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PD-1, PD-L1 Protein Expression in Non-Small Cell Lung Cancer and Their Relationship with Tumor-Infiltrating Lymphocytes

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Background: Immunotherapy targeting the programmed death-1 (PD-1)/programmed death ligand-1 (PD-L1) checkpoint has shown the good outcomes in non-small cell lung cancer (NSCLC). We investigated PD-1 and PD-L1 protein expression and their correlation with tumor-infiltrating lymphocytes (TILs), and association with survival in NSCLC.

Material/Methods: The expression of PD-1 (NAT105, Cell Marque) and PD-L1 (28-8, Dako) protein was assessed in 55 NSCLC cell lines by immunohistochemistry (IHC). PD-1 (NAT105, Cell Marque) and PD-L1 (22C3, Dako) protein expression was evaluated by IHC, and TIL percentage was scored, in 139 surgically resected specimens from patients with NSCLC.

Results: PD-1 was not expressed on NSCLC cell lines. PD-L1 was expressed on 20 NSCLC cell lines (36.4%). A total of 60 patient samples (43.2%) were positive for PD-1 on the TILs, and 25 (18.0%) were positive for PD-L1 on tumor cells. High expression of PD-1 on tumor cells was significantly correlated with higher expression of PD-L1 (P=0.026) and a higher percentage of TILs (P<0.001). In the Cox regression model, the odds ratio for PD-1 was 2.828 (95% CI: 1.325–11.165; P=0.013) and 8.579 (95% CI: 4.148–22.676; P<0.001) when PD-L1 and TILs were positive. Patients whose tumor cells were PD-L1 negative had a tendency for longer relapse-free survival (RFS) than patients who were PD-L1 positive (1.85 years, 95% CI: 0.77–2.93 *vs.* 0.97 years, 95% CI: 0.71–1.23; P=0.054).

Conclusions: PD-1 was expressed on TILs in tumor tissues in NSCLC patients. PD-L1 was expressed on both TILs and tumor tissues. PD-1 expression was correlated with PD-L1 on tumor cells and TILs. Patients who were PD-L1 positive tended to experience progression after surgery.

MeSH Keywords: Carcinoma, Non-Small-Cell Lung • Immunity, Active • Tumor Escape

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Background

Lung cancer is the leading cause of cancer death [1]. About 80% of lung cancers are non-small cell lung cancer (NSCLC) [2]. Chemotherapy is a standard first-line therapy for advanced stage disease and has a poor prognosis. Targeted therapy shows promise outcome for advanced NSCLC [3–5], but only patients who harbor driving mutations such as epidermal growth factor receptor (EGFR) can get benefit [6,7]. Moreover, acquired resistance of targeted therapy limits their ability to prolong survival [5]. Therefore, it remains urgent to find new, more effective therapeutic strategies for patients with advanced NSCLC.

Immunotherapy can reverse tumor immune escape [8]. The inhibition of checkpoints, such as cytotoxic T lymphocyte antigen-4 (CTLA-4), programmed death-1 (PD-1), and programmed death ligand-1 (PD-L1) [9–14], can activate T cells to eliminate tumors. Recently, anti-PD-1 or anti-PD-L1 antibodies have shown promising efficacy in different cancers [9,11,13–30]. Significantly, nivolumab and pembolizumab have been approved by the U.S. Food and Drug Administration (FDA) as second-line treatments for advanced NSCLC based on the CheckMate 017 and 057 and Keynote 010 trials [21,22,26,31,32].

In this study, we investigated PD-1 and PD-L1 protein expression in NSCLC cell lines and NSCLC patient tumor tissues by immunohistochemistry (IHC); analyzed the correlation between PD-1 and PD-L1, clinicopathological characteristics, and tumor-infiltrating lymphocytes (TILs); and conducted survival analysis of NSCLC patients.

Material and Methods

Patients

Primary tumor specimens were obtained from 139 NSCLC surgical patients at Department of Oncology and Radiotherapy, Medical University of Gdansk, Poland, from April 2008 to August 2010. The patients had not undergone radiation or chemotherapy before surgery. The surgical histology reports were reviewed, and the lymph node and lung cancer stages were categorized by the seventh edition International Association for the Study of Lung Cancer (IASLC) TNM staging system. The protocol was approved by the Shanghai Pulmonary Hospital, Tongji University (ethical number 15-235). All participants were competent to provide their consent.

Cell lines

A tissue micro array (TMA) containing 55 NSCLC cell lines was produced in the Hirsch Lab. Cell lines were grown in RPMI 1640 media with 10% fetal bovine serum. Cells were harvested, fixed overnight, mixed with 0.9% agarose, and then allowed to solidify at room temperature for at least 5 minutes. Each solidified agar pellet was gently placed in a cassette and submerged in 70% ethyl alcohol. The pellets were processed and embedded in paraffin blocks. Cores were taken from each cell line block to create the TMA, from which 4 mm sections were cut.

IHC for PD-1 by Ventana Benchmark XT®

Paraffin tissue sections were baked in a drying oven at 60°C for 1 hour. Heat-mediated antigen retrieval was performed in LAG-3 slides. All slides were labeled and put in a Benchmark XT® (Ventana Medical Systems, Inc.). After treating slides with standard cell conditioning 1 for 60 minutes (PD-1, predilute, NAT105, Cell marque) was applied, and the slides were incubated at 37°C for 1 hour. An UltraView DAB detection and amplification kit was used. Slides were counterstained with hematoxylin for 4 minutes and post-counterstained with bluing agent for 4 minutes. Slides were washed and then dehydrated in 70% to 100% reagent alcohol baths and then xylenes baths before applying coverslips.

IHC for PD-L1 by Dako

Paraffin tissue sections were baked in a drying oven at 60°C for 1 hour. Slides were loaded onto the Dako autostainer and treated with Proteinase K for 5 minutes. Primary antibody (PD-L1, 28-8, Dako) (PD-L1, 22C3, Dako) was applied by the Dako autostainer, and then the slides were incubated at room temperature for 30 minutes. After a wash in buffer, slides were incubated with a labeled polymer, HRP, at room temperature for 30 minutes. Next, DAB+ substrate-chromagen solution was applied for 10 minutes. Slides were counterstained with Dako automation hematoxylin for 5 minutes and post-counterstained with bluing agent for 1 minute. Slides were washed and then dehydrated in 3 washes of 70%, 95%, and 100% reagent alcohol and then 3 xylenes baths before applying coverslips.

Determination of PD-1, and PD-L1 IHC cutoff

All IHC results were checked independently by two pathologists. The cutoff for PD-1 was equal to or more than 8% staining [33]. The cutoff for PD-L1 on tumor cells (Dako, 22C3) (approved by FDA) was equal to or more than 50% staining. We use the same cutoff (50%) for PD-L1 (Dako, 28-8) in NSCLC cell lines.

Evaluation of TILs

We calculated the number of lymphocytes in each histospot, as described [34]. A score of 1+ (<30%) was low TILs; 2+ (30–60%) was moderate; and 3+ (>60%) was marked increase in TILs. Spots with discordance in TIL category between pathologists

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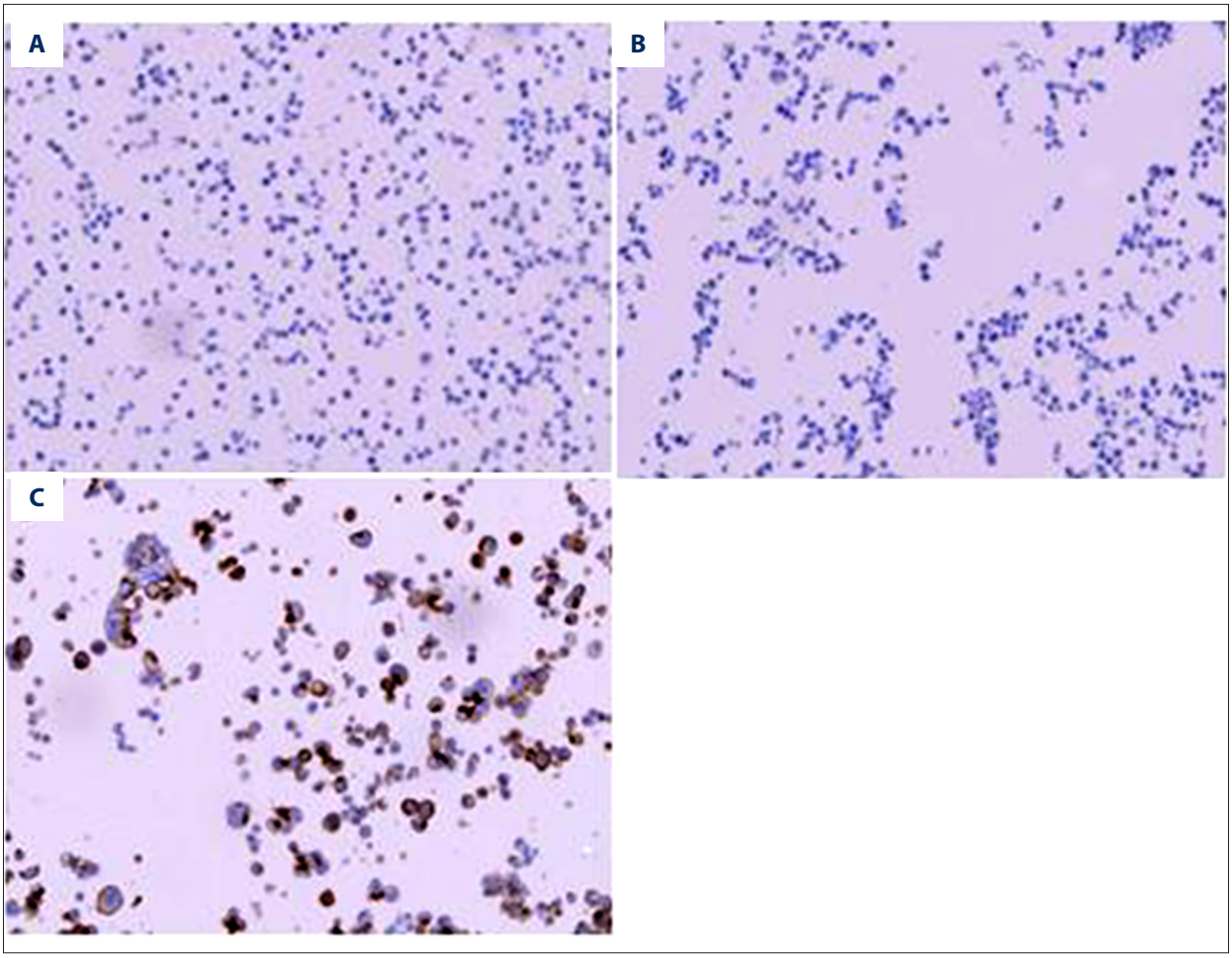

Figure 1. PD-1 and PD-L1 in NSCLC cell lines. (**A**) PD-1 negative expression on a NSCLC cell line (H1435) (20×); (**B**) PD-L1 negative expression on a NSCLC cell line (H520) (20×); (**C**) PD-L1 positive expression on a NSCLC cell line (H2444) (20×).

were reviewed jointly and a single consensus category was established [18].

Statistical analysis

We performed statistical analysis by using the SPSS statistical software package (version 17.0; SPSS, Inc.; Chicago, Illinois, USA). Chi-square tests were used to analyze the correlation between PD-1 and PD-L1 protein expression, clinicopathological variables, and TILs. The correlation between the expression of PD-1 and PD-L1 and TILs was analyzed by Spearman's rank correlation. The odds ratios for positive PD-1 in TILs and PD-L1 in tumor cell expression were calculated by logistic regression model for factors including age, gender, smoking history, pathology, lung cancer stage, grade, PD-L1 expression, and TILs. The survival curves were estimated by the Kaplan-Meier method. All statistics were 2-sided and statistical significance was defined as $P<0.05$.

Results

PD-1 and PD-L1 on NSCLC cell lines

PD-1 was not expressed on any of the 55 NSCLC cell lines. PD-L1 was expressed on 20 NSCLC cell lines (36.4%) (Figure 1).

Patient characteristics

NSCLC patient tissues were obtained from 139 patients undergoing surgery at the Department of Oncology and Radiotherapy, Medical University of Gdansk, Poland, from April 2008 to August 2010. Among them, 109 (78.4%) were male and 30 (21.6%) were female. The mean age was 64 years old. Six (4.3%) were never smokers. A total of 58 patients (43.7%) were stage I, 35 (25.2%) were stage II, 39 (28.1%) were stage III, and 7 (5.0%) were stage IV. Forty patients (28.8%) had adenocarcinoma, and 81 (52.3%) had squamous cell carcinoma (SCC) (Table 1).

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Table 1. Patient Characteristics (n=139).

Characteristic	Total	Characteristic	Total
Gender, n (%)		M stage, n (%)	
Male	109(78.4%)	0	132 (95.0%)
Female	30(21.6%)	1	7 (5.0%)
Age, median	64	Lung cancer staging, n (%)	
<70, n (%)	105(75.5%)	I	58 (41.7%)
≥70, n (%)	34(24.5%)	II	35 (25.2%)
Smoking status, n (%)		III	39 (28.1%)
Non-smoker	6(4.3%)	IV	7 (5.0%)
Smoker	133(95.7%)	Pathology, n (%)	
Surgery, n (%)		SCC	81 (52.3%)
Wedge	2(1.4%)	Adenocarcinoma	40 (28.8%)
Segmentectomy	3(2.2%)	Large cell carcinoma	4 (2.9%)
Lobectomy	73(52.5%)	NSCLC NOS/Mixed	12 (8.6%)
Bilobectomy	8(5.8%)	NSCLC others	2 (1.4%)
Pneumonectomy	47(33.8%)	Grade, n (%)	
Sleeve lobectomy	6(4.3%)	G1	16 (11.5%)
T stage, n (%)		G2	57 (41.0%)
1	25(18.0%)	G3	47 (33.8%)
2	80(57.6%)	Unknown	19 (13.7%)
3	24(17.3%)	Surgical margin, n (%)	
4	10(7.2%)	Complete	113 (81.3%)
N stage, n (%)		Macroscopic positive	11 (7.9%)
0	75(54.0%)	Unknown	15 (10.8%)
1	31(22.3%)		
2	33(23.7%)		

Characterization of PD-1, PD-L1, and TILs in NSCLC and their association with clinicopathological factors

Sixty samples (43.2%) stained positive for PD-1 on the TILs, and 25 (18.0%) had positive PD-L1 expression on tumor cells (Figure 2). Neither the expression of PD-1 on TILs nor that of PD-L1 on tumor cells had significant correlation with clinicopathological factors.

Relationship between checkpoints and TIL abundance

There was a correlation between the percentage of TILs and PD-1 (R^2=0.212, y=1.274x+0.030, P<0.001) (Figure 3). We also performed correlation analysis between PD-L1 and TIL percentage (R^2=0.005, y=12.441x+1.568; P=0.015), and PD-1 and PD-L1 (R^2=0.052, y=12.970x+0.125; P<0.001).

High expression of PD-L1 on tumor cells was significantly correlated with higher expression of PD-1 (P=0.026). The same relationship was also found between PD-1 and TIL expression (P<0.001) (Table 2).

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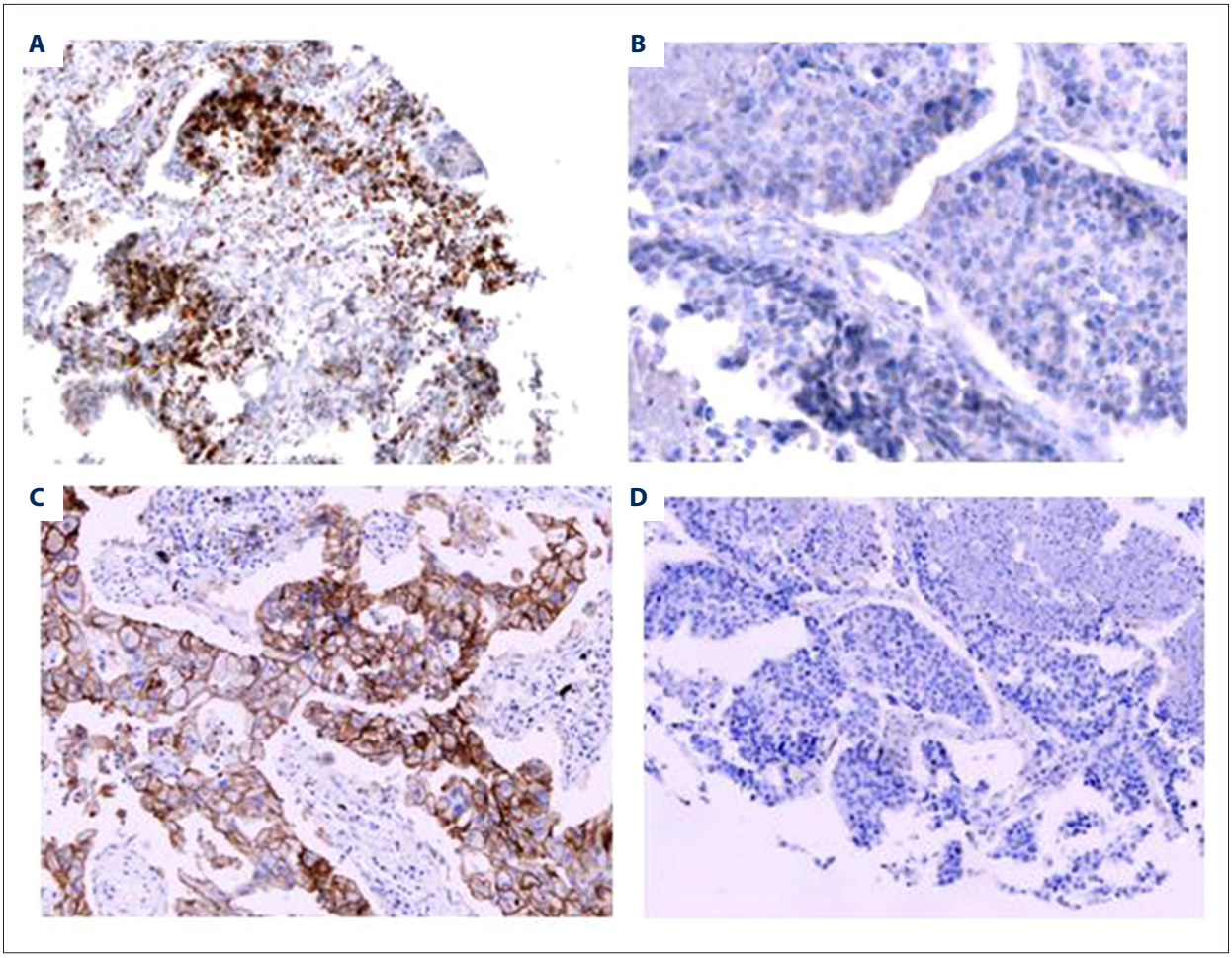

Figure 2. IHC staining for PD-1, and PD-L1 (20×). (**A**) IHC positivity for PD-1 on TILS; (**B**) IHC negative for PD-1 on TILS; (**C**) IHC positivity for PD-L1 on tumor cells; (**D**) IHC negative for PD-L1 on tumor cells.

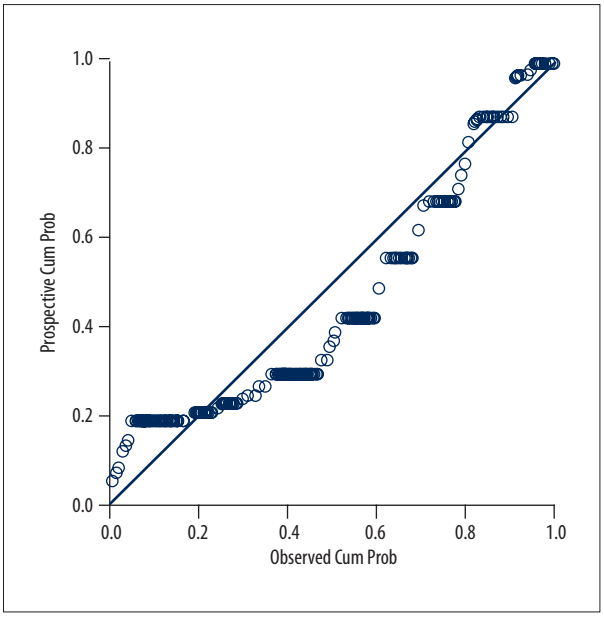

Figure 3. P-P plot for the correlation between PD-1 and PD-L1.

Univariate and multivariate logistic analysis for predicting PD-1 and PD-L1 expression

With the Cox regression model, after adjusting for age, gender, smoking history, pathology, lung cancer stage, grade, PD-L1 expression, and TILs, the odds ratio for PD-1 was 2.828 (95% CI: 1.325–11.165; P =0.013) and 8.579 (95% CI: 4.148–22.676; P<0.001) when PD-L1 and TILs are positive (Tables 3, 4).

Association between PD-1 and PD-L1 and RFS and OS in NSCLC patients

Relapse-free survival (RFS) and overall survival (OS) were not significantly different in PD-1 positive patients and PD-1 negative patients (1.470 years, 95% CI: 0.58–2.36 *vs.* 1.760 years, 95% CI: 1.28–2.24; P=0.881 for RFS) (2.29 years, 95% CI 1.04–3.54 *vs.* 2.97 years, 95% CI 1.36–4.58; P=0.558 for OS). Likewise, PD-L1 status did not significantly affect OS (2.96 years, 95% CI: 2.25–3.67 *vs.* 1.29 years, 95% CI 0.62–1.96; P=0.290). We found that patients with PD-L1-negative tumor cells had longer

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Table 2. Relationships between different checkpoints.

Characteristic	PD-L1 expression on tumor cells, n (%)			TILs, n (%)		
	<50%	≥50%	P	<30%	≥30%	P
PD-1 expression on TILs, n(%)						
<8%	70 (88.6%)	9 (11.4%)	**0.026**	52 (65.8%)	27 (34.2%)	**<0.001**
≥8%	44 (73.3%)	16 (26.7%)		11 (18.3%)	49 (81.7%)	
PD-L1 expression on tumor cells, n (%)						
<50%				52 (45.6%)	62 (54.4%)	1.000
≥50%				11 (44.0%)	14 (56.0%)	

Table 3. Univariate and multivariate analysis for prediction of PD-1 expression in all patients.

Variables	Univariate			Multivariate		
	Odds Ratio	95% CI	P	Odds Ratio	95% CI	P
Age (<70 *vs.* ≥70)	1.442	0.663–3.135	0.356			
Gender (Female *vs.* Male)	0.592	0.263–1.335	0.207			
Smoking status (Non-smoker *vs.* Smoker)	1.547	0.274–8.738	0.622			
Pathology (AD *vs.* non-AD)	0.720	0.339–1.528	0.392			
Stage (I–II *vs.* III–IV)	1.019	0.500–2.079	0.959			
Grade (1 *vs.* 2–3)	0.934	0.547–1,595	0.802			
PD-L1 on tumor cells (negative *vs.* positive)	2.828	1.150–6.953	**0.023**	3.846	1.325–11.165	**0.013**
TILs (<30% *vs.* ≥30%)	8.579	3.846–19.138	**<0.001**	9.698	4.148–22.676	**<0.001**

Table 4. Univariate and multivariate analysis for prediction of PD-L1 expression in all patients.

Variables	Univariate			Multivariate		
	Odds Ratio	95% CI	P	Odds Ratio	95% CI	P
Age (<70 *vs.* ≥70)	0.970	0.353–2.669	0.953			
Gender (Female *vs.* Male)	0.650	0.243–1.741	0.392			
Smoking status (Non-smoker *vs.* Smoker)	0.418	0.072–2.421	0.330			
Pathology (AD *vs.* non-AD)	0.564	0.196–1.625	0.289			
Stage (I–II *vs.* III–IV)	1.444	0.592–3.526	0.419			
Grade (1 *vs.* 2–3)	1.051	0.500–2.212	0.895			
PD-1 on tumor cells (negative *vs.* positive)	2.828	1.150–6.953	**0.023**	2.828	1.150–6.953	**0.023**
TILs (<30% *vs.* ≥30%)	1.067	0.447–2.552	0.883			

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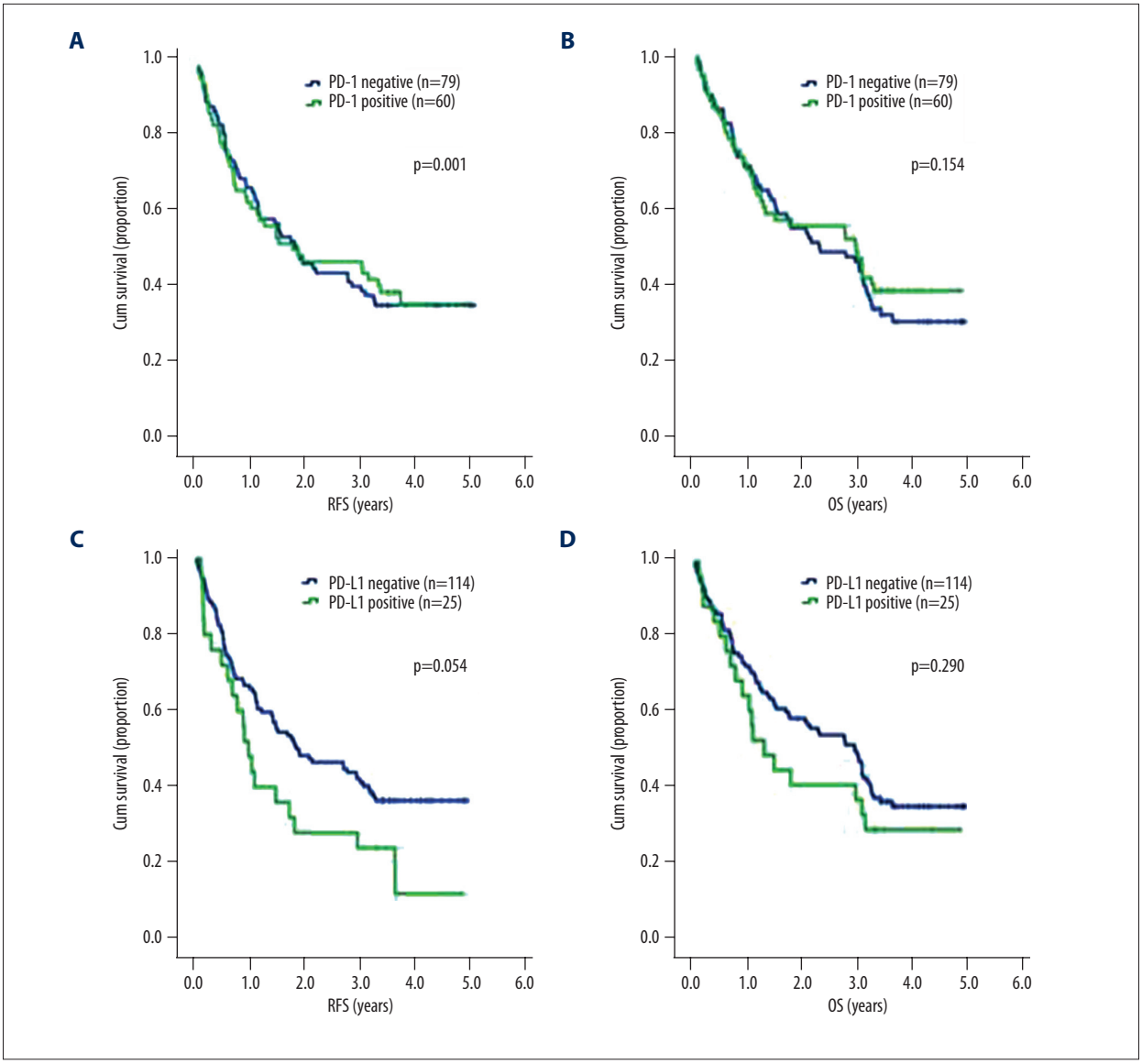

Figure 4. RFS, OS by PD-1 and PD-L1. (**A**) RFS by PD-1; (**B**) OS by PD-1; (**C**) RFS by PD-L1; (**D**) OS by PD-L1.

RFS than patients who were PD-L1 positive (1.85 years, 95% CI: 0.77–2.93 *vs.* 0.97 years, 95% CI: 0.71–1.23; P=0.054) (Figure 4).

Discussion

In this study, we analyzed PD-1 and PD-L1 in NSCLC cell lines and patient tumor tissues as well as described the correlations between TILs and survival.

PD-1 and PD-L1 have been described in several malignancies, including ovarian cancer [35], lung cancer [33,36], breast cancer [34], etc. In our study, PD-L1 was expressed on 36.4% of NSCLC cell lines. In tumor tissues, PD-1 was expressed on TILs, and PD-L1 was detected on both tumor cells and TILs. Among the samples, 43.2% stained positive for PD-1 on the TILs, and 18.0% stained positive for PD-L1 on tumor cells. PD-1 on TILs was significant correlated with PD-L1 expression on tumor cells. It was reported that there was no relationship between PD-L1 and clinicopathological factors in ovarian cancer [35]. In addition, PD-L1 did not correlate to clinical factors in lung cancer patients [33]. In our study, we also analyzed the correlation between PD-1 and PD-L1 and found there was no significant correlation between patient clinicopathological characteristics and PD-1 and PD-L1.

TILs were significant prognostic factors in cancer [35,37,38]. CD8 T cell infiltration was an independent prognostic factor indicating favorable outcome in esophageal carcinomas [37]. In human renal cell carcinoma, the activation of CD8 T cells

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could reflect effective antitumor immunity [38]. PD-L1 on tumor cells was correlated with TILs, which may down-regulate antitumor immune responses in NSCLC [33]. Our results showed that PD-1 expression, not PD-L1, was correlated with TILs in NSCLC, which was consistent with the other results of the past.

The prognostic value of PD-1 and PD-L1 in various cancers has also been discussed. PD-L1 on tumor cells correlates with poor clinical prognosis in ovarian cancer, renal cancer, lung cancer, and breast cancer [34,35,39,40]. In ovarian cancers, patients with lower expression of PD-L1 had a better outcome [35]. PD-L1 negatively regulated antitumor immunity, and was independently associated with poor outcome in clear cell renal cell carcinoma. Tumor PD-L1-positive clear cell renal cell carcinoma patients had a risk of progression and mortality [39]. High PD-L1 expression in NSCLC was an independent predictor of poor prognosis [41]. However, the prognostic function of PD-L1 still remains controversial. Cooper et al. revealed that high PD-L1 expression is independently associated with longer OS [42]. In our study, there was no significant difference in OS between PD-L1-positive and PD-L1-negative patients. PD-L1-positive NSCLC patients tended to have lower RFS after surgery.

The limits of this study were that it was retrospective research and had a relatively small number of patients. We are going to expand our samples and conduct a larger prospective study. As there are many ongoing PD-1 or PD-L1 clinical trials, we will add the treatment outcomes in our further study.

Conclusions

In this study, we analyzed the relationship between PD-1 and PD-L1 expression, clinical characteristics, and TILs. Furthermore, we also investigated the association between PD-1 and PD-L1 expression and postoperative survival. We have demonstrated the expression of PD-1 and PD-L1 in surgically resected specimens of NSCLC. PD-1 expression on TILs was correlated with PD-L1 and TIL percentage. Patients with PD-L1-positive NSCLC tended to have progression after surgery.

Conflicts of interest

There is no conflict of interest.

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