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ImmuneScore as a novel RNA-based prognostic signature superior to PD-L1 in advanced non-squamous NSCLC patients receiving chemotherapy combined with immune checkpoint inhibitor therapy

Zhihuan Lin[#], Jianhua Zhan[#], Li Zhang

Department of Medical Oncology, Sun Yat-sen University Cancer Center, State Key Laboratory of Oncology in South China, Collaborative Innovation Center for Cancer Medicine, Guangzhou 510030, Guangdong, China.
[#]Authors contributed equally.

Correspondence to: Dr. Li Zhang, Department of Medical Oncology, Sun Yat-sen University Cancer Center, State Key Laboratory of Oncology in South China, Collaborative Innovation Center for Cancer Medicine, 651 Dongfeng Road East, Guangzhou 510030, Guangdong, China. E-mail: zhangli@sysucc.org.cn

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Abstract

Aim: Our study aimed to explore the prognostic predictive potential of a novel RNA-based signature called ImmuneScore in advanced non-squamous NSCLC patients receiving combined immune checkpoint inhibitor (ICI) treatment and chemotherapy.

Methods: RNA-sequencing data of 113 patients screened out from ORIENT-11 trial were retrospectively analyzed. ImmuneScore was calculated by the ESTIMATE algorithm. The association of ImmuneScore with early tumor progression, progression-free survival (PFS), and overall survival (OS) was analyzed using chi-square test, Cox regression test, and log-rank test. Receiver operating characteristic (ROC) curves were generated, with higher values of area under the ROC curves (AUCs) indicating better prediction ability.

Results: ImmuneScore was negatively correlated with early tumor progression rate (4.3% *vs.* 18.6%, $P = 0.013$) while positively correlated with PFS (HR = 0.29, 95%CI: 0.16-0.53, $P < 0.001$) and OS (HR = 0.32, 95%CI: 0.18-0.58, $P < 0.001$), demonstrating higher AUCs than that of Programmed death-ligand 1 (PD-L1) tumor

proportion score (TPS) (early tumor progression: 0.64 *vs.* 0.68; PFS: 0.67 *vs.* 0.58; OS: 0.73 *vs.* 0.63). Nomograms integrating ImmuneScore and other significant variables (age and T-stage for PFS, gender and T-stage for OS) yielded good performance in PFS and OS prediction.

Conclusion: ImmuneScore serves as a novel RNA-based prognostic signature superior to PD-L1 in advanced non-squamous NSCLC patients receiving chemotherapy combined with ICI therapy. Higher ImmuneScore indicates lower early tumor progression rate, longer PFS, and longer OS.

Keywords: Non-squamous non-small cell lung cancer, immune checkpoint inhibitors, RNA-based signature, ImmuneScore

INTRODUCTION

Lung cancer remains the leading cause of cancer-related death worldwide[1] . Broadly, lung cancer is divided into small-cell lung cancer and non-small-cell lung cancer (NSCLC). At present, the chemotherapy and immune checkpoint inhibitor (ICI) combination has emerged as the standard first line of care for advanced NSCLC patients without sensitizing epidermal growth factor receptor (EGFR) or anaplastic lymphoma kinase (ALK) genomic aberrations[2-4]. However, clinically effective biomarkers in this combination chemoimmunotherapy population are still lacking[5,6]. Though Programmed death-ligand 1 (PD-L1) tumor proportion score (TPS) assessed by immunohistochemistry (IHC) serves as a well-recognized biomarker in predicting ICI therapy efficacy, contributing to selecting ideal ICI treatment candidates[7,8], its prognostic value in this combination therapy population remained to be suboptimal[9,10]. To overcome this dilemma, there have been some studies exploiting potential clinical factors like gender, performance status, and concomitant medication administration through meta-analysis[11-13]. In recent years, molecular biomarkers like DNA and RNA signatures developed based on high-throughput sequencing data have raised concerns[14-16], but studies remain insufficient.

Yoshihara developed the "Estimation of STromal and Immune cells in MAlignant Tumors using Expression data" (ESTIMATE) algorithm, via which the level of tumor-infiltrating immune cells could be quantified as the immune score (ImmuneScore), based on RNA expression profiles[17]. Its clinical application value has not been elucidated yet. The current study aimed to investigate the prognostic value of this RNA-based signature in advanced non-squamous NSCLC patients receiving chemotherapy combined with ICI therapy through a retrospective study containing 113 patients. Results indicated that ImmuneScore was significantly positively associated with prognosis in this population, with predictive ability superior to PD-L1.

METHODS

Inclusion and exclusion criteria

Patients included in our current study were screened out from those participating in ORIENT-11 trial. The selection process is depicted as a flowchart in Figure 1. As we know, ORIENT-11 trial is a double-blind, multicenter, phase 3 study conducted in China, which enrolled 397 advanced non-squamous NSCLC patients from August 23, 2018 to July 30, 2019 to randomly receive either combination chemoimmunotherapy (sintilimab immunotherapy plus platinum-pemetrexed standard chemotherapy) or platinum-pemetrexed chemotherapy (ClinicalTrials.gov: NCT03607539). More specific details of the study design were provided in our published article[18] and thus did not belabor here. Ethical permission and patient consent were obtained. Among the total 397 patients in ORIENT-11 trial, 171 had baseline Formalin-Fixed Paraffin-Embedded (FFPE) tumor samples that met the criteria for RNA sequencing. Of these, 113 patients from the sintilimab plus standard chemotherapy group were finally included in our current study and retrospectively analyzed. Clinical data like age, gender, and smoking history had been collected before the initial treatment.

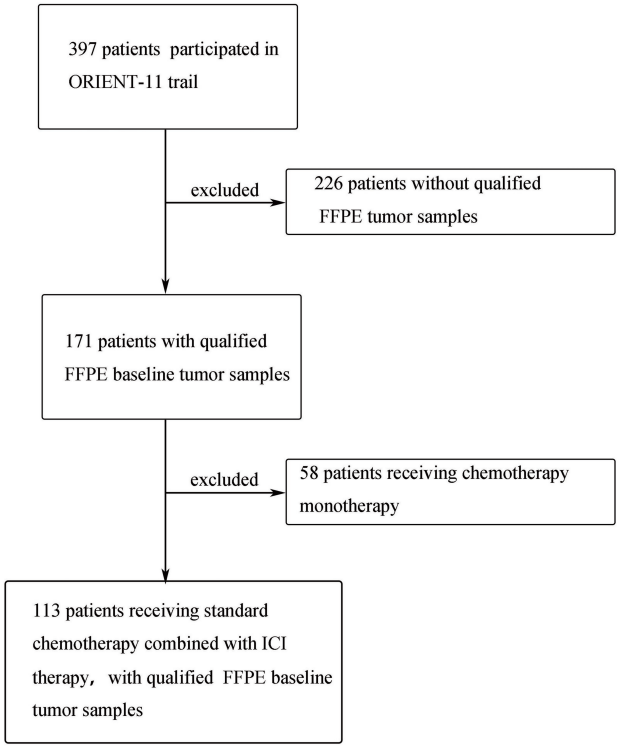

Figure 1. CONSORT flow diagram of the included patients. ICI: Immune checkpoint inhibitor.

Calculation of PD-L1 TPS

PD-L1 TPS, defined as the percentage of tumor cells with partial or complete membrane staining of PD-L1, was evaluated on baseline tumor tissues using PD-L1 immunohistochemical 22C3 pharmDx assay (Agilent Technologies). Samples were considered to be PD-L1-positive if PD-L1 TPS was ≥ 1%.

RNA sequencing and ImmuneScore calculation

Baseline FFPE tissues were obtained before treatment by bronchoscopic biopsy or percutaneous needle biopsy. RNA was isolated from 15-20 tissue slides (10-15 μm thick, about 0.25 cm^2). cDNA library was synthesized with the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB, USA). Subsequent RNA sequencing was conducted on the Illumina NovaSeq 6000 platform, and reads were mapped to human genome version 38 (GRCh38). The raw reads and counts were normalized to the number of transcripts per kilobase million. Based on gene expression profiles, the ImmuneScore for each of the 113 patients was calculated using ESTIMATE analysis with the "ESTIMATE" R package provided by Yoshihara *et al.* (https://sourceforge.net/projects/estimateproject/)[17].

Evaluation of clinical efficacy and study outcome

Treatment efficacy evaluation data had been generated during ORIENT-11 trial by a blinded independent radiographic review committee (BIRRC), based on Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 criteria. Efficacy was classified as complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD). Patients' survival status was followed up by regular phone calls by clinical research coordinators. Progression-free survival (PFS) was defined as the time from treatment assignment to disease progression or death from any cause, and overall survival (OS) was defined as the time from treatment

assignment to death from any cause. The PFS data were last updated on May 15, 2020 and the OS data were last updated on January 15, 2021. Details were described in our previous published articles[18,19]. PD that occurred within 6 weeks was defined as "early tumor progression" here; otherwise, it was defined as disease control. PFS and OS were set to be primary endpoint, and early disease control served as the secondary outcome

Evaluation of predictive accuracy

Receiver operating characteristic (ROC) analysis was performed to evaluate the predictive efficacy of variables, with larger area under the curve (AUC) values indicating better predictive accuracy. For early tumor progression prediction, ROC curves were generated with the "pROC" R package, which is used for analyzing dichotomic data. For PFS and OS prediction, ROC curves were generated via the "TimeROC" R package, which is specific for survival data. The optimal cut-off point of ImmuneScore was determined considering the highest Youden Index (sensitivity + specificity - 1).

Nomogram construction

Nomograms for PFS and OS prediction were established via the "rms" R package, based on significant prognostic variables identified by multivariate COX regression analysis. The total points for each patient were calculated as the sum of the points for each variable. Calibration curves and decision curves were performed to assess the calibration performance and clinical utility of nomograms.

Gene set enrichment analysis of the high- and low-ImmuneScore groups

Gene set enrichment analysis (GSEA) was carried out with the "fgsea" R package to identify the significant Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling pathways involved in the high- versus low-ImmuneScore group, utilizing "c2.cp.kegg.v7.1.symbols.gmt" downloaded from Molecular Signatures Database (MSigDB) as the reference gene set. Adjusting P-value < 0.05 was regarded as statistically significant.

Statistical analysis

Continuous variables were described as median (range/interquartile range) and were compared using Student's independent samples t-test. Qualitative variables were presented as frequencies and percentages and were examined with the chi-square test. Before statistical tests, missing values including three tumor T-stage and four tumor N-stage were imputed with the median. Cox regression analysis was performed to determine prognostic value. Variables with P values < 0.05 according to univariate Cox regression analysis were entered into multivariate analysis, using the enter selection method. Results were expressed as hazard ratio (HR) with a 95% confidence interval (95%CI). Kaplan-Meier (K-M) survival curves were employed for survival comparison with the log-rank test. All figures were generated with the R programming language (R version 3.6.1). Two-tailed P-value < 0.05 was considered to be statistically significant.

RESULTS

Demographic and clinical characteristics of the patient cohort

As shown in Figure 1, after excluding patients who did not meet the inclusion criteria, 113 non-squamous NSCLC patients receiving chemotherapy combined with the anti-PD-1 antibody sintilimab were retained and retrospectively analyzed. The demographic and clinical characteristics of these patients are shown in Table 1. Patients were diagnosed as stage IIIB to IV (in accordance with the eighth edition of the AJCC TNM classification) without EGFR and ALK mutation. The ESTIMATE algorithm was used to determine the ImmuneScore for each patient. Their ImmuneScores were distributed between -573 and 3,141 [Supplementary Table 1]. The median follow-up of PFS and OS were 15.0 months and 24.8 months, respectively. The median PFS was 9.2 months, while the median OS was 23.6 months.

Table 1. Demographic and clinical characteristics of the study cohort

Variables	Patients (*N* = 113)
Age (y)	61 (30-74)
Gender	
Male	87 (77.0%)
Female	26 (23.0%)
ECOG	
0	29 (25.7%)
1	84 (74.3%)
Smoking status	
Previous/Current smoker	42 (37.2%)
Never	71 (62.8%)
PD-L1 TPS	
< 1%	31 (27.4%)
≥ 1%	82 (72.6%)
T-stage	
T1	19 (16.8%)
T2	30 (26.5%)
T3	19 (16.8%)
T4	42 (37.2%)
Tx	3 (2.7%)
N-stage	
N0	7 (6.2%)
N1	2 (1.8%)
N2	30 (26.5%)
N3	70 (61.9%)
Nx	4 (3.5%)
M-stage	
M0	9 (8.0%)
M1	104 (92.0%)
TNM stage	
IIIB	7 (6.2%)
IIIC	2 (1.8%)
IV	104 (92.0%)
Initial response	
PR	35 (31.0%)
SD	67 (59.3%)
PD	11 (9.7%)
PFS (months)	9 (1-14)
OS (months)	24(3-37)
Survival endpoint	
Dead	53 (42.5%)
Alive	60 (57.5%)

ECOG: Eastern Cooperative Oncology Group performance score; PD-L1 TPS: programmed death-ligand 1 tumor proportion score; PR: partial response; SD: stable disease; PD: progressive disease; PFS: progression-free survival; OS: overall survival.

ImmuneScore as a predictor of early tumor progression

We classified patients into the PD-L1-positive group (82/113) or PD-L1-negative group (31/113) according

to the 1% threshold. In the PD-L1-positive group, 76 patients (92.7%) achieved disease control (SD + PR): 47 patients (57.3%) exhibited SD and 29 patients (35.4%) exhibited PR. In the PD-L1-negative group, 26 patients (83.9%) achieved disease control: 20 exhibited SD (64.5%) and 6 patients (19.4%) exhibited PR. Early tumor progression occurred in 6 patients (7.3%) and 5 patients (16.1%) in PD-L1 positive and negative groups, respectively, without significant difference (P = 0.159) [Figure 2A]. ROC analysis was utilized to evaluate the accuracy of PD-L1 in predicting early tumor progression; the AUC value (0.64, 95%CI: 0.54-0.72, P = 0.131) was not significant [Figure 2B].

ImmuneScore 1,060 corresponded to the highest Youden Index and thereby was set as the optimal cut-off point in predicting early tumor progression. According to this threshold,70 patients were assigned to the high-ImmuneScore group and the remaining 43 patients to the low-ImmuneScore group. ImmuneScores were significantly different between high and low-ImmuneScore groups, with median values of 1,867 (interquartile range: 1,400-2,170) and 505 (interquartile range: 115-928), respectively (P < 0.001). As shown in Figure 2C, in the high-ImmuneScore group, disease control was achieved in 67 patients, with 42 demonstrating SD (60.0%) and 25 demonstrating PR (35.7%). In the low-ImmuneScore group, disease control was achieved in 35 patients, with 10 demonstrating PR (23.3%) and 25 demonstrating SD (58.1%). Early tumor progression rate was significantly lower in high-ImmuneScore group than in low-ImmuneScore group [3 (4.3%) *vs.* 8 (18.6%), P = 0.013]. ImmuneScore demonstrated a better performance than PD-L1 (AUC = 0.68, 95%CI: 0.58-0.76, P = 0.021) [Figure 2D].

ImmuneScore as an independent prognostic signature of PFS

As shown in Figure 3A, ImmuneScore was an effective predictor for median PFS, with predictive accuracy (AUC = 0.67, 95%CI: 0.53-0.81) superior to that of PD-L1 TPS (AUC = 0.58, 95%CI: 0.44-0.72). As determined by the highest Youden Index, 1,060 was considered as the optimal cut-off point of ImmuneScore in predicting PFS.

As shown in Table 2, univariate COX regression revealed that high ImmuneScore was significantly associated with longer PFS (HR = 0.30, 95%CI: 0.17-0.54, P < 0.001). In addition, age (HR = 2.14, 95%CI: 1.20-3.80, P = 0.010) and tumor T-stage (HR = 1.65, 95%CI: 1.25-2.17, P < 0.001) were also significant. After adjusting for these two variables, multivariate COX regression determined ImmuneScore to be an independent prognosis signature (HR = 0.29, 95%CI: 0.16-0.53, P < 0.001).

Kaplan-Meier survival analysis was conducted to compare PFS between the high- and low-ImmuneScore groups. 48 patients experienced progression during the follow-up, while 65 patients without progression were censored. The high-ImmuneScore group had longer median PFS than the low-ImmuneScore group (7.2 *vs.* 11.4 months; HR = 0.25, 95%CI: 0.14-0.48, P < 0.001) [Figure 3B]. Similar results were obtained in subgroup analysis based on PD-L1 TPS expression (PD-L1 ≥ 1%: HR = 0.33, 95%CI: 0.14-0.80, P = 0.014; PD-L1 < 1%: HR = 0.25, 95%CI: 0.10-0.66, P = 0.005; PD-L1 ≥ 50%: HR = 0.30, 95%CI: 0.09-0.98, P = 0.045; PD-L1 < 50%: HR = 0.25, 95%CI: 0.12-0.53, P < 0.001) [Figure 3C and D].

A nomogram was constructed by incorporating three statistically significant variables identified in the multivariate COX regression analysis mentioned above, i.e., age, tumor T-stage and ImmuneScore [Figure 4A]. The nomogram exhibited good predictive performance in predicting 4-month PFS rate (AUC = 0.81, 95%CI: 0.72-0.90), 6-month PFS rate (AUC = 0.80, 95%CI: 0.71-0.89) and 9-month PFS rate (AUC = 0.77, 95%CI: 0.64-0.89) [Figure 4B]. The calibration curve and decision curve exhibited good performance [Supplementary Figure 1A].

Table 2. Results of univariate and multivariate COX regression analysis of variables for PFS prediction

Variables	Univariate analysis		Multivariate analysis	
	HR (95%CI)	***P*-value**	**HR (95%CI)**	***P*-value**
Age (y)				
< 65	Ref.		Ref.	
≥ 65	2.14 (1.20-3.80)	0.010*	2.21 (1.23-3.95)	< 0.001*
Gender				
Male	Ref.	-		
Female	0.67 (0.33-1.36)	0.271		
ECOG				
0	Ref.			
1	1.50 (0.74-3.01)	0.259		
Smoking status				
Never	Ref.	-		
Previous/Current smoker	1.10 (0.61-2.00)	0.758		
PD-L1 TPS		0.074		
< 1%	Ref.	-		
≥ 1%	0.59 (0.33-1.05)			
T-stage, per stage	1.65 (1.25-2.17)	< 0.001*	1.65 (1.25-2.19)	< 0.001*
N-stage, per stage	1.04 (0.74-1.47)	0.804		
M-stage M0	Ref.			
M1	3.12 (0.75-12.90)	0.117		
ImmuneScore				
Low	Ref.	-	Ref.	
High	0.30 (0.17-0.54)	< 0.001*	0.29 (0.16-0.53)	< 0.001*

PFS: Progression-free survival; HR: hazard ratio; ECOG: Eastern Cooperative Oncology Group performance score; PD-L1 TPS: programmed death-ligand 1 tumor proportion score. *$P < 0.05$.

ImmuneScore as an independent prognostic signature of OS

As shown in Figure 5A, ImmuneScore was an effective predictor for median OS, with predictive accuracy (AUC = 0.73, 95%CI: 0.61-0.85) higher than that of PD-L1 TPS (AUC = 0.63, 95%CI: 0.50-0.76). 1,005 was set as the optimal ImmuneScore cut-off point in predicting OS with the highest Youden index.

As shown in Table 3, univariate COX regression revealed that high ImmuneScore was significantly associated with longer OS (HR = 0.28, 95%CI: 0.16-0.48, $P < 0.001$). Additionally, gender (HR = 0.46, 95%CI: 0.21-0.98, $P = 0.044$), tumor T-stage (HR = 1.31, 95%CI: 1.01-1.69, $P = 0.036$), and PD-L1 TPS (HR = 0.56, 95%CI: 0.32-0.99, $P = 0.045$) were also significant. After adjusting for these three variables in multivariate COX regression, ImmuneScore remained statistically significant (HR = 0.32, 95%CI: 0.18-0.58, $P < 0.001$).

Kaplan-Meier survival analysis of OS was conducted. 53 patients died during the follow-up, while 60 were censored (34 alive and 26 lost to follow-up). Kaplan-Meier survival analysis revealed that the high ImmuneScore group had a longer median OS than the low ImmuneScore group (15.8 *vs.* 23.6 months; HR = 0.22, 95%CI: 0.12-0.40, $P < 0.001$) [Figure 5B]. Similar findings were obtained across subgroups with different PD-L1 levels (PD-L1 ≥ 1%: HR = 0.18, 95%CI: 0.08-0.42, $P < 0.001$; PD-L1 < 1%: HR = 0.37, 95%CI: 0.15-0.93, $P = 0.035$; PD-L1 ≥ 50%: HR = 0.16, 95%CI: 0.05-0.50, $P = 0.002$; PD-L1 < 50% (HR = 0.30, 95%CI: 0.15-0.62, $P = 0.001$) [Figure 5C and D].

Table 3. Univariate and multivariate COX regression analysis of variables for OS prediction

Variables	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P*-value	HR (95%CI)	*P*-value
Age (y)				
< 65	Ref.			
≥ 65	1.68 (0.97-2.89)	0.064		
Gender				
Male	Ref.	-	Ref.	-
Female	0.46 (0.21-0.98)	0.044*	0.35 (0.15-0.78)	0.010*
ECOG				
0	Ref.	-		
1	1.75 (0.85-3.60)	0.126		
Smoking status				
Never	Ref.	-		
Previous/Current smoker	1.48 (0.83-2.64)	0.187		
PD-L1 TPS				
< 1%	Ref.	-	Ref.	
≥ 1%	0.56 (0.32-0.99)	0.045*	0.79 (0.43-1.47)	0.455
T-stage, per stage	1.31 (1.01-1.69)	0.036*	1.43 (1.09-1.87)	0.009*
N-stage, per stage	0.97 (0.71-1.33)	0.869		
M-stage				
M0	Ref.			
M1	7.14 (0.98-51.81)	0.052		
ImmuneScore				
Low	Ref.		Ref.	
High	0.28 (0.16-0.48)	< 0.001*	0.32 (0.18-0.58)	< 0.001*

OS: Overall survival; HR: hazard ratio; ECOG: Eastern Cooperative Oncology Group performance score; PD-L1 TPS: programmed death-ligand 1 tumor proportion score. *$P < 0.05$.

A nomogram was constructed by incorporating three statistically significant variables identified in multivariate COX regression analysis, that is, gender, tumor T-stage, and ImmuneScore [Figure 4C]. The nomogram exhibited good ability in predicting 6-month OS rate (AUC = 0.82, 95%CI: 0.68-0.95), 12-month OS rate (AUC = 0.74, 95%CI: 0.62-0.86), and 24-month OS rate (AUC = 0.84, 95%CI: 0.75-0.93) [Figure 4D]. The calibration curve and decision curve exhibited good performance [Supplementary Figure 1B].

Enrichment of immune response-related pathways in patients with high ImmuneScores

As shown in Figure 6, several immune response-related pathways were significantly enriched in the high-ImmuneScore group, such as KEGG_hematopoietic_cell_lineage, KEGG_cytokine_cytokine_receptor_interaction, and KEGG_T_cell_ receptor_ signaling_ pathway. This suggested that compared with the low-ImmuneScore group, the high-ImmuneScore group had a more active antitumor immune response.

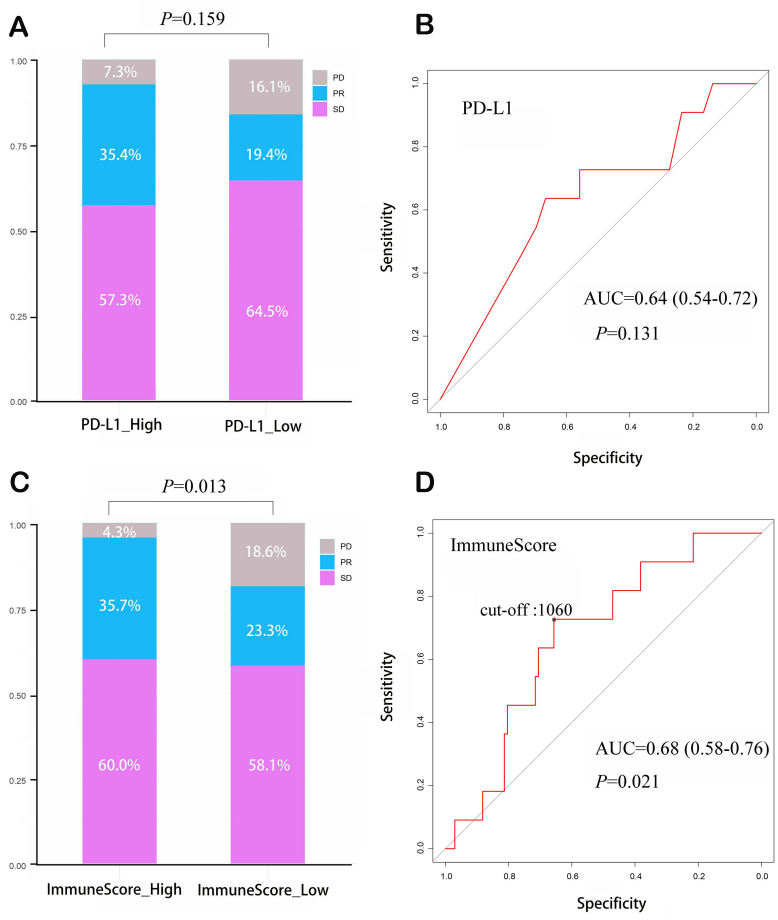

Figure 2. ImmuneScore as a predictor of early disease progression in patients receiving ICI therapy combined with chemotherapy. (A) PD-L1 TPS could not effectively predict early tumor progression; (B) ROC curve and corresponding AUC for PD-L1 TPS in predicting early disease progression; (C) ImmuneScore could predict early tumor progression; (D) ROC curve and corresponding AUC for ImmuneScore in predicting early disease progression. ICI: Immune checkpoint inhibitor; PD-L1 TPS: programmed cell death 1 ligand 1 tumor proportion score; ROC: receiver operating characteristic curve; AUC: area under the ROC curve; PD: progressive disease; PR: partial response; SD: stable disease.

DISCUSSION

Although PD-L1 TPS serves as the most commonly used biomarker in predicting ICI therapy efficacy currently[20], our present study revealed that PD-L1 TPS neither effectively predicts early tumor progression nor PFS in the combination chemoimmunotherapy population. With regard to OS, PD-L1 TPS demonstrated prognostic value in univariate COX regression, but its significance was lost after adjustment in the multivariate analysis. In agreement with our findings, previous studies found that the predictive power of PD-L1 is inadequate for patients receiving ICI therapy combined with chemotherapy[21,22]. Chemotherapy alters the tumor immune microenvironment, and combined immunotherapy and chemotherapy may have a synergistic effect[23,24]. We hence consider that in patients receiving combination therapy, PD-L1 is insufficient to reflect their antitumor immunity level, which may interpret its limited prognostic value in this population.

Some multigene RNA signature-based assays have been used to predict the prognosis of malignancies and assist in treatment decision-making. For instance, the 70-gene signature is used to aid treatment decisions in early-stage breast cancer[25]. However, as far as we know, no RNA-based signature has been clinically applied

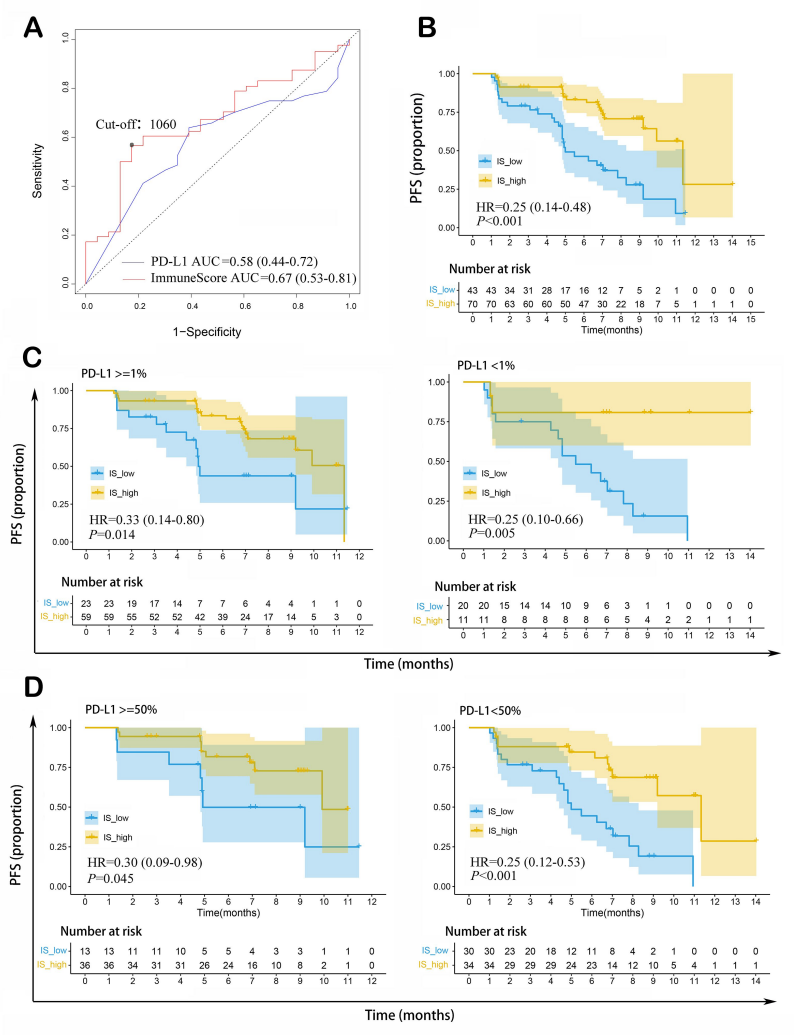

Figure 3. ImmuneScore as a prognostic indicator of PFS in patients with non-squamous NSCLC receiving ICI therapy combined with chemotherapy. (A) ImmuneScore could predict median PFS, with higher AUC than that of PD-L1 TPS; (B) Patients with high ImmuneScore had longer PFS than those with low ImmuneScore; (C) Higher ImmuneScore indicated longer PFS in PD-L1 TPS ≥ 1% and PD-L1 TPS < 1% subgroups; (D) Higher ImmuneScore indicated longer PFS in PD-L1 TPS ≥ 50% and PD-L1 TPS < 50% subgroups. NSCLC: Non-squamous cell lung cancer; ICI: immune checkpoint inhibitor; PFS: progression-free survival; PD-L1 TPS: programmed cell death 1 ligand 1 tumor proportion score; AUC: area under the ROC curve; IS: ImmuneScore; HR: hazard ratio.

in the field of lung cancer yet. In our current study, the ImmuneScore for each patient was calculated based on total RNA sequencing data, via the ESTIMATE algorithm proposed by Yoshihara *et al.* Briefly, ImmuneScore is calculated as the enrichment score of a gene set containing 141 immune-related genes, on the basis of the ssGSEA algorithm[17]. Our results revealed that ImmuneScore had prognostic value in advanced non-squamous NSCLC patients receiving chemotherapy combined with ICI therapy, with higher AUCs than that of PD-L1, indicating its relatively superior predictive ability. Specifically, patients with higher ImmuneScores had lower early tumor progression rates, longer PFS, and longer OS than those with lower ImmuneScores. The available evidence suggests that the tumor immune microenvironment is an important determinant of sensitivity to ICI treatment[26,27]. We consider that ImmuneScore seems to be a biomarker assisting in distinguishing so-called "immune-hot tumors" or "immune-cold tumors"[28]. According to the present GSEA results, patients with high ImmuneScore values are likely to have "immune-hot tumors", which are characterized by active antitumor immune response, high level of immune

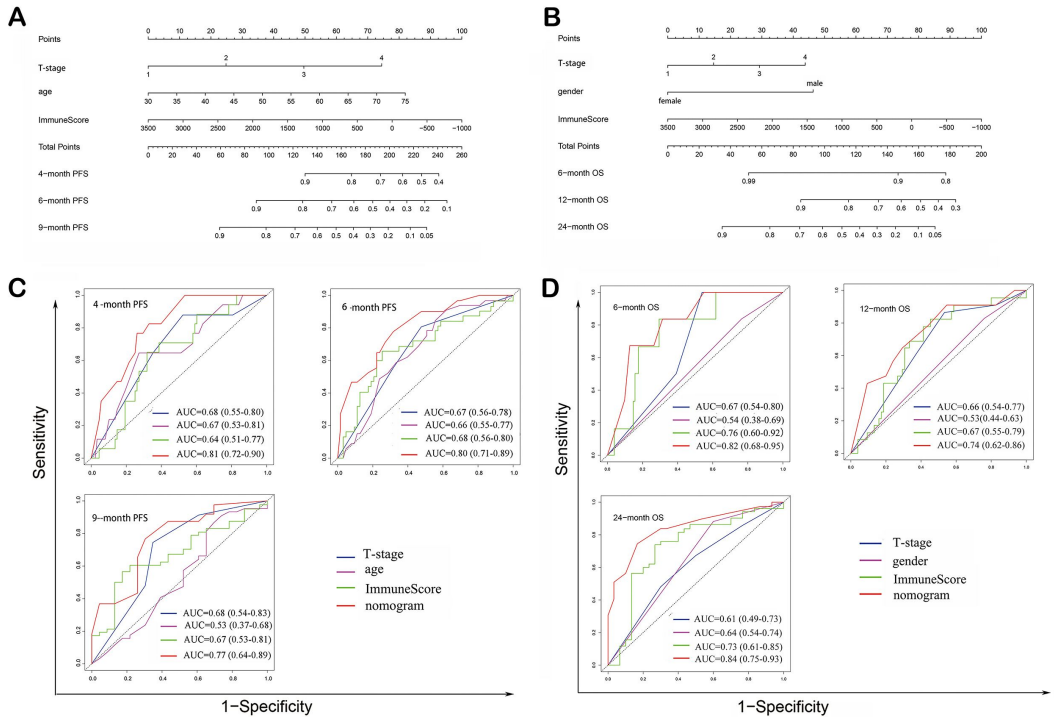

Figure 4. Construction of prognostic nomogram models. (A) Prognostic nomogram model construction for PFS prediction; (B) ROC curves and corresponding AUCs for variables in predicting PFS; (C) Prognostic nomogram model construction for OS prediction; (D) ROC curves and corresponding AUCs for variables in predicting OS. NSCLC: Non-squamous cell lung cancer; ICI: immune checkpoint inhibitor; PFS: progression-free survival; OS: overall survival.

infiltration, and sensitivity to immunotherapy. In contrast, those with low ImmuneScore values are likely to have "immune-cold tumors", which are characterized by inactive antitumor response, low level of immune infiltration, and insensitivity to immunotherapy. Additionally, in considering that the current RNA-based signatures are mostly based on absolute RNA expression levels and have limited reproducibility across platforms[29], ImmuneScore may possess specific advantages, since ImmuneScore is calculated by the ssGSEA algorithm, which is based on the rank order and empirical cumulative distribution functions of 141 targeted genes, rather than their absolute expression levels[17].

Based upon our findings, we suggest ImmuneScore be routinely detected in advanced NSCLC patients without sensitive mutation so as to guide clinical decision-making in the future. We are more inclined to recommend those with low ImmuneScore to receive chemotherapy rather than combination chemoimmunotherapy, since they are more likely to possess immune-cold tumors. In contrast, those with high ImmneScore are more likely to possess immune-hot tumors and thus would be recommended to receive combination chemoimmunotherapy. Multidisciplinary collaboration involving clinicians, pathologists and bioinformatics specialists is essential for the construction of "ImmuneScore platform" and quality control. As an RNA-based signature, the clinical application of ImmuneScore faces some challenges. For one, due to the limited stability of RNA and its susceptibility to degradation, cryopreservation and a shorter transportation time for specimens would be required. Further, despite continuing price reductions, the cost of traditional total RNA sequencing is still relatively high. A desirable alternative may be to adopt "gene panels" like tumor mutation burden (TMB) detection, which may accelerate the clinical application process. More cohorts need to be examined in the future to identify and optimize the suitable "RNA panel".

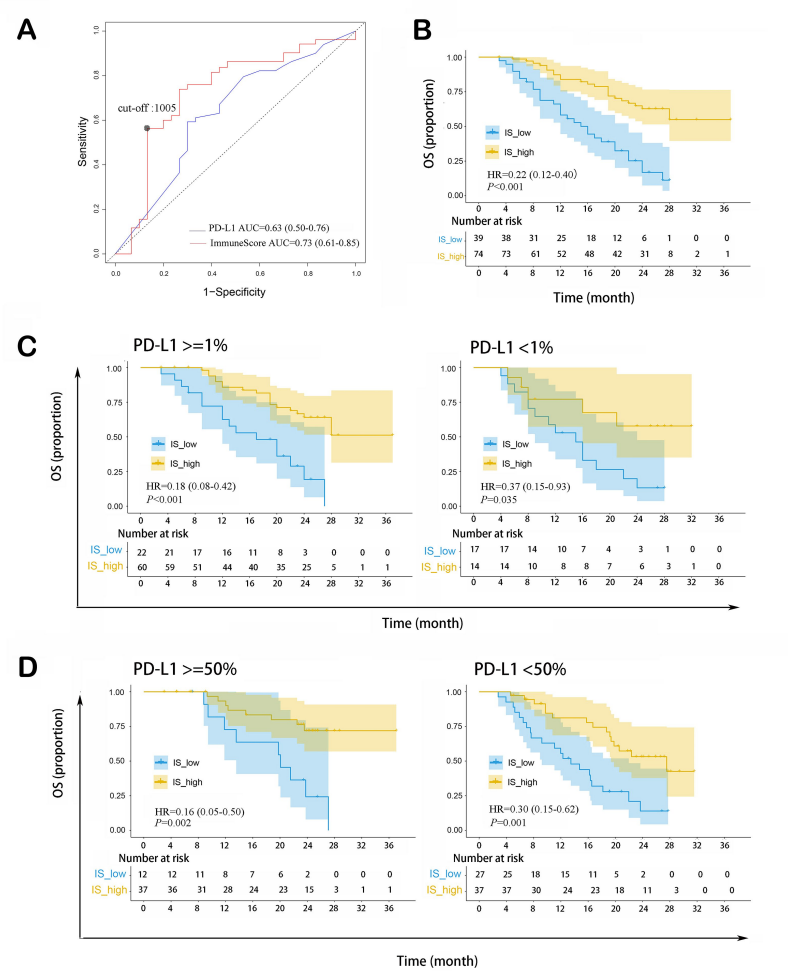

Figure 5. ImmuneScore as a prognostic indicator of OS in patients with non-squamous NSCLC receiving ICI therapy combined with chemotherapy. (A) ImmuneScore could predict median OS, with higher AUC than that of PD-L1 TPS; (B) Patients with high ImmuneScore had longer OS; (C) Higher ImmuneScore indicated longer OS in PD-L1 TPS ≥ 1% and PD-L1 TPS < 1% subgroups; (D) Higher ImmuneScore indicated longer OS in PD-L1 TPS ≥ 50% and PD-L1 TPS < 50% subgroups. NSCLC: Non-small cell lung cancer; ICI: immune checkpoint inhibitor; OS: overall survival; PD-L1 TPS: programmed cell death 1 ligand 1 tumor proportion score; AUC: area under the ROC curve; IS: ImmuneScore; HR: hazard ratio.

Apart from immuneScore, our study also identified several valuable clinical parameters including tumor T-stage, gender, and age. Tumor T-stage is mainly determined by tumor size. Previous studies revealed large tumors to be more immunosuppressive and less responsive to cytotoxic treatments compared to small tumors[30,31]. This fits well with our finding that T-stage was negatively associated with both PFS and OS. As to age, patients ≥ 65 years had shorter PFS time than those < 65 years. It may be due to the immunosuppression tumor phenotypes in elderly patients[32]. Their poor tolerance leading to susceptible to dose reduction or treatment delay may also worsen outcomes[33]. In the case of gender, this study found that female patients obtained longer OS time than their male counterparts, which was consistent with a previous meta-analysis[34]. This may account for the different immune features between males and females determined by genetics and sex hormones[35]. It was found that females possessed more abundant immune cell infiltration and more excellent anti-cancer immunity response than males and thus benefit more from immunotherapy[36]. Indeed, the impact of age or gender on immunotherapy or chemoimmunotherapy outcomes remained controversial, with published studies reaching inconsistent conclusions[32,37,38]. Although

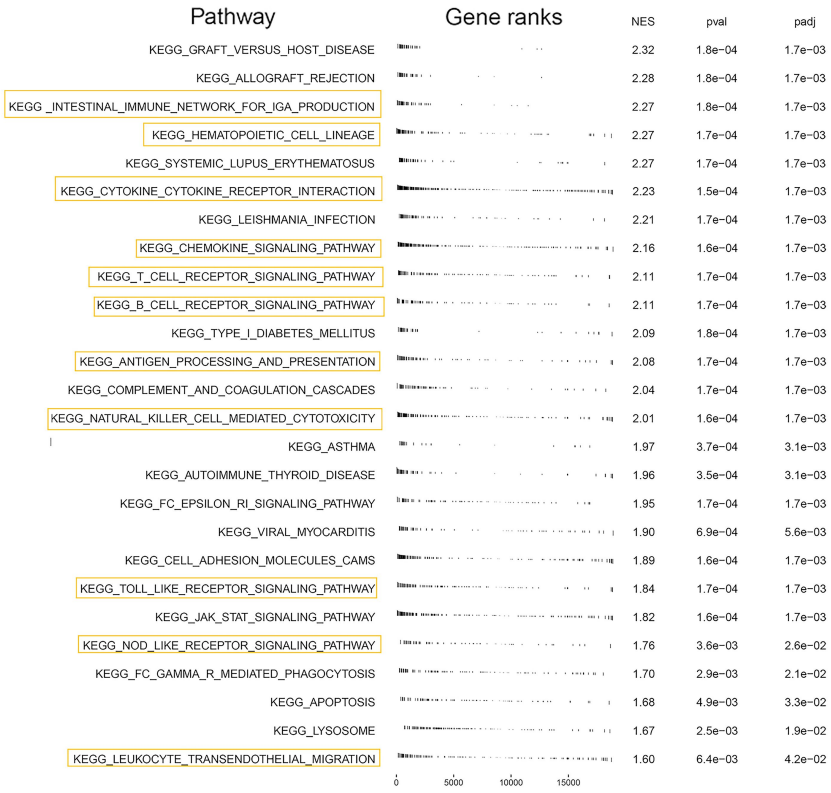

Figure 6. Significant KEGG signaling pathways involved in the high versus low ImmuneScore group. Several immune-associated pathways were enriched in the high ImmuneScore group, which were highlighted with yellow frame border.

our current study supported new evidence, prospective cohort studies and molecular biology studies are still needed to further elucidate it.

Some limitations of this study need to be considered. First, our cohort comprised only Chinese patients with non-squamous NSCLC and only sintilimab was used as the ICI therapy regimen. Therefore, generalizability to populations from other regions of the world, patients with squamous NSCLC, or patients receiving treatment with other immunotherapeutic agents needs to be further confirmed. Second, since TMB was not detected in our samples, we could not include this dynamic immunological marker in multivariate regression COX analysis. Third, since patients receiving ICI monotherapy were not available in this study, we could not verify the prognostic value of ImmuneScore in this population. Finally, due to the retrospective nature, we adopted the patients' efficacy evaluation data generated by BIRRC, based on RECIST 1.1 criteria rather than re-evaluating it by ourselves based on immune RECIST (iRECIST) criteria, which could not identify the so-called pseudoprogression due to immunotherapy[39]. Previous studies suggested that the pseudoprogression considered by iRECIST was rare in NSCLC, occurring in about 0%-5% of patients[40]. Thus, we considered that this limitation would not impact the reliability of our results.

In conclusion, ImmuneScore calculated by the ESTIMATE algorithm serves as a promising RNA-based prognostic signature for advanced non-squamous NSCLC patients receiving chemotherapy combined with ICI therapy, with predictive efficacy superior to that of PD-L1 TPS. Specifically, higher ImmuneScore

indicates lower rate of early tumor progression, longer PFS, and longer OS. Further, the nomogram integrating ImmuneScore and clinical parameters, such as tumor T-stage, age, and gender, may serve as a tool for prognostic prediction and screening the dominant population to receive combined ICI treatment and chemotherapy.

DECLARATIONS

Authors' contributions

Contributed to the conception and design of the study: Zhang L, Lin Z
Contributed to the data acquisition and statistical analysis: Lin Z, Zhan J
All authors wrote and approved the final manuscript.

Availability of data and materials

The data that support our findings in this study are available from the corresponding author upon reasonable request.

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Ethics approval and consent to participate

Given the retrospective nature of our current study and the fact that no personal identifiers were contained, the need for ethical approval and individual informed consent was waived by the Ethics Commission of Sun Yat-sen University Cancer Center. All methods were carried out in accordance with the Declaration of Helsinki.

Consent for publication

Given the retrospective nature of our current study and the fact that no personal identifiers were contained, the need for individual informed consent was waived by the Ethics Commission of Sun Yat-sen University Cancer Center. All methods were carried out in accordance with the Declaration of Helsinki.

Conflicts of interest

All authors declare that there are no conflicts of interest.

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