Original Article

Relationship of tumor PD-L1 expression with EGFR wild-type status and poor prognosis in lung adenocarcinoma

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Abstract

Background: Programmed death-ligand 1 is an immune modulator that promotes immunosuppression by binding to programmed death-1 of T-lymphocytes. Whereas programmed death-ligand 1 expression has been shown to be associated with the clinical response to anti-programmed death-ligand 1 antibody, the association of tumor programmed death-ligand 1 expression with clinicopathological/molecular features and with prognosis remains inconclusive in lung adenocarcinoma. We therefore examined the association of programmed death-ligand 1 expression with the clinicopathological/molecular features and prognosis of lung adenocarcinoma.

Methods: Using tissue microarrays of 268 consecutive cases of lung adenocarcinoma, we evaluated programmed death-ligand 1 expression by immunohistochemistry. We examined the association of programmed death-ligand 1 expression with clinicopathological/molecular features. We also examined the prognostic association of programmed death-ligand 1 expression, using the log-rank test as well as Cox proportional hazards regression models to compute the mortality hazard ratio (HR).

Results: Programmed death-ligand 1 immunoreactivity (at least 5% of the tumor cells) was observed in 43 (16%) of 268 cases of lung adenocarcinoma. Programmed death-ligand 1 positivity was associated with less tumor differentiation (P < 0.0001) and EGFR wild-type status (P = 0.0008). In a multivariable logistic regression analysis, less tumor differentiation was independently associated with programmed death-ligand 1 positivity (multivariable odds ratio, 6.54; 95% confidence interval [CI], 2.37–23.3; P = 0.0001). Programmed death-ligand 1 positivity was associated with a poor prognosis for lung cancer-specific survival (log-rank, P = 0.019; HR, 1.73; 95% CI, 1.06–2.72; P = 0.030) and overall survival (log-rank, P = 0.0014; HR, 1.88; 95% CI, 1.25–2.74).

Conclusion: Our study demonstrated that programmed death-ligand 1 positivity in lung adenocarcinoma was associated with less tumor differentiation and EGFR wild-type status, as well as a poor prognosis.

Key words: driver mutation, immune checkpoint, lung cancer, outcome, PD-L1
**Introduction**

Lung cancer, especially non-small cell lung cancer (NSCLC), is the leading cause of cancer death in men and women worldwide, accounting for more than 1.5 million deaths per year (1). Recent advances in molecular-targeted therapy have led to a major paradigm shift in the treatment for advanced NSCLC (2–6). However, accumulating evidence shows that tumors can evade host immune surveillance by several strategies, including down-regulation of cell surface major histocompatibility complex class I molecules, secretion of immunosuppressive factors, and expression of death ligands or negative ligands (7,8).

Emerging evidence indicates that the immune checkpoint mechanism plays a critical role in suppressing the anti-tumor T-cell-mediated immune response in the tumor microenvironment (9,10). Programmed death-ligand 1 (PD-L1; also known as B7-H1 and CD274) is an immune modulator that promotes immunosuppression by binding to programmed death-1 (PD-1; also known as PDCD1) of T cells. PD-L1 has been shown to engage in the negative regulation of the immune response through the PD-1 receptor, and it is thought to be an important strategy for cancer to evade the host immune surveillance. Therapeutic antibodies targeting PD-1 and PD-L1 have been shown to be effective in a number of cancer types, including lung cancer, and PD-L1 expression in tumor cells has been suggested as a predictive marker of the clinical response to PD-1/ PD-L1-targeted therapy (11–15).

Although various studies have reported the association of PD-L1 positivity with EGFR mutational status and with prognosis in lung adenocarcinoma, the results are conflicting and inconclusive (16–28). Because patients with EGFR wild-type lung adenocarcinoma cannot be treated with EGFR-targeted therapy, the association of EGFR status with PD-L1 positivity is important for the treatment selection. Therefore, we examined the association of PD-L1 expression with clinicopathological/molecular features, including EGFR status, and the prognostic association of PD-L1 expression in lung adenocarcinoma, using 268 consecutive cases of lung adenocarcinoma.

**Materials and methods**

**Patients**

On the basis of the availability of tumor PD-L1 expression status and survival data, we enrolled a series of 268 consecutive Japanese patients with lung adenocarcinoma surgically resected between April 1995 and January 2002 at The Cancer Institute Hospital, Japanese Foundation for Cancer Research (JFCR), Tokyo, Japan. Patients were observed until death or 1 December 2015, whichever came first. Smoking histories were obtained from a rigorous interview of each patient by experienced thoracic surgeons who were well aware of the tendency of patients to underreport smoking. In this study, ‘never smokers’ were strictly defined as patients without any smoking history, whereas all the other patients were included as ‘ever smokers.’ All patients included in this study provided informed consent for research and the study plan was approved by the institutional review board of the JFCR.

**Pathologic evaluation**

Pathologic diagnosis was made by an expert pulmonary pathologist (YI) essentially on the basis of the 2004 WHO classification of lung tumors (29) with the additional use of a newly proposed classification (30). Tumor differentiation grades were defined according to the Japanese Lung Cancer Society criteria (31). In brief, well-differentiated tumors are composed mainly of glands lined by one-layer of tumor cells. Adenocarcinoma in situ is also included in this category. Moderately-differentiated tumors comprise glands showing a cribriform pattern, glands fused with one another, or glands lined by tumor cells showing evident piling-up. Poorly-differentiated tumors show mainly solid growth and only occasionally glandular/papillary patterns and/or mucus production. When two or more patterns were observed, the predominant grade was considered. All patients were staged pathologically according to the 7th edition of the AJCC-TNM staging system (32).

**Immunohistochemistry for PD-L1**

Tumor PD-L1 expression was evaluated by immunohistochemistry using tissue microarrays. We constructed tissue microarrays as described previously (33). In brief, we punched selected points of the donor paraffin blocks with a 2-mm-diameter coring needle and transferred the material to the array in the recipient block using a manual tissue arrayer (KIN-1; Azumaya, Tokyo, Japan). For each tumor, experienced pulmonary pathologists (KI and HN) selected one site, which showed the most representative histology in the tumor. Sections of 4 μm thickness were immunostained for PD-L1 with an anti-PD-L1 rabbit monoclonal antibody (clone: E1L3N, Cell Signaling Technology, Danvers, MA, USA; diluted 1:50), using the Leica Bond III automated system (Leica Biosystems Melbourne Pty Ltd, Australia). The sections were incubated at pH 9 for 20 min at 100°C. The expression of PD-L1 detected by the antibodies was interpreted blindly by a pathologist (KI). The percentages of tumor cells with membranous PD-L1 positive staining were recorded. A score of 5% or more was categorized as ‘PD-L1-positive’ (Fig. 1a) and a score of less than 5% as ‘PD-L1-negative’ (Fig. 1b) based on recent studies of tumor PD-L1 expression (15,21,34). A random subset of 126 cases were blindly examined by a second pathologist (YY). The agreement between the two pathologists for the tumor membranous PD-L1 expression was good with a kappa of 0.70 (95% confidence interval [CI], 0.55–0.86), indicating substantial agreement.

We conducted a validation of the PD-L1 antibody (clone: E1L3N) for specificity and sensitivity, because PD-L1 is also known as B7-H1, one of the B7 subfamilies. We used a B7 subfamily cell array (provided by Daiichi Sankyo Co., Ltd, Tokyo, Japan), consisting of CHO-K1 cells overexpressing the B7 subfamily. The CHO-K1 cells transiently overexpressing PD-L1 (B7-H1), B7-H2, PD-L2 (B7-DC), B7-H3, B7-H4, B7-1, or B7-2, and the mock-transfected control cells, were fixed with 10% neutral buffered formalin and processed to produce a paraffin-embedded cell block array. Using this B7 subfamily cell array, we verified the specificity and sensitivity of the PD-L1 antibody (clone: E1L3N) by confirming that the PD-L1 antibody (clone: E1L3N) exclusively recognized B7-H1 (PD-L1) among the B7 subfamilies tested.

**Detection of EGFR and KRAS mutations and the ALK fusion**

The tumor specimens were snap-frozen in liquid nitrogen within 20 min after surgical removal and stored at −80°C until use. DNA was extracted by standard proteinase K digestion and phenol-chloroform extraction. For EGFR mutational analysis, four exons that code for the tyrosine kinase domain of the EGFR gene (exons 18–21) were examined. For exons 18 (G719X), exon 20 (S768I and T790M), and exon 21 (L858R and L861Q), the TaqMan™ SNP Genotyping Assay
(Applied Biosystems, Foster City, CA, USA) was performed according to the manufacturer’s instructions. Fragment analysis was conducted for the exon 19 deletion and the exon 20 insertion mutations as described previously (35). For the mutational analysis of KRAS (codons 12, 13 and 61), direct sequencing assays were performed as described previously (35). For the ALK fusion, ALK immunohistochemistry was performed using an anti-ALK mouse monoclonal antibody (clone: 5A4, Leica Biosystems Newcastle Ltd, UK; diluted 1:50) and the Leica Bond III automated system (Leica Biosystems Melbourne Pty Ltd). The sections were incubated at pH 9 for 30 min at 100°C. The ALK fusions in the ALK-immunohistochemically positive cases were confirmed by fluorescence in situ hybridization as described previously (36).

Statistical analysis
All statistical analyses were conducted using the JMP statistical software package 12 (SAS Institute Inc., Cary, NC, USA) and Excel 2013 software (Microsoft, Redmond, WA, USA). All P values were two-sided, and statistical significance was set at \( P = 0.05 \). To investigate the associations of tumor PD-L1 positivity with clinicopathological and molecular factors in lung adenocarcinoma, we performed the chi-square test or Fisher’s exact test as appropriate. For multiple hypothesis testing, we used a simple Bonferroni correction. As a multiple-comparison correction, we divided the two-sided \( \alpha \) level by the number of comparisons being performed. We adjusted the two-sided \( \alpha \) level to 0.0050 (\( = 0.05/10 \)) by a simple Bonferroni correction for multiple hypothesis testing. The relationships of the clinicopathological and molecular features with PD-L1 positivity were also assessed by univariable and multivariable logistic regression models. For the multivariable analysis, we initially included age (\( \geq 60 \) vs \(< 60 \)), sex (male vs female), smoking habit (ever vs never smokers), tumor size (\( > 30 \) vs \( \leq 30 \) mm), differentiation grade (moderate to poor vs well), pathological stage (p-stage) (II-IV vs I), EGFR mutation status (wild type vs mutant), KRAS mutation status (wild type vs mutant), and ALK rearrangement status (negative vs positive). We adjusted the two-sided \( \alpha \) level to 0.0056 (\( = 0.05/9 \)) by a simple Bonferroni correction for multiple hypothesis testing. A backward stepwise elimination with a threshold of \( P = 0.05 \) was used to select variables in the final model to avoid overfitting. We created missing categories for missing cases for each variable, if applicable.

The Kaplan–Meier method and log-rank test were used for survival analysis. For the analyses of lung cancer-specific mortality, deaths as a result of other causes were censored. We also used a univariable Cox proportional hazards regression model to calculate a hazard ratio (HR) for mortality according to the PD-L1 expression status.

Results
Tumor PD-L1 expression in lung adenocarcinoma
Among 268 lung adenocarcinomas, we observed PD-L1 positivity (defined as 5% or more of tumor cells with membranous staining) in 43 tumors (16%) by immunohistochemistry (Fig. 1a). Table 1 shows the clinicopathological and molecular characteristics according to PD-L1 expression status (negative vs positive). Based on the Bonferroni-corrected significance level of \( P = 0.0050 \) (\( = 0.05/10 \)), PD-L1 positivity was significantly associated with less differentiation.

![Figure 1](https://example.com/image1.png)

**Figure 1.** Immunohistochemical analysis of tumor programmed death-ligand 1 (PD-L1) membranous expression. (a) PD-L1-positive lung adenocarcinoma. (b) PD-L1-negative lung adenocarcinoma.

![Figure 2](https://example.com/image2.png)

**Figure 2.** Kaplan–Meier curves for lung cancer-specific survival (a) and overall survival (b) according to tumor PD-L1 expression status (negative vs positive).
Logistic regression analysis to assess relations with PD-L1 positivity

In a univariable logistic regression analysis to assess relations with PD-L1 positivity, we found that less differentiation grades (moderate to poor), EGFR wild type, higher p-stage, and male gender were associated with PD-L1 positivity (all \( P < 0.0056 \), given the two-sided \( \alpha \) level of 0.0056, Table 2). Because several interrelated variables were associated with PD-L1 positivity, we performed a multivariable logistic regression analysis to assess the independent relations of those variables with PD-L1 positivity. Less tumor differentiation was independently associated with PD-L1 positivity (odds ratio, 6.54; 95% CI, 2.37–23.3; \( P = 0.0001 \); Table 2).

PD-L1 positivity and survival of lung adenocarcinoma patients

Among 268 patients, there were 148 overall deaths, including 108 lung cancer-specific deaths, during a median follow-up of 90 months (interquartile range: 36–156 months) for those who were censored. As shown in Fig. 2a, PD-L1 positivity was associated with shorter lung cancer-specific survival (Log-rank, \( P = 0.019 \)). In a univariable Cox regression analysis, PD-L1 positivity was associated with higher lung cancer-specific mortality (HR, 1.73; 95% CI, 1.06–2.72; \( P = 0.030 \)). As shown in Fig. 2b, PD-L1 positivity was associated with shorter overall survival (Log-rank, \( P = 0.0014 \), Table 3). In a univariable Cox regression analysis, PD-L1 positivity was associated with higher overall mortality (HR, 1.88; 95% CI, 1.25–2.74; \( P = 0.0031 \), Table 3).

As an exploratory analysis, multivariable Cox regression analyses were conducted (Table 3). PD-L1 positivity was not independently associated with lung cancer-specific (multivariable HR, 0.88; 95% CI, 0.53–1.41; \( P = 0.61 \)) or overall survival (multivariable HR, 0.94; 95% CI, 0.61–1.40; \( P = 0.75 \)).
The association between other covariates and patient mortality in univariable and multivariable Cox regression analyses is provided in Supplementary Table S1.

**Discussion**

We conducted this study to examine the association of tumor PD-L1 expression with clinicopathological/molecular features and with prognosis in lung adenocarcinoma, using 268 consecutive cases. We have shown that PD-L1 positivity was associated with higher lung cancer-specific and overall mortality. We have also shown that PD-L1 positivity was associated with the EGFR wild type and independently associated with less tumor differentiation. This study provides useful information about the characteristics of PD-L1 expression in lung adenocarcinoma, clarifying the role of PD-L1 immunohistochemistry in clinical and pathology practice.

A number of studies have reported conflicting results regarding the association of PD-L1 positivity with EGFR mutational status and with prognosis in lung adenocarcinoma (14-28). The varying results may suggest a diverse nature of PD-L1 and the complicated interactions of tumor PD-L1 expression with immune cells in the tumor microenvironment. In addition, the differences between the results of other studies and ours are possibly due to the limited sample size, heterogeneity of the study populations, or biological differences between models involving mice, cell lines, or human patients. Because experimental systems cannot thoroughly recapitulate the complexities of human tumors or the immune system, analyses of human subjects are essential in translational medicine. Furthermore, there has been no standardized method to evaluate PD-L1 expression immunohistochemically, which might result in conflicting results of studies of PD-L1 expression. As a validation of the anti-PD-L1 antibody used in this study, we confirmed its specificity and sensitivity by using a B7 subfamily cell array, comprising the B7 subfamily (PD-L1 [B7-H1], PD-L2 [B7-DC], B7-H2, B7-H3, B7-H4, B7-1, and B7-2) overexpressed CHO-K1 cells, and mock-transfected control cells. Furthermore, to assess the replicability of our immunohistochemical assessment, we conducted blinded and independent assessments of PD-L1 immunohistochemical expression by two pathologists, which yielded good interobserver agreement (a kappa of 0.70).

We have shown the association of PD-L1 positivity with clinicopathological and molecular features in lung adenocarcinoma. Less tumor differentiation was independently associated with PD-L1 positivity. In the univariable analysis, the EGFR wild type and male gender was associated with PD-L1 positivity. In addition, PD-L1 positivity was associated with a higher rate of smoking history. Smoking is known to promote oncogenesis of less differentiated adenocarcinomas (37), and it has been suggested that PD-L1 expression is inducible by smoking (38). Smoking-associated lung cancers have a higher mutational load, resulting in the creation of more
tumor neoantigens and increased immunogenicity (39,40), which are associated with PD-L1 expression (41,42). Smoking history, male gender, and less tumor differentiation are all associated with the EGFR wild type. The current study demonstrated that all four factors were associated with PD-L1 positivity. Because PD-L1 positivity was associated with the EGFR wild type, patients with EGFR wild-type lung adenocarcinomas may have a better chance of a good response to anti-PD-L1 therapy.

There are several limitations of this study. First, there is no standardized anti-PD-L1 antibody and cutoff level of tumor PD-L1 expression. Several different clones are available for the assessment of PD-L1 expression. Nonetheless, we confirmed the specificity and sensitivity of the PD-L1 antibody (clone: E1L3N), using a B7 subfamily cell array with CHO-K1 cells overexpressing B7 subfamily genes and mock-transfected cells as controls. Second, we used tissue microarrays to assess the tumor PD-L1 expression. Because intratumoral heterogeneity exists in lung adenocarcinoma, a subset of tumors with heterogeneous PD-L1 positivity in whole sections might have been scored as negative in tissue microarray cores (43). This potential misclassification of tumors in terms of PD-L1 expression would be expected to be distributed nearly at random, and hence would have yielded null results. Despite this limitation, we were able to observe significant associations of PD-L1 positivity with less tumor differentiation, EGFR wild-type status, and inferior prognosis. In addition, because experienced pulmonary pathologists chose each core site in the most representative histology of the tumor, core sites might not substantially affect the results. Third, there is a possibility that long-term storage (14–21 years) might affect PD-L1 immunoreactivity. However, there was no significant difference of PD-L1 expression by the year of diagnosis. Therefore, it is likely that long-term storage did not substantially affect PD-L1 immunoreactivity. Fourth, we evaluated PD-L1 expression only in tumor cells. PD-L1 expression in tumor cells, tumor-associated immune cells, or a combination of the two, might have biological or prognostic significance. Fifth, the total number of patients (N = 268) was relatively small, and the statistical power was therefore limited. Sixth, our dataset was collected retrospectively. Finally, this study may not be generalizable because we only used cases of Japanese patients at a single cancer hospital. Therefore, further research is required to confirm our findings.

In conclusion, we showed the association of PD-L1 positivity with less tumor differentiation, EGFR wild-type status, and poor survival in lung adenocarcinoma patients. Additional large-scale prospective studies should confirm our findings.

Supplementary data
Supplementary data are available at http://www.jjco.oxfordjournals.org.

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Conflict of interest statement
Yusuke Yokouchi is an employee of Daiichi Sankyo Co., Ltd, Tokyo, Japan. Yuichi Ishikawa received research grants from Daiichi Sankyo Co., Ltd, Chugai Pharmaceutical Co. Ltd, Sony Corp., and is a consultant for Fujirebio Inc. All other authors declare no conflict of interest.

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