Implication of PD-L1 polymorphisms rs2297136 on clinical outcomes of patients with advanced NSCLC who received PD-1 blockades: A retrospective exploratory study

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Abstract. Clinically, programmed death-1 (PD-1) blockades have demonstrated promising therapeutic outcomes for patients with advanced non-small cell lung cancer (NSCLC). The present study aimed to examine the impact of programmed death-ligand 1 (PD-L1) polymorphism on clinical outcomes of patients with advanced NSCLC who were treated with PD-1 blockades therapy. The present study was designed as a retrospective analysis, where a consecutive screening of 89 patients with advanced NSCLC who received PD-1 blockades monotherapy were screened. Biological specimens were collected to determine the presence of polymorphism and PD-L1 mRNA expression through genotyping. The analysis focused on examining the relationship between the genotype status of PD-L1 polymorphism and clinical outcomes. Among the 89 patients with advanced NSCLC, the use of PD-1 blockades monotherapy resulted in objective response rate (ORR) of 22.5%, a median progression-free survival (PFS) of 3.4 months [95% Confidence Interval (CI): 1.80-5.00) and a median overall survival (OS) of 11.3 months (95% CI: 7.93-14.67). The analysis of polymorphism indicated that only rs2297136 had clinical significance. Among the 89 patients with NSCLC, the prevalence of rs2297136 was as follows: A total of 58 cases (65.2%) had the AA genotype, 28 cases (31.5%) had the AG genotype and 3 cases (3.4%) had the GG genotype. This resulted in a minor allele frequency of 0.19, which was in consistent with Hardy-Weinberg Equilibrium (P=0.865). The correlation analysis between genotype status of rs2297136 and clinical outcomes indicated that patients with the AA genotype had an ORR of 19.0%, while those with the AG/GG genotype had an ORR of 29.0% (P=0.278). Additionally, the median PFS for the AA genotype was 2.95 months, compared with 5.30 months for the AG/GG genotype (P=0.038). Accordingly, median OS of the AA and AG/GG genotypes was 8.8 and 18.4 months, respectively (P=0.011). The mRNA expression of PD-L1 was significantly higher in patients with AG/GG genotype compared with those with AA genotype (P<0.001). In clinical practice, PD-1 blockades demonstrated promising effectiveness in treating patients with advanced NSCLC. The presence of the rs2297136 variant in PD-L1 gene could potentially be used as a biomarker to predict the clinical outcomes of PD-1 blockades.

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Introduction

Lung cancer is a prevalent type of solid tumors worldwide and non-small cell lung cancer (NSCLC) makes up ~85% in lung cancer cases (1). In China alone, there are almost 688,000 new cases and 604,000 deaths from lung cancer each year (2). In recent years, significant progress has been made in identifying various driver genes in NSCLC and developing targeted drugs specifically for these mutations. This has led to NSCLC with positive driver gene mutations becoming one of the most successful cancers to be treated with precision medicine (3). Furthermore, the use of programmed death-1 (PD-1) blockades has shown promising and long-lasting therapeutic effects for patients without driver gene mutations, resulting in a significant increase in the 5-year survival rate of advanced NSCLC patients ranging from 5 to >15% currently (4). Over the past few years, the use of PD-1 blockade monotherapy as standard treatment for patients with advanced NSCLC in the second-line or subsequent-line therapy has been established (5). The combination of pembrolizumab, nivolumab and atezolizumab with chemotherapy has emerged as the first-line therapy for patients with advanced NSCLC; as evidenced by the Keynote, Checkmate, and Impower series clinical trials (6). Similarly in China, sintilimab, camrelizumab, tislelizumab and other PD-1 blockades are also approved for patients with advanced NSCLC (7) and widely used with improved results (8). However, it is important to note that the overall response of patients with advanced NSCLC who received PD-1 blockades is still considered disappointing. While PD-1 blockades have shown promise in some patients, there is still need to identify efficacious biomarkers that can help refine the selection of patients who are likely to benefit from this therapy.

Nevertheless, accurately predicting the therapeutic response of PD-1 blockades remained challenging, particularly

in cases where these blockades were administered with single agent, as there were no specific biomarkers available in clinical practice (9). A significant number of patients with advanced NSCLC did not exhibit any response to PD-1 blockades, resulting in an ~20% objective response rate (ORR) for patients without available biomarkers (10). Currently, only programmed death-ligand 1 (PD-L1) expression, DNA mismatch repair (MMR) and tumor mutation burden (TMB) status can potentially be beneficial prediction markers for PD-1 blockades (11). Unfortunately, these are still not satisfactory biomarkers because of genetic heterogeneity and expression in tumors (12). Therefore, ORR of pembrolizumab in patients with high PD-L1 expression (PD-L1 ≥50%) and dMMR patients was found to be below 45% (44.8 and 43.8%) in the Keynote 024 and Keynote 177 clinical trials, respectively (13,14). This indicated the need to further investigate alternative biomarkers for more precise guidance in the use of PD-1 blockades.

The PD-L1 gene, located at chromosome 9p24.1, consists of 8 exons and demonstrates significant ethnic differences even within the Chinese population (15). Furthermore, the level of PD-L1 expression can vary among different cancer patients, including those with the same type of cancer (16). Previous studies have shown the correlation of various PD-L1 mutations with susceptibility and prognosis different cancers such as 901A>G with hepatocellular carcinoma (17), rs822339, rs1411262 rs2282055 and rs4143815 with prognosis of NSCLC patients with NSCLC who received nivolumab (18,19). This indicated that PD-L1 polymorphisms potentially contribute to the clinical outcomes of patients with NSCLC who received PD-1 blockades therapy. However, there is currently no consensus on the correlation between PD-L1 polymorphism and the effectiveness of PD-1 blockades among Chinese patients with advanced NSCLC currently.

Therefore, the present study aimed to retrospectively examine the clinical outcomes of patients with advanced NSCLC who received PD-1 blockades specifically focusing on the clinical significance of PD-L1 polymorphisms.

Patients and methods

Design of the study and eligibility criteria. In recent years, the Chinese National Medical Products Administration has approved PD-1 blockades for the treatment of patients with advanced NSCLC. Therefore, a significant number of patients with advanced NSCLC have undergone PD-1 blockade monotherapy in clinical practice. The present study aimed to retrospectively include patients with advanced NSCLC who underwent PD-1 blockade monotherapy (any PD-1 blockade licensed in China) at the Department of Thoracic Surgery of the Affiliated Hospital of Hebei University (Baoding, China) from July 2018 to June 2022. The inclusion criteria were as follows: i) Diagnosis of NSCLC with pathological staging of IIIb or IV confirmed by pathological expert; ii) age ≥18 years; iii) patients with previously-treated advanced NSCLC who received PD-1 blockades monotherapy for at least one cycle in clinical practice; and iv) at least one measurable target lesion. The main exclusion criteria were: i) Patients with a history of autoimmune disease or clinical symptoms unsuitable for PD-1 blockades therapy; ii) patients diagnosed with one or more tumors or serious diseases that might compromise their living status; and iii) insufficient availability of demographic characteristics or efficacy assessment data according to the investigators' judgment. Eventually, a total of 89 patients with advanced NSCLC met the eligibility criteria and were included in the present study. The primary objective of the present study was to identify the association between PD-L1 polymorphisms and clinical outcomes of PD-1 blockades, including ORR, disease contrail rate (DCR), progression-free survival (PFS) and overall survival (OS).

The protocol and additional materials for the present study were approved (approval no. 2022-KY-11053) by the Ethics Committee of the Affiliated hospital of Hebei university (Baoding, China). Each enrolled patient provided written informed consent in accordance to the recommendations of the Declaration of Helsinki (1964).

Therapeutic regimens of PD-1 blockades in clinical practice. All 89 patients with advanced NSCLC received PD-1 blockade monotherapy for a minimum of one cycle in clinical practice. The PD-1 blockades utilized in the present study were approved in mainland China and clinically available for Chinese patients. These included nivolumab (Bristol-Myers Squibb Co.), pembrolizumab (Merck KGaA), camrelizumab (Jiangsu Hengrui Pharmaceuticals Co., Ltd.), sintilimab (Innovent) and tislelizumab (BeiGene, Inc.). Camrelizumab, sintilimab, pembrolizumab and tislelizumab were administered intravenously at a dose of 200 mg on day 1, while nivolumab was given intravenously with a dose of 360 mg on day 1. A treatment cycle of 21 days was completed. (20). The administration of these five PD-1 blockades persisted until either disease progression or intolerable adverse reactions in the patients.

Assessment of response and protocol of follow-up. Since PD-1 blockades monotherapy was used in the present study, the iRECIST criteria were utilized to evaluate the therapeutic response of the patients (21). As aforementioned, all 89 patients included in the present study had at least one measurable target lesion, assessed through radiological scans such as CT or MRI at baseline and throughout PD-1 blockades treatment. ORR and DCR were calculated according to the best overall response during administration of PD-1 blockades. Specifically, ORR was defined as the proportion of patients with complete response (CR) and partial response (PR) among the 89 patients. DCR was determined as the proportion of patients with CR, PR and stable disease (SD) among the 89 patients.

Furthermore, clinical and demographic characteristics of the 89 patients were retrieved from the electronic medical record system at the Affiliated Hospital of Hebei University (Baoding). Additionally, the disease progression status of each patient was assessed, and follow-up was conducted through phone communication to gather prognostic data. The therapeutic regimens of patients who experienced progression after PD-1 blockades monotherapy were documented and their health status was primarily obtained accordingly. PFS was defined as the duration from the initiation of PD-1 blockade

Table I. Association between genotypes status of the four polymorphisms and PFS of the 89 patients with advanced non-small cell lung cancer.

Polymorphisms	Primer sequence (5'→3')	Location	Minor allele frequency	Median PFS (months)	P-value
rs2297136	GGAGGAGACGTAATCCAGCA CCAGGCTCCCTGTTTGACT	Non-coding region	0.19	2.95 vs. 5.3 (AA vs. AG/GG)	0.038
rs17718883	GGACAGCATCAAGCTATGTACG CTCTTGGAATTGGTGGTGGT	Coding region	0.00	NA	NA
rs822339	TAACTCTGGCCCAAGGAAAA TTTTGGTCTGTTTATGTCACTGG	Intron region	0.39	3.2 vs. 3.5 (AA vs. AG/GG)	0.315
rs1411262	TGGTTTTGGGATTGAGTTCAG TCCTGTGGGGAAGCTATGTT	Intron region	0.42	3.5 vs. 3.1 (TT vs. TC/CC)	0.536

PFS, progression-free survival.

treatment to the date of disease progression or death, whichever occurred first. OS was defined as the initiation from the date of PD-1 blockades treatment to the date of death from any cause.

Genotyping of PD-L1 gene polymorphism. Concerning the analysis of PD-L1 gene polymorphism, the DNA specimens of each patient were primarily extracted from their respective peripheral blood or cancer tissue biopsies, obtained before the initiation of PD-1 blockade treatment, according to a previous study (22).

Additionally, single nucleotide polymorphisms in PD-L1 gene of the present study were adopted from a previous study (23), including rs2297136, rs17718883, rs822339 and rs1411262. The preliminary analysis comparing the genotype status of these polymorphisms and PFS of the 89 patients is presented in Table I. Notably, only rs2297136 exhibited clinical significance. Therefore, the present study primarily focused on the results of rs2297136.

Genotyping of rs2297136 polymorphism was carried out using PCR-RFLP methods derived from a previous study (23). The forward primer for the PCR products of rs2297136 was 5'-GGAGGAGACGTAATCCAGCA-3', and the reverse primer was 5'-CCAGGCTCCCTGTTTGACT-3', resulting in a PCR product of 216 bp. PCR product was disposed using *PspOMI* restriction enzyme. The genotyping of rs2297136 was determined based on the following criteria: AA (216 bp stripe); AG (216 bp stripe, 104 bp stripe and 112 bp stripe); GG (104 bp stripe and 112 bp stripe).

Analysis of PD-L1 gene mRNA expression. Aiming to investigate the potential association between PD-L1 polymorphism and PD-L1 gene mRNA expression, available fresh specimens of peripheral blood mononuclear cells (PBMC) were initially collected from 89 patients with advanced NSCLC. Unfortunately, 8 patients failed to obtain the qualified RNA specimens. Ultimately, a total of 81 patients were included in PD-L1 gene mRNA expression analysis. The methodology of PD-L1 mRNA expression analysis was adopted from a previous study (23). Total RNA samples were extracted with TRIzol® (Thermo Fisher Scientific, Inc.) using RNAiso Plus reagents (Takara Biotechnology Co., Ltd.) as the RNA extraction buffer according to the manufacturer's instructions, and stored at -80˚C for mRNA expression analysis. A total of 500 ng RNA extracted from the PBMC was used as the templates for reverse-transcription polymerase chain reaction to prepare the first-stand of cDNA with the PrimeScript RT reagent Kit. Relative quantitative analysis of PD-L1 gene mRNA expression was carried out with Roche LightCycler 480 (Roche Diagnostics, Ltd.) using a SYBR Premix EX Taq system. The forward primer of PD-L1 was 5'-TTCAATGTG ACCAGCACACTGAG-3', the reverse primer was 5'-TTTTCA CATCCATCATTCTCCCT-3'. The amplification system was comprised of a 20 μl containing 10 μl SYBR Premix EX Taq, 0.2 μl of each primer (20 μM), 7.6 μl double distilled water (ddH2O) and 2 μl cDNA. PD-L1 mRNA expression level was calculated by comparative Cq ($2^{-\Delta\Delta Cq}$) method (24), with GAPDH mRNA expression serving as an endogenous control. The forward primer sequences used for GAPDH mRNA expression was 5'-GAAGGTGAAGGTCGGAGTCAAC-3', the reverse primer was 5'-CAGAGTTAAAAGCAGCCCTGG T-3' (25). The thermocycling conditions were as follows: 50˚C for 2 min and 95˚C for 2 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 30 sec.

Statistical analysis. The statistical analysis presented in the present study was conducted using SPSS software version 25.0 (IBM Corp.). The conformity of the genotype status of the rs2297136 polymorphism with Hardy-Weinberg equilibrium was assessed through the chi-square test (22). Regarding the analysis of baseline characteristics, the distribution between proportion variables and genotype status of rs2297136 was carried out using the Mann-Whitney U non-parametric test, between the two groups. Data in the present study were presented as median (range) and the number of patients in percentages based on corresponding data category. PFS and OS were defined as aforementioned. Survival curves were generated using Stata 14.0 to illustrate survival data according to rs2297136 genotype status, with a log-rank test used to determine significant differences. Cox analysis was used for OS in multivariate analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Baseline characteristics and genotyping of PD-L1 gene rs2297136 polymorphism. Baseline characteristics of the 89 patients with advanced NSCLC were shown in Table II. It was revealed that all the 89 patients included in the present study were individuals commonly encountered in clinical practice with previously treated advanced NSCLC. Notably, 38.2% of the patients had an Eastern Cooperative Oncology Group (ECOG) performance status score of 2-3. Interestingly, 18 cases (20.2%) exhibited positive EGFR mutation, and 4 patients (4.5%) exhibited anaplastic lymphoma kinase positive rearrangement. Among the patients, 19 received PD-1 blockades as second-line therapy, while 70 underwent third-line or subsequent treatments. The present study utilized five PD-1 blockades, including camrelizumab (28.1%), sintilimab (23.6%), tislelizumab (22.5%), pembrolizumab (16.9%) and nivolumab (8.9%).

As outlined in the methods section, only rs2297136 demonstrated clinical significance in the preliminary analysis, as demonstrated in Table I. The prevalence of rs2297136 among the 89 patients with advanced NSCLC is detailed as follows: The AA genotype was observed in 58 cases (65.2%), the AG genotype was found in 28 cases (31.5%), and the GG genotype was noted in 3 cases (3.4%), resulting in a minor allele frequency (MAF) of 0.19, consistent with Hardy-Weinberg Equilibrium (P=0.865). Given the rarity of patients with GG genotype, patients with GG and AG were combined into one group in the subsequent analysis. The association between genotype status of rs2297136 and baseline characteristics is presented in Table II. Evidently, baseline characteristics of patients with AA and AG/GG genotypes were comparable and well-balanced (P>0.05).

Association between efficacy of PD-1 blockades and genotype status of rs2297136. Radiological evidence for the target lesions of the 89 patients with advanced NSCLC who received PD-1 blockade treatment was collected and assessed. According to iRECIST criteria, the best overall response during PD-1 blockade treatment indicated a CR in one patient (1.1%), PR in 19 patients (21.3%), SD in 37 patients (41.6%) and progressive disease (PD) in 32 patients (36.0%). This resulted in an ORR of 22.5% (95% CI: 14.3-32.6%) and a DCR of 64.0% (95% CI: 53.2-73.9%; Fig. 1). Specifically, the changes in the target lesions of the 89 patients after PD-1 blockade treatment according to genotype status of rs2297136 are depicted in Fig. 1. Target lesions of some patients shrunk significantly after the PD-1 blockade treatment. It was noteworthy that even patients with AG/GG genotype numerically demonstrated a higher ORR compared with patients with AA genotype [ORR of AG/GG vs. AA: 29.0% (95% CI: 14.2-48.0%) vs. 19.0% (95% CI: 9.9-31.4%)]. However, this difference did not reach statistical significance (χ^2=1.18, P=0.278). Additionally, the DCR for patients with AA and AG/GG genotype was 58.6% (95% CI: 44.9-71.4%) and 74.2% (95% CI: 55.3-88.1%), respectively (χ^2=2.13, P=0.145).

Association between prognosis of PD-1 blockades and genotype status of rs2297136. Regular follow-up was performed for the 89 patients with advanced NSCLC included in the present study, resulting in a mature prognostic data ultimately. The data cut-off date of the present study was November 15, 2022 and the median follow-up duration was 10.2 months (follow-up range: 0.9-32.5 months). Among these patients, 75 were observed to experience progression or death events, providing a maturity of 84.3% for PFS data. The PFS survival curve is presented in Fig. 2, revealing a median PFS of 3.4 months (95% CI: 1.80-5.00) for the 89 patients treated with PD-1 blockade monotherapy. Notably, a total of 15 patients experienced a sustained PFS benefit lasting over 12 months.

Additionally, 61 patients were documented to have succumbed, resulting in a maturity of OS data of 68.5%. The OS survival curve, also depicted in Fig. 2, revealed a median OS of 11.3 months (95% CI: 7.93-14.67) for the 89 patients with advanced NSCLC treated with PD-1 blockades. Interestingly, 10 patients achieved a sustainable OS benefit lasting over 24 months. Furthermore, as shown in Table SI, 49 patients received subsequent treatment upon progression during PD-1 blockade therapy. Among them, 21 received anlotinib regimen, 13 underwent chemotherapy, 9 were administered traditional Chinese medicine and the remaining 6 received PD-1/PD-L1 related therapy.

In exploring the connection between prognosis and the genotype status of rs2297136, additional survival analysis was conducted. As demonstrated in Fig. 3, patients with AG/GG genotype showed a tendency towards improved PFS compared with those with AA genotype [median PFS: 5.30 months (95% CI: 3.42-7.18) vs. 2.95 months (95% CI: 2.58-3.32)], reaching marginal statistical significance (χ^2=4.30, P=0.038). Furthermore, the association between OS and genotype status of rs2297136 was separately examined. As illustrated in Fig. 4, patients with AG/GG genotype exhibited a longer OS compared with those with AA genotype [median OS: 18.4 months (95% CI: 8.61-28.19) vs. 8.8 months (95% CI: 4.82-12.78)] and this difference was statistically significant (χ^2=6.43, P=0.011).

Additionally, to investigate the independent prognostic implication of rs2297136 for patients with advanced NSCLC, multivariate Cox analysis for OS was adopted subsequently. Initially, association analysis between OS and baseline characteristic subgroups in univariate analysis was carried out separately. The median OS and 95% CI according to baseline characteristic subgroups in univariate analysis were presented in Table III. Notably, it appeared that almost all patients might uniformly benefit from PD-1 blockades monotherapy uniformly. However, ECOG performance status and number of metastatic lesions exhibited a significant association with OS in the univariate analysis, as shown in Table III. This suggested that patients with ECOG performance status 0-1 score had a longer OS than that of patients with 2-3 score (median OS: 15.5 vs. 9.5 months, P=0.008), and patients with number of metastatic lesions ≤3 demonstrated improved OS than those >3 metastatic lesions (median OS: 13.5 vs. 9.5 months, P=0.019). Interestingly, patients with EGFR positive mutation demonstrated a trend towards inferior OS compared with those with EGFR negative mutation, although the difference was not statistically significant (median OS: 9.5 vs. 13.8 months, P=0.131). Subsequently, variables significantly associated with OS were incorporated into multivariate Cox analysis furthermore. As illustrated in Table III, after multivariate adjustment, the Cox multivariate analysis demonstrated that ECOG performance status (HR=0.63, P=0.011), the number of metastatic lesions

Table II. Baseline characteristics of the 89 patients with advanced non-small cell lung cancer according to genotype status of programmed death-ligand 1 rs2297136.

Baseline characteristics	Total, N=89 (%)	rs2297136 genotype status		χ^2	P-value
		AA (N=58)	AG/GG (N=31)		
Age, years					
Median (range)	66 (21-79)	66 (21-78)	66 (24-79)	NA	0.573
Sex				0.067	0.796
Male	59 (66.3)	39 (67.2)	20 (64.5)		
Female	30 (33.7)	19 (32.8)	11 (35.5)		
ECOG PS score				0.149	0.700
0-1	55 (61.8)	35 (60.3)	20 (64.5)		
2-3	34 (38.2)	23 (39.7)	11 (35.5)		
Pathological stage					
IIIb	9 (10.1)	6 (10.3)	3 (9.7)	0.010	0.920
IV	80 (89.9)	52 (89.7)	28 (90.3)		
Smoking status				1.384	0.239
Non-smoker	17 (19.1)	9 (15.5)	8 (25.8)		
Former smoker/smoker	72 (80.9)	49 (84.5)	23 (74.2)		
EGFR mutation status				0.495	0.482
Positive	18 (20.2)	13 (22.4)	5 (16.1)		
Negative	71 (79.8)	45 (77.6)	26 (83.9)		
Anaplastic lymphoma kinase rearrangement				0.608	0.434
Positive	4 (4.5)	2 (3.4)	2 (6.5)		
Negative or not available	85 (95.5)	56 (96.6)	29 (93.5)		
History of surgical resection				0.021	0.885
Yes	25 (28.1)	16 (27.6)	9 (29.0)		
No	64 (71.9)	42 (72.4)	22 (71.0)		
Histological category				0.149	0.700
Adenocarcinoma	55 (61.8)	35 (60.3)	20 (64.5)		
Squamous cell carcinoma	34 (38.2)	23 (39.7)	11 (35.5)		
Number of metastatic lesions				0.726	0.394
≤3	52 (58.4)	32 (55.2)	20 (64.5)		
>3	37 (41.6)	26 (44.8)	11 (35.5)		
Therapeutic Lines of PD-1 blockades				0.563	0.453
Second-line	19 (21.3)	11 (19.0)	8 (25.8)		
Third-line or more	70 (78.7)	47 (81.0)	23 (74.2)		
PD-1 blockades				0.123	0.726
Camrelizumab	25 (28.1)	17 (29.3)	8 (25.8)		
Sintilimab	21 (23.6)	14 (24.1)	7 (22.6)		
Tislelizumab	20 (22.5)	13 (22.4)	7 (22.6)		
Pembrolizumab	15 (16.9)	9 (15.5)	6 (19.4)		
Nivolumab	8 (8.9)	5 (8.6)	3 (9.7)		

PD-1, programmed death-1.

(HR=0.73, P=0.026) and PD-L1 rs2297136 genotype status (HR=2.01, P=0.018) were all independent risk factors for OS.

Association between PD-L1 gene mRNA expression and genotype status of rs2297136. Ultimately, mRNA analysis was conducted on a total of 81 patients. The prevalence of rs2297136 polymorphism among these patients was as follows: The AA genotype was observed in 53 cases (65.4%), the AG genotype was noted in 26 cases (32.1%), the GG genotype was found in 2 cases (2.5%). The MAF was 0.19, aligning with Hardy-Weinberg Equilibrium (P=0.567) and demonstrating similarity with the genotype distribution frequency among the 89 patients with

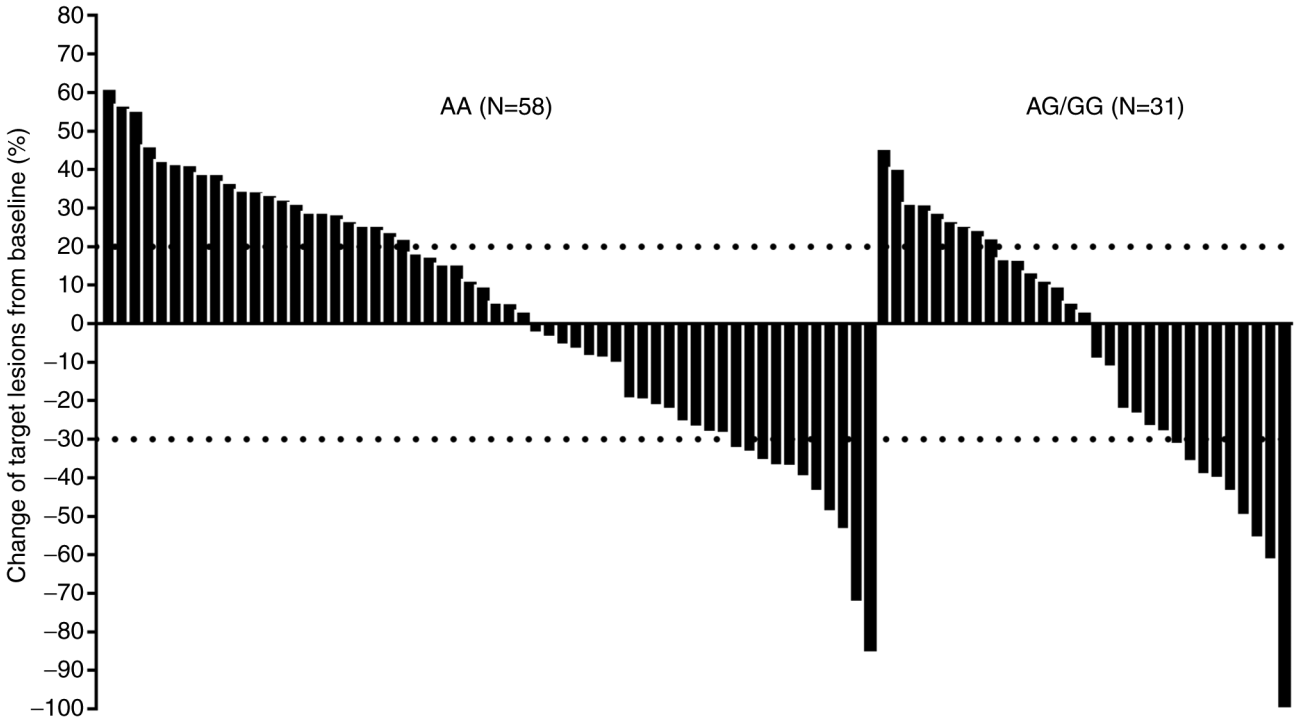

Figure 1. Waterfall plot for the best percentage change in target lesion of the 89 patients with advanced non-small cell lung cancer according to programmed death ligand-1 rs2297136 genotype status.

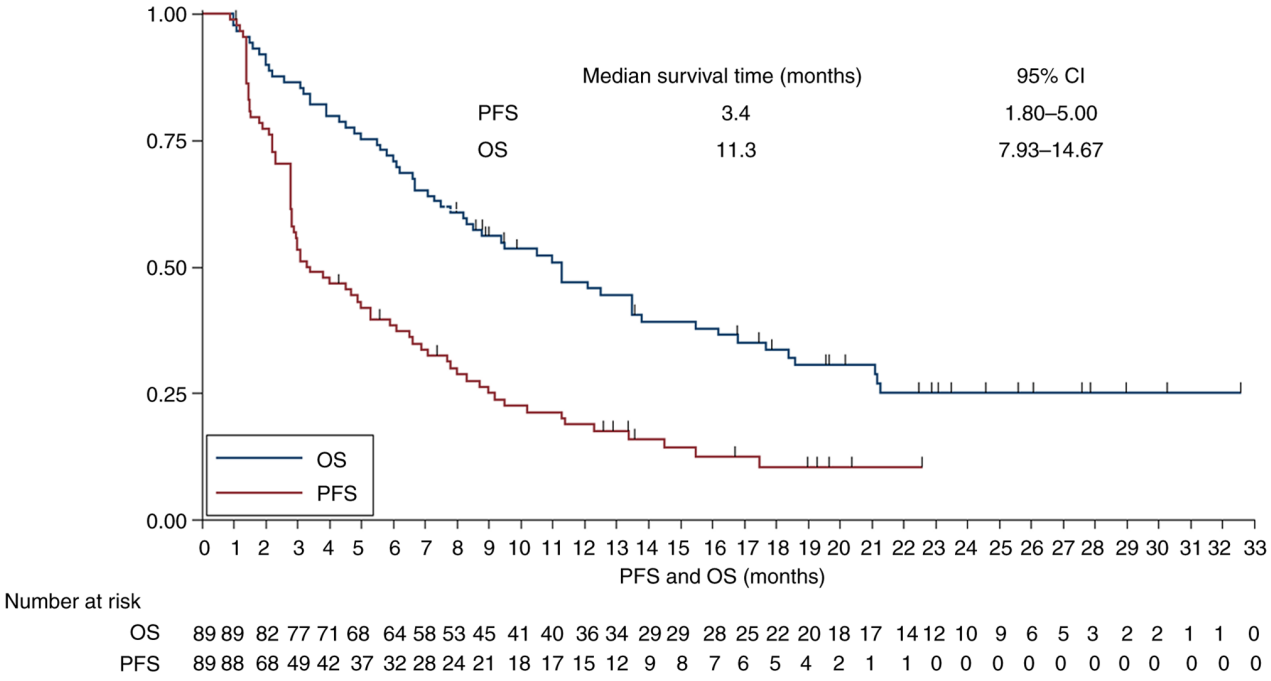

Figure 2. PFS and OS of the 89 patients with advanced non-small cell lung cancer who received programmed death-1 blockades. PFS, progression-free survival; OS, overall survival; CI, Confidence interval.

advanced NSCLC. The median relative expression level of PD-L1 mRNA was 3.30 (ranging from 1.55 to 5.32) and the mean relative expression level was 3.24±0.835 in 81 PBMC specimens. Subsequently, the association between PD-L1 mRNA expression and genotype status of rs2297136 is illustrated in Fig. 5. In comparison with AA genotype, AG/GG genotypes of rs2297136 exhibited a higher relative expression of PD-L1 mRNA in PBMC specimens (4.09±0.538 vs. 2.59±0.644), demonstrating statistical significance (P<0.001).

PD-L1 immunohistochemical expression results in two patients among the present study and matched with PD-L1 mRNA expression status is shown in Figure S1. PD-L1 mRNA expression status was divided into PD-L1 high expression (PD-L1 H) and PD-L1 low expression (PD-L1 L) according

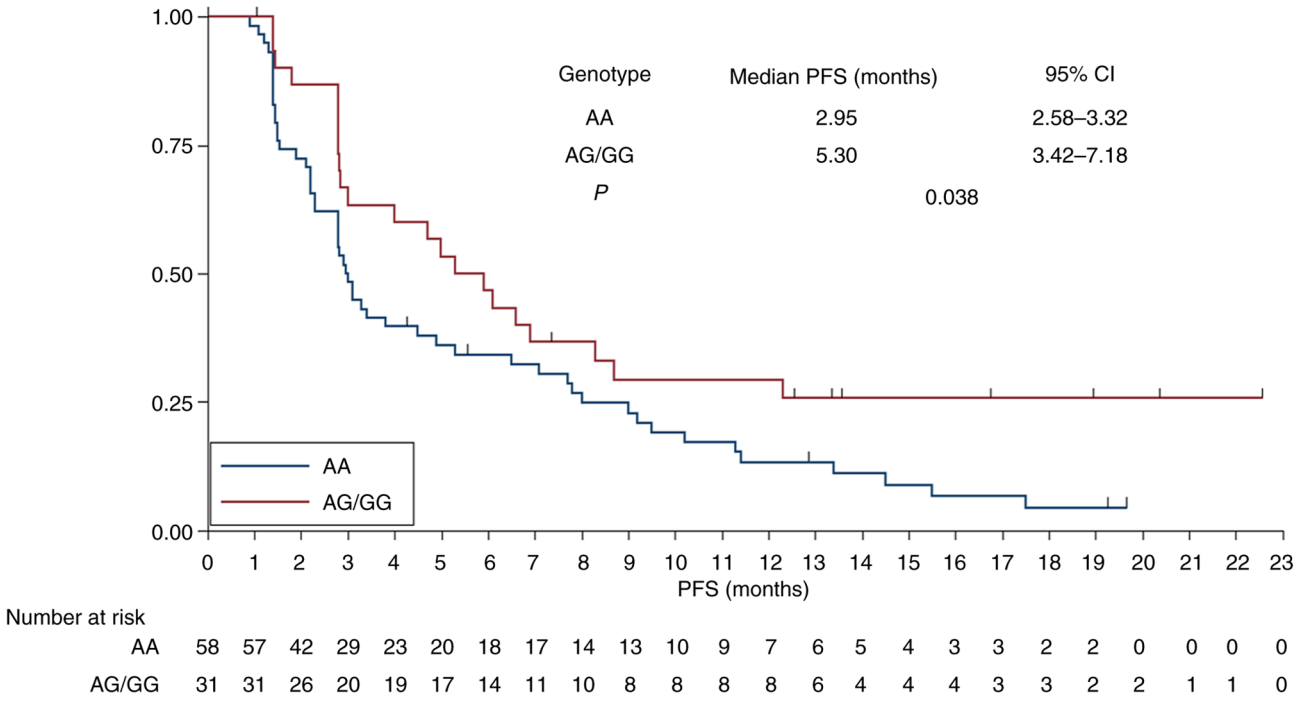

Figure 3. PFS of the 89 patients with advanced non-small cell lung cancer who received PD-1 blockades according to PD-L1 rs2297136 genotype status. PFS, progression-free survival; PD-L1, programmed death ligand-1; CI, Confidence interval.

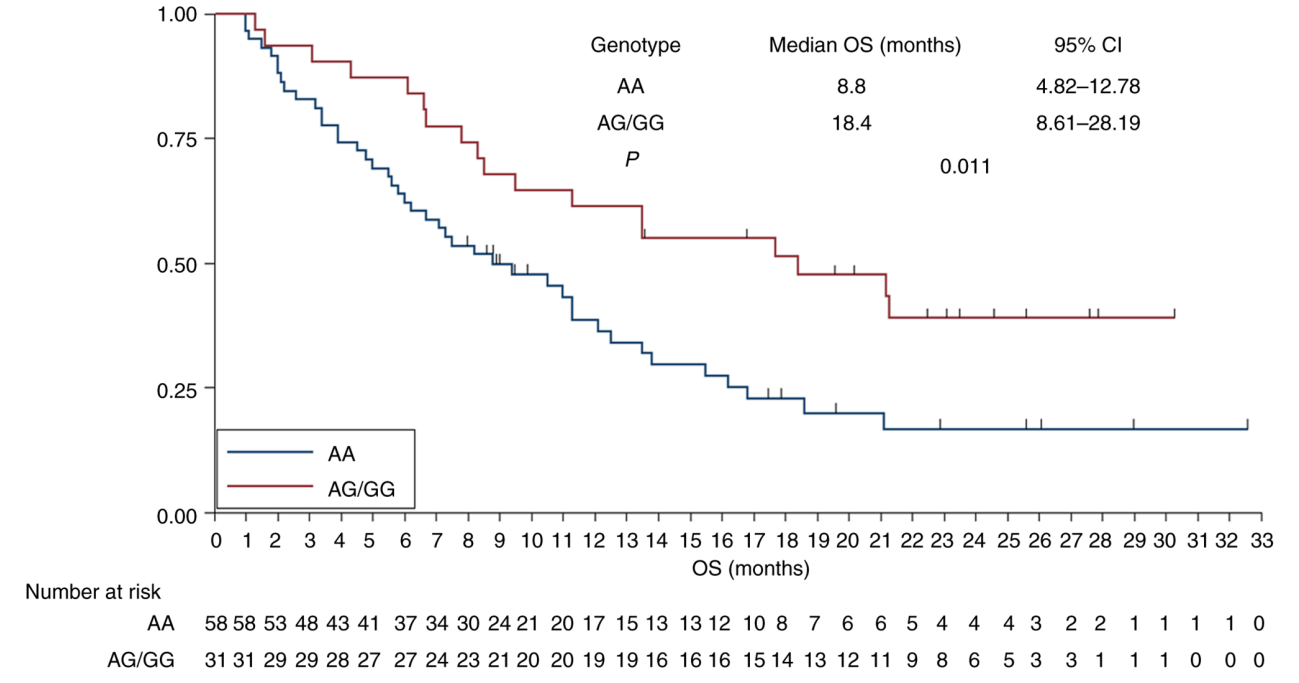

Figure 4. OS of the 89 patients with advanced non-small cell lung cancer who received PD-1 blockades according to PD-L1 rs2297136 genotype status. OS, overall survival; PD-L1, programmed death ligand-1; CI, Confidence interval.

to the median expression threshold value (3.30). Patients with PD-L1 H and PD-L1 L were observed in 41 and 40 cases, respectively. As exhibited in Fig. S2, patients with PD-L1 H conferred a trend for superior PFS compared with those with PD-L1 L (median PFS: 5.3 vs. 2.8 months), although the difference was not statistically significant (χ^2=3.438, P=0.064). Furthermore, as shown in Fig. S3, patients with PD-L1 H conferred a significantly improved OS compared with those with PD-L1 L (median OS: 13.5 vs. 7.8 months), demonstrating statistical significance (χ^2=4.559, P=0.033).

Additionally, some patients with advanced NSCLC examined the expression the of immunohistochemistry (IHC) of PD-L1 using biopsy cancer tissue samples in the third-party testing agency to predict the efficacy of PD-1 blockades. These test results were collected and matched with mRNA expression results correspondingly. As shown in Fig. S1, the relative

Table III. OS of the 89 patients with advanced non-small cell lung cancer according to baseline characteristic subgroups in univariate analysis and multivariate Cox analysis.

Characteristics	Median OS (95% CI)	Univariate analysis P-value	Multivariate analysis	
			Hazard ratio (95% CI)	P-value
Age, years		0.513		
<66	12.5 (8.56-16.44)			
≥66	10.5 (8.02-12.98)			
Sex		0.331		
Male	11.3 (8.12-14.48)			
Female	13.5 (9.35-17.65)			
ECOG PS score		0.008	0.63 (0.29-0.88)	0.011
0-1	15.5 (10.12-20.88)			
2-3	9.5 (7.14-11.86)			
Pathological stage	13.5 (7.81-19.19)	0.637		
IIIb				
IV	11.3 (8.02-14.58)			
Smoking status		0.535		
Non-smoker	11.3 (7.73-14.87)			
Former smoker/smoker	11.3 (8.31-14.29)			
EGFR mutation status		0.131		
Positive	9.5 (7.18-11.82)			
Negative	13.8 (9.12-18.48)			
History of surgical resection		0.618		
Yes	12.1 (9.22-14.98)			
No	11.0 (8.79-13.21)			
Histological category		0.561		
Adenocarcinoma	10.5 (8.45-12.55)			
Squamous cell carcinoma	12.5 (9.03-15.97)			
Number of metastatic lesions		0.019	0.73 (0.41-0.92)	0.026
≤3	13.5 (8.23-18.77)			
>3	9.5 (7.21-11.79)			
Lines of PD-1 blockades		0.572		
Second-line	12.5 (8.67-16.33)			
Third-line or later	11.3 (8.83-13.77)			
PD-1 blockades		0.582		
Camrelizumab	10.5 (8.63-12.37)			
Sintilimab	11.0 (7.91-14.09)			
Tislelizumab	12.5 (9.51-15.49)			
Pembrolizumab	11.3 (8.97-13.63)			
Nivolumab	12.1 (9.34-14.86)			
PD-L1 rs2297136 genotype status		0.011	2.01 (1.12-3.32)	0.018
AA	8.8 (4.82-12.78)			
AG/GG	18.4 (8.61-28.19)			

OS, overall survival; CI, confidence interval.

mRNA expression level of PD-L1 gene was correlated with the PD-L1 IHC expression consistently, the relatively high mRNA expression level was associated with high TPS score of PD-L1 IHC expression.

Discussion

The present study contributes real-world evidence regarding the viability of PD-1 blockade monotherapy for patients with

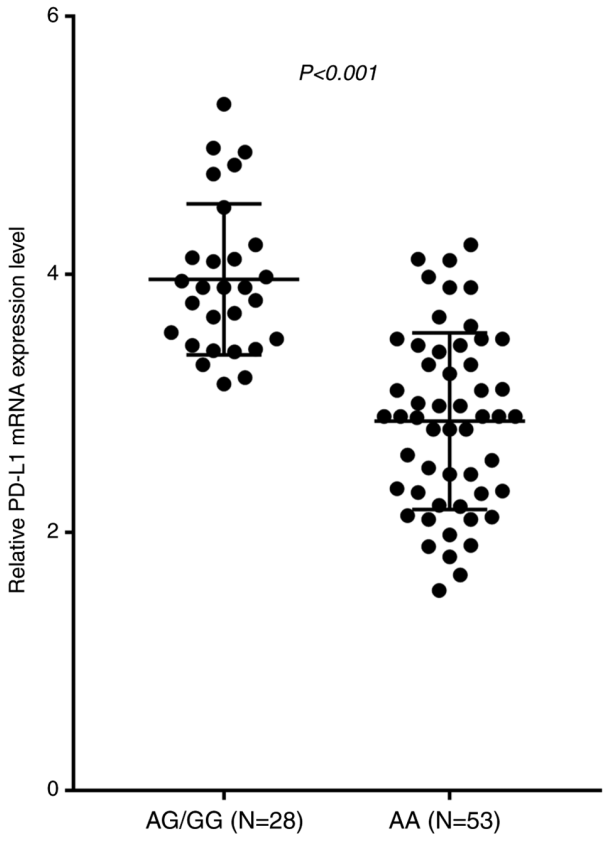

Figure 5. Relative expression level of PD-L1 mRNA according to PD-L1 rs2297136 genotype status. PD-L1, programmed death ligand-1.

previously treated advanced NSCLC, assessed retrospectively. Simultaneously, the investigation of the present study highlights the clinical significance of rs2297136 in the PD-L1 gene for predicting the prognosis of the 89 patients. In aggregate, rs2297136 in the PD-L1 gene holds potential as a biomarker for prognostic prediction in clinical settings for patients with advanced NSCLC undergoing PD-1 blockade monotherapy.

To the best of the authors' knowledge, PD-1 blockades have demonstrated enduring responses and promising efficacy in patients with previously-treated advanced NSCLC, establishing themselves as the standard second-line treatment for patients with advanced NSCLC over the past years (26). However, the overall response to PD-1 blockade monotherapy in patients with advanced NSCLC remains suboptimal. Despite the clinical significance of factors such as PD-L1 expression, DNA MMR status and TMB in predicting PD-1 blockade efficacy to some extent, a substantial number of patients still do not respond to these regimens (27). There is an ongoing need to explore additional potential biomarkers to identify patients who may benefit from subsequent PD-1 blockade administration. (28). In this context, other potential biomarkers were observed, such as the neutrophil-to-lymphocyte ratio and gut microbiota, have recently emerged as potentially clinically significant predictors of PD-1 blockade efficacy (29). However, these alternatives also lack conclusive evidence.

Among the 89 patients enrolled in the present study, a total of 19 were administered PD-1 blockades as second-line therapy, while the remaining 70 received PD-1 blockades as third-line treatment or beyond. Considering that certain PD-1 blockades (specifically, tislelizumab and nivolumab) had indications for use as second-line therapy in patients with advanced NSCLC in China, the administration of PD-1 blockades monotherapy in the present study appears to be reasonable and ethical. All 89 NSCLC patients included in the present study were typical cases of advanced NSCLC, making the sample representative (30).

Overall, the therapeutic outcomes exhibited that the ORR and DCR for the 89 patients with advanced NSCLC treated with PD-1 blockades monotherapy were 22.5 and 64.0%, respectively. The median PFS was 3.4 months. The efficacy of the present study and PFS outcomes closely aligned with the ORR and PFS of Checkmate-017 and Checkmate-057 trials, where nivolumab served as second-line treatment for squamous cell and non-squamous cancers, respectively (ORR was ~20%, median PFS was almost 3 months) (31,32). Additionally, the present study's therapeutic outcomes were consistent with the ORR and DCR observed in the RATIONALE-303 trial, where tislelizumab was utilized as second-line therapy for advanced NSCLC (ORR=22.6%, DCR=55.7%) (33). Interestingly, it is noteworthy that the PFS and OS in RATIONALE-303 trial were slightly longer than those in the present study. It was hypothesized that this discrepancy may be attributed in two aspects: Firstly, all patients in the RATIONALE-303 trial had an ECOG performance status of 0-1, whereas the present study included 38.2% of patients with a status of 2-3. Clearly, ECOG performance status emerged as an independent factor influencing the prognosis of patients with advanced NSCLC (34). Additionally, the present study's retrospective design may have impacted the management of patients compared with well-designed phase III clinical trials, potentially compromising the efficacy and prognosis to some extent. This notion is supported by a prior retrospective study among advanced NSCLC patients (35). These two factors could potentially explain why the prognosis in the present study was inferior to that in RATIONALE-303 trial. Significantly, the present study included 18 patients with positive EGFR mutation who received PD-1 blockades as third-line or subsequent treatment. These patients, having undergone extensive prior treatments with EGFR-TKI and chemotherapy, had limited therapeutic options, making immunotherapy a viable consideration (36). An association analysis between EGFR mutation status and OS suggested that patients with positive EGFR mutation might not benefit significantly from PD-1 blockades monotherapy, even though the statistical difference was not significant (P=0.131). However, caution is warranted in interpreting this finding. All 18 patients with positive EGFR mutation underwent intensive treatment and received PD-1 blockades as third-line or subsequent therapy, indicating a relatively worse prognosis regardless of the therapeutic regimens (37). A recent study indicated that a subset of patients with positive EGFR mutation and high PD-L1 expression may derive benefits from PD-1 blockades administration (38). Another study suggested that subjects with a short PFS on EGFR tyrosine kinase inhibitor (TKI) might exhibit an improved response to immunotherapy, and combined PD-1 blockades treatment might be a promising option compared with chemotherapy in second-line setting for patients with worse PFS on EGFR TKI therapy and no T790M mutation (39). In conclusion, the question whether patients with EGFR positive mutation might

benefit from PD-1 blockades treatment should be thoroughly explored in prospective clinical trials.

Remarkably, a recent study indicated that genetic variation in the pathogenic gene might contribute to the therapeutic outcomes of PD-1 blockades in metastatic melanoma (40). In a recent investigation led by Parakh *et al* (40), comprising 318 patients undergoing PD-1/PD-L1 blockade treatment, the clinical significance of key genes associated with tumor immunity was explored. Their findings identified immunogenetic polymorphisms including ATG7 rs7625881, CD274 rs2297136 and TLR4 rs1927911 as potential predictors of response to PD-1/PD-L1 blockade in tumor patients. These studies suggested that gene polymorphism may play a role in the clinical outcomes of PD-1/PD-L1 blockades. The conclusion drawn from the present study regarding PD-L1 gene polymorphism suggested that AG/GG genotype of rs2297136 is associated to a relatively favorable prognosis among Chinese patients with advanced NSCLC undergoing PD-1 blockades, aligning with the previous study initiated by Yoshida *et al* (18). The aforementioned study, which included 133 patients treated with nivolumab, identified an association between prognostic outcomes and PD-L1 polymorphisms. Among the 7 polymorphisms investigated, rs822339 and rs1411262 were suggested to predict the prognosis of patients receiving nivolumab therapy but not those undergoing non-PD-1 blockades therapy. While the concept and design of the aforementioned study are consistent to the present study, the present study did not establish the clinical significance of rs822339 and rs1411262, as outlined in the preliminary analysis in Table I. This discrepancy was attributed to ethnic variations in the two polymorphisms, where the MAF of rs822339 and rs1411262 ranged from 0.11 to 0.55 among different population, potentially contributing to the differences in efficacy of PD-1 blockades (41). Additionally, another exploratory study initiated by Nomizo *et al* (19), also investigated the influence of polymorphism in PD-L1 on the response to nivolumab among patients with advanced NSCLC. Involving 50 patients who received nivolumab monotherapy, the aforementioned study identified rs2282055 and rs4143815 as associated with distinct ORR and PFS among NSCLC patients treated with nivolumab. Despite the alignment in study design with the present study, it is important to note that the sample size of Nomizo's *et al* study was limited, necessitating confirmation of their conclusions in a larger patient cohort. Furthermore, the present study is in line with another previous study initiated by Minari *et al* (42), which investigated the clinical significance of PD-L1 polymorphism as potential biomarker, predicting the prognosis of 166 patients with advanced NSCLC who received PD-1/PD-L1 blockades. The findings of the aforementioned study indicated that rs4143815 in PD-L1 gene appeared to be marginally correlated with clinical outcomes in NSCLC undergoing PD-1/PD-L1 blockades. Collectively, all these studies suggested that PD-L1 polymorphisms may contribute to the potential interactions between PD-1 and PD-L1, thereby influencing the therapeutic efficacy of PD-1/PD-L1 blockades clinically.

Additionally, PD-L1 gene mRNA expression analysis suggested that AG/GG genotype of rs2297136 was associated to higher PD-L1 mRNA expression, consistent with findings of a previous study initiated by Su *et al* (23). The aforementioned study, which involved 86 PBMC specimens, aimed to uncover the association between the genotype status of rs2297136 and PD-L1 mRNA expression, confirming that patients with the AG/GG genotype of rs2297136 exhibited elevated PD-L1 mRNA expression. Interestingly, the present study shared a similar conclusion to previous research, indicating that higher expression of PD-L1 mRNA could predict superior efficacy for patients undergoing PD-1 blockades. This is in contrast to studies suggesting that higher PD-L1 mRNA expression predicts worse prognosis for patients receiving capecitabine-based adjuvant chemotherapy (43,44). However, it should be noted that the present study highlighted that higher expression of PD-L1 mRNA might predict superior efficacy of patients who received PD-1 blockades. It was hypothesized that this discrepancy may attribute to the therapeutic regimens received. To the best of the authors' knowledge, PD-L1 gene was a hot spot gene in the field of tumor immunotherapy at present and considerable clinical trials confirmed that higher expression level of PD-L1 could predict the superior efficacy of PD-1/PD-L1 blockades (45). Unfortunately, since the IHC results of PD-L1 expression were not available in the present study, an analysis of the association between PD-L1 mRNA expression and IHC expression could not be performed. Fortunately, PD-L1 IHC expression results in two patients matched with the PD-L1 mRNA expression correspondingly, suggesting that the results of PD-L1 mRNA expression in the present study might also reflect the results of PD-L1 IHC expression to some extent. Therefore, further in-depth investigations are necessary to validate the clinical significance of PD-L1 polymorphism and PD-L1 mRNA expression. Given that rs2297136 is located at the 3'-untranslated regions of PD-L1 gene, potentially modifying miRNA binding and altering the interaction between miRNAs and target mRNAs, could result in increased mRNA expression of PD-L1. Previous studies have validated that miR-324-5p and miR-632 possess the potential to bond to rs2297136, altering mRNA expression and influencing susceptibility to cancer occurrence (46,47). Therefore, it was hypothesized that the genotype status of rs2297136 may have different binding capacities to miR-324-5p and miR-632, leading to changes in PD-L1 mRNA expression. Regarding the association between PD-L1 expression and efficacy of PD-1 blockades, Keynote-010 and Checkmate 057 clinical trials have previously affirmed that increased PD-L1 expression predicts superior clinical outcomes for both pembrolizumab and nivolumab among patients with advanced NSCLC (32,48). As a result, increased PD-L1 mRNA expression might serve as a positive prognostic biomarker for PD-1 blockades in the present study.

The present study, however, does have certain limitations. Firstly, due to its retrospective nature, the sample size in the present study was relatively small with only 89 patients included in polymorphism analysis. The conclusion that rs2297136 is associated with effectiveness of PD-1 blockades requires further clarification in larger subject cohorts. Secondly, various PD-1 blockades were used in the present study, potentially resulting in heterogeneous and diverse efficacy outcomes. Thirdly, the present study was unable to detect the PD-L1 IHC expression, compromising the utility of PD-L1 mRNA expression to some extent. Nonetheless, the present study highlights the potential significance of rs2297136 in predicting the effectiveness of PD-1 blockades for patients with

advanced NSCLC, suggesting that rs2297136 in the PD-L1 gene could be a valuable biomarker for predicting therapeutic outcomes in clinical practice.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QG and HTJ designed the study, conducted data analysis and drafted the manuscript. SYD collected data, performed the experiment and participated in the patients' follow-up. HLQ and HTJ provided guidance in designing the study and supervised the study's result. QG and HTJ confirm the authenticity of all the raw data. All authors have read and approved the manuscript, agreeing to be accountable for all aspects of the research and ensuring that the accuracy and integrity of the work were appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved (approval no. 2022-KY-11053) by the Ethics Committee of the Affiliated Hospital of Hebei University (Baoding, China). Written informed consent was obtained from each enrolled patient according to the recommendations of the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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