognostic significance in colorectal cancer. *J Clin Oncol* 2009; 27:186-92.
11. Chen L, Flies DB. Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nat Rev Immunol* 2013; 13:227-42.
12. Hirai A, Yoneda K, Shimajiri S, et al. Prognostic impact of programmed death-ligand 1 expression in correlation with human leukocyte antigen class I expression status in stage I adenocarcinoma of the lung. *J Thorac Cardiovasc Surg* 2018; 155:382-92.
13. Hanagiri T, Shigematsu Y, Shinohara S, et al. Clinical significance of expression of cancer/testis antigen and down-regulation of class-I in patients with stage I non-small cell lung cancer. *Anticancer Res* 2013; 33:2123-8.
14. Goldstraw P, Crowley J, Chansky K, et al, International Association for the Study of Lung Cancer International Staging Committee; Participating Institutions. 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; 2:706-14.
15. Salgado R, Denkert C, Demaria S, et al, International TILs Working Group 2014. The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. *Ann Oncol* 2015; 26:259-71.
16. Alexandrov LB, Nik-Zainal S, Wedge DC, et al. Signatures of mutational processes in human cancer. *Nature* 2013; 500:415-21.
17. Zhuo C, Xu Y, Ying M, et al. FOXP3+ Tregs: heterogeneous phenotypes and conflicting impacts on survival outcomes in patients with colorectal cancer. *Immunol Res* 2015; 61:338-47.
18. Toyokawa G, Takada K, Okamoto T, et al. Relevance between programmed death ligand 1 and radiologic invasiveness in pathologic stage I lung adenocarcinoma. *Ann Thorac Surg* 2017; 103:1750-7.
19. Chan AWH, Tong JHM, Kwan JSH, et al. Assessment of programmed cell death ligand-1 expression by 4 diagnostic assays and its clinicopathological correlation in a large cohort of surgical resected non-small cell lung carcinoma. *Mod Pathol* 2018; 31:1381-90.
20. Casey SC, Tong L, Li Y, et al. MYC regulates the antitumor immune response through CD47 and PD-L1. *Science* 2016; 352:227-31.
21. Usó M, Jantus-Lewintre, Bremnes RM, et al. Analysis of the immune microenvironment in resected non-small cell lung cancer: the prognostic value of different T lymphocyte markers. *Oncotarget* 2016; 7:52849-61.
22. Asamura H, Hishida T, Suzuki K, et al, Japan Clinical Oncology Group Lung Cancer Surgical Study Group. Japan Clinical Oncology Group Lung Cancer Surgical Study Group radiographically determined noninvasive adenocarcinoma of the lung: survival outcomes of Japan Clinical Oncology Group 0201. *Thorac Cardiovasc Surg* 2013; 146:24-30.
23. Tsutani Y, Miyata Y, Okada M, et al. Appropriate sublobar resection choice for ground glass opacity-dominant clinical stage IA lung adenocarcinoma: wedge resection or segmentectomy. *Chest* 2014; 145:66-71.
24. Serra P, Petat A, Maury JM, et al. Programmed cell death-ligand 1 (PD-L1) expression is associated with RAS/TP53 mutations in lung adenocarcinoma. *Lung Cancer* 2018; 118:62-8.
25. Huynh TG, Morales-Oyarvide V, Campo MJ, et al. Programmed cell death ligand 1 expression in resected lung adenocarcinomas: association with immune microenvironment. *J Thorac Oncol* 2016; 11:1869-78.
26. 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; 12:208-22.

Relation Between PD-L1 and Immune Status

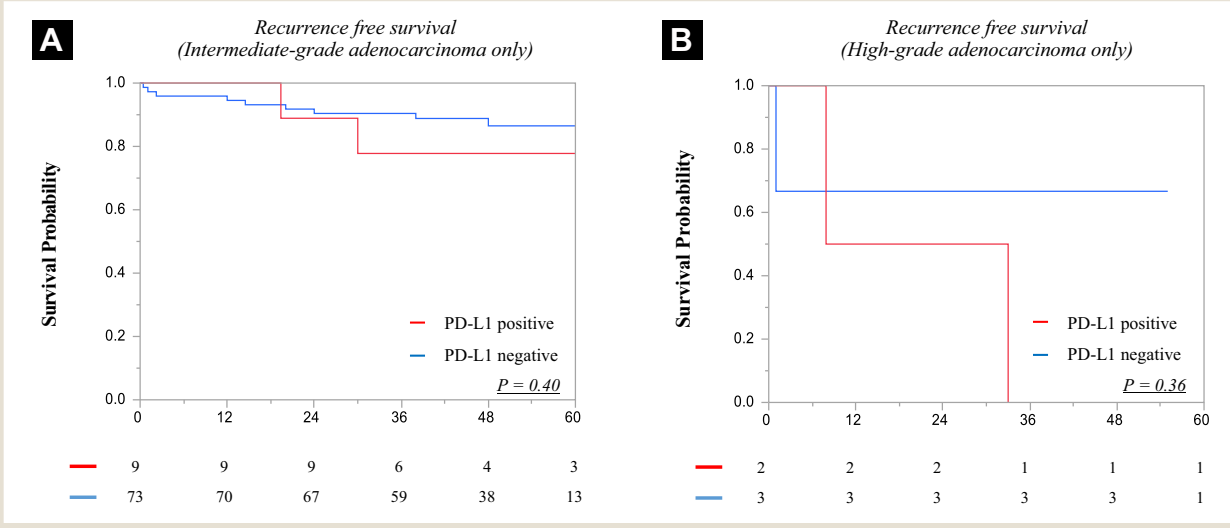
Supplemental Data

Supplemental Figure 1 Study Profile



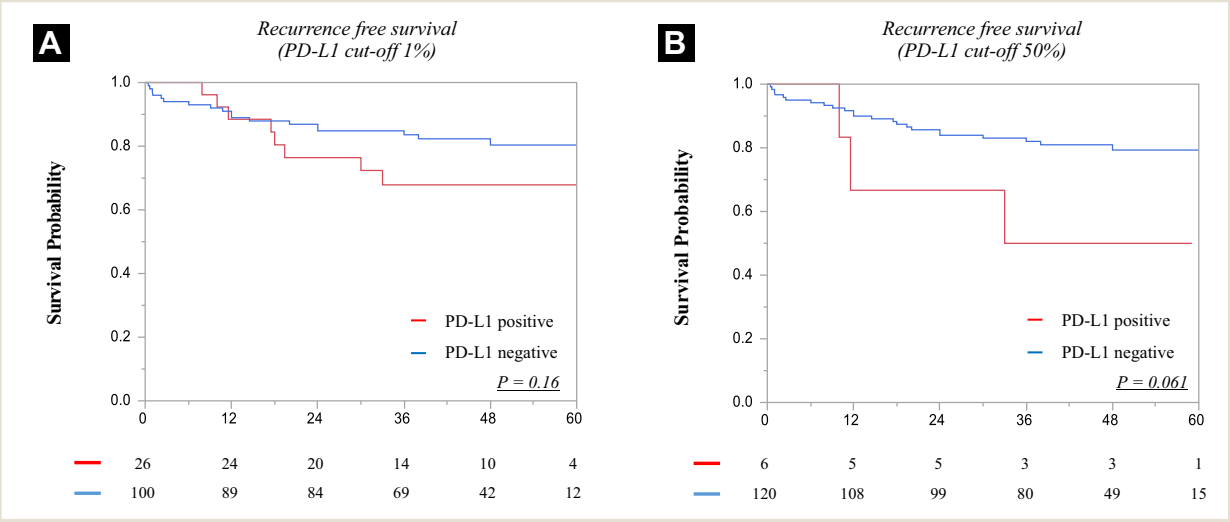
Abbreviations: NSCLC = non–small-cell lung cancer; PD-L1 = programmed death-ligand 1.

Supplemental Figure 2 Recurrence-free Survival Curves of Patients Who Are Positive (*red line*) and Negative (*blue line*) for PD-L1. The X-axis Shows the Survival Time in Months, and the Y-axis Shows the Survival Probability. The Number of Patients at Risk is Listed at the Bottom of the Figure. A, Analysis of Intermediate-grade (Acinar and Papillary) Adenocarcinoma Patients. B, Analysis of High-grade (Micropapillary and Solid) Adenocarcinoma Patients



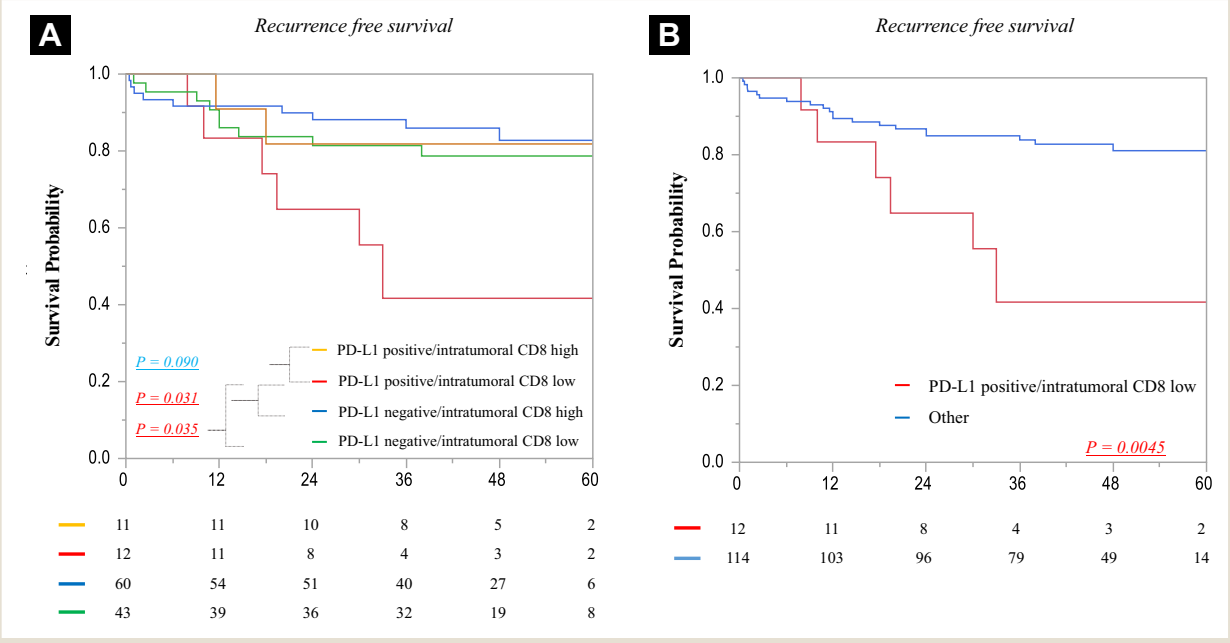
Abbreviation: PD-L1 = programmed death-ligand 1.

Supplemental Figure 3 Recurrence-free Survival Curves of Patients Who Are Positive (*red line*) and Negative (*blue line*) for PD-L1. The X-axis Shows the Survival Time in Months, and the Y-axis Shows the Survival Probability. The Number of Patients at Risk is Listed at the Bottom of the Figure. A, Analysis With the Cutoff Value Set at 1%. B, Analysis With the Cutoff Value Set at 50%



Abbreviation: PD-L1 = programmed death-ligand 1.

Supplemental Figure 4 Recurrence-free Survival Curves of Patients Who are Positive (*red line*) and Negative (*blue line*) for PD-L1 Stratified by Intratumoral CD8 Expression Status. The X-axis Shows the Survival Time in Months, and the Y-axis Shows the Survival Probability. The Number of Patients at Risk is Listed at the Bottom of the Figure. A, Analysis Between the PD-L1-Positive/Intratumoral CD8-Low Group and Each of the Other Groups. B, Analysis Between PD-L1-Positive/Intratumoral CD8-Low and the Other Group (as a Single Group)



Abbreviation: PD-L1 = programmed death-ligand 1.

Supplemental Table 1 Univariable Logistic Regression Analysis for PD-L1 Positivity in Patients With Stage I Lung Cancer

Variables	Univariable		
	Odds Ratio	95% Confidence Interval	*P* Value
Age: $\geq$ 68.5 y[a] (vs. $<$ 68.5 y)	0.56	0.22-1.39	.21
Gender: male (vs. female)	2.48	0.91-7.97	.078
Smoking: yes (vs. never)	2.89	0.99-10.5	.051
Surgical procedure: lobectomy (vs. sublobar resection)	2.38	0.87-7.66	.093
Adjuvant chemotherapy: yes (vs. no)	1.43	0.53-3.67	.47
Histology: non-adenocarcinoma (vs. adenocarcinoma)	3.07	1.21-7.89	.018
Visceral pleural invasion: positive (vs. negative)	1.92	0.62-5.44	.25
Lymphatic invasion: positive (vs. negative)	2.38	0.81-6.59	.11
Vascular invasion: positive (vs. negative)	4.28	1.68-11.2	.0024
Stromal CD4: high (vs. low)	1.92	0.77-5.00	.16
Intratumoral CD4: high (vs. low)	1.11	0.45-2.78	.82
Stromal CD8: high (vs. low)	0.73	0.29-1.79	.49
Intratumoral CD8: high (vs. low)	0.66	0.26-1.63	.36
Stromal FOXP3: high (vs. low)	1.16	0.46-2.89	.75
Intratumoral FOXP3: high (vs. low)	1.54	0.63-3.94	.34
HLA class I: normal (vs. reduced)	1.72	0.69-4.32	.24

Abbreviations: HLA = human leukocyte antigen; PD-L1 = programmed death-ligand 1.
[a]Continuous variables were dichotomized and converted to categorical variables using the mean values.

Supplemental Table 2 Patient and Tumor Characteristics of PD-L1–positive and Intratumoral CD8-low Patients and Other

Variables	PD-L1-positive and Intratumoral CD8-low N = 12	Other N = 114	*P* Value
Age, y	65.0 (59.3-75.5)	70.0 (63.8-74.0)	.48
Gender			.53
Male	9 (75.0)	70 (61.4)	
Female	3 (25.0)	44 (38.6)	
Smoking			1.00
Yes	8 (66.7)	75 (65.8)	
Never	4 (33.3)	39 (34.2)	
Surgical procedure			.21
Lobectomy	10 (83.3)	70 (61.4)	
Sublobar resection	2 (16.7)	44 (38.6)	
Adjuvant chemotherapy	5 (41.7)	31 (27.2)	.32
Histology			.75
Adenocarcinoma	9 (75.0)	78 (68.4)	
Non-adenocarcinoma	3 (25.0)	36 (31.6)	
Visceral pleural invasion	3 (25.0)	19 (16.7)	.44
Lymphatic invasion	3 (25.0)	20 (17.5)	.46
Vascular invasion	6 (50.0)	31 (27.2)	.11
Stromal CD4			1.00
High	6 (50.0)	54 (47.4)	
Low	6 (50.0)	60 (52.6)	
Intratumoral CD4			.36
High	8 (66.7)	55 (48.3)	
Low	4 (33.3)	59 (51.7)	
Stromal CD8			.36
High	4 (33.3)	59 (51.8)	
Low	8 (66.7)	55 (48.2)	
Stromal FOXP3			.24
High	8 (66.7)	54 (47.4)	
Low	4 (33.3)	60 (52.6)	
Intratumoral FOXP3			.77
High	5 (41.7)	55 (48.3)	
Low	7 (58.3)	59 (51.7)	
HLA class I			1.00
Normal	5 (41.7)	47 (41.2)	
Reduced	7 (58.3)	67 (58.8)	

Values are median (interquartile range) or n (%).
Abbreviations: HLA = human leukocyte antigen; PD-L1 = programmed death-ligand 1.