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Relationship Between Programmed Death-ligand 1 and
Clinicopathological Characteristics in Non-small
Cell Lung Cancer Patients

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Objective To evaluate the correlation between programmed death-ligand 1 (PD-L1) expression in primary lung cancer cells, tumor associated macrophages (TAM) and patients' clinicopathological characteristics.

Methods From 2008 to 2010, 208 non-small cell lung cancer patients who underwent surgery or CT-guided biopsy were recruited from Huadong Hospital, Fudan University. Immunohistochemistry staining was performed to evaluate the PD-L1 expression in both primary lung cancer cells and CD68 positive TAM. The relationship between PD-L1 expression and the clinical pathology was evaluated using χ^2 test. Spearman's rank correlations were used to determine the correlation between PD-L1 expression in tumor cells and macrophages.

Results Positive PD-L1 expression in primary cancer cells was found in 136 (65.3%) patients, which were negatively correlated with lymph node metastasis ($P=0.009$) and smoking history ($P=0.036$). Besides, TAM with PD-L1 expression (found in 116 patients) was positively associated with smoking history ($P=0.034$), well-differentiation ($P=0.029$) and negative lymph node metastasis ($P=0.0096$). A correlation between PD-L1 expression in primary tumor cells and non-small cell lung cancer associated macrophages was found ($r=0.228$, $P=0.021$).

Conclusion PD-L1, secreted from TAM, might induce cancer cells apoptosis, and decrease lymph node metastasis.

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MORE than 1 500 000 lung cancer patients are diagnosed every year, and non-small cell lung cancer (NSCLC) accounts for 80 percent of all lung cancer cases.

Programmed death-ligand 1 (PD-L1), known as B7-H1 or CD274, was the fourth member of ultra-family of immunoglobulin, which was firstly identified in 1999.^{1, 2} Human PD-L1 gene, located at 9p24-2, encodes I-type transmembrane protein including 290 amino acids. PD-L1 in combination with its receptor, programmed death-1 (PD-1), mediates important regulation of immunological responses through immunoreceptor tyrosine-based inhibitory motif (ITIM) in the cytoplasmic region of PD-1 molecule. PD-L1 is widely expressed on immune and nonimmune cells, including T cells, B cells, macrophages and dendritic cells, which induces T-cell anergy or apoptosis via PD-1 expressed on activated T cells. Gao *et al*^{3, 4} found that high expression of PD-L1 on T cells and hepatocellular carcinoma cells is associated with poorer prognosis. Meanwhile, the correlation between tumor PD-L1 expression and poor prognosis has also been revealed in variety of human cancers, such as urothelial carcinoma and ovarian cancer.⁵ Chen *et al*⁶ found that up-regulated PD-L1 expression in NSCLC is related to the degree of tumor cell differentiation and TNM stage. Therefore in this retrospective study, we aimed to evaluate the relationship between clinicopathologic characteristics and PD-L1 expressed in tumor cells as well as tumor associated macrophages (TAM), which present in the micro-environment of tumors have immunosuppressive effect and tumor-promoting action,⁷ in NSCLC patients.

PATIENTS AND METHODS

Patients

From 2008 to 2010, 208 NSCLC patients (116 men, age 35-82 years) who underwent surgery or CT-guided biopsy, were recruited from Huadong Hospital, Fudan University. The clinical data of 208 patients were retrospectively analyzed. The pathological staging was performed according to UICC 2009 criterion.

Immunohistochemical staining

Anti-PD-L1 rabbit polyclonal antibody and anti-human PD-1 antibody were purchased from Abcam (Cambridge, UK). Anti-CD68 antibody, which is the specific biomarker of macrophages was purchased from Santa Cruz Biotechnology (CA, USA).

Immunohistochemical staining was performed as previously described.^{3, 4} Briefly, NSCLC samples embedded

in paraffin were sliced sequentially, then slices were de-paraffined routinely and incubated in 3% H₂O₂ at the room temperature for 10 minutes to inactivate androgenic peroxides. Then the slices were washed with distilled water and soaked in PBS three times, 5 minutes each time. Then the slices were sealed in 10% normal goat serum, incubated at the room temperature for 10 minutes and then serum was poured out. The slices were incubated with the first antibodies overnight at 4°C. Then the slices were washed with PBS three times, 5 minutes each time. Two-stage immunohistochemical kit (ChemMate™ Envision Detection Kit, Peroxidase/DAB, DAKO, Denmark) was used. The slices were added with a proper amount of ChemMate™ Envision Detection+HRP, incubated for 30 minutes at room temperature, then washed with PBS three times, 5 minutes each time. The slice was developed with DAB for 2-5 minutes, then washed with tap water, re-stained with HE, dehydrated, hyalinized, and sealed. In case yellow-brown granules of PD-L1 appeared in membrane or cytoplasm, regarded as PD-L1 positive.

PD-L1 expression in tumor cells

Slices were evaluated by two independent pathologists, using a two-grade scoring system. The staining intensity was divided into 0-3 scores: not stained, 0 score; yellowish, 1 score; yellow-brown, 2 scores; brown, 3 scores. Slices were divided into 4 categories according to percentage of positive cells that were scored as 0, 1, 2, and 3, respectively. 0 score, positive cells ≤2%; 1 score, positive cells 3%-20%; 2 scores, positive cells 21%-60%; 3 scores, positive cells >60%. The final score was the product of the staining intensity with the corresponding percentage, arranged from 0 to 9. The final score >3 is considered as positive expression.^{7, 8}

PD-L1 expression in TAM

The paraffin-embedded samples were sliced consecutively into slice series at 4 μm in thickness. The first slice was used for identification of macrophages by CD68 antibody. Then the second slice incubated with PD-L1 antibody was used to identify PD-L1 expression in macrophages via the aid of the location for macrophages by the first slice.^{7, 8}

Statistical analysis

SPSS, version 13.0, was used for all statistical analysis. The relationship between PD-L1 expression and the clinical pathology was evaluated using χ^2 test. Spearman's rank correlations were used to determine the correlation between PD-L1 expression in tumor cells or macrophages. A *P*-value

<0.05 was considered to be statistically significant.

RESULTS

Clinicopathological characteristic of NSCLC patients

Totally, 208 NSCLC patients were collected in this study including 130 squamous cancers, 46 adenocarcinoma, 6 adeno-squamous carcinoma, 12 large cell lung cancers, 4 sarcoid carcinoma, and 10 non- or low-differentiated carcinoma. TNM staging was as follows: 114 cases at TNM stage I, 30 cases stage II, 48 cases stage III, and 16 cases stage IV (Table 1).

Relationship between the PD-L1 expression in tumor cells and the clinicopathological factors

Of 208 patients, 136 (65.3%) were PD-L1 positive in the tumor cells, which is expressed either in cytoplasm or membrane (Fig. 1). Significantly increased expression of PD-L1 was also found in non-smoking patients ($P=0.036$, Table 2). In patients with lymphatic metastasis, only 35.5% were PD-L1 positive, compared to 58.3% in non-metastatic patients ($P=0.009$).

Relationship between PD-L1 expression in TAM and the clinicopathological factors

Macrophages, which were identified by CD68 antibody, were successfully observed in 206 cases. We found 116 patients with PD-L1 positive macrophages, including 84 cases both in tumor cells and macrophages (Fig. 1). And the expression of PD-L1 in macrophages did not relate to sex, age, pathological type, pleural infiltration, tumor size

and staging (Table 3). Significant correlation between PD-L1 expression in tumor cells and in macrophages ($r=0.228$, $P=0.021$) were also found. Increased expression of PD-L1 in macrophages was found in non-smoking, non-lymphatic metastasis and well differentiated cancer patients (all P values < 0.05).

Table 1. Clinicopathological characteristics of 208 NSCLC patients

Pathological factors	Case no. (%)
Sex	
Male	116 (55.7)
Female	92 (44.3)
Age (yr)	63.5±11.6
Smoking	
Smoker	50 (24.0)
Nonsmoker	158 (76.0)
Pathological type	
Adenocarcinoma	130 (62.5)
Squamous carcinoma	46 (22.2)
Squamous-adenocarcinoma	6 (2.9)
Large cell carcinoma	12 (5.7)
Others*	14 (6.7)
TNM stage	
I	114 (54.8)
II	30 (14.4)
III	48 (23.1)
IV	16 (7.7)

NSCLC: non-small cell lung cancer; *means undifferentiated or poorly differentiated NSCLC.

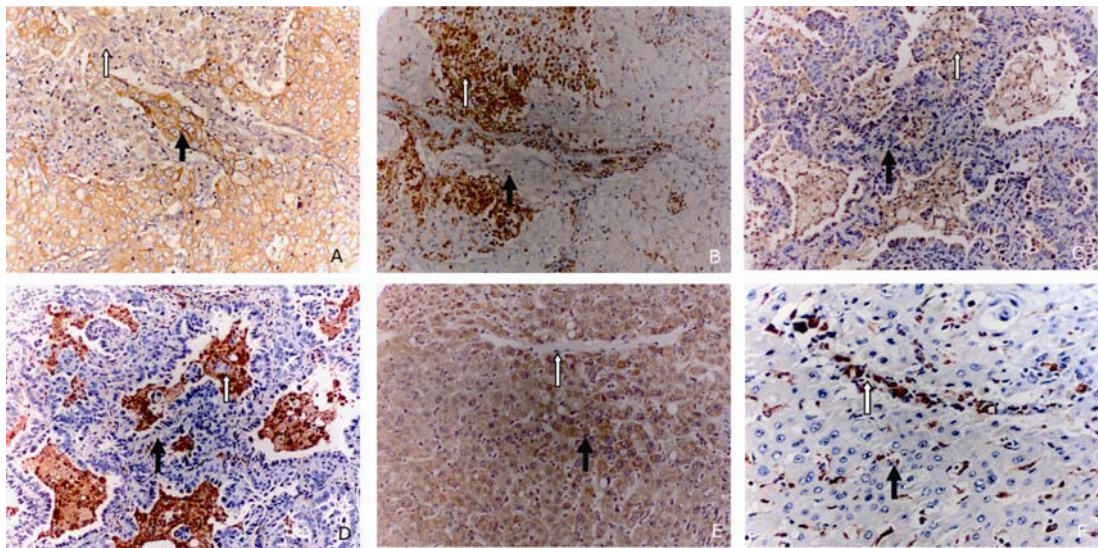


Figure 1. Results of immunohistochemical staining of PD-L1 (A, C, E) and CD68 (B, D, F) in NSCLC cells and TAM. HE staining × 200
A, B. PD-L1 was highly expressed in both tumor cells and macrophages.
C, D. Macrophages expressed PD-L1 at high level and tumor cells expressed PD-L1 at lower level.
E, F. Tumor cells expressed PD-L1 at high level and macrophages expressed PD-L1 at lower level.
PD-L1: programmed death-ligand 1; TAM: tumor associated macrophages.
The black arrows indicate tumor cells and the white arrows indicate macrophages.

Table 2. Relationship between the expression of PD-L1 in tumor cells and the clinicopathological factors of NSCLC patients

Pathological factors	Case no.	Tumor cells		P
		PD-L1 (+)	PD-L1 (-)	
Sex				
Male	116	74	42	0.607
Female	92	62	30	
Age(yr)				
>70	78	56	22	0.921
≤70	130	80	50	
Smoking				
Smoker	50	24	26	0.036
Nonsmoker	158	112	46	
Pathological type				
Adenocarcinoma	130	40	90	0.757
Squamous carcinoma	44	12	32	
Size of tumor (cm)				
<4	156	106	50	0.341
≥4	52	30	22	
Differentiation				
Poorly	46	18	28	0.093
Moderately-highly	110	34	76	
Pleural metastasis				
No	158	60	98	0.243
Yes	48	12	36	
Lymph nodes metastasis				
No	144	84	60	0.009
Yes	62	22	40	
TNM stage				
I	114	82	32	0.149
II-IV	94	54	40	

Table 3. Relationship between expression of PD-L1 in TAM and the clinicopathological factors of NSCLC patients

Pathological factors	Case no.	TAM		P
		PD-L1 (+)	PD-L1 (-)	
Sex				
Male	116	64	52	0.157
Female	90	48	42	
Age(yrs)				
>70	76	44	32	0.583
≤70	130	68	62	
Smoking				
Smoker	50	22	28	0.034
Nonsmoker	156	90	66	
Pathological type				
Adenocarcinoma	130	60	70	0.156
Squamous carcinoma	42	12	30	
Size of tumor (cm)				
<4	154	84	70	0.951
≥4	52	28	24	
Differentiation				
Poorly	46	16	30	0.029
Moderately-highly	110	68	42	
Pleural metastasis				
No	158	86	72	0.736
Yes	48	28	20	
Lymph nodes metastasis				
No	144	86	58	0.0096
Yes	62	26	36	
TNM stage				
I	112	58	54	0.692
II-IV	94	54	40	

DISCUSSION

PD-L1 is expressed widely in both lymphatic and non-lymphatic tissues for example heart, spleen, kidneys, liver, skin, muscle and placenta in addition to lung. High expression of PD-L1 was found in CD8-positive T cells, leading to less secretion, poorer proliferation and subsequent apoptosis. While the CD8-positive T cells may restore the secretion and proliferation function after PD-L1 was blocked by monoclonal antibodies.⁹

Furthermore, it is reported that increased expression of PD-L1 was also found in various human tumors compared

to normal tissues, including thymus tumor, breast cancer, hepatic cancer, colonic cancer, cystic cancer and tongue squamous cancer, as well as lung cancer.^{10, 11} However, the predictive role of PD-L1 in NSCLC patients remains controversial. Mu *et al*¹² reported that high expression of PD-L1 in the primary foci of NSCLC was an independent predictor of poor prognosis, whereas Konishi *et al*¹³ also reviewed that PD-L1 and PD-L2 did not correlate to the prognosis of NSCLC, nor did they relate to other clinically pathological factors. In our study, 65.3% of total NSCLC patients were found to be PD-L1 positive in tumor cells, which was negatively correlated with lymph node metastasis.

Therefore, we think that PD-L1 may promote tumor cell apoptosis and in turn reduce metastasis.

Furthermore, we also detected the expression of PD-L1 in the TAM. We observed that PD-L1 was expressed not only in NSCLC cells, but also in TAM. Interestingly, increased expression of PD-L1 in TAM was negatively correlated to lymphatic metastasis. Based on our results, patients with PD-L1 high expression TAM would had less lymph node metastasis ($P=0.0096$).

Our researches revealed that TAM may promote metastasis of tumors through inhibiting the function of T cells. TAM with PD-L1 high expression was more liable to development apoptosis, thereby inhibiting the pro-metastatic potency of TAM and in turn reduced lymph node metastasis. However, the specific mechanism remains to be confirmed by more pre-clinical and clinical experiments.

Nearly 50% of patients who undergo surgery would develop metastatic disease and less than 25% of them would obtain treatment and benefit from chemotherapy. Thus new therapeutic targets for lung cancer treatment are urgently needed. And several targets have recently been found, such as Recombinant Human Epidermal Growth Factor. If we regard tumors as the product in the micro-environment of tumors and may conclude that molecular signals participate in the information exchange between tumors and their hosts, then the top key abnormal pathway in the research of lung cancer may include receptor of tyrosine kinase, epithelial-mesenchymal transition, tumor angiogenesis and stem cell labeling; the research on the targeted therapy aiming at the above links is under way;¹⁴ mesenchymal therapy may be used to prevent and interfere with the development of tumors. Mesenchymal therapy is an early, dynamic and targeted therapy; therefore it may be used in multiple links in the therapeutic stage, including chemical prophylactic therapy, therapy of recurred tumors and therapy of metastatic tumors. Mesenchymal therapy of low density may reverse the signal transfer between the tumor and its hosts while the tumor is small and in a critically unbalanced state, thereby reducing as much as possible its toxic action on other tissues. The result of our researches presented in this paper offered a certain experimental basis for the targeted therapy based on the mesenchyma of tumors.

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