A Study on the Clinical Significance of Blood Exosomal PD-L1 in Non-Small Cell Lung Cancer Patients and its Correlation with PD-L1 in Tumor Tissues

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ABSTRACT

Exosomal programmed cell-death ligand 1 (ePD-L1) can influence immune inhibition and dysfunction. We were dedicated to unearthing the relation between ePD-L1 in blood and pathological characteristics as well as PD-L1 in tumor tissues. We recruited 65 non-small cell lung cancer (NSCLC) patients for exosome extraction and detected the blood ePD-L1 expression in these patients by enzyme-linked immunosorbent assay (ELISA) method. Besides, the correlation between blood ePD-L1 and patients' pathological characteristics was also analyzed. The expression of PD-L1 in tumor tissues was tested by immunohistochemistry (IHC) and its correlation with blood ePD-L1 expression level was analyzed by Spearman correlation coefficient. No significant correlation was observed in PD-L1 expression levels between blood-derived exosome and tumor tissue. Altogether, high blood ePD-L1 expression was relevant to NSCLC progression, while no such relevance to PD-L1 expression in tumor tissue.

Introduction

To date, routine therapies for lung cancer are surgery, chemoradiotherapy, and targeted therapy [1]. Immune therapies based on immune checkpoint programmed cell death protein 1 (PD-1)/programmed cell death ligand 1 (PD-L1) serve as novel options and show fantastic application prospect [2–5]. However, some patients

respond insensitively to these therapies [2–5]. Thus, we need to explore reliable biomarkers, which can predict the sensitivity of therapeutic strategy to lung cancer [6]. At present, many associated biomarkers have been identified, such as PD-L1 expression [7–9], mismatch repair (MMR) [10], microsatellite instability (MSI) [11], and tumor mutation burden (TMB) [12]. The efficacy and reactivity of immune therapy vary from individual to individual. Thus, it is necessary to use biomarkers to screen people who are suitable for immunotherapy.

Prediction of immunotherapy outcomes driven by biomarkers has the potential to revolutionize cancer immunotherapy [13]. Using various detection methods and platforms to monitor tumor immune status can provide effective treatment basis for tumor immunotherapy [14]. For example, biomarkers can be used to classify patients between monotherapy and combination immunotherapy, as well as to determine the effectiveness of immunotherapy in cancer patients. Additionally, patients who do not respond to current checkpoint immunotherapy can be predicted by biomarkers to avoid toxicity during treatment [15]. Immune cells can play an imperative role in immunotherapy as a biomarker. Immune inflammatory tissues are highly sensitive to immunotherapy because immune checkpoint inhibitors can activate the immune response and inhibit immune evasion and play a therapeutic role [13]. Neoantigens are truncated proteins produced by mutations in DNA protein-coding regions that help immune cells target and eliminate tumor cells, which are potential biomarkers of clinical activity of immune checkpoint inhibitors [13]. Anti-PD-1/PD-L1 is also a biomarker. Programmed cell-death ligand 1 (PD-L1), also called B7-H1, is a member of B7 family. PD-L1 is highly expressed in varying tumors and inactivates T cells to lessen anti-tumor immune response through binding PD-1 receptors [2, 3, 16]. PD-L1 level in tumor tissue serve as a biomarker to predict clinical efficacy of PD-1/PD-L1 checkpoint inhibitor [9, 17]. Objective response rate (ORR) of non-small cell lung cancer (NSCLC) patients with high PD-L1 levels is also high after pembrolizumab treatment, suggestive of increased benefits of immune monotherapy on lung cancer [18]. This detection approach is inapplicable to patients whose tumor tissue is not easily accessible. Hence, IHC examining PD-L1 is yet limited.

In addition to PD-L1 on the surface of cytomembrane, tumor cells also release circulating forms of PD-L1, which is further divided into exosome and soluble forms [19]. Exosomes are small membrane-bound vesicles 50–150 nm in diameter, capable of protecting bioactive molecules (i. e., nucleic acid and protein) from degradation in vivo [20, 21]. These vesicles are deemed as novel cross-talk circuits built by tumor cells and tumor microenvironment [22–24]. Exosomes PD-L1 (ePD-L1) level contributes to progression of assorted tumors. Metastatic melanoma patients display noticeably higher ePD-L1 level in comparison with healthy people [25]. Level of exosome-carried PD-L1 is pertinent to lymphatic metastasis of head and neck squamous cell cancer [26]. Several investigations have suggested ePD-L1 surveillance as a harbinger for immune therapy response [25, 27]. Cordonnier et al. [28] revealed that melanoma patients with complete/partial response had a slight change of ePD-L1 (ΔExoPD-L1; no statistical significance) after 4.5 months of immune therapy, whereas those with progressive disease had notably elevated ΔExoPD-L1 (evidently correlates with efficacy). In Chen and his co-worker's report, higher level of circulating ePD-L1 indicated poorer clinical outcomes [25]. Interferon-γ (IFN-γ) stimulation hampers $CD8^+$ T cell functions and accelerates tumor growth via elevating ePD-L1 expression. Circulating ePD-L1 level positively correlates with tumor load. Thus, blood-circulating ePD-L1 can act as an effective biomarker for prediction of tumor progression and immune therapy response.

This investigation was conducted to unravel clinical significance of blood-derived ePD-L1 to NSCLC patients. The relevance between PD-L1 expression in patients' tumor tissues and blood-derived ePD-L1 expression was investigated to present a rationale for suitable indicators.

Patients and Methods

Patient selection

This research enrolled 65 NSCLC patients receiving treatment in Yuncheng Central Hospital from January 2020 to January 2021. Patients were diagnosed with stage III–IV NSCLC and tumor tissues from patients were suitable for IHC detection. Lung tumor tissue was classified according to the 2015 World Health Organization (WHO) Classification [29]. The research was approved by the Ethics Committee of Yuncheng Central Hospital. Patients were notified of all information required and signed informed consent in written form. Information encompassed age, sex, smoking history, Eastern Cooperative Oncology Group (ECOG) performance status, lymph node status, tumor stage, and distant metastasis.

Treatment of peripheral blood and tumor sample

Patients' peripheral blood was collected with EDTA anticoagulant tubes before treatment. Centrifugation was performed at 1000 × *g* for 10 minutes. Serum samples were then carefully transferred to new tubes. All serum samples were retained at –80 °C. Stage III–IV NSCLC patients' tumor tissues were fixed with formalin and embedded with paraffin for IHC.

Serum exosome isolation

In brief, the viscosity of the serum was reduced by diluting it with PBS (1:2) and centrifuging it at 4 °C at 10 × *g* for 4 minutes. Following gentle blending, serum was incubated for 30 minutes at 4 °C and centrifuged at 10 000 × *g* for 10 minutes. The supernatant was abandoned, and the precipitation was the exosome we needed. The final exosome precipitates were re-suspended in 1 × PBS and stored at –20 °C for the use in following experiments.

Transmission electron microscopy (TEM) observation

The separated exosome was resuspended by phosphate buffered saline (PBS). The suspension was negatively stained with uranyl acetate on chloroform coated copper mesh. Images were captured with JEM-1011 TEM (JEOL Ltd, Japan).

Western blotting method

Total exosomal proteins were fabricated by resuspending exosome in Radio Immunoprecipitation Assay (RIPA) buffer. Concentration quantification was undertaken using bicinchoninic acid (BCA). Separated proteins (40 µg from each sample) were loaded on 10 % so-

dium dodecyl sulfate polyacrylamide gel electrophoresis. Following a gap of protein transfer on a polyvinylidene fluoride (PVDF) membrane, overnight incubation was performed on the membrane and primary antibodies anti-CD9 (1:1000, ab236630, Abcam, UK), anti-CD63 (1:1000, ab134045, Abcam, UK), and anti-TSG101 (1:1000, ab125001, Abcam, UK) at 4 °C. Afterwards, the membrane was washed three times with PBS containing 0.1 % Tween 20 for 10 minutes each. Horseradish peroxidase-labeled goat anti-rabbit secondary antibody (1:2000, ab6721, Abcam, UK) was applied for membrane incubation at room temperature for 1 hour. Protein bands were developed with optical photometer (GE Medical Systems, USA) and protein levels were tested on Image Pro Plus 6.0 Software (Media Cybernetics, USA). The results of western blotting are depicted in **Supplemental Fig. 1S**.

Enzyme-linked immunosorbent assay (ELISA)

Isolated exosomes were re-suspended by PBS and PD-L1 level was quantified by PD-L1 ELISA Kit (ab214565, Abcam, UK). Protein concentration was calculated based on standard curve.

Immunohistochemistry (IHC)

The paraffin-embedded slices underwent xylene dewaxing, gradient ethanol treatment, and 30 minutes of blocking with 5 % fetal bovine serum (FBS). Afterwards, tissue samples were incubated with primary antibody rabbit anti-PD-L1 (1:500, ab205921, Abcam, UK) at 4 °C overnight and then with secondary antibody (1:1000, ab6721, Abcam, UK) described as above for 1 hour. Staining was visualized with diaminobenzidine followed by counterstaining with hematoxylin for nucleus. After washing and dehydration, sample staining was observed with a microscope. PD-L1 positive rate was observed in 5 random visual fields. The results of IHC are shown in **Supplemental Fig. 2S**.

Analysis of data

Data were processed on SPSS 18.0 (SPSS Inc., USA) and GraphPad Prism 7 (GraphPad Software Inc., USA). Data were denoted as mean ± standard deviation. Mann–Whitney U-test was used for intergroup comparison. The agreement of IHC was evaluated by Cohen's kappa coefficient. Chi-square test was applied to assess the relation between ePD-L1 and clinical pathological characteristics. Correlation between PD-L1 expression in tumor tissues and blood ePD-L1 expression was evaluated by Spearman coefficient. $p < 0.05$ was of statistical significance.

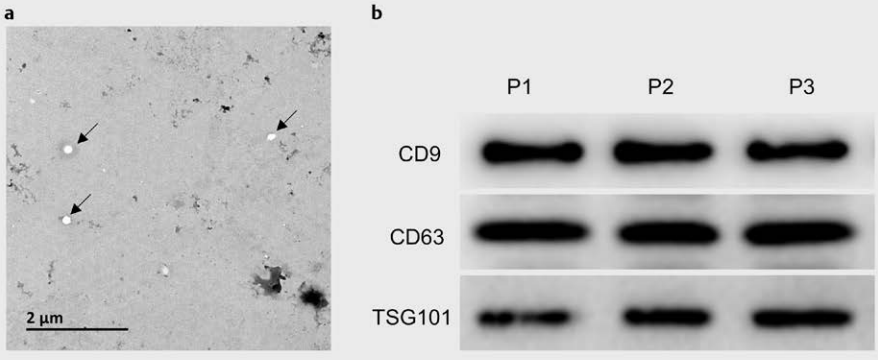
Results

Isolation and identification of exosomes from NSCLC patients' blood

The isolated exosomes were observed by TEM. Exosomes are small membrane-bound vesicles 50–150 nm in diameter (▶ **Fig. 1a**). Western blotting method was utilized to test the levels of exosome specific marker proteins (CD63, CD9, and TSG101) [30] (▶ **Fig. 1b**), which suggested successful isolation exosomes. The isolated exosomes were used for the subsequent analyses.

The expression of blood ePD-L1 in NSCLC patients and its correlation with clinical characteristics

Blood ePD-L1 level in 65 NSCLC patients were determined by ELISA. Patients were split into low ePD-L1 group and high ePD-L1 group based on median ePD-L1 levels (827 pg/ml). Afterwards, we further unraveled the correlation between ePD-L1 expression and several clinical characteristics (age, sex, smoking history, ECOG performance status, lymph node status, distant metastasis and TNM stage) (▶ **Table 1**). To be specific, no relevance was found between blood-derived ePD-L1 level and patient's age, sex, smoking status and ECOG performance status. Blood ePD-L1 level was associated with tumor TNM stages. ePD-L1 level was remarkably lower in stage III patients than that in stage IV patients (with significant differences) (▶ **Fig. 2a**). Moreover, lymph node positive patients had higher blood ePD-L1 expression than lymph node negative patients (▶ **Fig. 2b**). The ePD-L1 level of patients with distant metastasis was significantly increased than those without distant metastasis (▶ **Fig. 2c**). Taken together, high expression level of ePD-L1 was associated with patients' stage IV, positive lymph node status, and distant metastasis.

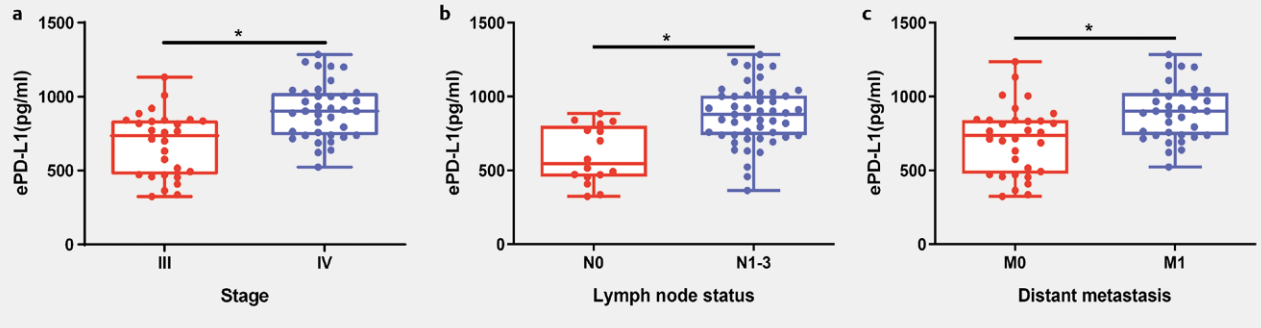


▶ **Fig. 1** Characteristics of blood-derived ePD-L1 from NSCLC patients: **a**: Exosomes (black arrows) in TEM images (50–150 nm in diameter; bar = 100 nm); **b**: The protein expression levels of CD63, CD9, and TSG101 in 3 random NSCLC patients.

▶ **Table 1** NSCLC patient's clinical pathological characteristics.

Characteristics	Cases (%)	ePD-L1		
		Low N (%)	High N (%)	p-value
Age (years)				0.371
≤60	28 (43.08)	16 (24.62)	12 (18.46)	
>60	37 (56.92)	17 (26.15)	20 (30.77)	
Sex				0.543
Male	37 (56.93)	20 (30.77)	17 (26.15)	
Female	28 (43.07)	13 (20.00)	15 (23.08)	
Smoking status				0.722
Smoking	48 (73.8)	25 (38.46)	23 (35.38)	
No-smoking	17 (26.2)	8 (12.31)	9 (13.85)	
ECOG performance status				0.685
0	26 (40.00)	14 (21.54)	12 (18.46)	
1	39 (60.00)	19 (29.23)	20 (30.77)	
TNM stage				0.017*
III	28 (43.08)	19 (29.23)	9 (13.85)	
IV	37 (56.92)	14 (21.54)	23 (35.38)	
Lymph node status				0.026*
N0	16 (24.61)	12 (18.46)	4 (6.15)	
N1–3	49 (75.38)	21 (32.31)	28 (43.08)	
Distant metastasis				0.018*
M0	30 (46.15)	20 (30.77)	10 (15.38)	
M1	35 (53.85)	13 (20.00)	22 (33.85)	

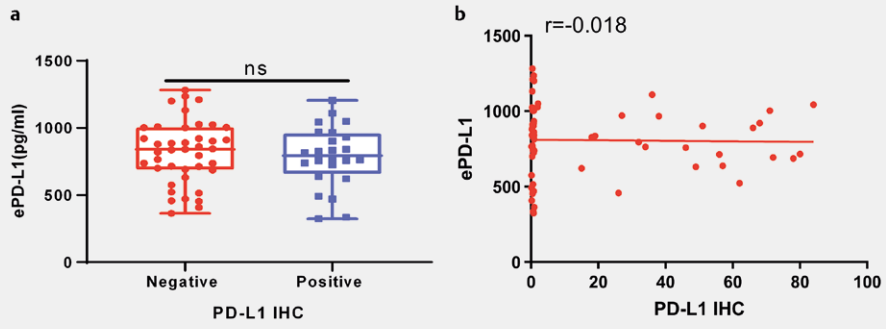
* $p<0.05$.



▶ **Fig. 2** The correlation of blood--derived ePD-L1 level and clinical characteristics of NSCLC patients: **a**: ePD-L1 level was highly expressed in blood of stage IV NSCLC patients; **b**: ePD-L1 level was highly expressed in blood of lymph node positive NSCLC patients (N1-N3); **c**: ePD-L1 was highly expressed in blood of NSCLC patients with tumor distant metastasis (M1). * $p<0.05$.

Correlation analysis of PD-L1 IHC staining and blood ePD-L1 expression level

According to the relationship between PD-L1 expression threshold in tumor tissues and clinical response of anti-PD-L1 therapy, PD-L1 positive staining < 1 % in tumor tissues was defined as PD-L1 negative, while that ≥ 1 % was defined as PD-L1 positive [31]. Afterwards, PD-L1 negative/positive patient's blood ePD-L1 expression was analyzed. The result showed that average levels of blood ePD-L1 in PD-L1 negative patients were lower but had no significant differences with that in PD-L1 positive patients (▶ **Fig. 3a**). Thereafter, correlation between PD-L1 IHC status in paired samples and blood ePD-L1 was investigated. As shown in ▶ **Fig. 3b**, their correlation



▶ **Fig. 3** Correlation between PD-L1 expression in tumor tissues and ePD-L1 expression in blood: **a**: Blood ePD-L1 expression level of NSCLC patients in PD-L1 negative/positive groups (n = 24 in each group); **b**: Relevance of PD-L1 expression in tumor tissues and blood ePD-L1 expression (p = 0.88). * p < 0.05.

was not remarkable. Thus, we extrapolated blood ePD-L1 expression was irrelevant to PD-L1 expression of tumor tissues in NSCLC patients.

Discussion

In recent years, PD-1/PD-L1 checkpoint inhibitor-directed immune therapy has demonstrated favorable efficacy during NSCLC treatment. The therapy prolongs patient's survival and has been in the limelight for tumor treatment [32–34]. In comparison with chemotherapy and targeted therapy, it is an approved plan based on biomarkers solely, for the first time, instead of referring to tumor location and histological type. However, benefits of immune therapy are only available for few NSCLC patients. Thus, it is noteworthy to find corresponding biomarkers to screen eligible patients.

As crucial messengers, exosomes encompass abundant bioactive molecules, like proteins, nucleic acids, and lipids capable of changing recipient cell functions [35]. Cumulative reports revealed that PD-L1 expression in circulating exosomes was involved in tumor progression, patients' prognosis and immune therapy efficacy [25, 36, 37]. This investigation analyzed the relevance between blood ePD-L1 expression and patients' pathological characteristics as well as clinical efficacy of ePD-L1 level on patients. Notably, positive correlation was found between blood ePD-L1 expression and lymph node status, distant metastasis and TNM stage, suggestive of its role as an indicator for tumor progression. Likewise, ePD-L1 correlates with disease progression and glioblastoma tumor volume [26]. In head and neck squamous cell carcinoma, ePD-L1 level was associated with patient's lymph node status, Union for International Cancer Control (UICC) stage and disease activity [26]. As testified by Gang Chen, ePD-L1 level positively correlated with tumor size and poor prognosis [25]. Integrative references demonstrated clinical significance of ePD-L1 level to NSCLC patients.

Here, we also compared the association between blood ePD-L1 of NSCLC patients and PD-L1 level in tumor tissues to present more rationale for predictive markers. ePD-L1 expression was not different in blood between PD-L1 negative/positive patients. This may be due to two mutually exclusive pathways where PD-L1 expresses on cell surface and secreted blood PD-L1 via exosomes [25]. The blood ePD-L1 levels of NSCLC patients were irrelevant to PD-L1 expression in tumor tissues.

All in all, this investigation suggests notable relevance between blood ePD-L1 level of NSCLC patients and tumor lymph node metastasis and distant metastasis. Besides, PD-L1 expression in tumor tissues was not associated with blood ePD-L1 level. However, limitations here shall be acknowledged: relatively few samples; late-stage NSCLC patients included; and possible bias in correlation analysis. The included patients did not receive PD-1 or PD-L1 immune therapy, thus we cannot research how blood ePD-L1 antagonizes PD-1/PD-L1 immune therapy. In the future, abundant samples are needed to make up for this deficiency to testify clinical potential of blood ePD-L1.

Conflict of Interest

The authors declare that they have no conflict of interest.

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