



PD-L1 mRNA derived from tumor-educated platelets as a potential immunotherapy biomarker in non-small cell lung cancer

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Background: To date, the role of programmed death ligand-1 (PD-L1) messenger RNA (mRNA) derived from tumor-educated platelets (TEPs) has not been well investigated in patients with advanced non-small cell lung cancer (NSCLC). A few reports have examined whether mRNA in TEPs can predict the clinical responses of patients with advanced NSCLC following immunotherapy. This study aimed to identify novel biomarkers to improve the clinical benefits and outcomes of NSCLC patients.

Methods: Advanced NSCLC patients receiving a combination of immunotherapy and chemotherapy, or immunotherapy alone as a first- or second-line treatment at the Fudan University Shanghai Cancer Center were enrolled in this study. All the patients had wild-type epidermal growth factor receptor/anaplastic lymphoma kinase. The patients were enrolled in clinical trials for immune checkpoint inhibitors (ICIs), including nivolumab, pembrolizumab, atezolizumab, durvalumab, tremelimumab, and camrelizumab. Tumoral PD-L1 expression was tested by immunohistochemistry (PD-L1 22C3 pharmDx kit, Agilent, Santa Clara, CA, USA) in archived tissue samples, when available, to calculate the tumor proportion scores (TPSs). RNA and exosomal RNA of blood were isolated before immunotherapy using the Yunying RNA extraction kit (Yunying Medicine, Shanghai, China). The concentration and quality of the RNA was determined using a Qubit fluorometer (Life Technologies, Carlsbad, CA, USA). Finally, we analyzed the predictive value of TEP-derived PD-L1 mRNA expression and association with the level of the tumoral PD-L1 expression.

Results: In total, 72 patients were enrolled in this study. Most of the patients were male (n=54, 75.0%), had adenocarcinoma (n=49, 68.1%). We found there was no significant correlation between the TEP-derived mRNA of PD-L1 and tumoral PD-L1 expression based on the results of the Pearson Correlation test ($r=-0.19$, $P=0.233$). Based on the median of PD-L1 mRNA, 72 patients were divided into a high PD-L1 group and a low PD-L1 group. We found that 19 patients (44.4%) responded to immunotherapy [partial response or progression-free survival (PFS) >6 months] in the high PD-L1 group, but only five patients (13.9%) responded to immunotherapy in the low PD-L1 group ($P<0.01$). The median PFS of the low PD-L1 group was lower than that of the high PD-L1 group (2.8 vs. 8.3 months, $P<0.001$). For the patients who were treated with immunotherapy alone (n=64), a similar PFS advantage was observed in the high PD-L1 group (2.8

vs. 8.0 months, $P=0.002$).

Conclusions: This article presented the first data on TEP-derived PD-L1 mRNA in advanced NSCLC patients following immunotherapy and showed the potential advantage of using it as the surrogate biomarker for predicting the PFS and overall survival of patients following immunotherapy.

Keywords: Immunotherapy; biomarker; tumor-educated platelet-derived programmed death ligand-1 messenger RNA (TEP-derived PD-L1 mRNA); non-small cell lung cancer (NSCLC)

Submitted Jan 09, 2024. Accepted for publication Feb 15, 2024. Published online Feb 28, 2024.

doi: 10.21037/tlcr-24-29

View this article at: <https://dx.doi.org/10.21037/tlcr-24-29>

Introduction

Lung cancer is the primary cause of cancer-related death worldwide (1). Every year, many people are diagnosed with or die from lung cancer (1). Lung cancer includes non-small cell lung cancer (NSCLC) (which accounts for 85% of all lung cancer cases) and small cell lung cancer (SCLC) (which

accounts for 15% of all lung cancer cases) (2,3). Recently, unprecedented survival benefits have been achieved in selected patients using small molecule tyrosine kinase inhibitors or immunotherapy; however, the survival and overall cure rates of NSCLC patients remain low. Thus, continued research needs to be conducted into new therapy strategies to improve the clinical benefits and outcomes of NSCLC patients (4).

Immune checkpoint inhibitors (ICIs) have demonstrated encouraging success in the treatment of NSCLC patients, including locally advanced and metastatic patients without driver mutations. ICIs are now a primary part of the first- and second-line treatment armamentarium as monotherapy, combined with chemotherapy, or after definite chemoradiotherapy in advanced NSCLC patients (3). ICI monotherapy produced better clinical results than traditional chemotherapy in an unselected population of patients with advanced NSCLC (5). Further, extensive research has been conducted to identify predictive biomarkers for the initial ICI response. Tumor programmed death ligand-1 (PD-L1) expression, the tumor mutational burden (TMB), deficient mismatch repair, tumor infiltrating lymphocytes, and related gene expression signatures have been identified as potential predictors, and various other markers are under investigation (6-12). However, due to tumor heterogeneity, sampling bias, antibody discrepancy, and the poor feasibility of dynamic monitoring, obvious limitations exist among these biomarkers. Conversely, liquid biopsy has many advantages, as it enables the non-invasive overview of real-time changes in cancer and other diseases, including circulating tumor cells, circulating tumor DNA, circulating messenger RNA (mRNA), microRNA, exosomes, and tumor-educated platelets (TEPs) (13,14).

Circulating exosomal PD-L1 has been shown to be immunosuppressive and predict an anti-programmed death-1 (PD-1) response in patients with metastatic

Highlight box

Key findings

- This research presents the data of tumor-educated platelet (TEP)-derived programmed death ligand-1 (PD-L1) messenger RNA (mRNA) in advanced non-small cell lung cancer (NSCLC) patients following immunotherapy, and discusses the potential advantage of using it as a surrogate biomarker for predicting the progression-free survival (PFS) and overall survival (OS) of NSCLC patients following immunotherapy.

What is known and what is new?

- It has been reported that the platelet PD-L1 protein in NSCLC could reflect collective tumor PD-L1 expression, play important roles in tumor immune evasion, and overcome the limitations associated with the histological quantification of often heterogeneous intratumoral PD-L1 expression. TEP-derived mRNA has been employed for cancer diagnosis, mutation detection, and treatment monitoring.
- In this study, we prospectively detected pre-treatment PD-L1 mRNA and analyzed its prediction value for immunotherapy in advanced NSCLC patients.

What is the implication, and what should change now?

- In the present study, we demonstrated that TEP-derived PD-L1 mRNA could predict the response to immunotherapy, PFS, and OS of advanced NSCLC patients. The relationship between the level of TEP-derived PD-L1 and immune checkpoint inhibitor (ICI) resistance needs to be investigated further in the future. Additionally, the differences between TEP-derived PD-L1 and other biomarkers, such as tumor mutational burden and exosome PD-L1, also need to be studied.

melanoma, which highlighted the potential of circulating biomarkers for cancer immunotherapy (15,16). Similarly, as the second most abundant cells in the blood, TEPs were studied in cancers by various ways, which could transfer information between tumors and immune cells (17). One study suggested that the platelet PD-L1 protein in NSCLC could reflect collective tumor PD-L1 expression, play important roles in tumor immune evasion, and overcome the limitations associated with the histological quantification of often heterogeneous intratumoral PD-L1 expression (18). The mRNA in TEPs has been employed for cancer diagnosis, mutation detection, and treatment monitoring (19-22). The role of PD-L1 mRNA derived from TEPs has not yet been well investigated. However, few reports have indicated that the mRNA in TEPs can predict the clinical response of advanced NSCLC patients after immunotherapy. In this study, we prospectively detected the pre-treatment PD-L1 mRNA and analyzed its prediction value for immunotherapy in advanced NSCLC patients. We present this article in accordance with the REMARK reporting checklist (available at <https://tlcr.amegroups.com/article/view/10.21037/tlcr-24-29/rc>).

Methods

Patient population and study design

Advanced NSCLC patients receiving a combination of immunotherapy and chemotherapy, or immunotherapy alone as the first- or second-line treatment at the Fudan University Shanghai Cancer Center (FUSCC) from January 2016 to July 2018 were enrolled in this study. All the patients had wild-type epidermal growth factor/anaplastic lymphoma kinase receptor. The patients were enrolled in clinical trials for ICIs, including nivolumab, pembrolizumab, atezolizumab, durvalumab, tremelimumab, and camrelizumab. The ICIs were administrated as per the study protocols, and the therapeutic effects were evaluated as a complete/partial response (CR/PR), stable disease (SD), or progressive disease (PD) according to the response evaluation criteria in solid tumors (RECIST) 1.1. This study was performed in accordance with the principles of the Declaration of Helsinki (as revised in 2013). The Institutional Review Board of Fudan University Shanghai Cancer Center approved the study (Date 2012-12-24; No. 050432-4-1212B), and all patients provided informed consent.

Tumoral PD-L1 expression was tested by immunohistochemistry (PD-L1 22C3 pharmDx kit,

Agilent, Santa Clara, CA, USA) in archived tissue samples, when available, to calculate the tumor proportion scores (TPSs). 5 mL of peripheral blood samples were collected before immunotherapy from all the patients.

Platelet collection and purification

Platelets and plasma were isolated from the same sample of whole blood in tubes containing ethylenediamine tetra acetic acid anti-coagulant. The red cells, white cells, and aggregates were removed by centrifugation at room temperature for 20 min at 1,200 r/min, resulting in platelet-rich plasma. 10 µL of each obtained sample was used for the subjecting Swiss Giemsa staining. Samples with high purity, devoid of impurities such as leukocytes in the field of view, proceeded to the next steps of the operation. Thereafter, the platelets were isolated from the platelet-rich plasma by centrifugation for 20 min at 2,100 r/min. Platelet RNA and exosomal RNA were isolated using the Yuning RNA extraction kit (Yuning Medicine, Shanghai, China). The concentration and quality of the RNA was determined using a Qubit fluorometer (Life Technologies, Carlsbad, CA, USA). As a standard control, three healthy subjects provided equivalent blood samples for mRNA detection.

PD-L1 mRNA detection

Total RNA was stored at -80 °C awaiting use, and the complementary DNA was synthesized using a RT MasterMix kit (Yuning Medicine). The quantitative polymerase chain reaction (PCR) was run on the ABI7500 (Applied Biosystems, Foster City, CA, USA) for 10 min at 95 °C followed by 50 cycles of 10 s at 95 °C and 50 s at 60 °C. The expression of human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the normalization control in the real-time PCR reactions. The following primers were used: PD-L1 forward, 5'-AGCACTGACATTCATCTTCCG-3'; PD-L1 reverse, 5'-GCTGGATTACGTCTCCTCCA-3'; PD-L1 probe, 5'-FAM-AATGTGGCATCCAAGATAC-MGB-3'; GAPDH forward, 5'-CTCTGCTCC T C C T G T T C G A C - 3', GAPDH forward, 5'-ATGGTGTCTGAGCGATGTGG-3'; GAPDH probe, 5'-FAM-CGTCGCCAGCCGA-MGB-3'. The cycle threshold (Ct) value was recorded as the threshold cycle of the PCR reaction, and the final mRNA expression value was generated from the PD-L1 Ct and GAPDH Ct value.

Table 1 Clinical characteristics of the study patients

Characteristics	Values
Gender	
Male	54 (75.0)
Female	18 (25.0)
Age (years)	61 [42–75]
Pathology	
Adeno	49 (68.1)
Squamous	23 (31.9)
PD-L1 TPS (%)	
<1	25 (34.7)
1–49	9 (12.5)
≥50	7 (9.7)
Unknown	31 (43.1)
Treatment line	
1st	14 (19.4)
2nd	58 (80.6)
Chemo combined	
No	64 (88.9)
Pemetrexed/carboplatin	5 (6.9)
Paclitaxel/carboplatin	3 (4.2)
Checkpoint inhibitors	
Nivolumab	50 (69.4)
Atezolizumab	11 (15.3)
Pembrolizumab	1 (1.4)
Camrelizumab	4 (5.6)
Tremelimumab	6 (8.3)
Best response	
Partial response	21 (29.2)
Stable disease	24 (33.3)
Progressive disease	23 (31.9)
Unevaluable	4 (5.6)

Data are presented as n (%) or median [range]. PD-L1, programmed death ligand-1; TPS, tumor proportion score.

Statistical methods

Progression-free survival (PFS) and overall survival (OS) were estimated using the Kaplan-Meier method. PFS was defined as the time from ICI initiation until the date of

disease progression or death. OS was defined as the time from ICI initiation until the date of death or last follow-up date. Patients were deemed to have a clinical benefit if they achieved an objective response (CR/PR) or had a SD duration of over 6 months. The median value was used as the PD-L1 mRNA cut-off value to define high PD-L1 expression or low PD-L1 expression. Using Cox regression analysis, we also analyzed the relationship between prognosis and clinical-pathological characteristics, such as age, gender, pathology, TPS (PD-L1 expression), with or without chemotherapy, lines of therapy, and expression level of TEP-derived PD-L1 mRNA. A two-sided P value <0.05 level was considered statistically significant.

Results

Patient characteristics

Of the 72 patients enrolled in this study, 64 (88.9%) received immunotherapy only, while the others were treated with immunotherapy in combination with platinum-contained chemotherapy. In this study, most of the patients were male (n=54, 75.0%), had adenocarcinoma (n=49, 68.1%), and received single-agent immunotherapy as a second-line therapy (n=58, 80.6%). The median follow-up was 6.3 months. Nivolumab was the most commonly used inhibitor (n=50, 69.4%) followed by atezolizumab (n=11, 15.3%) (*Table 1*).

According to the RECIST 1.1 criteria, 21 patients achieved PR, with an objective response rate (ORR) of 29.2% as shown in *Table 1*. Seven patients (9.7%) had a SD duration of over 6 months. After a median follow-up of 15.8 months, there were 52 events of PD or death. The median PFS was 3.81 months (95% confidence interval: 2.0 to 5.6 months). There were 20 deaths (34.5%) in the second-line cohort; however, there was only one death (7.1%) in the first-line cohort (P=0.015).

TEP-derived PD-L1 mRNA was uncorrelated with PD-L1 TPS

Blood samples were collected from all patients. The range of PD-L1 mRNA expression of TEPs detected in 72 patients was from 1.7 to 9.9, in which the median value was 3.17. PD-L1 expression was examined successfully in the archived tissue of 41 patients (56.9%). Based on the review by two independent pathologists on PD-L1 TPS, the rate of <1%, 1–49%, ≥50% was 56.8%, 20.5% and 15.9%, respectively. The range of the PD-L1 TPS was 0–75%, in which the

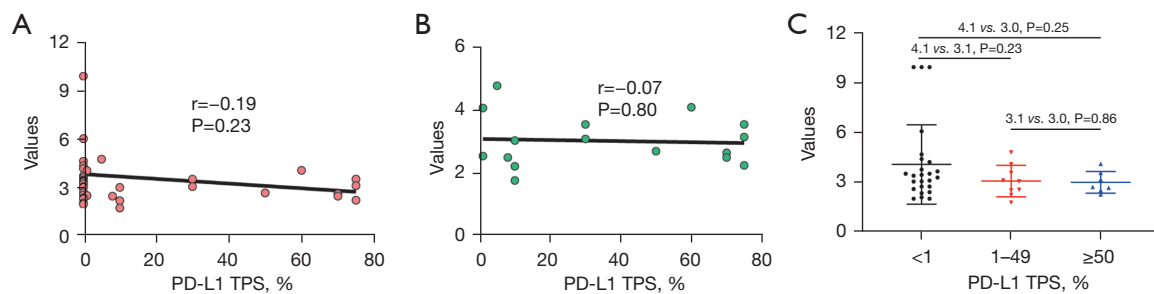


Figure 1 The correlation of PD-L1 TPS (X-axis) and TEP-derived mRNA value (Y-axis). (A) PD-L1 TPS and TEP-derived mRNA in all patients. (B) PD-L1 TPS ($\geq 1\%$) and corresponding TEP-derived mRNA. (C) The level of TEP-derived mRNA could not be used to stratify PD-L1 TPS across these three levels. PD-L1, programmed death ligand-1; TPS, tumor proportion score; TEP, tumor-educated platelet; mRNA, messenger RNA.

median TPS was 0.

The mRNA derived from TEP showed a mild inverse relationship with PD-L1 TPS (*Figure 1*). There was no significant correlation between the TEP-derived mRNA of PD-L1 and tumoral PD-L1 expression based on the results of the Pearson Correlation test ($r=-0.19$, $P=0.233$). For those with positive tumoral PD-L1 ($TPS \geq 1\%$), the correlation was also insignificant ($r=-0.066$, $P=0.8$).

PD-L1 mRNA predicts patient survival after immunotherapy

Based on the median of TEP-derived PD-L1 mRNA, all patients were allocated to the high PD-L1 group ($n=36$) or the low PD-L1 group ($n=36$). *Table 2* shows patient background and the effect of ICI treatment for each group. In the high PD-L1 group, 16 patients achieved PR, which was more than five patients in the low PD-L1 group (44.4% *vs.* 13.9%, $P<0.01$). We also found that 19 patients (44.4%) responded to immunotherapy (PR or PFS >6 months) in the high PD-L1 group, but only five patients responded to immunotherapy in the low PD-L1 group (52.8% *vs.* 13.9%, $P<0.01$).

As *Figure 2* shows, the median PFS of the low PD-L1 group was significantly shorter than that of the high PD-L1 group (median PFS: 2.8 *vs.* 8.3 months, $P<0.001$, *Figure 2A*). The median OS was not reached in the high PD-L1 group, while it was 13.5 months in the low PD-L1 group ($P=0.0047$, *Figure 2B*). For the patients who were treated with immunotherapy alone, a similar PFS advantage was observed in the high PD-L1 group (2.8 *vs.* 8.0 months, $P=0.002$, *Figure 2C*). In the *Figure 2D*, the median OS of patients treated only with immunotherapy in the high PD-

L1 group was greater than that in the low PD-L1 group ($P=0.0126$, *Figure 2D*). What is more, the median PFS of the high PD-L1 group with negative TPS, was also significantly longer than that of the low PD-L1 group ($P=0.0074$, *Figure 2E*). In the population, the median OS was not reached in the high PD-L1 group, while it was 13.47 months in the low PD-L1 group ($P=0.0344$, *Figure 2F*).

We also analyzed the difference in the expression of TEP-derived mRNA across different clinical responses (*Figure 3*). We found that patients with clinical benefit (PR or PFS >6 months) had higher TEP-derived mRNA expression than the other patients (median: 2.97 *vs.* 3.44 months, $P=0.012$).

Univariate analysis showed that female, lines of therapy are associated with worse prognosis, while high expression level of TEP-derived mRNA relates to longer survival (*Tables S1,S2*). By using Cox's hazard regression model, we found that the pathologic type of NSCLC, expression of PD-L1 protein and high expression level of TEP-derived mRNA constitute the independent risk factors.

Discussion

In the era of immunotherapy, biomarkers are becoming increasingly important in patient selection to avoid unnecessary costs and toxicity (23). To date, most immunotherapy biomarkers took only tumor biological information (e.g., the TPS, TMB, or mismatch repair) or host factors (e.g., patient smoking status or immune cell PD-L1) into account (23,24). However, the therapeutic effects of ICIs are obviously affected by factors related both to the cancer and patient. In the present study, we showed that TEP-derived PD-L1 mRNA could predict

Table 2 Patient characteristics and the effect of ICI treatment for the high and low PD-L1 mRNA group

Characteristics	Total (n=72)	High PD-L1 group (n=36)	Low PD-L1 group (n=36)	P value (χ^2 test)
Gender				0.414
Male	54	29	25	
Female	18	7	11	
Age (years)	61 [42–75]	61 [48–75]	60 [42–72]	0.131
Pathology				0.613
Adeno	49	23	26	
Squamous	23	13	10	
PD-L1 TPS (%)				0.037
<1	25	14	11	
1–49	9	3	6	
≥50	7	2	5	
Unknown	31	17	14	
Treatment line				0.372
1st	14	9	5	
2nd	58	27	31	
Chemo combined				0.196
No	64	31	33	
Pemetrexed/carboplatin	5	2	3	
Paclitaxel/carboplatin	3	3	0	
Response				0.019
Partial response	21	16	5	
Stable disease	24	7	17	
<6 months	7	4	3	
≥6 months	17	3	14	
Progressive disease	23	11	12	
Unevaluable	4	2	2	

Data are presented as n or median [range]. ICI, immune checkpoint inhibitor; PD-L1, programmed death ligand-1; mRNA, messenger RNA; TPS, tumor proportion score.

the immunotherapy response, PFS, and OS of advanced NSCLC patients.

A substantial information exchange occurs in the circulation between tumor cells, immune cells and circulating platelets (TEPs) (25–27). One study reported that PD-L1 mRNA expression in plasma-derived exosomes could help predict the clinical responses of melanoma or NSCLC patients who received anti-PD-1 therapy (28). Colarusso *et al.* reported that PD-L1 on platelets

represent an easy-to-use clinical approach to predict ICI responsiveness (29). Taken together, the predictive value of TEP PD-L1 in immunotherapy is supported by its biological process and related research findings (including those of the present study). TPSs are commonly used in clinic to guide ICI treatment (e.g., the companion diagnostic antibody DAKO 22C3 is used for pembrolizumab) (30). However, the predictive value of the TPS has not been validated in combination therapies of some ICIs and

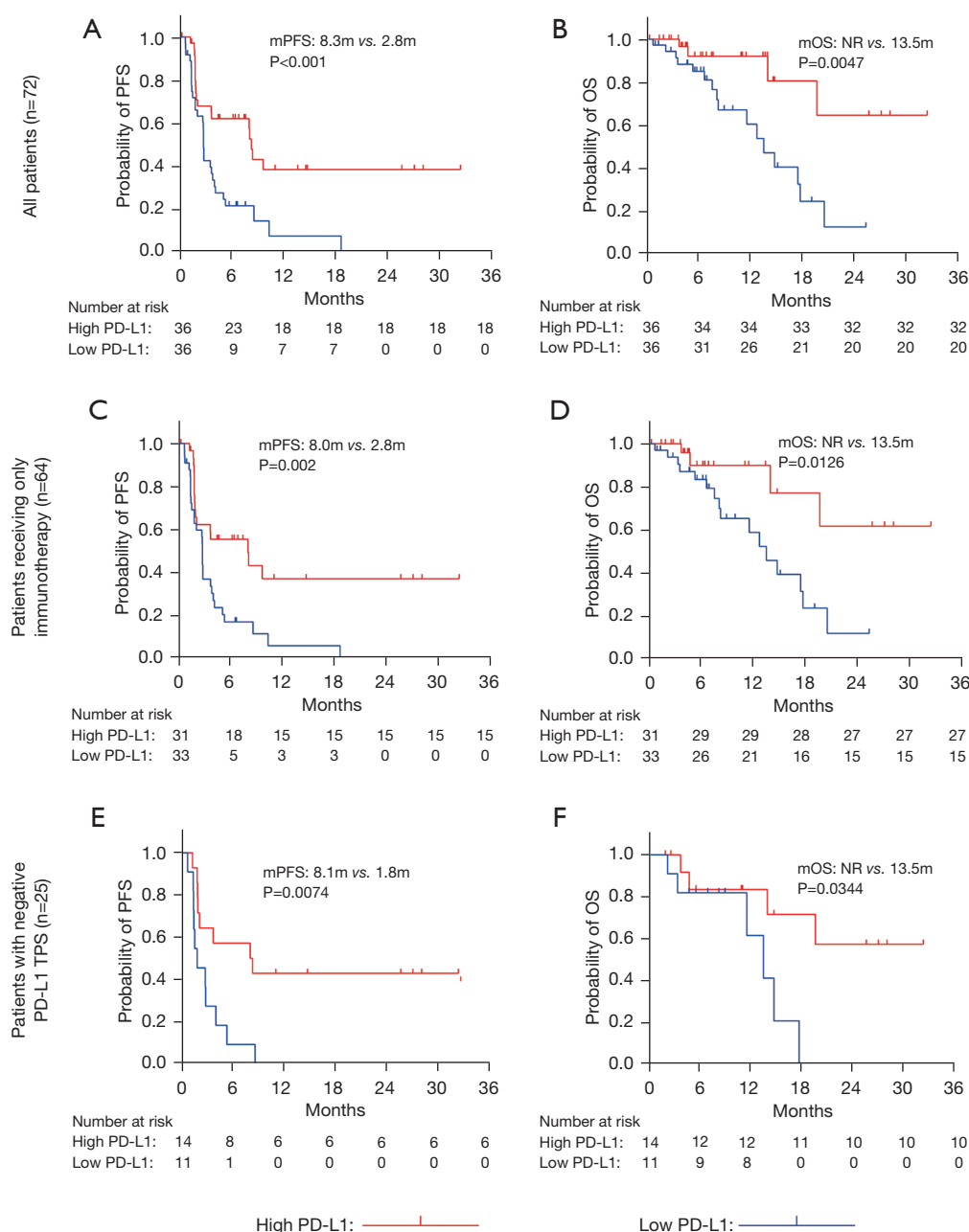


Figure 2 TEP-derived mRNA of PD-L1 predicts patient survival. For (A,B), the PFS and OS of all patients in the high PD-L1 group (n=36) and low PD-L1 group (n=36). For (C,D), the PFS and OS of patients receiving only immunotherapy in the high PD-L1 group (n=31) and low PD-L1 group (n=33). For (E,F), the PFS and OS of patients with negative PD-L1 TPS in the high PD-L1 group (n=14) and low PD-L1 group (n=11). mPFS, median progression-free survival; PD-L1, programmed death ligand-1; mOS, median overall survival; NR, not reached; TPS, tumor proportion score; TEP, tumor-educated platelet; mRNA, messenger RNA.

chemotherapy. In the present study, we found that TEP-derived PD-L1 was not correlated with the TPS, which suggests that TEP PD-L1 mRNA has independent value in predicting immunotherapy outcomes for advanced NSCLC

patients. These findings had strongly promoting influence on current treatment protocols or future research directions of NSCLC. For example, it is worth to study the potential value of TEP PD-L1 mRNA in other treatment, such as

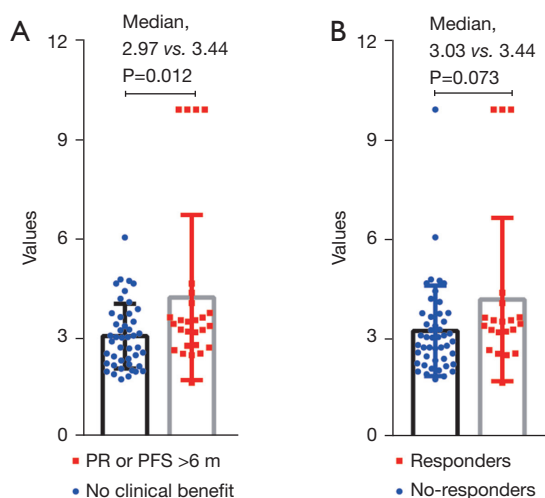


Figure 3 The expression of TEP-derived mRNA (Y-axis) across different clinical responses. (A) PR or PFS >6 months, and no clinical benefit. (B) Responders and non-responders. PR, partial response; PFS, progression-free survival; TEP, tumor-educated platelet; mRNA, messenger RNA.

targeted therapy or the combination of chemotherapy and immunotherapy. Additionally, it is also valuable to explore the predictive capacity of TEP PD-L1 mRNA in early stage of lung cancer and other cancers.

For the biological mechanism of this observation in the study, we think it may be related to the function of blood platelets. Platelets come from megakaryocytes and circulate in peripheral blood, which plays their strong role in hemostasis (31,32). Meanwhile, platelets have the capacity of to transfer tumor-associated biomolecules, which includes mRNA from cancer (33). Thus, platelets have potential applicability to predict the cancer diagnostics and therapy, which is consistent with our conclusions. However, one study reported that platelet RNA may have small capacity for predicting anti-PD-1 nivolumab therapy (34). The unexpected difference might be due to the distinct races, machines and limited therapeutic.

It is interesting that significant correlation between TEP-derived mRNA and tumoral PD-L1 expression was not detected. There are several reasons possibly as followed. Firstly, it is different for the samples tested. The tumoral PD-L1 expression was tested in archived tissue samples, while TEP-derived mRNA was isolated from blood. Secondly, the study should include more samples to define the conclusion in the study. Thirdly, TEP-derived mRNA and tumoral PD-L1 are distinct matters affected

by different conditions. Finally, the time interval between tissue collection and platelet collection was within one year, which might influence the negative correlation. Therefore, it is rational that there was a mild inverse relationship between TEP-derived mRNA and PD-L1 TPS.

The mechanisms of acquired resistance to ICIs have been extensively studied recently (35). For example, overactive PI 3-kinase in cancer and immune-dysregulation was reported to account for immunotherapy resistance (36). We intend to study the relationship between the level of TEP-derived PD-L1 and ICI resistance in the future. This study had several limitations. First, it was a study with a moderate sample size, which limits the confidence in determining the prediction potential of TEP-derived PD-L1. Thus, further research needs to be conducted to confirm the predictive value of TEP-derived PD-L1. Second, we did not compare it with other biomarkers, such as the TMB or exosome PD-L1. Third, we did not investigate the presence of PD-L1 in blood platelets using fluorescence in situ hybridization (FISH)/confocal microscopy. However, these limitations do not affect the conclusions of this study. Nevertheless, this study identified a surrogate biomarker that can be used in future studies to help select or stratify patients undergoing different immunotherapies. Further, our present study showed that TEP could be used in liquid biopsy-based analyses and thus guide clinical decisions about immunotherapy in advanced NSCLC patients.

Conclusions

In summary, this article presented the first data of TEP-derived PD-L1 mRNA of advanced NSCLC patients who underwent immunotherapy and showed the potential advantage of using it as a surrogate biomarker to predict PFS and OS following immunotherapy.

Acknowledgments

Funding: This work was supported financially by the Shanghai Municipal Health Commission Project (No. 2020CXJQ02) and the Huilan Public Welfare Fund Project (No. HL-HS2020-121).

Footnote

Reporting Checklist: The authors have completed the REMARK reporting checklist. Available at <https://tclr.amegroups.com/article/view/10.21037/tclr-24-29/rc>

Data Sharing Statement: Available at <https://tlcr.amegroups.com/article/view/10.21037/tlcr-24-29/dss>

Peer Review File: Available at <https://tlcr.amegroups.com/article/view/10.21037/tlcr-24-29/prf>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://tlcr.amegroups.com/article/view/10.21037/tlcr-24-29/coif>). S.W. receives payment or honoraria from Lilly, Novartis Pharma, Chugai Pharma, Bristol-Myers, Ono Pharmaceutical, Daiichi Sankyo, Taiho Pharmaceutical, Nippon Kayaku, Kyowa Kirin, Merck, Takeda Pharmaceutical, Celltrion and AstraZeneca. D.Z. and Z.G. are from Yunying Medicine Inc., Shanghai, China. The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was performed in accordance with the principles of the Declaration of Helsinki (as revised in 2013). Approval was granted by the Ethics Committee of Fudan University Shanghai Cancer Center (Date 2012-12-24; No. 050432-4-1212B). Informed consent was obtained from all the individual participants included in the study.

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Cite this article as: Hu Z, Wang N, Zhang Y, Zhang D, Sun S, Yu H, Lin Y, Zhao X, Wang H, Wu X, Ichiki Y, Watanabe S, Gong Z, Chang J, Wang J. PD-L1 mRNA derived from tumor-educated platelets as a potential immunotherapy biomarker in non-small cell lung cancer. *Transl Lung Cancer Res* 2024;13(2):345-354. doi: 10.21037/tlcr-24-29