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ORIGINAL RESEARCH

Protein expression of programmed death 1 ligand 1 and ligand 2 independently predict poor prognosis in surgically resected lung adenocarcinoma

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Background: The clinicopathologic characteristics of tumors expressing programmed death (PD-1) ligands (PD-Ls) PD-L1 or PD-L2 and their associations with common driver mutations in lung adenocarcinoma are not clearly defined, despite the progression of anti-PD-1/PD-L1 immunotherapy.

Methods: PD-L1 and PD-L2 expression was measured by immunohistochemistry in 143 surgically resected lung adenocarcinomas and was correlated with clinical variables, histologic subtypes, and the mutational status of *EGFR*, *KRAS*, *HER2,* and *ALK*.

Results: Positive PD-L1 expression was significantly associated with more advanced T status, N status, and pathologic stage. Histologically, lung adenocarcinomas with positive PD-L1 staining were less likely to be adenocarcinoma in situ or minimally invasive adenocarcinoma and more likely to have solid predominant subtype. Both PD-L1 expression (odds ratio =1.984, 95% confidence interval =1.010–3.894; P=0.047) and PD-L2 expression (odds ratio =2.328, 95% confidence interval =1.201–4.512; P=0.012) were independent predictors of poor overall survival. When the combined PD-L expression and pathologic stage were used together to predict overall survival, the concordance index increased to 0.763, and the Akaike information criteria value decreased to 356.08.

Conclusion: We defined the clinicopathologic features of lung adenocarcinomas with high expression of PD-L1 and PD-L2. We further demonstrated the role of PD-L expression as a useful prognostic marker for lung adenocarcinoma.

Keywords: immunotherapy, lung cancer, prognostic markers, oncogenic driver mutations

Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide.[1] Despite significant advances in multidisciplinary cancer therapies, the overall prognosis for lung cancer patients remains poor. Novel treatment strategies, including immunotherapy, are under investigation to improve patients' prognosis.

Programmed death 1 (PD-1) is a coinhibitory receptor induced on activated T and B cells,[2] and it plays a crucial role in tumor immune escape.[3,4] Two ligands of PD-1, PD-L1 (B7-H1) and PD-L2 (B7-DC), have been identified as negative immune regulators by engaging PD-1 receptor.[5] Several Phase I clinical trials demonstrated remarkable antitumor activity of both anti-PD-1 and anti-PD-L1 antibodies.[6–8] An objective response rate of 10%–20% was observed in non-small-cell lung cancer patients.[6,7] More interestingly, the responses were durable even in these heavily pretreated patients with

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lung cancer.[6,7] Furthermore, immunohistochemical analysis of tumor specimens revealed a positive correlation between PD-L1 expression and objective responses.[6,8]

We carried out an immunohistochemical study of PD-L1 and PD-L2 expression in surgically resected lung adenocarcinoma and correlated their expression with clinicopathologic and molecular parameters, including adenocarcinoma histologic subtypes, patient prognosis, and common driver mutations.

Materials and methods

Patients and samples

Lung adenocarcinoma samples were collected from patients who underwent surgical resection with curative intent in our institution from January 2008 to October 2009. Eligible patients were required to have sufficient tissue for immunohistochemical staining and comprehensive mutational analyses. Patients who received neoajuvant chemotherapy or had a history of malignant tumors were excluded.

Clinicopathologic variables collected for analyses included sex, age at diagnosis, smoking history, type of surgical resection, tumor histology, tumor differentiation, pathologic tumor-node-metastasis (TNM) stage in line with the seventh edition of the lung cancer staging system,[9] adenocarcinoma subtypes according to the new International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society International Multidisciplinary Classification of Lung Adenocarcinoma,[10] and postoperative adjuvant chemotherapy/radiotherapy. Disease recurrence and survival were observed in the follow-up clinic or obtained by telephone.

Immunohistochemistry and interpretation

Briefly, sections were deparaffinized and rehydrated, and were then treated with 3% H_2O_2 to block endogenous peroxidase activity. Antigen retrieval was done by immersing slides in citrate buffer and microwaving. Nonspecific immunoglobulin binding was blocked using 10% goat serum in phosphate-buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO, USA). Sections were incubated with primary anti-PD-L1 antibody (SAB2900365; Sigma-Aldrich) at 1:300 or anti-PD-L2 antibody (HPA013411; Sigma-Aldrich) at 1:100. After incubation with the primary antibody, the slides were then washed by PBS and incubated with secondary antibodies followed by incubation with 3, 3′-diaminobenzidine (DAB). Sections were counterstained with hematoxylin.

The expression levels of PD-L1 and PD-L2 were measured by the semiquantitative quickscore method.[11] Briefly, a quickscore for each sample was calculated by multiplying the general staining intensities throughout the whole section (0: negative; 1: weak staining; 2: intermediate staining; 3: strong staining) by the proportions of tumor cells staining positively throughout the section (1: 0%–4%; 2: 5%–19%; 3: 20%–39%; 4: 40%–59%; 5: 60%–79%; 6: 80%–100%), and ranged from 0–18.[11] Two authors who were blinded to the clinical data assessed the immunostaining independently, and discrepancies in quickscores were resolved by reviewing the corresponding sections and by discussion.

Mutational analysis

Comprehensive mutational analyses of *EGFR*, *KRAS*, *ALK,* and *HER2* were performed in lung adenocarcinomas as previously described.[12–14] Briefly, ribonucleic acid was extracted as per standard protocol after frozen tissues were dissected into TRIzol® (Life Technologies, Carlsbad, CA, USA), and was reverse transcribed into complementary deoxyribonucleic acid (cDNA). *EGFR* (exons 18–22), *KRAS* (exons 2–3), and *HER2* (exons 18–21) were amplified by polymerase chain reaction (PCR) using cDNA. Primers were as follows: *EGFR* (forward: 5′-TGAAGGCTGTCCAACGAATG-3′; reverse: 5′-AGGCGTTCTCCTTTCTCCAG-3′), *KRAS* (forward: 5′-GAGAGGCCTGCTGAAAATGACTG-3′; reverse: 5′-TGGTGAATATCTTCAAATGATTTAGT-3′), and *HER2* (forward: 5′-CCCTCTGACGTCCATCATCT-3′; reverse: 5′-GCAGGGTCTGGACAGAAGAA-3′). Amplified products were analyzed by direct dideoxynucleotide sequencing. A combined strategy of quantitative real-time PCR (qRT-PCR) and reverse transcriptase PCR (RT-PCR) were used to detect *ALK* fusions, with validations using fluorescence in situ hybridization.[13]

Statistical analysis

Pearson's chi-squared test or Fisher's exact test was used to assess correlations between different immunoreactivity and clinicopathologic variables as well as mutational status. Kaplan–Meier method was used to draw the survival curves. Relapse-free and overall survival of patients with positive or negative immunostaining was compared using the log-rank test. Independent prognostic factors were identified through the Cox proportional hazards regression (forward likelihood ratio model). The predictive accuracy for overall survival was determined by the Harrell's concordance index (C-index) which ranges from 0.5 (no predictive power) to 1 (perfect prediction).[15] The discriminatory ability of a prognostic

model was measured by the Akaike information criterion (AIC) value (a smaller value indicates a better discriminatory ability).[15] The statistical analyses were done using SPSS 16.0 (IBM Corporation, Armonk, NY, USA) and Stata 11.1 (StataCorp LP, College Station, TX, USA). All tests were two tailed. Statistical significance was set as $P<0.05$.

Results

A total of 143 lung adenocarcinoma samples were collected from 84 females and 59 males. The mean age of the patients was 58.6 years, ranging from 36–79 years. Detailed clinicopathologic and molecular characteristics are shown in Table 1. The expression of PD-L1 and PD-L2 was mainly located in the cell membrane and cytoplasm of tumor cells. Scattered expression of PD-L1 (weak to intermediate) and PD-L2 (weak) was also shown in macrophages. The median quickscores for PD-L1 and PD-L2 were 8 (range: 0–18) and 5 (range: 0–18), respectively, and were used as the cutoff values between positive and negative protein expression. Representative images of staining intensities of PD-Ls are shown in Figure 1.

Correlation between PD-L expression and clinicopathologic and molecular features

Positive PD-L1 expression was significantly associated with more advanced tumor (T) status, node involvement (N) status, and pathologic stage. Histologically, lung adenocarcinomas with positive PD-L1 staining were less likely to be adenocarcinoma in situ or minimally invasive adenocarcinoma, and more likely to have solid predominant subtype. No significant correlation was observed between PD-L2 expression and clinicopathologic variables. PD-L expression was not correlated with any of the common driver mutations.

Survival analysis

In univariate analysis, PD-L1-positive patients had significantly poorer relapse-free survival (RFS) ($P<0.001$) and overall survival (OS) ($P=0.002$) than did PD-L1-negative patients (Figure 2). There was significant difference in OS ($P=0.014$) but only borderline significant difference in RFS ($P=0.071$) between PD-L2 positive and PD-L2 negative patients. To correlate the combined PD-L expression with survival, we further divided the patients into three groups: (I) both PD-L1 and PD-L2 negative (n=41); (II) either PD-L1 or PD-L2 positive (n=63); and (III) both PD-L1 and PD-L2 positive (n=39). Significant differences in RFS and OS were found between Group I and Group III ($P<0.001$ for RFS; $P<0.001$ for OS) as well as Group II and Group III ($P=0.002$ for RFS; $P=0.010$ for OS). RFS and OS of patients positive for either PD-L1 or PD-L2 tended to be worse than that of patients who were negative for both, although statistical significance was not achieved. T stage (T2–T4 versus T1; $P<0.001$ for RFS; $P=0.034$ for OS), N stage (N1/N2 versus N0; $P<0.001$ for RFS; $P<0.001$ for OS), and pathologic stage (stage II/III versus stage I; $P<0.001$ for RFS; $P<0.001$ for OS) were also significantly associated with survival.

Table 1 Clinicopathologic and molecular characteristics according to PD-L1 and PD-L2 expression

Variable	PD-L1			PD-L2		
	+	–	*P*	+	–	*P*
Age (years)			0.153			0.677
≤58	40	33		35	38	
>58	30	40		36	34	
Sex			0.162			0.436
Female	37	47		44	40	
Male	33	26		27	32	
Smoking history			0.728			0.127
Never smokers	47	47		51	43	
Ever smokers	23	26		20	29	
Differentiation			0.376			0.332
Poor	29	25		24	30	
Moderate or well	41	48		47	42	
T status			**0.034**			0.053
T1	26	40		27	39	
T2–T4	44	33		44	33	
N status			**0.024**			0.677
N0	29	44		35	38	
N1/N2	41	29		36	34	
Pathologic stage			**0.005**			0.553
I	24	42		31	35	
II–III	46	31		40	37	
Histologic subtypes						
AIS	0	1	1.000	0	1	1.000
MIA	0	6	**0.028**	1	5	0.209
Lepidic	4	4	1.000	2	6	0.275
Papillary	12	15	0.603	16	11	0.268
Acinar	31	33	0.912	37	27	0.079
Solid	21	11	**0.032**	14	18	0.449
Micropapillary	0	1	1.000	0	1	1.000
IMA	1	2	1.000	1	2	1.000
Enteric	1	0	0.490	0	1	1.000
Mutational status						
EGFR mutation	37	39	0.946	36	40	0.561
KRAS mutation	4	3	0.715	4	3	0.719
HER2 mutation	2	5	0.442	4	3	0.719
ALK fusion	3	6	0.494	6	3	0.326

Notes: The median quickscores for PD-L1 (8) and PD-L2 (5) were used as the cutoff values between positive (+) and negative (–) protein expression. Bold indicates significant at $P<0.05$. Values in + and – columns represent n.

Abbreviations: AIS, adenocarcinoma in situ; IMA, invasive mucinous adenocarcinoma; MIA, minimally invasive adenocarcinoma; PD-L, programmed death 1 ligand.

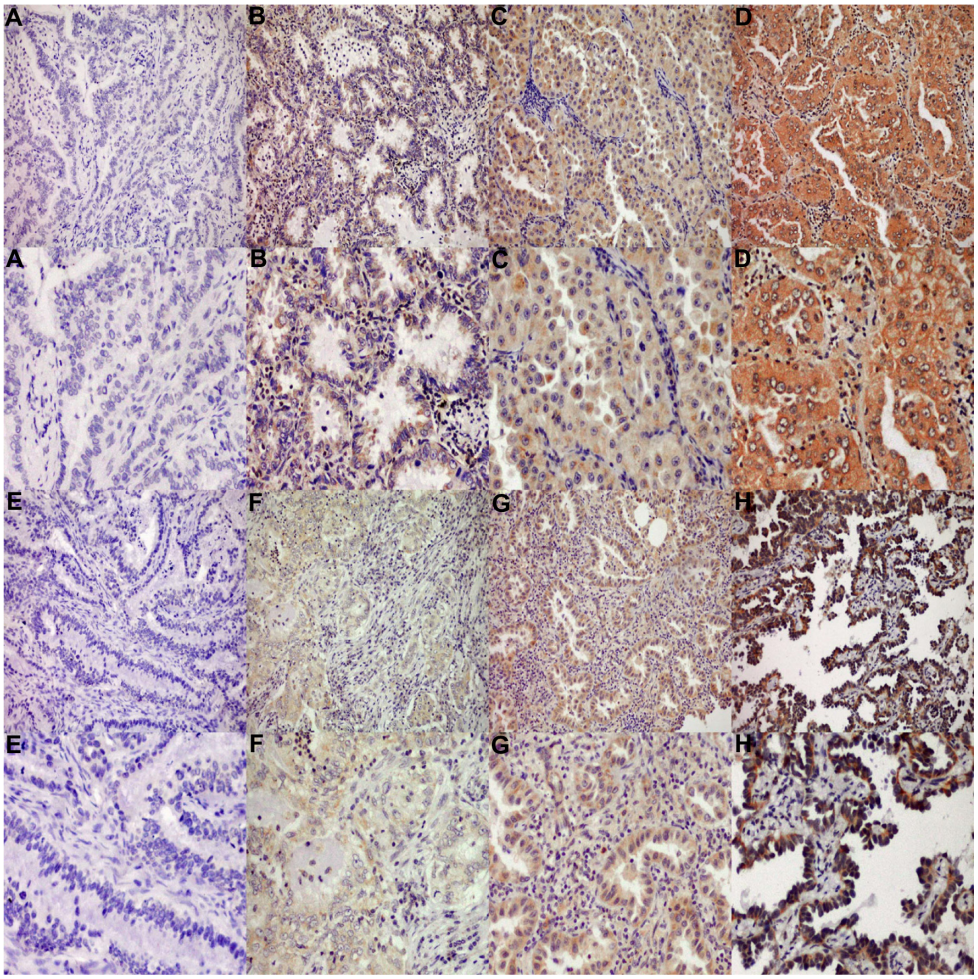

Figure 1 Representative images of staining intensities of PD-Ls.
Notes: Representative images of negative (**A** and **E**), weak (**B** and **F**), intermediate (**C** and **G**), and strong (**D** and **H**) staining intensities of PD-L1 **(A–D)** and PD-L2 **(E–H)** in lung adenocarcinoma.
Abbreviation: PD-L, programmed death 1 ligand.

Mutational status of *EGFR*, *KRAS*, *HER2,* or *ALK* was not significantly associated with RFS or OS.

To determine the prognostic accuracy of PD-L expression, we used multivariate Cox regression model adjusted for age, sex, smoking history, type of surgical resection (lobectomy versus bi-lobectomy/pneumonectomy), differentiation, TNM stage, histologic subtypes, mutational status, and postoperative chemotherapy/radiotherapy. Both PD-L1 (odds ratio [OR] =1.984, 95% confidence interval [CI] =1.010–3.894; *P*=0.047) and PD-L2 expression (OR =2.328, 95% CI =1.201–4.512; *P*=0.012) were independent predictors of poor overall survival. We also assessed the combined prognostic value of PD-Ls expression (Group III versus Group I/II) in multivariate analysis, and found that both PD-L1 and PD-L2 positive expression status (OR =2.540, 95% CI =1.347–4.791; *P*=0.004) and pathologic stage (stage II/III versus stage I, OR =4.971, 95% CI =2.188–11.294) were the only two independent predictors of poor overall survival.

We further calculated the C-index and AIC value to measure the prognostic accuracy of PD-L expression in conjunction with the current lung cancer staging system (Table 2). Pathologic stage alone had a C-index of 0.694 and an AIC value of 362.74. Higher C-index values (0.741 for PD-L1; 0.742 for PD-L2) and lower AIC values (359.54 for PD-L1; 358.71 for PD-L2) can be achieved by adding PD-L1 or PD-L2 expression to disease stage. When the combined PD-L expression and stage were used together to predict overall survival, the C-index increased to 0.763, and the AIC value decreased to 356.08.

Discussion

Lung adenocarcinoma is a disease characterized by driver mutation-defined molecular subsets, each with distinct

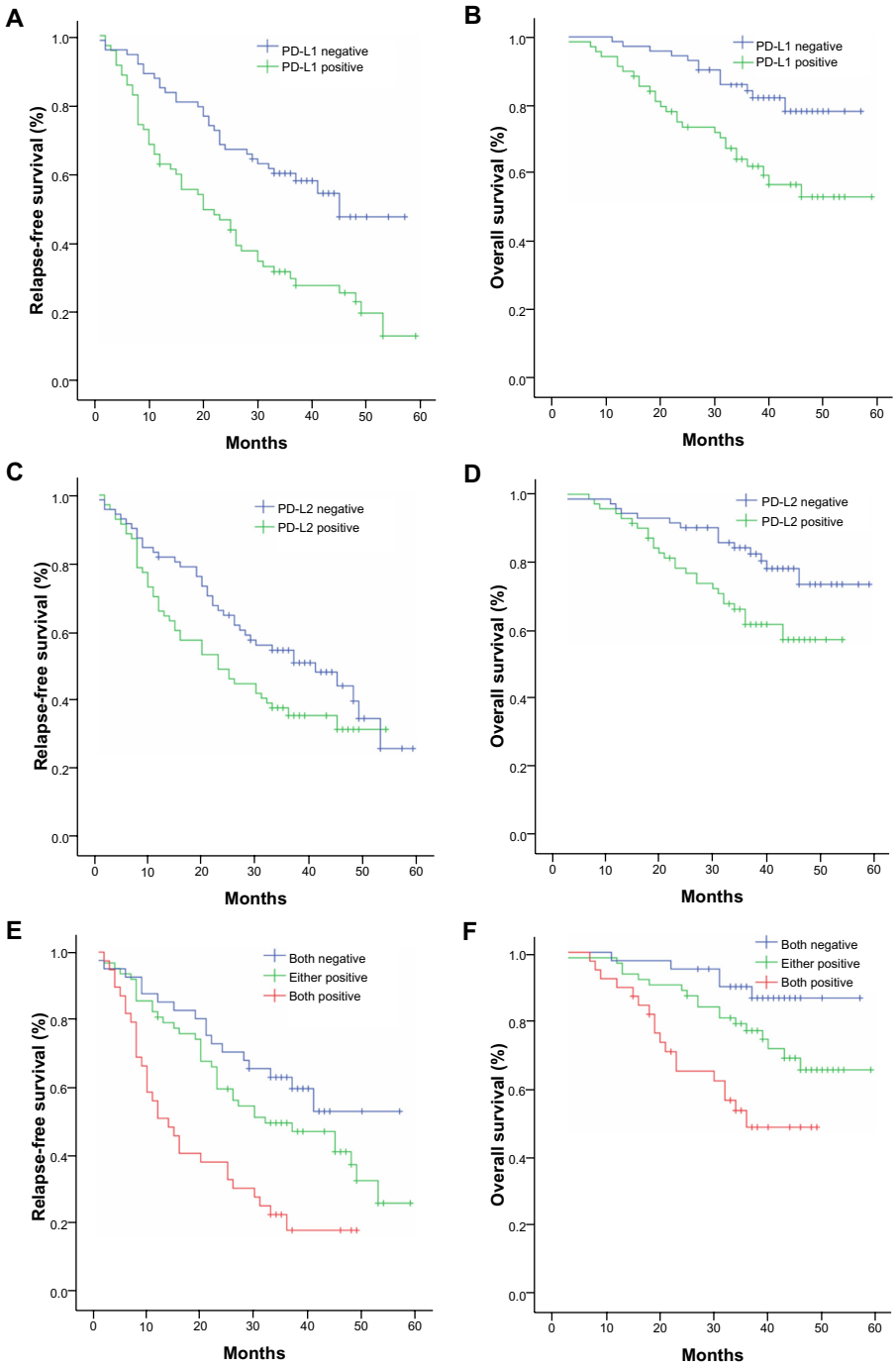

Figure 2 Relapse-free survival and overall survival in PD-L1 and PD-L2 positive and negative patients.
Notes: (**A**) Relapse-free survival according to PD-L1 expression ($P<0.001$). (**B**) Overall survival according to PD-L1 expression ($P=0.002$). (**C**) Relapse-free survival according to PD-L2 expression ($P=0.071$). (**D**) Overall survival according to PD-L2 expression ($P=0.014$). (**E**) Relapse-free survival according to PD-L1 and PD-L2 expression ($P<0.001$ for "both positive" versus "both negative"; $P=0.002$ for "both positive" versus "either positive"). (**F**) Overall survival according to PD-L1 and PD-L2 expression ($P<0.001$ for "both positive" versus "both negative"; $P=0.010$ for "both positive" versus "either positive").
Abbreviation: PD-L, programmed death 1 ligand.

clinicopathologic features and potentials for targeted therapies. Now, anti-PD-1/PD-L1 antibodies add to the weapons against this dreadful cancer type. Cancer cell-expressed PD-L1 was showed to induce apoptosis of antigen-specific T cells.[16] Inhibition of the PD-1/PD-L interaction enhanced immune responses in vitro[17] and mediated preclinical antitumor activity.[16,18] Besides the objective response rate of 10%–20% reported by the two clinical trials treating non-small-cell lung cancer patients with anti-PD-1/PD-L1 antibodies, the most intriguing fact is that the responses

Table 2 Comparison of the prognostic accuracies of pathologic stage and PD-L expression

Model	C-index	AIC
Stage	0.694	362.74
PD-L1 expression + stage	0.741	359.54
PD-L2 expression + stage	0.742	358.71
Combined PD-L expression + stage	0.763	356.08

Abbreviations: AIC, Akaike information criteria; C-index, Harrell's concordance index; PD-L, programmed death 1 ligand.

were durable even in those patients highly pretreated with conventional chemotherapy and tyrosine kinase inhibitors.[6,7] Therefore, it is appealing to explore the clinicopathologic characteristics and molecular associations of lung adenocarcinomas expressing PD-L1 or PD-L2, which might be candidates for anti-PD-1/PD-L1 immunotherapy.

Two previous studies investigated the expression of PD-L1 (or in combination with PD-L2) in non-small-cell lung cancer.[19,20] However, both studies had relatively small samples that were not limited to lung adenocarcinoma. To our knowledge, the current study is the first to assess the expression of PD-L1 and PD-L2 in lung adenocarcinoma together with a comprehensive panel of molecular and clinicopathologic variables, including common driver mutations and histologic subtypes of lung adenocarcinoma.

Although no significant correlations were found between PD-L expression and common driver mutations in lung adenocarcinoma, distinct histologic patterns were observed in lung adenocarcinomas with high expression of PD-L1. No adenocarcinoma in situ or minimally invasive adenocarcinoma showed high expression of PD-L1, whereas PD-L1 immunostaining was positively associated with solid predominant subtype. These data indicated that PD-L1 might be preferably highly expressed in more invasive/aggressive adenocarcinoma subtypes,[21] offering implications for selecting candidates for immunotherapy.

Positive PD-L1 expression was found to be correlated with more advanced T status, N status, and pathologic stage, suggesting its role as a marker of disease progression. The most interesting finding was that both PD-L1 and PD-L2 were independent predictors of poor prognosis for lung adenocarcinoma patients, which is consistent with previous reports on malignant melanoma,[22] urothelial cancer,[23] ovarian cancer,[24] hepatocellular carcinoma,[25] esophageal cancer,[26] and pancreatic cancer.[27] However, Konishi and colleagues[20] investigated 52 surgically resected specimens of non-small-cell lung cancer and found no significant association between PD-L1 or PD-L2 expression and patient survival. This discrepancy might be explained by the relatively small number of patients and the inclusion of histological types other than adenocarcinoma in their study.

Although TNM staging is a reliable prognostic factor, we showed that the addition of PD-L status, especially a combination of PD-L1 and PD-L2 status, markedly improved the prognostic accuracy in lung adenocarcinoma patients. This finding suggested that PD-L status might be used as a predictor for prognosis as well as a predictor for anti-PD-1/PD-L1 antibodies treatment, which provided a strengthened rationale for immunotherapy for lung adenocarcinomas with high PD-L expression.

In conclusion, we defined the clinicopathologic features of lung adenocarcinomas with high expression of PD-L1 and PD-L2. We further demonstrated the role of PD-L expression as useful prognostic markers for lung adenocarcinoma. These data have implications for anti-PD-1/PD-L1 immunotherapy for lung adenocarcinoma patients.

Disclosure

The authors report no conflicts of interest in this work.

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