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Research Article

Research Article

Exosomal PD-L1 predicts response with immunotherapy in NSCLC patients

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Abstract

Immune Check-Point Inhibitors (ICIs) have shown remarkable promise in treating tumors, including non-small cell lung cancer (NSCLC). Nevertheless, the treatment response rate is low. Studies have found that the high expression of exosomal PD-L1 is one of the reasons for the low treatment response. Therefore, this study focused on the relationship between the exosomal PD-L1 and the clinical response to immunotherapy in NSCLC patients to evaluate whether it could be used as a biomarker to predict the efficacy of ICIs. In this study, clinical information and blood samples of 149 NSCLC patients receiving ICIs were collected. The expression level of exosomal PD-L1 was detected by enzyme-linked immunosorbent assay method, and the relationship between exosomal PD-L1 and the efficacy of ICIs was explored. Overall, our study found that the expression level of exosomal PD-L1 was lower at pre-treatment, or the max fold increasing change higher at 3–6 weeks had a higher disease control rate and longer progression-free survival. It revealed that the exosomal PD-L1 was associated with the treatment response of patients using ICIs and provided a new tool for the evaluation of clinical efficacy of lung cancer immunotherapy.

Keywords: lung cancer, Immunity, Exosomes, PD-L1

Abbreviations: CR: complete response; CTLA-4: Cytotoxic T-lymphocyte associated protein 4; DCR: disease control rate; ELISA: enzyme linked immunosorbent assay; ICIs: immune checkpoint inhibitors; NSCLC: non-small cell lung cancer; NTA: nanoparticle tracking analysis; PBS: phosphate buffered saline; PD: progression disease; PD-1: programmed cell death-1; PD-L1: programmed cell death ligand 1; PFS: progression-free survival; PR: partial response; SD: stable disease; TEM: transmission electron microscopy; WB: western blot.

Introduction

Lung cancer is still a common malignancy with an incidence rate of 11.4% and ranks as the leading cause of cancer-related death accounting for 18% of the total deaths worldwide [1]. Immune checkpoint inhibitors (ICIs), especially antibodies targeting the programmed cell death-1 (PD-1)[2–6]/programmed cell death ligand 1 (PD-L1) [7, 8] pathways, have revolutionized the treatment of non-small cell lung cancer (NSCLC). However, taking nivolumab, for example, the patient response rate in NSCLC is still low [9]. Studies have found that the high expression of exosomal PD-L1 is one of the reasons for the low response rate of patients [10].

Exosomes, with a diameter of about 30–150 nm in lipid bilayer membrane structure, are widely present in biological fluids, such as blood, tears, urine, saliva, breast milk, ascites, and pleural fluid [11]. They contain nucleic acids (including mRNA, tRNA, microRNA, lncRNA, circRNA, and so on), transmembrane proteins, and cholesterol. The surface markers mainly include CD63, CD9, CD81, HRS, ALIX, TSG101, and HSP27 [12–14]. They have been studied in cancer diagnostics because tumor-derived exosomes play an important role in metastatic cascades, such as invasion, migration, and the priming of metastatic niches [15–17].

PD-L1 is found on the surface of the exosomes and can bind to PD-1 through its extracellular domain to inactivate T cells [18]. In 2018, scientists found that PD-L1 on metastatic melanoma-derived exosomes can suppress the function of $CD8^+$ T cells and facilitate tumor growth, and these effects are disrupted by anti-PD-1 antibodies [19]. In addition, exosomal PD-L1 was associated with anti-PD-1 response in melanoma [19].

In this study, we aimed to analyze the relationship between exosomal PD-L1 and the efficacy of ICIs to provide a new tool for the evaluation of the clinical efficacy of lung cancer immunotherapy.

Materials and methods

Patients and specimen collection

A total of 149 non-small cell lung cancer patients were enrolled for treatment with immune check-point inhibitor

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monotherapy or combination therapy (chemotherapy, targeted therapy, or immunotherapy) (Supplementary Table S1). The study was approved by the Ethics Committee of Shanghai Chest Hospital (Number KS1732). All patients were informed of the study and consented to the enrollment. All the procedures were conducted in accordance with the Declaration of Helsinki. Peripheral blood was obtained in sodium heparin tubes before each therapy every 2 weeks (Nivolumab therapy) or every 3 weeks (other therapies) for 12 weeks or until the progression disease (PD).

Clinical response was determined as 180 days of efficacy evaluation based on RECIST1.1 [20] using unidimensional measurements. Responders included those with complete response (CR) or partial response (PR) or stable disease (SD) lasting at least 6 months. Non-responders were patients with PD for 6 months [21]. The assessment of clinical responses for patients was performed independently in a double-blind fashion.

Exosome isolation

Venous blood from NSCLC patients was centrifuged at 2000g for 10 min at 4°C to obtain cell-free plasma. Then, 2 ml of the obtained plasma was centrifuged at 10 000g for 30 min. The collected supernatants were centrifuged at 100 000g for 70 min at 4°C to pellet the exosomes. The pelleted exosomes were diluted with PBS and collected by ultracentrifugation at 100 000g for 70 min at 4°C again, and finally resuspended in PBS.

Transmission electron microscopy (TEM)

An aliquot of 5 μl of purified exosomes dissolved in PBS were dropped on a copper grid. After sedimentation for 1 min, the remaining liquid was absorbed by the air-laid paper. Then 5 μl of 2% uranyl acetate solution (Merck) was applied to the same copper grid for negative staining for 1 min. Then the staining solution was removed with air-laid paper. Finally, the samples on the copper grid were imaged on a TecnaiG2 spirit Biotwin TEM.

Nanoparticle tracking analysis

Exosomes were measured by nanoparticle tracking analysis (NTA), using the ZetaView instrument (Particle Metrix, Germany). The exosomes were suspended in PBS at proper ratios. The size distribution and concentration of purified exosomes were analyzed by the software (ZetaView 8.03.04.01).

Western blots

Exosomal proteins were separated by 10% SDS–PAGE gels and transferred to nitrocellulose membranes (Millipore) through a semi-dry electrotransfer method. After being blocked in tris-buffered saline and Tween-20 (TBST) containing 5% non-fat milk, the membranes were incubated overnight at 4°C with the following primary antibodies: CD9 (CST), Calnexin (CST), PD-L1 (CST), HRS (CST), and GAPDH (CST). The membranes were washed with TBST and incubated with HRP-conjugated secondary antibodies (CST) at room temperature for 1 h. The blots on the membranes were developed with ECL detection reagents (Pierce) and visualized by Imaging LabTM software (Bio-Rad).

Flow cytometry

Collected samples stained with standard antibody mix. Surface markers: human CD3: BV510 (564713), human CD8: PE-Cy7 (557746), human CD45RO: FITC (555492), and human CD27: APC-Cy7 (560222). Stained cells were acquired on a BD Biosciences LSRFortessa and analysed by FlowJo software.

ELISA

An Invitrogen human PD-L1 ELISA Kit was used to measure the concentration of PD-L1 on exosomes. Each sample, standard, blank, and optional control sample, should be assayed in duplicate. First, 100 μl of standard dilution was added to the microwell plate in duplicate and 100 μl of sample diluent was added to the blank wells in duplicate. An aliquot of 50 μl of sample diluent and 50 μl of sample were applied to the sample wells in duplicate. Plates were covered with microwell strips and incubated for 2 h at room temperature (18–25°C) on a microplate shaker. Then, 100 μl of diluted Biotin-Conjugate was added to all wells and the plates were incubated for 1 h. Next, 100 μl of diluted Streptavidin-HRP was added and the plates were incubated for 30 min. Also, 100 μl of TMB substrate solution was added and the plates were incubated in the microwell strips for about 30 min protected from light. Finally, 100 μl of stop solution was added to all wells and the plates were read at 450 nm.

Results

The patients' demographics

The blood samples were collected from 29 June 2019 to 14 July 2020 in Shanghai Chest Hospital, and the subjects were followed up till 15 January 2021. A total of 149 patients were enrolled in our study (Supplementary Fig. S1). Their basic characteristics are summarized in Table 1. There were 100 patients with the history of smoking. Four patients were in stage II, 34 patients in stage III, and 111 patients in stage IV of lung cancer according to the TNM classification

Table 1: NSCLC patients clinical information

	Classification	Number
Sex	Male	119
	Female	30
Smoking history	Yes	100
	No	49
Stage	II	4
	III	34
	IV	111
Pathology	Lung adenocarcinoma	94
	Lung squamous cell carcinoma	47
	Not specifically classified as non-small cell lung cancer	8
Lines of treatment	First	64
	Second	50
	More	35
PD-L1 expression level	Negative	38
	1–50%	25
	50–100%	26
	Unknown	60
Treatment	ICI monotherapy	70
	ICI combination therapy	79

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of lung cancer. The number of patients with pathology lung adenocarcinoma, lung squamous cell carcinoma, and not specifically classified non-small cell lung cancer was 94, 47, and 8, respectively. The number of patients for first, second, and more lines of treatment was 64, 50, and 35 respectively. A total of 26 patients were found with 50–100% level of PD-L1 expression, 25 patients were found in the 1–50% range of PD-L1 expression level, while 38 patients were found negative for PD-L1 expression level. In the remaining 60 patients, the PD-L1 expression level was unknown. There were 70 patients receiving immune check-point inhibitor monotherapy treatment and 79 patients accepting immune check-point inhibitor combination therapy (chemotherapy, targeted therapy, or immunotherapy).

Isolation and characterization of exosomes derived from NSCLC patients

Purified exosomes from NSCLC patients were identified by transmission electron microscopy (TEM) [15, 22–24], which were spherical vesicles with a diameter of about 30–150 nm and bilayer membranes (Fig. 1A). Nanoparticle tracking analysis (NTA) displayed nanoparticles derived from NSCLC patients were mostly in a range of 50–200 nm in diameter (Fig. 1B). So, the diameter range size of isolated vesicles was basically coincided with TEM and NTA. The presence of common exosome markers, including HRS and CD9, were observed by Western blot (WB) (Fig. 1C). WB also revealed the presence of PD-L1 in exosomes (Fig. 1C).

The level of pre-treatment exosomal PD-L1 stratifies clinical responders and non-responders

In the mono-immunotherapy group of NSCLC patients (n = 70), the pre-treatment level of circulating exosomal PD-L1 in responders (n = 23) was lower than in non-responders (n = 47) (P<0.001; Fig. 2A). ROC analysis determined that the pre-treatment level of circulating exosomal PD-L1 of 0.54 ng/ml stratified patients by clinical response to immunotherapy (AUC = 0.889, P < 0.001, sensitivity = 80.9%, specificity = 95.7%; Fig. 2B). A higher level of circulating exosomal PD-L1 before the treatment was associated with lower disease control rate (DCR) (P < 0.001, Kappa value = 0.703; Fig. 2C) and shorter progression-free survival (PFS) median PFS in low group vs. high group: 238 (166.11, 309.89) days vs. 43 (38.11, 47.89) days, P < 0.0001, HR: 0.20 (0.11, 0.36; Fig. 2D). In the combination immunotherapy group (n = 79), we obtained the same conclusion. The pre-treatment level of circulating exosomal PD-L1 in non-responders (n = 37) was higher than in responders (n = 42) (P < 0.001; Fig. 2E). ROC analysis showed that the pre-treatment level of circulating exosomal PD-L1 stratifying patients by clinical response to immunotherapy was 0.55 ng/ml (AUC = 0.796, P < 0.001, sensitivity = 67.6%, specificity = 90.5%; Fig. 2F), which was similar to the value in the mono-immunotherapy. Furthermore, the lower pre-treatment level of circulating exosomal PD-L1 had a higher DCR (P < 0.001, Kappa value = 0.563; Fig. 2G) and longer PFS (median PFS in low group vs. high group: 311 (225.14, 396.87) days vs. 56 (42.58, 69.42) days, P < 0.0001, HR: 0.26 (0.13, 0.50); Fig. 2H).

The level of the maximum fold increasing change of exosomal PD-L1 stratifies clinical responders and non-responders

To investigate the level of circulating exosomal PD-L1 undergoing immunotherapy, the NSCLC patients were followed up. In clinical responders, the level of PD-L1 on circulating exosomes was increased, mostly within 3–6 weeks of therapy (Supplementary Fig. S2). In the mono-immunotherapy group (n = 41), the responders (n = 16) showed a larger increase in the level of circulating exosomal PD-L1 at 3–6 weeks following the initial treatment (P < 0.01; Fig. 3A). ROC analysis determined that a fold change of 1.96 in exosomal PD-L1 at week 3–6 stratified patients by clinical response to immunotherapy (AUC=0.815, P = 0.001, sensitivity = 68.8%, specificity = 84%; Fig. 3B). A fold change in circulating exosomal PD-L1 greater than 1.96 at week 3–6 had a higher DCR (P < 0.001, Fig. 3C) and longer PFS (median PFS in high group vs. low group: 195 (165.6, 224.4) days vs. 51 (37.94, 64.06) days, P = 0.016, HR: 0.45 (0.23, 0.89); Fig. 3D). In the combination immunotherapy group (n = 59), we also observed the same conclusion. The responders (n=34) showed a larger increase in the level of circulating exosomal PD-L1 at 3–6 weeks following the initial treatment (P < 0.01; Fig. 3E). ROC analysis determined that a fold change of 2.08 in exosomal PD-L1 at week 3–6 stratified patients by clinical response to

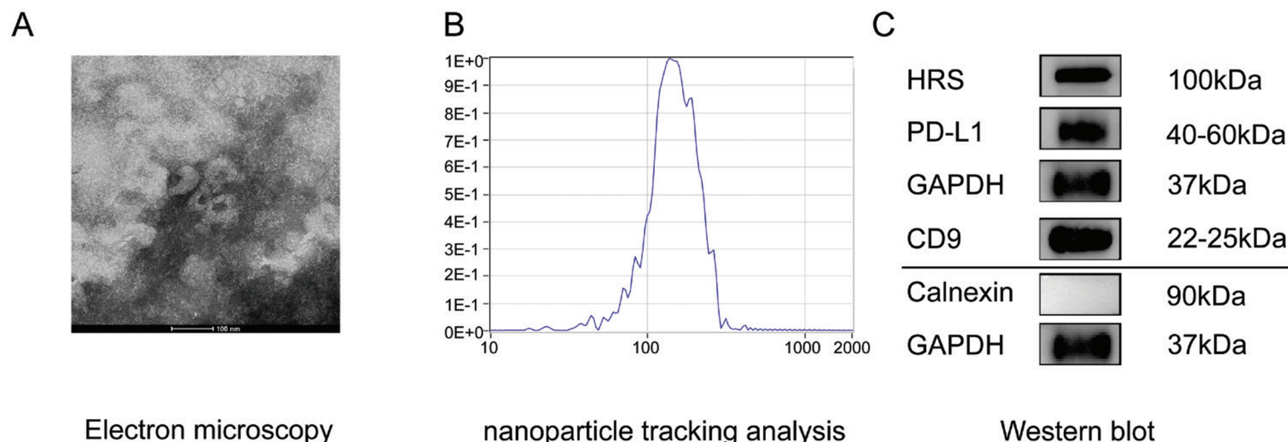

Figure 1: Characterization of exosomes derived from the NSCLC patients. A, Electron microscopy image of purified exosomes from the NSCLC patients. Scale bar = 100 nm. B, The particle size of purified exosomes was measured by nanoparticle tracking analysis (NTA). C, Western blot for exosome markers and PD-L1 in exosomes from the NSCLC patients.

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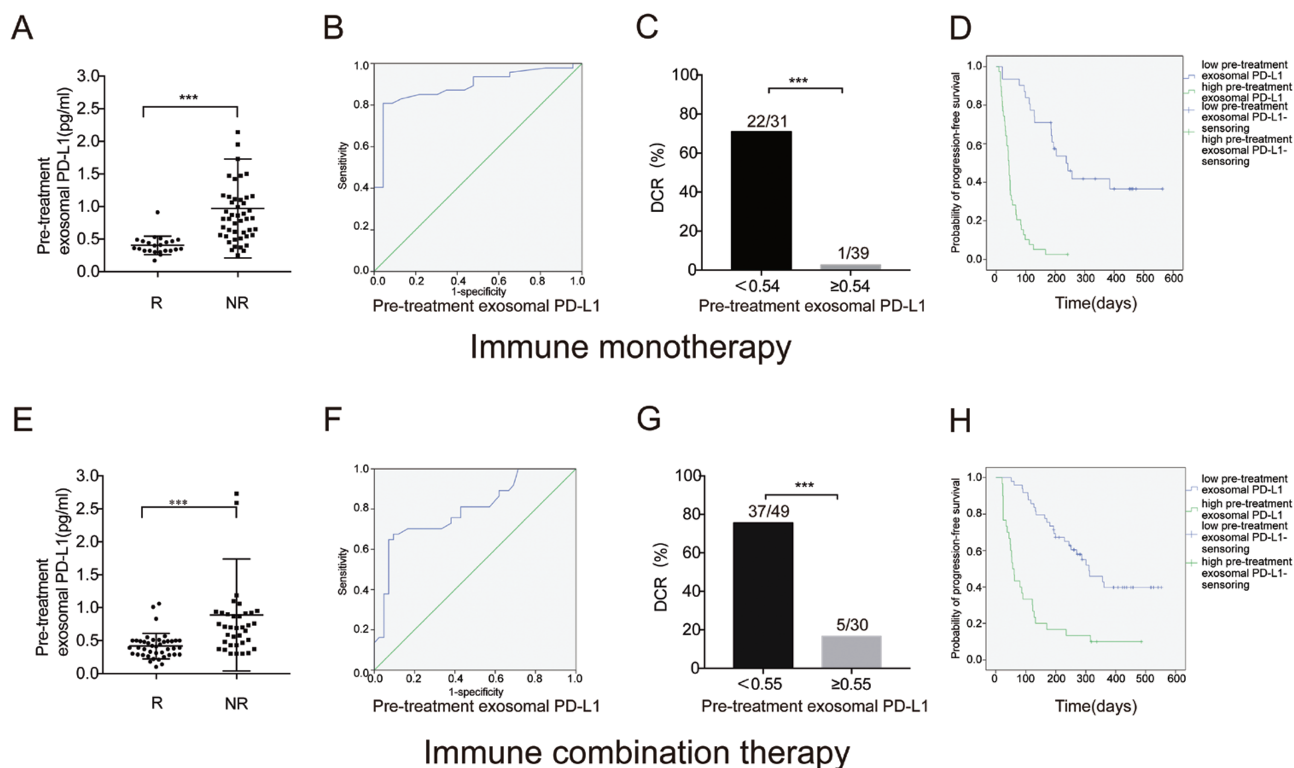

Figure 2: The level of pre-treatment exosomal PD-L1 stratifies clinical responders and non-responders. In a mono-immunotherapy group of NSCLC patients: A, Comparison of the pre-treatment exosomal PD-L1 between clinical responders and non-responders. In 70 NSCLC patients, R, responders; n = 23; NR, non-responders; n = 47. B, ROC curve analysis for the pre-treatment exosomal PD-L1 in clinical responders compared to non-responders. AUC = 0.889, P < 0.001, sensitivity = 80.9%, specificity = 95.7%, cut-off value = 0.54 pg/ml. C, Disease control rate (DCR) for patients with low and high pre-treatment exosomal PD-L1, Kappa value = 0.703. D, Progression-free survival (PFS) analysis for the pre-treatment exosomal PD-L1 in low group compared to high group. In the low pre-treatment exosomal PD-L1 group, median PFS was 238 (166.11, 309.89) days, while in the high pre-treatment exosomal PD-L1 group, median PFS was 43 (38.11, 47.89) days, P < 0.0001, HR: 0.20 (0.11, 0.36). In combination immunotherapy group of NSCLC patients: E, Comparison of the pre-treatment exosomal PD-L1 between clinical responders and non-responders. In 79 NSCLC patients, R, responders; n = 42; NR, non-responders; n = 37. F, ROC curve analysis for the pre-treatment exosomal PD-L1 in clinical responders compared to non-responders. AUC = 0.796, P < 0.001, sensitivity = 67.6%, specificity = 90.5%, cut-off value = 0.55 pg/ml. G, DCR for patients with low and high pre-treatment exosomal PD-L1, Kappa value = 0.563. H, PFS analysis for the pre-treatment exosomal PD-L1 in low group compared to high group. In the low pre-treatment exosomal PD-L1 group, median PFS was 311 (225.14, 396.87) days, while in the high group, median PFS was 56 (42.58, 69.42) days, P < 0.0001, HR: 0.26 (0.13, 0.50). Dates are mean ± S.D. P values are from a two-sided unpaired t-test (A–B) or two-sided Fisher's exact test (E–F). (*P < 0.05; **P < 0.01; ***P < 0.001; ****P<0.0001).

immunotherapy (AUC = 0.832, P < 0.001, sensitivity=64.7%, specificity = 92%; Fig. 3F). High max fold change in exosomal PD-L1 group had a higher DCR (P < 0.001, Fig. 3G) and longer PFS (median PFS in high group vs. low group: 313 (294.54, 331.46) days vs. 117 (72.96, 161.05) days, P = 0.0001, HR: 0.36 (0.19, 0.67); Fig. 3H).

Exploration mechanism of pre-treatment exosomal PD-L1 and immunotherapy

We explored the mechanism of pre-treatment exosomal PD-L1 and immunotherapy. Flow cytometry showed there was no difference between $CD8^+$ T cells in the high and low pre-treatment exosomal PD-L1 groups (Fig. 4A, Supplementary Fig. S3). $CD8^+$ T effector cells were higher in the low pre-treatment exosomal PD-L1 group than in the high pre-treatment exosomal PD-L1 group (Fig. 4B). Correlation analysis indicated that $CD8^+$ T effector cells were negatively correlated with pre-treatment exosomal PD-L1, but the correlation was very weak (Fig. 4C). Sadly, there was also no difference between $CD8^+$ T central memory cells, $CD8^+$ T effector memory cells, and $CD8^+$ T naïve cells (Fig. 4D–F).

Discussion

In the past few decades, immunotherapy has shown tremendous progress and plays an important role in the treatment of cancer. Scientists thought immunotherapy could be broadly divided into two different types: somatic immunotherapy and immune checkpoint inhibitor treatment [25]. Immune checkpoint inhibitor treatments mainly included anti-CTLA-4 antibodies [2], anti-PD-1 antibodies, and anti-PD-L1 antibodies [26]. Though the PD-1/PD-L1 pathway blockaded could activate T cells [27], little is known about the role of exosomal PD-L1 in anti-PD-1/PD-L1 therapy [28]. In 2018, a study showed exosomal PD-L1 harbored an active defense function to suppress T cells and promote tumor growth in breast cancer [18]. Another study displayed exosomal PD-L1 could contribute to immune suppression and was regulated by ALIX [29]. Furthermore, scientists found exosomal PD-L1 inhibited the activation of $CD8^+$ T cells and facilitated tumor growth, and was associated with anti-PD-1 response in melanoma [19].

We believe that exosomal PD-L1 is vital because exosomes are widespread, attach to their target cells easily and act as

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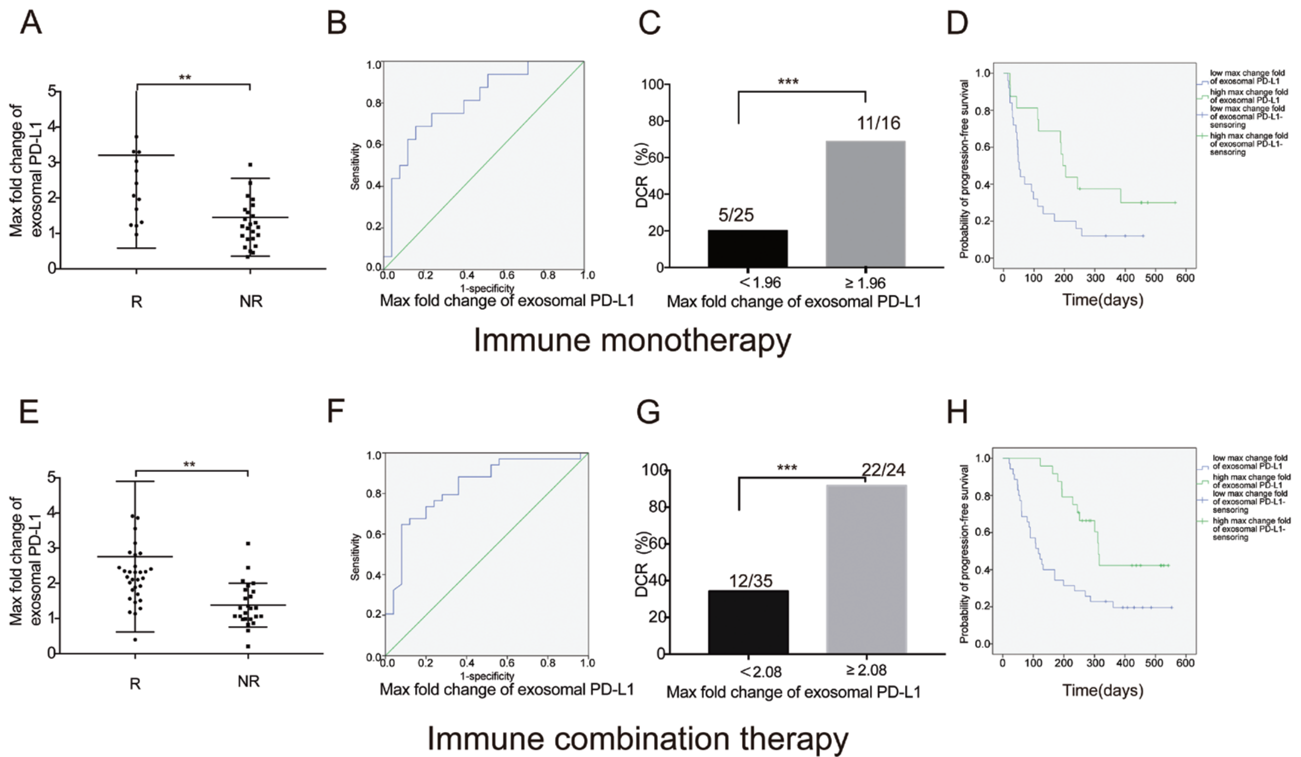

Figure 3: The level of the maximum fold increasing change of exosomal PD-L1 stratifies clinical responders and non-responders. In mono-immunotherapy group of NSCLC patients: A, Comparison of the maximum fold increasing change of exosomal PD-L1 at weeks 3–6 between clinical responders and non-responders. In 41 NSCLC patients, R, responders; n = 16; NR, non-responders; n = 25. B, ROC curve analysis for the maximum fold increasing change of the exosomal PD-L1 at week 3–6 in clinical responders compared to non-responders. AUC = 0.815, P = 0.001, sensitivity = 68.8%, specificity = 84%, cut-off value = 1.96. C, DCR for patients with high and low fold increasing change of exosomal PD-L1 at week 3–6. D, PFS analysis for high and low fold increasing change of exosomal PD-L1 at week 3–6. In the high max fold change of exosomal PD-L1 group, the median PFS was 195 (165.6, 224.4) days, while in the low max fold change of the exosomal PD-L1 group, the median PFS was 51 (37.94, 64.06) days, P = 0.016, HR: 0.45 (0.23, 0.89).In combination immunotherapy group of NSCLC patients: E, Comparison of the maximum fold increasing change of the exosomal PD-L1 at week 3–6 between clinical responders and non-responders. In 59 NSCLC patients, R, responders; n = 34; NR, non-responders; n = 25. F, ROC curve analysis for the maximum fold increasing change of exosomal PD-L1 at week 3–6 in clinical responders compared to non-responders. AUC = 0.832, P < 0.001, sensitivity = 64.7%, specificity = 92%, cut-off value = 2.08. G, DCR for patients with high and low fold increasing change of exosomal PD-L1 at week 3–6. H, PFS analysis for high and low fold increasing change of exosomal PD-L1 at week 3–6. In the high max fold change of exosomal PD-L1 group, the median PFS was 313 (294.54, 331.46) days, while in the low group, the median PFS was 117 (72.96, 161.05) days, P = 0.0001, HR: 0.36 (0.19, 0.67). Dates are mean ± S.D. P values are from a two-sided unpaired t-test (A–B) or two-sided Fisher's exact test (E–F). (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001).

the messenger of cell communication. In our study, we found exosomal PD-L1 may be related to the CD8$^+$ T effector cells, but we have yet not investigated the underlying mechanism. To better understand the role of exosomal PD-L1 in tumor immunity, there is still a lot of work to be done in different immune cell clusters and various immune cells, such as T cells, B cells, dendritic cells, NK cells, and macrophages [30]. Besides PD-L1, other exosomal proteins such as FasL [31], TGF-β, CD39, CD73, and exosomal miRNA may also contribute to immunosuppressive effects [32]. Although anti-PD-1/PD-L1 therapy has already been applied to clinical studies, there are still a lot of questions still remain to be further explored.

Tumor PD-L1 expression is currently the most useful biomarker correlating with immunotherapy efficacy for NSCLC [33]. However, tumor PD-L1 expression may be different due to tumor heterogeneity, time and site of the biopsy, and previous therapies administered to the patient. In addition, a repeated biopsy is very difficult. Considering these problems, liquid biopsies are very promising because they are noninvasive, quickly, and convenient, can be collected at multiple time points, and can overcome the problems associated with tumor heterogeneity [30]. Detecting exosomes, as one of the ways of liquid biopsies, is very useful to guide diagnosis and treatment. Although we evaluated the PD-L1 expression in tissues, in the remaining 60 patients PD-L1 expression level was unknown. In the mono-immunotherapy group of NSCLC patients, patients with PD-L1 negative in tissue may have high pre-treatment exosomal PD-L1 but the samples available for analysis were small. In patients with tissue PD-L1 positive, no significant relationship was found with the exosomal PD-L1 (Supplementary Table S2). The relationship between exosomal PD-L1 and PD-L1 expression in lung tissues needs further research.

Our study found the pre-treatment level of circulating exosomal PD-L1 in responders was lower than in non-responders. Since exosomes could inhibit T cell proliferation and function, patients with higher pre-treatment exosomal PD-L1 levels are in a state of T cell exhaustion, which may be accounting for the poor efficacy of subsequent immunotherapy. The responders showed a larger increase in the level of circulating exosomal PD-L1 at 3–6 weeks after undergoing therapy. An increase in the level of exosomal PD-L1 at weeks 3–6 may reflect the presence of a successful anti-tumor immunity elicited by the immunotherapy. Although the

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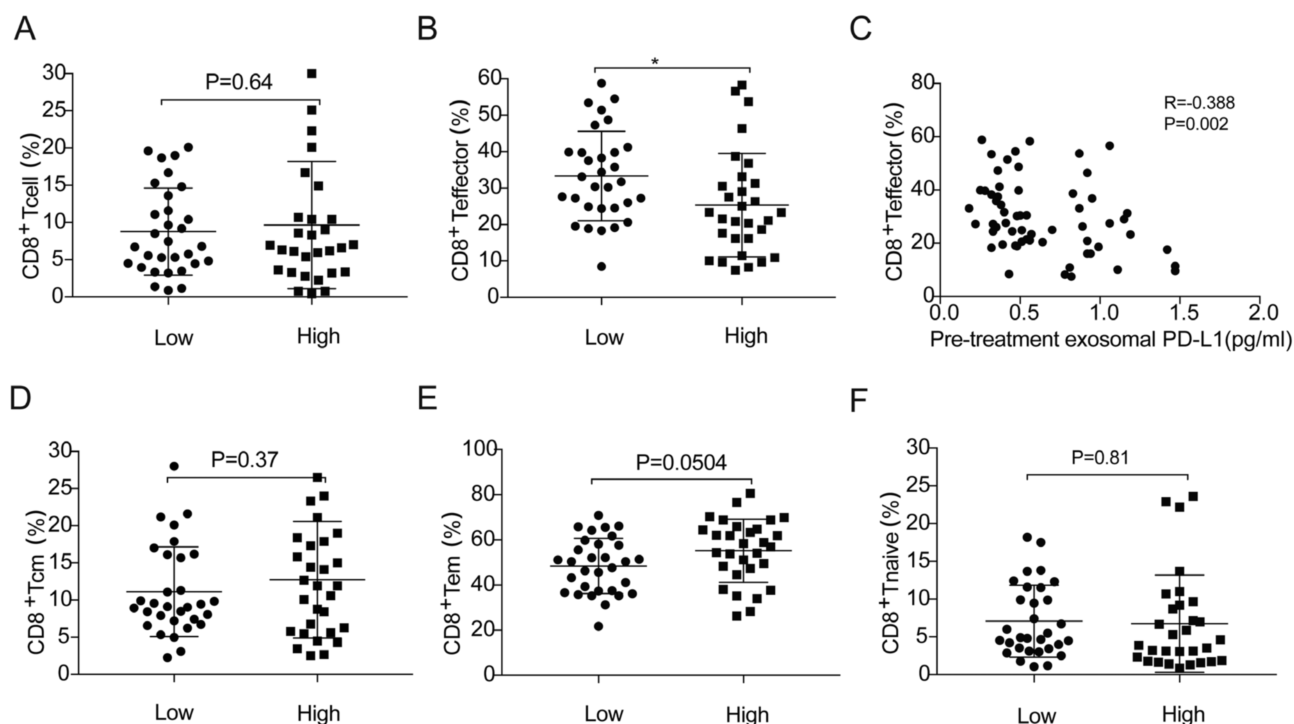

Figure 4: Exploration mechanism of pre-treatment exosomal PD-L1 and immunotherapy. A, Comparison of the CD8+ T cells between low and high pre-treatment exosomal PD-L1 in NSCLC patients. B, Comparison of the CD8+ T effector cells between low and high pre-treatment exosomal PD-L1 in NSCLC patients. C, Pearson correlation of the CD8+ T effector cells to the pre-treatment exosomal PD-L1 in NSCLC patients. D, Comparison of the CD8+ T central memory cells between low and high pre-treatment exosomal PD-L1 in NSCLC patients. E, Comparison of the CD8+ T effector memory cells between low and high pre-treatment exosomal PD-L1 in NSCLC patients. F, Comparison of the CD8+ T naïve cells between low and high pre-treatment exosomal PD-L1 in NSCLC patients. *P* values are from a two-sided unpaired *t*-test (A–B, D–F). (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).

increasing exosomal PD-L1 could inhibit T cell function, it may be blocked by immunotherapy. Hence, exosomal PD-L1 as one of the liquid biopsies may be a biomarker for clinical immunotherapy.

Nowadays, a combination immunotherapy is very common in clinical practice. In our study, in addition to mono-immunotherapy, we also concluded that NSCLC patients with combination immunotherapy. In the combination immunotherapy group, the same conclusion was reached as in the mono-immunotherapy group. Furthermore, we concluded that patients with combination immunotherapy had a high median PFS than patients with mono-immunotherapy, suggesting that combination therapy may be favorable. In this condition, we could still use exosomal PD-L1 to predict the efficacy.

Supplementary data

Supplementary data is available at *Clinical and Experimental Immunology* online.

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Conflicts of interest

The authors declare that they have no conflict of interest.

Author contributions

Y.T.W., X.M.N., S.L., and W.L.X. conceived the project and designed the experiments. Y.T.W., X.M.N., and S.L. provided human samples and associated clinical data. Y.T.W. and Y.R.C. purified and characterized exosomes. Y.T.W. and Y.S.Z. performed Western blot experiment. Y.T.W. and L.L.X performed the flow cytometry. Y.T.W performed the ELISA experiments. Y.T.W. and X.M.N. wrote the paper. Y.T.W., X.M.N., L.L.X., S.L., and W.L.X. edited the paper. All authors have read and approved the final manuscript.

Ethical approval

This study was approved by the Ethics Committee of Shanghai Chest Hospital (Number KS1732). All the procedures were conducted in accordance with the Declaration of Helsinki.

Patient consent

All patients were informed of the study and consented to the enrollment.

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Data availability

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

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