

Original Article

Association of PD-L1 expression with clinicopathologic features and overall survival in patients with non-small-cell lung cancer

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Abstract: Multiple lines of evidence showed that non-small cell lung cancer (NSCLC) patients had improved clinical outcomes when immune checkpoint was blocked. Programmed death receptor 1 (PD-L1) is one of the most extensively studied molecules that are highly implicated in the response to immunotherapy in NSCLC. However, studies that report the prevalence of PD-L1 expression in NSCLC and address their association are still scarce. This study was aimed to complement this vacancy by determining the prevalence of PD-L1 expression in NSCLC and analyzing its associations with clinical pathologic parameters and outcome. Totally, 139 operated patient at stage II-III NSCLC were enrolled. For malin-fixed and paraffin-embedded (FFPE) tumour sections were reviewed and stained with PD-L1 antibody. The correlations between expression of PD-L1 and clinical pathologic features and overall survival (OS) were analyzed. PD-L1 expression was detected in 61.8% of NSCLC. No significant relationship was found between PD-L1 expression and patients' gender, age, smoking history, stage, subtype or EGFR status. Furthermore, the median OS of PD-L1 positive patients (49.3 months, 95% CI: 44.5-54.2) was longer than PD-L1 negative patients (40.9 months, 95% CI: 35.3-46.5), $P = 0.028$. PD-L1 expression is correlated with a longer survival time, indicating its potential diagnostic value in assessment for operated patients with stage II-III NSCLC. Given the inconsistency in other related studies, some other factors beyond this study's scope may also affect immune response of NSCLC and thus more comprehensive researches are needed.

Keywords: Lung cancer, PD-1, PD-L1, EGFR

Introduction

Non-small cell lung cancer (NSCLC), is one of the most lethal malignancies and extracts the majority of death toll of lung cancer [1]. The overall 5-year survival rate remained at approximately 15%, which had not been substantially improved in recent decades [2, 3]. Intensive efforts have been dedicated to study a few independent prognostic factors, most of which, however, were not able to discriminate prognostic subclasses, which limits their predictive values [4, 5].

Last few decades have witnessed enormous efforts dedicated to studying the interaction between immune system and immunotherapy in NSCLC. Several studies suggest that the interaction between Programmed death recep-

tor-1 (PD-1) and its ligand PD-L1 suppressed activation, expansion and maturation of virus specific CD8+ T cells, as well as TCR driven stop signals, thereby inhibiting immune response [6, 7]. Several studies suggest PD-L1 contributed to the evasion of tumor cells from the host's immune system [8, 9]. The association of PD-1/PD-L1 with the relatively unfavorable prognosis and survival of a wide variety of cancers, including gastric cancer, breast cancer, hepatocellular carcinoma, esophageal cancer, ovarian cancer, malignant melanoma, urothelial cancer, pancreatic cancer, and renal carcinoma, have been reported [10-12].

In lung cancer, PD-1/PD-L1 holds promise for serving as a therapeutic target due to their inhibition on anti-tumor immune response. Currently, clinical results were improved using anti-

PD-L1 is correlated with overall survival

PD-1 and anti-PD-L1 monoclonal antibodies [13-17]. In era of precision medicine, decision making for treatment involves selecting biological agents targeting relevant biological processes in cancer and biomarkers that are able to predict response to therapy. Currently, a number of therapeutic agents targeting PD-L1/PD-1 are at all stages of development, and the immunohistochemistry-based (IHC-based) assessment of PD-L1 expression appears to be a preferred biomarker. However, to date, the prevalence of PD-L1 in NSCLC and whether there exists association between PD-L1 and clinicopathological variables and prognostic prediction remain uncertain. High PD-L1 was implicated as an unfavorable prognostic indicator in NSCLC [18-21], whereas opposite results were shown that PD-L1 negative patients had poor prognosis [22, 23], which makes conclusive ascertainment difficult.

This study retrospectively focused on patients atresected stage II-III NSCLC and evaluated the association between PD-L1 positivity and clinical pathologic features, clinical outcomes, as well as the driver mutations located in epidermal growth factor receptor (EGFR) in our single centre.

Materials and methods

Patients selection

Patients were chosen randomly from those whose formalin-fixed and paraffin-embedded (FFPE) tumor tissue and adjacent non-tumor tissue samples were both available. Complete clinical pathological data and follow-up information for enrolled patients were accessible. Finally, a cohort of 139 NSCLC patients who had undergone primary surgery at Shanghai Chest Hospital (Shanghai, China) from January to December of 2008 were selected, none of which had received anti-tumor therapy prior to or had other severe diseases on diagnosis. In all, 48 were with Stage II adenocarcinoma, 24 were with Stage II squamous cell carcinoma; 42 had Stage IIIA adenocarcinoma, 19 had Stage IIIA squamous cell carcinoma and 6 were other cases (small cell or large cell carcinoma). The approval from Ethics Committee of Shanghai Chest Hospital and written informed consents from all patients were obtained.

Clinicopathologic features

All original pathological diagnosis and description were confirmed by pathologists after surgery. Clinical pathological features including gender, age, tumor size, subtype and histologic grade were investigated. Histologic grading was conducted by a histologist from Shanghai Chest Hospital.

Treatment and follow-up

All the enrolled patient had undergone standard surgery (lobectomy and lymph node dissection). Follow-up visits were performed for each patient in October 2014 to conduct overall survival analysis.

Immunohistochemistry staining for PD-L1

All archived FFPE tissues were carefully reviewed by a histologist. Before IHC staining, sequential 10 µm specimens from archived FFPE tissue were harvested on super defrosted slides (Fisher) and placed at 58°C for one hour. IHC staining was conducted following the standard Avidin Biotin Complex method. (Vector Labs. Cat# PK-6102). Tissue sections were then dimmed in xylene to dewax followed by rehydration through a spectrum of decreasing concentration of ethanol solution to distilled water (1 min). Quench of endogenous peroxidase was done by immersion in 0.3% H₂O₂ at room temperature for 15 min. Slides were incubated in sodium citrate buffer solution (pH 6.0) (Dako, Cat# S1699) for 35 min to retrieve antigen. The antibody of PD-L1 was purchased from Sigma (Cat# SAB2900365). The background of sections were blocked using 10% normal horse serum, followed by incubation with primary antibody (1:300) at 4°C overnight. After incubation, secondary antibody (Vector Laboratory. Cat# PK-6100) was added into the reaction, and incubated at room temperature for 30 min, followed by application of tertiary reagent as per the affixed manual. FFPE lymph node tissues were subject to above same procedures with mouse IgG isotype and monoclonal antibody, which serve as negative and positive controls, respectively.

Evaluation of slides

Our pathologist examined the IHC- and H&E-stained slides and evaluated the results. The

PD-L1 is correlated with overall survival

Table 1. Correlation of PD-L1+ tumor cells with the clinicopathologic parameters

Characteristics	PD-L1 Negative	PD-L1 Positive	P value
Age			
< 70 y	49	58	0.159
≥70 y	10	22	
Gender			
Male	46	55	0.253
Female	13	25	
Smoking			
Yes	37	49	1.000
No	22	31	
Stage			
IIB	27	45	0.162
IIIA	30	30	
Histology			
Adeno	38	52	0.853
Square	19	24	

Notes: The 5% staining threshold was chosen to distinguish positive and negative expression of PD-L1, Fisher's exact test was used for these parameters.

evaluation was done blinded to the clinical pathologic characteristics and patient outcome. For each IHC-sample, more than four non intersecting 200× microscopic field which represent the most effective staining were chosen for evaluation. Both the number of stained cells located within tumor and the stroma immediately adjacent to tumor nests were manually counted.

PD-L1 immunostaining was classified into two groups according to intensity and extent: (a) negative, when no staining was observed or positive staining was detected in < 5% of the cells; and (b) positive, when staining was present in over 5% of the cells. The 5% staining threshold was chosen for keeping with previous study [15]. Immunostaining was assessed separately by two pathologists who were unaware of clinical data. Any discrepancies were resolved through review and discussion.

Statistical analysis

The association between positively stained cell counts and clinical pathologic features were analyzed with Fisher's exact test. Survival curves were drawn with Kaplan-Meier method. Log-rank test was employed to evaluate the overall survival time of PD-L1 positive patients

and PD-L1 negative patients. All statistics were conducted by SPSS 13.0 (IBM Corporation, Armonk, NY, USA), and significance level *p*-value < 0.05 (two-sided).

Results

Clinical information

Table 1 summarized clinical pathologic characteristics of all patients. The average age of enrolled patients was 59.8 years [range: 31-85 years; standard deviation (SD): 12.3 years]. Of all, 72 patients (54.1%) were at stage II, and 61 (45.9%) at stage III cancer. For histological subtypes, 90 of them were with adenocarcinoma, 43 with squamous carcinoma, and 6 with others. Patients were followed-up for 6 years, and by their latest follow-up, 81 patients (58%) were dead. Excluding 8 unreadable slides, 131 slides were examined in total. In the remaining 131 slides, 81 (61.8%) specimens showed positive PD-L1 staining (≥5% positively staining cells), and 50 (38.2%) specimens together with the tumor adjacent normal tissue were negative (**Figure 1**).

Comparing the PD-L1 expression of samples from patient at different levels within each category, no significant relationship were observed between PD-L1 expression and age, gender, smoking history, stage or subtype of tumor.

EGFR mutation in NSCLC patients

Further, EGFR mutation testing using ARMS (amplification refractory mutation system) was performed on 131 specimens and 33.3% of them (50 in a total 131 specimens) carried EGFR mutation, which is in accord with high EGFR mutate rate reported in Asian people [24]. In particular, 14 patients carried a deletion in exon 19 of EGFR and 36 patients had L858R mutation in exon 21. No significant differences were found between EGFR mutational status and PD-L1 expression level (**Table 2**).

Overall survival

The overall survival (OS) of the 131 patients with readable slides was assessed. By the time when the analysis was conducted, 76 (58%) were dead and 55 (42%) alive. The median OS of PD-L1 positive patients and PD-L1 negative patients were 49.3 months (95% CI: 44.5-54.2)

PD-L1 is correlated with overall survival

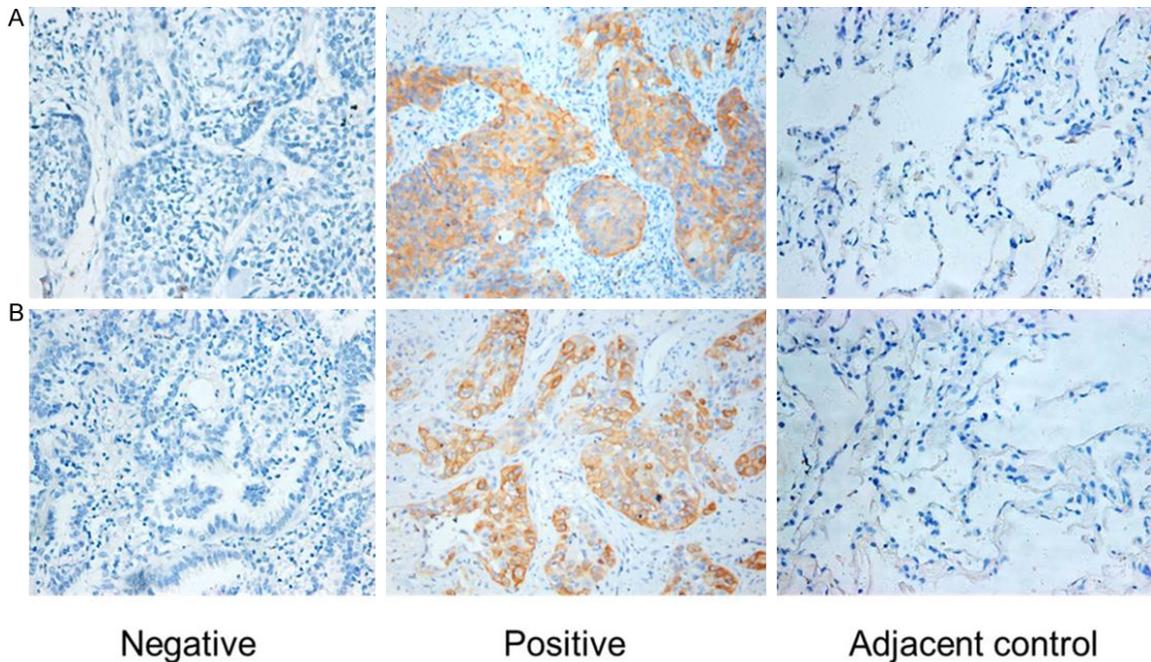


Figure 1. Representative tumor tissue to PD-L1 Immunohistochemistry. A: Squamous cell carcinoma; B: Adenocarcinoma. ($\times 200$) Notes: Ratios of PD-L1+ cells varied substantially among tumor samples.

Table 2. EGFR status in NSCLC patients and their relationship with PD-L1 expression

EGFR status	PD-L1 Negative	PD-L1 Positive	Relativity test
EGFR mutant type	15	35	0.098
EGFR wild type	37	44	

Notes: Fisher's exact test was used for EGFR status.

and 40.9 months (95% CI: 35.3-46.5), respectively. Overall survival analysis combining all 131 patients' record and PD-L1 expression showed that positive expression implies a longer survival time, $P = 0.028$ (Figure 2).

Discussion

Membrane staining was regarded as most significant and performed in most studies that evaluated the expression of PD-L1 in cancer cells [25]. Several lines of evidence showed that the percentage of PD-L1 expression in NSCLC presented by IHC range widely from 21% to 95% [18, 26-29]. The 5% of membrane positively stained tumor cells used as a cut-off to determine the positivity of PD-L1 staining in this study was in keeping with previous study that evaluated clinical response to anti-PD-1 therapy [15].

We found PD-L1 was over-expressed in 61.8% NSCLC samples, which appears to support the study by Chen et al. [19] reporting that 57.5% of patients with NSCLC overexpress PD-L1 in Chinese population. Consistent with most previous studies [19-22, 29-32], our data showed PD-L1 expression negatively correlate with clinicopathologic parameters including ages, gender, smoking history, histological subtypes and tumor stage. However, conflicting results had been reported. Some studies demonstrated higher PD-L1 expression in higher stage [18, 19] and older patients, while others showed that PD-L1 overexpression was related to younger age [18, 23]. In addition, whether PD-L1 was preferentially expressed in adenocarcinoma or in SCC is still controversial [18, 20-22]. The discrepancy between different studies may be due to several limitations. Firstly, the clinicopathologic heterogeneity in the population, such as distinct histology, pathological stage and diverse sizes of samples among different studies, may affect outcome analysis. Secondly, difference between antibodies (we used rabbit polyclonal anti-human PD-L1 (Sigma) while others used different ones such as 5H1 monoclonal antibody) and immunohistochemical technique used in studies may in part account for the variations in results.

PD-L1 is correlated with overall survival

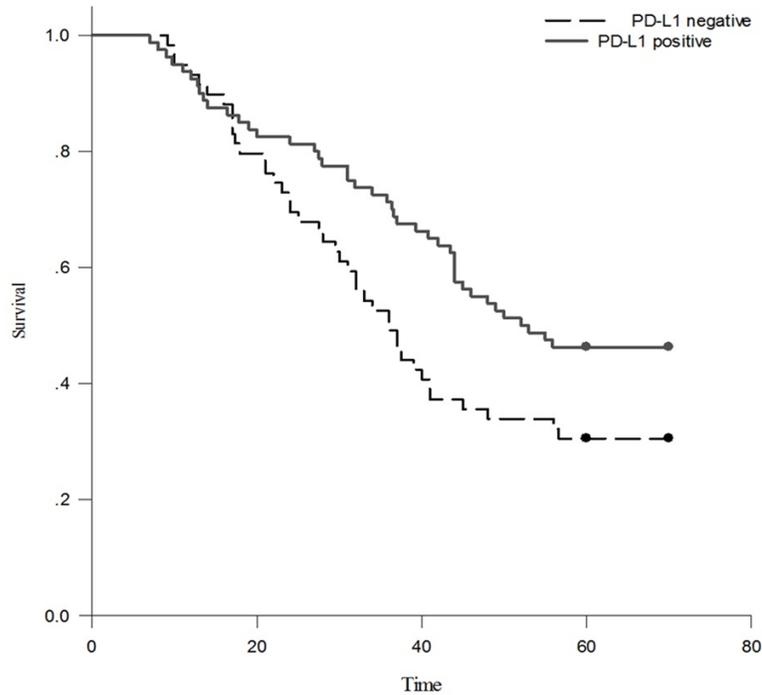


Figure 2. Kaplan-Meier survival curves based on PD-L1 expression in two cohorts. Overall survival in patients with PD-L1 positive showed statistically longer compared with patients with PD-L1 negative ($P = 0.028$). The OS was 49.3 months (95% CI: 44.5-54.2) and 40.9 months (95% CI: 35.3-46.5), respectively.

Multiple different tests in different scoring system used by oncologists or pathologists may constitute “a recipe for confusion”. Thirdly, the threshold of IHC ‘positive’ PD-L1 expression differed among studies. Unstandardized interpretation for the staining intensity and distribution, together with various cut-off percentages by which to define PD-L1 that ranges from $\geq 1\%$ to $\geq 50\%$, compounded on the potential contradiction. In particular, the comparability of the outcomes has been compromised for the use of diversified proprietary PD-L1 IHC assay, which, together with the lack of definitive criteria of positive tumor PD-L1 results, render the limitation on proper data interpretation. Because of the heterogeneity and inconsistent results in different studies, a concerted, multicenter, or even international effort should be in place to develop a standardized scoring system, taking into account of various types of test platforms, we consider that this paradigm may also work for a number of checkpoint blockade therapies.

The present study showed no association between PD-L1 expression and mutational sta-

tus of EGFR. However, previous studies showed that EGFR activation increased the expression of PD-L1 through MAPK and ERK1/2c-Jun signaling pathway [33], and this elevated expression was found to be reversed by EGFR-TKIs in EGFR mutant NSCLC cell lines [21], indicating that apart from killing tumor cells, EGFR-TKIs may also stimulate immune enhancement in EGFR mutant NSCLC patients. Consequently, the combinatorial therapy might be a scientific rationale-based strategy for NSCLC patients carrying EGFR mutation whereas no evidence-based verifications were available. To reasonably clarify this, more delicately and sophisticatedly designed *in vivo* and *in vitro* studies are urgently required to explore the efficacy of the rapyintegrating EGFR-TKIs and anti-PD-1/PD-L1 antibodies.

Currently, the clinical utility of PD-L1 positivity in prognostic assessment of lung cancer is still controversial [34, 35], due to the inconsistent results of PD-L1 expression and patient outcome in different studies. It is well recognized that interaction between PD-1 and its ligand PD-L1 leads to apoptosis or inactivation of activated T-cells, where as some evidence showed that immunosuppressive PD-1/PD-L1 axis induces T-cell tolerance in NSCLC [36]. These indicate that PD-L1 overexpression appear to be associated with poor prognostic factors. On the contrary, the overall survival of PD-L1 positive NSCLC patients in our cohort was significantly longer than that of PD-L1 negative NSCLC patients. Ribas *et al.* have found that PD-L1 positive tumors seemed to be concordantly localized closely adjacent to tumor infiltrating lymphocytes (TILs) that are adjacent to tumors, and TILs may secrete cytokines to produce inhibitory effect, which also drives tumor PD-L1 expression [37]. Therefore, the induced activation of the PD-1/PD-L1 pathway may result from an immune resistance activity stimulated by tumor cells to antagonize endogenous antitumor activity, which might account for the

PD-L1 is correlated with overall survival

inexorable immune escape in NSCLC even at the presence of endogenous antitumor immune response. It can be inferred from those observations that blockage of PD-1/PD-L1 pathway may be conducive to treatment for PD-L1 positive patients.

However, nowadays many tumors deemed PD-L1 positive did not respond to anti-PD-1 therapies and some responses occurred in PD-L1-negative tumors [37, 38]. The aforementioned issues that resulted in inconsistency among relevant researches posed substantial limitation in utilizing IHC assay of PD-L1 as a prognostic biomarker. Additionally, antitumor immune response should be regarded as a complicated activity involving de-/activation of various pathways, therefore, any single biomarker alone may not be able to adequately reflect the prognosis. A recent study showed that several neoantigens were overexpressed in mismatch repair-deficient tumor microenvironment. The active immune microenvironment was diminished by immune inhibitory signals-stimulated thereof, which antagonizes tumor elimination [39, 40]. This reinforces the novel idea that recognition of mutation-associated neoantigen is critical for the endogenous antitumor immune response.

Our study detected a significant correlation between better survival and PD-L1 overexpression in stage II-III NSCLC patients, independent of age, smoking history, histological subtypes, tumor stage and EGFR status. Due to inconsistencies between different researches, further studies are warranted to determine the prognostic significance of PD-L1 expression. In addition, with the guidance of precision medicine, cancer genomic studies might hold the promise for immunotherapy in lung cancer, which should entail prospective studies more adequately designed to address this issue.

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Disclosure of conflict of interest

None.

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PD-L1 is correlated with overall survival

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