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Scoring of PD-L1 expression intensity on pulmonary adenocarcinomas and the correlations with clinicopathological factors

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ABSTRACT

Introduction: The contribution of programmed cell death ligand-1 (PD-L1) immune checkpoint molecule toward progression of non-small cell lung cancer (NSCLC) has not yet been elucidated, in part, because of lack of a standardised method to evaluate PD-L1 expression. In this study, we developed a novel method for the evaluation of PD-L1 expression on NSCLC cells and examined its correlation with clinicopathological characteristics.

Methods: After immunohistochemical examination of PD-L1 expression for surgically resected pulmonary adenocarcinomas (n=106), based on the findings that PD-L1 are consistently expressed on alveolar macrophages, PD-L1 staining intensity of tumour cells was classified into four levels relative to PD-L1 staining intensity in alveolar macrophages; PD-L1 expression scores (range, 0–300) were semiquantitatively assessed. An analysis of statistical association between PD-L1 expression score and clinicopathological characteristics was performed.

Results: Almost all of the alveolar macrophages in the specimens were moderately to strongly stained with PD-L1, serving as an internal positive control in the immunohistochemistry of PD-L1. PD-L1 expression score (median, 52.3) was significantly higher in tumours with G2/3 differentiation than in those with G1 (p=0.022) and higher in those with lymphatic invasion than in those without invasion (p=0.032). Postoperative relapse-free survival was significantly shorter in patients with a high PD-L1 expression score than in those with low PD-L1 expression score (p=0.035). Smoking habits, histological subtype, and *epidermal growth factor receptor* mutation status were not associated with PD-L1 expression score.

Conclusions: Given the heterogeneous distribution of PD-L1 expression in pulmonary adenocarcinoma cells, the scoring of PD-L1 expression on tumour cells relative to that in alveolar macrophages appears to be a valid indicator of PD-L1 status of patients with pulmonary adenocarcinomas, demonstrating a significant correlation with several factors associated with tumour progression.

Key questions

What is already known about this subject?

- Immune checkpoint mechanisms, such as the programmed cell death 1 (PD-1) and programmed cell death ligand-1 (PD-L1) axis, is a promising therapeutic target in non-small cell lung cancer (NSCLC).
- Several studies have reported a correlation between PD-L1 expression on NSCLC cells and clinicopathological features of the patients; however the methods to evaluate PD-L1 expression on NSCLC cells are different among those studies and has not yet been standardised.
- Inappropriate handling of surgical tumour samples would influence the PD-L1 staining of tumour cells on immunohistochemistry. Thus, an internal control or standard for PD-L1 staining is needed when we try to compare the PD-L1 staining intensity of tumour cells between patients.

What does this study add?

- Alveolar macrophages are proven to be consistently stained with anti-PD-L1 antibody, serving as a positive control on PD-L1 immunohistochemistry.
- A novel method to evaluate PD-L1 expression on NSCLC cells was developed, where PD-L1 staining intensity on tumour cells was classified into four levels relative to that of alveolar macrophages and scored.
- The scoring method for PD-L1 expression appears to be a valid indicator of PD-L1 status of patients with pulmonary adenocarcinoma, showing significant correlation with low-grade differentiation, lymphatic invasion and postoperative relapse-free survival.

INTRODUCTION

Non-small cell lung cancer (NSCLC) currently is a leading cause of cancer death worldwide.[1] Therapeutic strategies, cytotoxic

Key questions

How might this impact on clinical practice?

- Through the scoring method, the tumour status with heterogeneous PD-L1 expression can be semiquantitatively evaluated.
- The method to evaluate PD-L1 expression on NSCLC cells is standardised, contributing to development of biomarkers for clinical responses of immune checkpoint inhibitors.

chemotherapy, radiotherapy and surgery have improved the survival of patients with NSCLC over the past few decades.[2–5] Furthermore, breakthroughs in molecular-targeted therapy directed at mutations of driver oncogenes, such as epidermal growth factor receptor (EGFR), has improved prognosis of patients with NSCLC.[6–9] However, novel therapeutic strategies must be developed to help prolong the survival of patients with NSCLC.

Immune checkpoint mechanisms in the functional reaction between tumour and immune cells are novel therapeutic targets in NSCLC and other malignant tumours.[10] Tumour cells are known to evade immunological surveillance of effector immune cells through immune checkpoint mechanisms such as, the programmed cell death 1 (PD-1)-programmed cell death-ligand 1 (PD-L1) axis.[11 12] PD-L1 expressed on tumour cells interacts with PD-1 expressed on activated effector immune cells like cytotoxic T lymphocytes, which induces apoptosis, anergy and exhaustion of effector immune cells.[13 14] Research on the immune escape system of tumour cells has led to the development of PD-1-targeted therapies and PD-L1-targeted therapies; several clinical trials of anti-PD-1 and anti-PD-L1 antibodies have revealed their antitumour effects with significant durable responses in patients with NSCLC.[15 16] Inhibition of the PD-1–PD-L1 axis is a promising therapeutic target for NSCLC. However, therapeutic biomarkers in these molecular-targeted therapies remain to be established, and an actual role of PD-L1 in the progression of NSCLC is yet to be elucidated.

Several studies have demonstrated a correlation between PD-L1 expression on NSCLC cells and clinicopathological characteristics of patients with NSCLC.[17–21] However, these results have not been consistent and definitive conclusions have yet to be drawn. These discrepancies may be due to the lack of standardised methods to evaluate PD-L1 expression on NSCLC cells.

The goal of this study was to establish a standard evaluation method for PD-L1 expression in NSCLC. We conducted a semiquantitative evaluation of PD-L1 expression of pulmonary adenocarcinomas, and compared the staining intensity with that of alveolar macrophages; an analysis of the correlation between PD-L1 expression intensity and clinicopathological characteristics is presented.

MATERIALS AND METHODS

NSCLC tissue samples

Tumour tissue samples were obtained from surgically resected specimens from patients with pulmonary adenocarcinoma at the Shiga University of Medical Science Hospital, between January 2008 and December 2013. Those patients did not receive anticancer therapy such as neo-adjuvant chemotherapy prior to the surgery for their pulmonary adenocarcinoma. Data on clinicopathological variables of patients with *EGFR* mutation status was obtained from their medical records. The study design was approved by the Ethical Committee of Shiga University of Medical Science; written informed consent was obtained from all patients.

Immunohistochemistry

Whole tissue sections rather than tissue microarrays were used for immunohistochemistry in this study. The 4 µm thick sections of formalin-fixed paraffin-embedded tissue specimens were stained by standard indirect immunoperoxidase procedures, according to the manufacturer's protocol (Cell Signaling Technology, Danvers, Massachusetts, USA). Briefly, each tissue section was deparaffinised in xylene, and rehydrated in ethanol and distilled water. Antigen retrieval was performed by microwave treatment in 10 mM sodium citrate buffer (pH 6.0) for 10 min; endogenous peroxidase activity was blocked by treatment with 3% H_2O_2 for 10 min. After blocking with 5% normal goat serum in Tris-buffered saline with Tween 20 for 1 hour at room temperature, the sections were incubated overnight with anti-human PD-L1 monoclonal antibody (clone: E1L3N, diluted at 1:200) (Cell Signaling Technology) at 4°C. On the following day, the sections were incubated with SignalStain boost IHC detection reagent (Cell Signaling Technology), and visualised using the SignalStain DAB substrate kit (Cell Signaling Technology) for 1 min, followed by counterstaining with hematoxylin.

We confirmed by flow-cytometric analysis that lung cancer cell line H-1975 cells (American Type Culture Collection, Manassas, Virginia, USA) are positive and A549 cells (American Type Culture Collection) are negative for PD-L1 (data not shown). Based on the finding, paraffin-embedded cell-blocks of H-1975 and A549 cells were utilised for positive and negative controls of PD-L1 immunohistochemistry, respectively. Rabbit IgG monoclonal antibody (Cell Signaling Technology) was used as a negative control of anti-human PD-L1 monoclonal antibody.

PD-L1 expression intensity scoring

Following PD-L1 immunohistochemistry, tumour tissue sections were independently examined by two researchers, including a pathologist. PD-L1 staining intensity of each tumour cell was classified into four levels relative to that of alveolar macrophages (AMs) in the same section (figure 1A). Level 0, non-stained tumour cell (figure 1B);

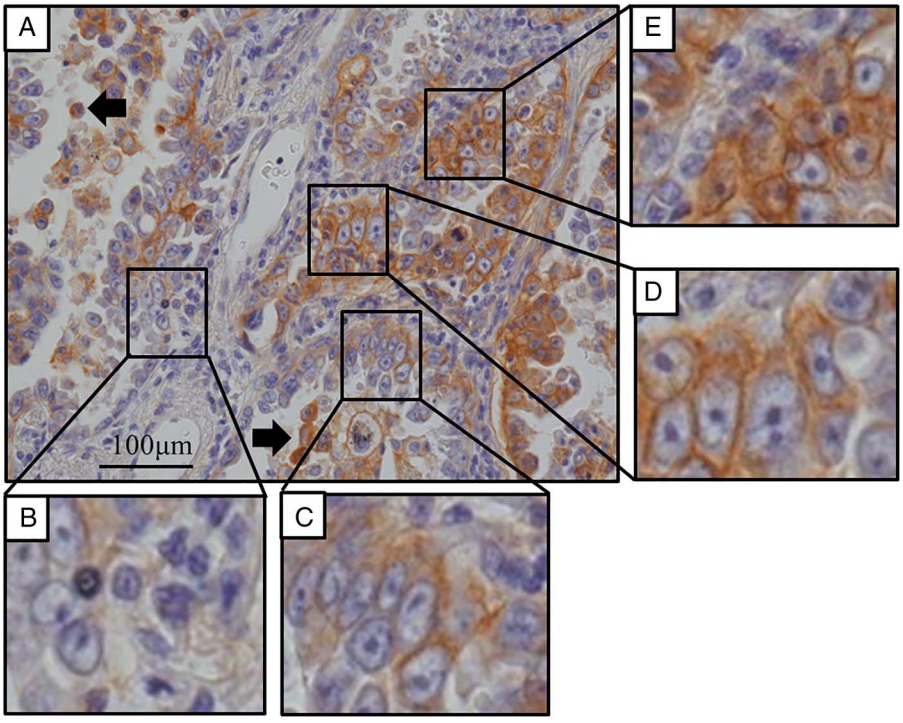

Figure 1 Programmed cell death ligand-1 (PD-L1) immunohistochemistry for pulmonary adenocarcinoma tissues. (A) Heterogeneous distribution of PD-L1 staining intensity of adenocarcinoma cells. PD-L1 staining intensity was classified relative to that of alveolar macrophages (arrow) into four levels: (B) non-stained, (C) weakly stained, (D) moderately stained and (E) strongly stained (magnification, ×200).

level 1: weakly stained tumour cell (staining intensity of tumour cell lower than that of AMs) (figure 1C); level 2: moderately stained tumour cell (staining intensity of tumour cell similar to that of AMs) (figure 1D); and level 3: strongly stained tumour cells (staining intensity of tumour cells stronger than that of AMs) (figure 1E). The number of tumour cells in three randomly selected fields was counted under 200-fold magnification; the number (%) of tumour cells in each PD-L1 staining level was counted. PD-L1 expression score (H score) was calculated for each case according to the following formula:

PD-L1 expression score (H score) (range, 0–300) =0×% of non-stained tumour cells +1×% of weakly stained tumour cells +2×% of moderately stained tumour cells +3×% of strongly stained tumour cells.

Statistical analysis

Correlation between PD-L1 expression score of tumour cells and clinicopathological characteristics of patients were statistically analysed by Mann-Whitney U test. Receiver operating characteristics (ROC) curve analysis was performed to determine the optimal cut-off level of PD-L1 expression score (H score) associated with postoperative recurrence of pulmonary adenocarcinoma. The cut-off level was determined as the point closest to (0, 1) on the ROC curve; this was calculated as '$(1-\text{sensitivity})^2+(1-\text{specificity})^2$,' or the point associated with maximum Youden index (calculated as 'sensitivity +specificity−1').[22] Median relapse-free survival after surgery was calculated using Kaplan-Meier analysis; the relapse-free survival in different groups was compared with log-rank test. p Values of less than 0.05 were considered statistically significant. All analyses were performed using SPSS Statistics V.22.0 software (IBM, Armonk, New York, USA).

RESULTS

Patients' characteristics

A total of 106 (62 men (58.5%) and 44 women (41.5%)) patients with pulmonary adenocarcinoma who underwent surgical resection were included in this study (table 1). The median age of patients at the time of surgery was 66 years (range, 36–87 years). Sixty-two patients (58.5%) were habitual smokers. The most frequent histological subtype was papillary adenocarcinoma (N=38, 35.8%), followed by adenocarcinoma in situ or minimally invasive adenocarcinoma (AIS/MIA) (N=25, 23.6%), acinar adenocarcinoma (N=15, 14.2%), micropapillary adenocarcinoma (N=12, 11.3%), lepidic adenocarcinoma (N=10, 9.4%) and solid adenocarcinoma (N=6, 5.7%).

Postoperative pathological staging of pulmonary adenocarcinoma was IA in 45 cases (42.5%), IB in 14 (13.2%), IIA in 10 (9.4%), IIB in 3 (2.8%), IIIA in 22 (20.8%), IIIB in 5 (4.7%), and IV in 8 (6.6%). Among these, 46 patients (43.3%) had received postoperative adjuvant

Table 1 Patients' characteristics

Characteristic	
Total, N	**106**
Median age (range)	66 (36–87)
Gender, N (%)	
Male	62 (58.5)
Female	44 (41.5)
Smoking status, N (%)	
Current/former	62 (58.5)
Never	42 (39.6)
Unknown	2 (1.9)
Histology, N (%)	
AIS/MIA	
Lepidic	25 (23.6)
Papillary	10 (9.4)
Acinar	38 (35.8)
Solid	6 (5.7)
Micropapillary	12 (11.3)
Pathological stage, N (%)	
IA	45 (42.5)
IB	14 (13.2)
IIA	10 (9.4)
IIB	3 (2.8)
IIIA	22 (20.8)
IIIB	5 (4.7)
IV	8 (7.5)
EGFR status, N (%)	
Mutation	37 (34.9)
Wild-type	69 (65.1)

AIS, adenoma in situ; MIA, minimally invasive adenocarcinoma.

chemotherapy (uracil/tegafur or platinum-based doublet) ~1 month after surgery. Thirty-seven patients (34.9%) exhibited *EGFR* mutations including L858R point mutation on exon 21 or deletion mutation in exon 19.

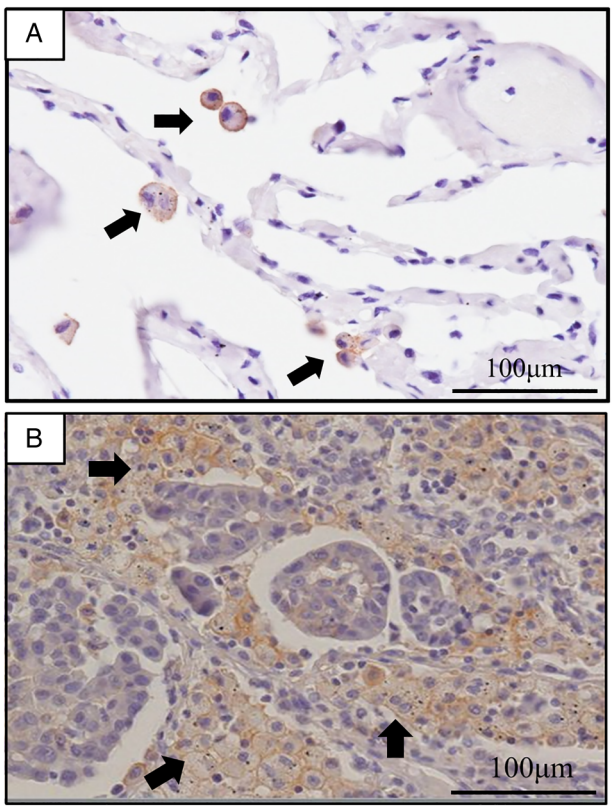

Figure 2 Programmed cell death ligand-1 (PD-L1) expression of alveolar macrophages. (A) Alveolar macrophages in the normal lung tissue are expressing PD-L1 on their membrane and/or cytoplasm. (B) Alveolar macrophages in the pulmonary adenocarcinoma tissue are expressing PD-L1 on their membrane and/or cytoplasm (arrows). Some multinucleated giant cells are also observed around the PD-L1-expressing tumour cells. (magnification, ×200).

PD-L1 expression on adenocarcinoma cells and AMs

On preliminary PD-L1 immunohistochemical analysis, we confirmed that PD-L1 was usually expressed at the membrane of pulmonary adenocarcinoma cells, and in some cases, in the cytoplasm. However, variability was observed in the levels of PD-L1 staining intensity in tumour cells. Heterogeneous distribution of PD-L1 staining intensity was observed within a single section of tumour tissue (figure 1A), with some areas being dominated by cells with strong PD-L1 staining intensity, whereas other areas by cells lacking PD-L1 expression. In some cases, tumour cells with various levels of PD-L1 staining intensity coexisted within one area of the tissue section. These findings suggest that all areas in a tissue section should be observed to appropriately evaluate PD-L1 expression on NSCLC cells. In addition, we observed that AMs in pulmonary adenocarcinoma tissues consistently stained positive for PD-L1 (figure 2). Tumour-associated macrophages were also found to be positive for PD-L1 staining, therefore we need to morphologically discriminate tumour-associated macrophages from tumour cells and AMs. AMs usually existed in the air space of pulmonary alveoli around the tumour cells, which suggests that PD-L1 staining intensity of AMs could serve as an internal positive control in immunohistochemical studies of PD-L1 expression.

Semiquantitative analyses of PD-L1 expression

Based on these preliminary findings, PD-L1 staining intensity of each pulmonary adenocarcinoma cell was classified into four levels by comparing it with the staining intensity of AMs in the same tissue section (figure 1). A semiquantitative PD-L1 expression score (H-score) was obtained for each case as described in the Methods section. The median PD-L1 expression score of pulmonary adenocarcinomas was 52.3 (range, 0.1–273.3). When both 'moderately stained' and 'strongly stained' tumour cells were considered as 'PD-L1-positive', the median percentage of 'PD-L1-positive' tumour cells was 6.6%. The frequency of cases in which more than 1%, 5%, 10% and 50% of tumour cells were 'PD-L1-positive' was 94.3% (100/106), 82.1% (87/106), 73.5% (78/106), 48.1% (51/106), respectively.

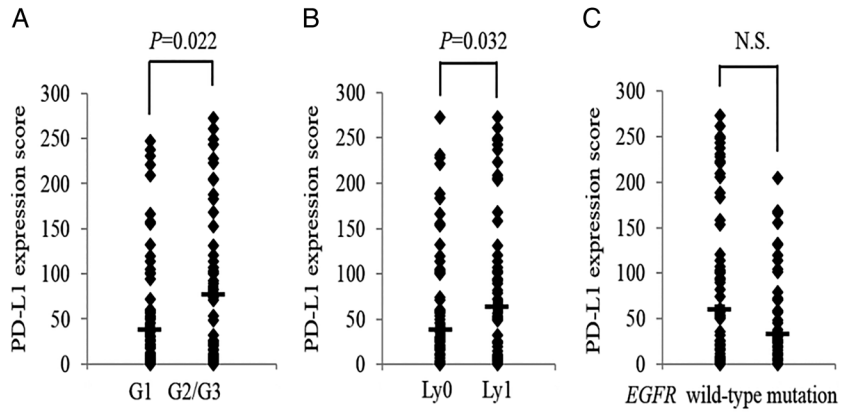

Figure 3 (A) Programmed cell death ligand-1 (PD-L1) expression score was significantly higher in pulmonary adenocarcinomas with grade G2/G3 differentiation as compared to those with grade G1 differentiation (36.7 vs 101.0, p=0.022) and (B) pulmonary adenocarcinomas with lymphatic invasion as compared with those without lymphatic vessels invasion (36.7 vs 64.0, p=0.032). (C) No correlation was observed between the score and *EGFR* mutation status of tumour cells (30.8 vs 58.8, p=0.140). EGFR, epidermal growth factor receptor; NS, not significant.

Correlation between PD-L1 expression score and clinicopathological characteristics

The PD-L1 expression score of pulmonary adenocarcinomas with grade G2 and G3 differentiation was significantly higher than those with grade G1 differentiation (101.0 vs 36.7; p=0.022) (figure 3A). In addition, PD-L1 expression score of those with lymphatic vessel invasion was higher than those without invasion (36.7 vs 64.0; p=0.032) (figure 3B). Patient gender, age, smoking habits, histological subtype, pathological stage, pleural invasion and vascular invasion were not associated with PD-L1 expression score (table 2). Further, no significant correlation was observed between PD-L1 expression score and *EGFR* mutation status (p=0.140; figure 3C).

PD-L1 expression score and relapse-free survival

Of the 106 patients, 12 patients were excluded from the analysis of relapse-free survival time (eight patients had pathological stage IV after surgery; lack of clinical records for four patients). In the 94 patients included in this analysis, median postoperative follow-up duration was 27.2 months (range, 0.1–83.7 months). Using 71.5 as the cut-off level for PD-L1 expression score, median PD-L1 expression in patients with PD-L1 expression score above the cut-off level (N=54) was 131.5 (range, 72.2–272.8), while those with PD-L1 expression score below the cut-off level (N=40) was 17.8 (range, 0.1–70.9). Relapse-free survival after surgery was 24.1 months (95% CI 19.7 to 28.4) in patients with PD-L1 expression score above the cut-off level, and 36.4 months (95% CI 29.1 to 39.3) in those below the cut-off level. The findings demonstrate that postoperative relapse-free survival in patients with high PD-L1 expression score was significantly shorter than that in patients with low PD-L1 expression score (p=0.035; figure 4).

DISCUSSION

Given the heterogeneous distribution of PD-L1 expression in pulmonary adenocarcinomas cells, we established a scoring method to evaluate PD-L1 expression status in patients with pulmonary adenocarcinomas. PD-L1 staining intensity of tumour cells was semiquantitatively scored relative to that of AMs; high PD-L1 expression score of tumour cells significantly correlated with low grade tumour differentiation, lymph vessel invasion, and postoperative relapse-free survival.

Several studies have investigated the correlation between PD-L1 expression on NSCLC cells and clinicopathological characteristics of patients.[17–21] However, the reagents used for immunohistochemical analyses, such as anti-PD-L1 antibodies, have varied among the studies. Standardised evaluation of PD-L1 expression on NSCLC cells requires positive and negative controls for PD-L1 staining.[23] In this study, we aimed to determine a positive control for PD-L1 immunohistochemical examination for NSCLC tissues.

In our preliminary study, we noticed that AMs in NSCLC tissues were strongly stained with anti-PD-L1 antibody. AMs are localised in the air space of pulmonary alveoli, and are relatively larger in size (20–50 µm diameter) than other types of leukocytes. Given, both, the role of AMs and the functional nature of PD-L1 in the immune system, it is consistent that PD-L1 is expressed on AMs to converge immune responses.[24 25] We observed around 20 AMs on average per field of view at 200-fold magnification, and were able to detect them easily due to their typical localisation and size of the cells. In addition, PD-L1 staining intensity of AMs was stable, and did not vary between cells. In PD-L1 immunohistochemistry, inappropriate handling of surgical tumour samples would influence the PD-L1 staining intensity of tumour cells. Thus, an internal control or standard for PD-L1 staining is required, especially when we try to compare PD-L1 staining intensity of tumour cells between patients. Our findings demonstrate that PD-L1 staining intensity of AMs can serve as a valid positive internal control in PD-L1 immunohistochemistry,

Table 2 Correlation between PD-L1 expression score and clinicopathological characteristics

	N (%)	H-score±SD	p Value
Gender			
Male	62 (58.5)	47.6±83.0	NS
Female	44 (41.5)	56.5±72.4	
Smoking status			
Current/former	62 (58.5)	57.6±84.4	NS
Never	42 (39.6)	49.2±69.7	
Unknown	2 (1.9)		
Histology			
AIS/MIA	25 (23.6)	50.1±67.6	
Lepidic	10 (9.4)	33.6±38.6	
Papillary	38 (35.8)	55.9±87.6	
Acinar	15 (14.2)	48.6±66.1	
Solid	6 (5.7)	84.2±106.0	
Micropapillary	12 (11.3)	107.0±88.4	
EGFR status			
mutation	37 (34.9)	30.8±57.3	NS
wild-type	69 (65.1)	58.8±86.0	
Cellular differentiation			
G0	53 (50.0)	36.7±68.7	0.022
G2/G3	52 (49.1)	101.0±93.0	
Unknown	1 (0.9)		
Microblood vessels invasion			
v0	45 (42.5)	50.1±74.6	NS
v1	61 (57.5)	53.6±81.6	
Lymphatic vessels invasion			
ly0	55 (51.9)	36.7±70.7	0.032
ly1	50 (47.2)	64.0±86.4	
Unknown	1 (0.9)		
Pleural invasion			
p0	87 (82.1)	51.3±77.9	NS
p1	18 (17.0)	51.7±84.9	
Unknown	1 (0.9)		
Pathological stage			
IA	45 (42.5)	51.0±62.0	
IB	14 (13.2)	29.3±78.1	
IIA	10 (9.4)	36.8±62.0	
IIB	3 (2.8)	158.0±16.1	
IIIA	22 (20.8)	93.4±105.0	
IIIB	5 (4.7)	52.5±63.8	
IV	8 (7.5)	76.3±86.5	

AIS, adenoma in situ; MIA, minimally invasive adenocarcinoma; NS, not significant; PD-L1, programmed cell death-ligand 1.

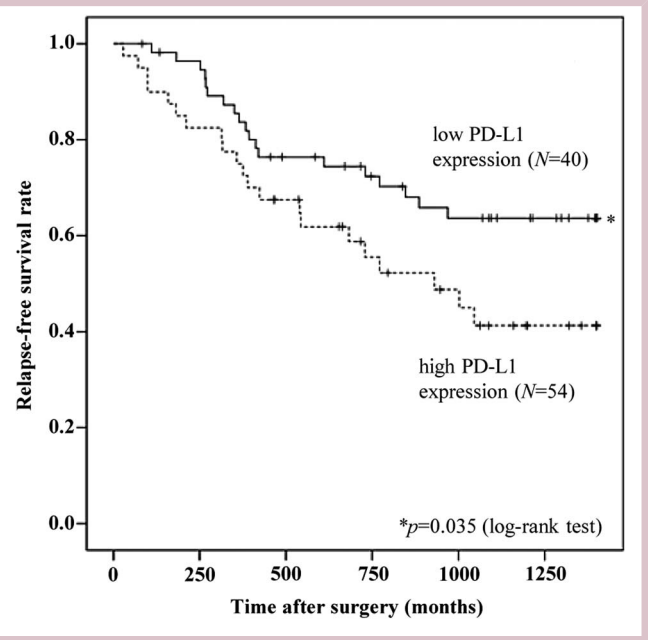

Figure 4 Kaplan-Meier analysis showing postoperative relapse-free survival. Patients in pulmonary adenocarcinomas with high Programmed cell death ligand-1 (PD-L1) expression (N=54) had a significantly shorter relapse-free survival time (N=40) (24.1 vs 36.4 months for those with low expression; p=0.032).

and that PD-L1 staining intensity of NSCLC cells can be assessed relative to that of AMs. Our findings also suggest that automatic analysis of PD-L1 expression on NSCLC cells using image analysing software is liable to count PD-L1-expressing AMs as PD-L1-positive cells, which amounts to a miscount of PD-L1-positive NSCLC cells.

In the preliminary study, we also observed variability in PD-L1 staining intensity among NSCLC cells.[26] In some cases, cells with various levels of PD-L1 staining intensity coexisted in a single section of NSCLC tissue. These findings suggest that all areas in a tissue section should be observed for evaluation of PD-L1 expression. Therefore, in this study, we used a whole-tissue section of NSCLC specimens for PD-L1 immunohistochemistry, and randomly selected the fields of view for assessment of PD-L1 staining intensity of pulmonary adenocarcinoma cells. In 89 of the 106 cases (83.9%), PD-L1-expressing tumour cells and PD-L1-non-expressing cells were mixed in a single tissue section, with different areas tending to be dominated by either PD-L1-expressing cells or PD-L1-non-expressing cells. This finding indicates that a case can be categorised as PD-L1-positive or PD-L1-negative case according to the field of view selected for evaluation. In addition, in terms of evaluation of PD-L1 expression on NSCLC cells, a small fragment of tissue section, such as small specimen obtained by needle biopsy and tissue microarray, is unlikely to be representative of the tumour issue. Given the heterogeneous distribution of NSCLC cells with various levels of PD-L1 staining intensity, we consider that PD-L1 expression on NSCLC cells needs to be evaluated using whole-tissue sections.

In most previous studies, an NSCLC case was defined as PD-L1-positive when more than 5% of NSCLC cells were positive for PD-L1 staining.[15 27 28] However, we questioned the validity of this cut-off level because of lack of a rationale for its setting. In the present study, PD-L1 staining intensity was classified into four levels. When NSCLC cells with PD-L1 staining intensity other than 'non-staining' were defined as 'PD-L1-positive' NSCLC cells, the median percentage of 'PD-L1-positive' NSCLC cells was calculated to be 47.6%. If a case with

more than 5% of 'PD-L1-positive' NSCLC cells was defined as 'PD-L1-positive' , 82.1% of cases (87/106) would have been categorised as 'PD-L1-positive' NSCLC cases in our study. In some studies, the cut-off level of PD-L1 positivity was set as more than 10% or 50% of PD-L1-positive NSCLC cells;[16 29 30] however, the rationale for this cut-off level is not clear. In other studies, only moderate-to-strong 'PD-L1-positive' staining NSCLC cells were considered while weakly stained cells were excluded.[20] However, the reason why 'weakly stained cells' were excluded in the analyses is unclear. Given the heterogeneous expression levels, the frequency of 'PD-L1-positive' NSCLC cells is likely to vary according to the field of view observed, even if only moderately-to-strongly stained cells are counted as 'PD-L1-positive' NSCLC cells. Based on these findings, we suggest that PD-L1-positivity in NSCLC should be determined by semiquantitative analysis through observation of whole-tissue sections.

We propose that the correlation between PD-L1 expression and clinicopathological characteristics should be disaggregated by histological type, given the variability in clinicopathological characteristics between pulmonary adenocarcinomas, squamous cell carcinomas and other pathological types of NSCLC. Therefore, distinct from previous papers,[18 20 21] in the present study, we used a single histological type of NSCLC, pulmonary adenocarcinomas, for investigating the association between clinicopathological features and PD-L1 expression score. Based on our findings, PD-L1 expression intensity of tumour cells does not appear to be associated with gender, age or smoking habits of the patients, which indicates that these variables may not contribute to the regulation of PD-L1 expression responsible for immune checkpoint mechanisms. In addition, histological subtypes of pulmonary adenocarcinomas, pathological stages, pleural invasion and blood vessel invasion were not associated with PD-L1 expression intensity of tumour cells, which indicates that PD-L1 expression may not necessarily contribute to the aggressive property of adenocarcinoma cells. On the other hand, we observed significant correlation between PD-L1 expression intensity and lymphatic invasion (p=0.032) and low-grade differentiation (G2 and 3) (p=0.022). These data are partly consistent with a recent study.[17]

In contrast to previously reported findings,[20 21] we did not observe any correlation between PD-L1 expression intensity and frequency of *EGFR* mutation in pulmonary adenocarcinoma. For cancer cells with *EGFR* mutation, PD-L1 expression should, theoretically, be upregulated via activation of stat-3, PI3K-AKT or RAS-RAF-MAPK pathways by constant signals from EGFR.[31–37] Therefore, the case with up-regulated PD-L1 expression by *EGFR* mutation would be included in pulmonary adenocarcinomas. However, our findings appear to negate the main contribution of *EGFR* mutation of tumour cells towards PD-L1 expression intensity of pulmonary adenocarcinomas, although a relationship between PD-L1 expression and oncogene addiction including various signal pathway cascades remains to be elucidated in future studies. In addition, additional immunohistochemical analysis and/or more sensitive techniques for PD-L1 expression using larger sets of lung adenocarcinomas with *EGFR* mutation could clarify this discrepancy.

In conclusion, this is the first report to establish a positive control for immunohistochemical examination of PD-L1 expression and semiquantitative assessment of PD-L1 expression intensity of pulmonary adenocarcinoma cells. Given the heterogeneous distribution of PD-L1 expression in pulmonary adenocarcinomas cells, assessment of PD-L1 expression relative to that of AMs appears to be valid for the evaluation of PD-L1 expression status in patients with pulmonary adenocarcinomas. High PD-L1 expression score of tumour cells showed a significant correlation with low-grade differentiation, lymphatic invasion and postoperative relapse-free survival.

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Contributors KT and YD took part in concept and study design. TI, MI and JH acquired the data for study. TI and KT analysed and interpreted the data, and drafted the manuscript. YD critically revised the manuscript.

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Competing interests None declared.

Patient consent Obtained.

Ethics approval The Institutional Review Board of Shiga University of Medical Science.

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