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PathwayTMB: A pathway-based tumor mutational burden analysis method for predicting the clinical outcome of cancer immunotherapy

Xiangmei Li,[1,3] Yalan He,[1,3] Ying Jiang,[2,3] Bingyue Pan,[1] Jiashuo Wu,[1] Xilong Zhao,[1] Junling Huang,[1] Qian Wang,[1] Liang Cheng,[1] and Junwei Han[1]

[1]College of Bioinformatics Science and Technology, Harbin Medical University, Harbin 150081, China; [2]College of Basic Medical Science, Heilongjiang University of Chinese Medicine, Harbin 150040, China

Immunotherapy has become one of the most promising therapy methods for cancer, but only a small number of patients are responsive to it, indicating that more effective biomarkers are urgently needed. This study developed a pathway analysis method, named PathwayTMB, to identify genomic mutation pathways that serve as potential biomarkers for predicting the clinical outcome of immunotherapy. PathwayTMB first calculates the patient-specific pathway-based tumor mutational burden (PTMB) to reflect the cumulative extent of mutations for each pathway. It then screens mutated survival benefit-related pathways to construct an immune-related prognostic signature based on PTMB (IPSP). In a melanoma training set, IPSP-high patients presented a longer overall survival and a higher response rate than IPSP-low patients. Moreover, the IPSP showed a superior predictive effect compared with TMB. In addition, the prognostic and predictive value of the IPSP was consistently validated in two independent validation sets. Finally, in a multi-cancer dataset, PathwayTMB also exhibited good performance. Our results indicate that PathwayTMB could identify the mutation pathways for predicting immunotherapeutic survival, and their combination may serve as a potential predictive biomarker for immune checkpoint inhibitor therapy.

INTRODUCTION

With the advent of the era of precision medicine, immunotherapy with immune checkpoint inhibitors (ICIs) is one of the most promising therapy methods for a range of tumors. However, only a minority of patients benefit from immunotherapy. Thus, there is an urgent need to discover biomarkers that allow for more precise identification of patients who can benefit from ICI treatment.

Accumulating evidence indicates that high tumor mutational burden (TMB) is a leading candidate biomarker to predict the immunotherapy response,[1,2] and a recent study confirmed that TMB can be used as a potential biomarker of benefit to non-small cell lung cancer (NSCLC) patients treated with nivolumab plus ipilimumab.[3] However, the tumor heterogeneity limits its clinical application. Moreover, Prasad and Addeo reported that the US Food and Drug Administration approval of pembrolizumab for patients with 10 mut/Mb was an unwise decision because of the high cost and the arbitrariness of the cutoff of 10 mut/Mb.[4] McGrail et al. reported that their research failed to support the application of TMB as a biomarker for treatment with ICIs in all solid cancer types,[5] and TMB remains a controversial biomarker in some cancer types.[6] Programmed death ligand-1 (PD-L1) expression is another potential biomarker, but Brahmer et al. performed a clinical trial of immunotherapy, and their results showed that the PD-L1 expression level was neither prognostic nor predictive of efficacy in squamous-cell NSCLC patients treated with nivolumab.[7] Moreover, the tumor heterogeneity and dynamic changes have also limited the clinical application of PD-L1 expression.[8] Thus, there is an urgent need to identify more effective and precise biomarkers for immunotherapy.

Mounting evidence shows that specific gene or pathway mutations are related to the clinical outcome of immunotherapy.[9,10] For example, the ZFHX3 mutation was found to be a protective biomarker for ICIs in NSCLC.[11] Pan et al. developed a 52-gene mutation signature that could predict immunotherapy benefits in patients with NSCLC.[12] Furthermore, recent research has revealed that mutations tend to occur within a core group of pathways.[13] Wang et al. demonstrated that co-mutations in the DNA damage repair (DDR) pathways could serve as potential biomarkers for ICI treatment.[10] Although they provided a potentially convenient approach for the clinical practice of immunotherapy, they only focused on the DDR pathways and neglected the other important pathways that may be associated with cancer progression and clinical outcome. Mutations in the important pathways, such as signaling

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[3]These authors contributed equally

Correspondence: Liang Cheng, College of Bioinformatics Science and Technology, Harbin Medical University, Harbin 150081, China.
E-mail: liangcheng@hrbmu.edu.cn
Correspondence: Junwei Han, College of Bioinformatics Science and Technology, Harbin Medical University, Harbin 150081, China.
E-mail: hanjunwei@ems.hrbmu.edu.cn

pathways that control cell-cycle progression, apoptosis, and cell growth, are common hallmarks of cancer.[14] Mutations in these pathways may not only produce neoantigens but may also functionally affect the outcome of immunotherapy.[12] Therefore, there is an urgent need to identify the key pathways whose mutations may influence the response to immunotherapy.

Here, we developed a pathway analysis method named PathwayTMB to identify the pathways with genomic mutations that serve as potential biomarkers for ICIs. PathwayTMB first defines the patient-specific pathway-based TMB (PTMB) to reflect the cumulative extent of mutations for each pathway. We screened the survival benefit-related pathways based on PTMB and constructed an immune-related prognostic signature based on these pathways (IPSP) to predict the response to immunotherapy and the clinical outcome. In a melanoma cohort, we identified a three-pathway IPSP, and IPSP-high patients presented a longer overall survival (OS) and a higher objective response rate (ORR) than IPSP-low patients. The predictive power of IPSP with respect to the clinical benefits of immunotherapy was found to be superior to that of TMB. Moreover, the prognostic and predictive value of IPSP was consistently validated in two independent validation cohorts. To test if the PathwayTMB method could predict immunotherapy responsiveness in other cancer types, we applied the method to a multi-cancer cohort and obtained promising results. Finally, we implemented PathwayTMB as a freely available R-based package to expand its usage (https://CRAN.R-project.org/package=pathwayTMB).

RESULTS

Construction of the immune-related prognostic signature based on PTMB

We developed the PathwayTMB method to identify potential biomarkers for predicting the clinical outcome of immunotherapy. A detailed flowchart of the PathwayTMB method is shown in Figure 1. In the training cohort of 104 metastatic melanoma patients treated with CTLA-4 inhibitors,[15] the genes mutated in at least 1% of cancer patients were retained. Next, the survival benefit-related mutations were identified and mapped to the pathways (see materials and methods). To obtain the prognostic-related mutated pathways, we first calculated the patient-specific PTMB to reflect the cumulative extent of TMB at the pathway level. Through the Wilcoxon rank-sum test and fold change (FC) values, 32 differential PTMB pathways between alive and deceased samples were identified with p value <0.01 and $|\log_2(FC)| > 1$ (Figure 2A). We then applied random forest with nested cross-validation to select the important survival-related pathways based on PTMB by calculating the important score for each pathway (see materials and methods), and seven pathways were obtained. Finally, the LASSO-Cox regression model was constructed and the log rank test was performed to find the most critical prognosis-related significant pathways (Figure 2B). We thus obtained three candidate pathways, namely, the JAK-STAT signaling pathway, signaling pathways regulating pluripotency of stem cells (PSC), and the adipocytokine signaling pathway (Table S2).

To analyze if mutations in these three candidate pathways could predict ICI treatment efficacy, we first classified each pathway as mutation or wild type (WT) based on the presence or absence of a pathway mutation (at least one gene mutated in the pathway) and then divided 104 metastatic melanoma patients of the training cohort into the following three subgroups: (1) those without mutations in the three pathways (WT group), (2) those with only one of the three pathways mutated (single mutation group), and (3) those with two or more of the three pathways mutated (compound mutation group). Through comparing the OS among the three subgroups (Figure 2C), our results showed that the patients in the compound mutation group showed significantly better prognosis than the single mutation and WT groups (Kaplan-Meier survival analysis, log rank test compound vs. single, p = 1.14e−03; compound vs. WT, p = 9.85e−03). Therefore, the three mutation pathways may jointly influence the clinical outcome of immunotherapy.

To predict the clinical outcome of immunotherapy by using the three pathways jointly, a risk model defined as IPSP was calculated using a formula derived from the PTMBs of the three pathways weighted by their multivariate Cox proportional hazards regression coefficient: IPSP score = (0.020 × JAK-STAT signaling pathway) + (0.094 × adipocytokine signaling pathway) + (0.043 × signaling pathways regulating PSC). The median value of the IPSP score was used to separate the patients into the IPSP-high (>0.162) and IPSP-low ($\leq$ 0.162) groups in the training cohort. The Kaplan-Meier survival curves showed that patients in the IPSP-high group had significantly longer OS than those in the IPSP-low group (median OS, 21.55 months vs. 6.93 months, log rank test, p < 0.0001; Figure 2D). Then, univariable and multivariable analyses of clinicopathological factors (TMB, sex, age, and stage) in the training cohort for OS were performed. The results showed that the IPSP score could serve as an independent prognostic factor for OS after multivariable adjustment by clinicopathological variables (Table 1).

Prediction of clinical benefit from immunotherapy based on IPSP

Recently, TMB has been proposed as a potential biomarker for ICI therapy in metastatic melanoma.[16] We thus evaluated the relationship between the pathway mutation signature and TMB in predicting the patient response to ICI treatment. For each cohort, we calculated the TMB as the total number of non-synonymous somatic mutations in the coding region per megabase.[17] Patients with mutations in two or more candidate pathways (compound mutation group) possess significantly higher average TMB than the single mutation group and the WT group (Wilcoxon rank-sum test, p = 0.041 and p = 2e−08; Figure 3A). It was also observed that IPSP-high patients had a significantly higher average TMB than IPSP-low patients (Wilcoxon rank-sum test, p = 1.20e−04; Figure 3B). Moreover, IPSP showed a significant positive correlation with TMB (Pearson correlation R = 0.49, p = 8.4e−07; Figure S1A).

We next compared the relative predictive powers of the IPSP vs. TMB in the metastatic melanoma patient cohort. According to the median value

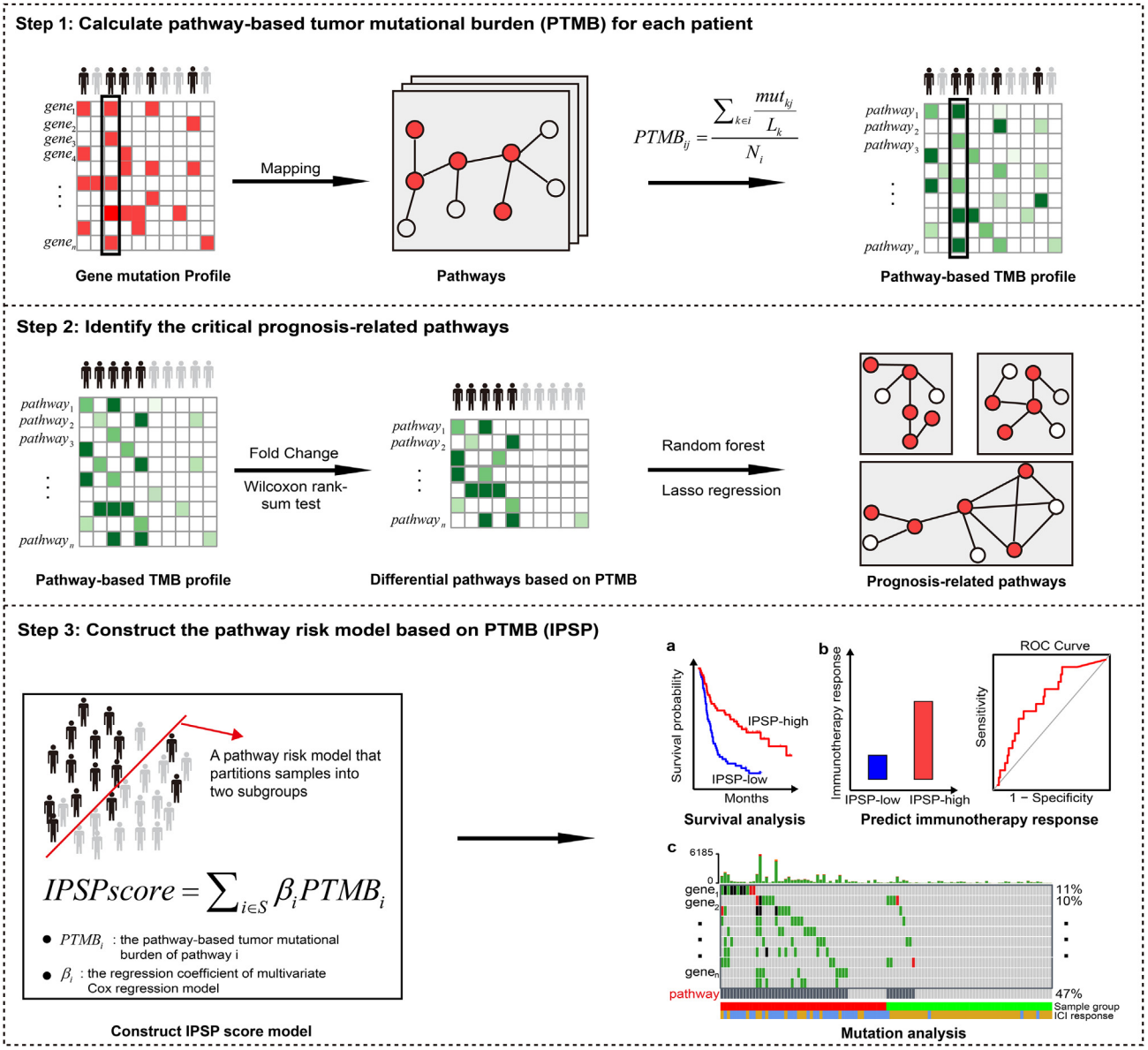

Figure 1. Flowchart of the PathwayTMB method.

of the TMB, the patients were classified into a TMB-high and a TMB-low group. TMB-high patients seem to have a clear survival benefit compared with TMB-low patients, although this difference was not statistically significant (Figure S1B). To compare the IPSP with TMB, we stratified patients with high or low TMB based on the IPSP and compared their OS levels (Figure 3C). We observed that IPSP-high patients with low TMB (median OS, 21.23 months) had a significant survival advantage compared with IPSP-low patients with either high TMB (median OS, 5.58 months) or low TMB (median OS, 7.43 months) (log rank test, IPSP-high and TMB-low vs. IPSP-low and TMB-high, p = 3.31e−03; IPSP-high and TMB-low vs. IPSP-low and TMB-low, p = 9.96e−03), which seems to contradict the previous results that patients with high TMB levels had better immunotherapy efficacy. But that may not be the case because previous studies indicated that high tumor mutational burden failed to predict immune checkpoint blockade response across all cancer types,[5,18,19] suggesting even tumors with low TMB levels may respond to immunotherapy. These findings demonstrated that our PathwayTMB approach can identify potential immunotherapy responders in patients with low TMB levels, revealing the superiority of the IPSP in predicting ICI treatment benefits compared with TMB in patients with metastatic melanoma.

We further tested if the IPSP could predict the response of immunotherapy in patients with metastatic melanoma. A significantly higher

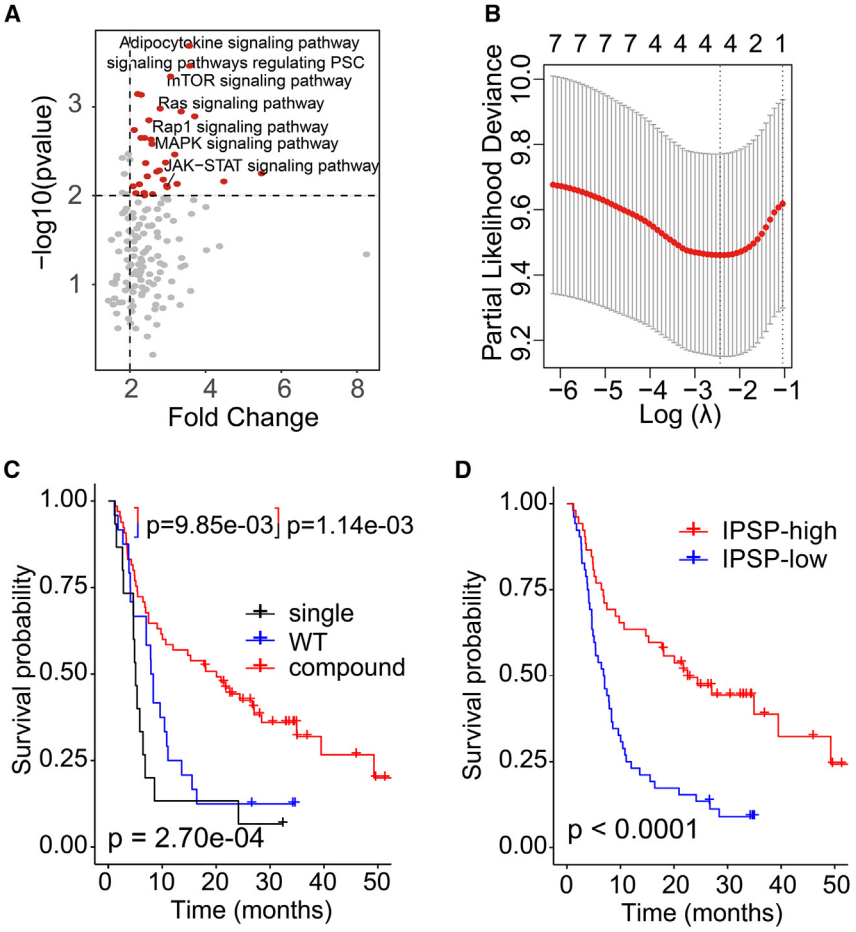

Figure 2. Construction of the immune-related prognostic signature based on PTMB

(A) Dot plot of differential PTMB pathways between deceased and alive samples ($p < 0.01$ and $|\log_2(FC)| > 1$). (B) Plot of 10-fold cross-validation via minimum criteria for selection of the optimal value of tuning parameter. The two dotted vertical lines are drawn at the optimal values by minimum criteria (right) and 1-SE criteria (left). (C) Kaplan-Meier survival curves of OS comparing the three subgroups with different pathway mutation signature components from the melanoma training cohort. (D) Kaplan-Meier survival curves of OS comparing the IPSP-high and IPSP-low groups from the melanoma training cohort.

response rate was displayed in the IPSP-high group compared with the IPSP-low group (25% vs. 7.7%, chi-squared test, p = 0.03; Figure 3D). Although the TMB-high group also showed a higher response rate compared with the TMB-low group, this difference was not significantly different (21.2% vs. 11.5%, chi-squared test, p = 0.29; Figure 3E). Moreover, receiver operating characteristic (ROC) curve analysis revealed that the IPSP has a better predictive power of the response to immunotherapy than TMB (area under the ROC [AUROC] of IPSP = 0.70 vs. AUROC of TMB = 0.59; Figure 3F).

Validation of the predictive value of IPSP in the independent validation cohorts

To validate the predictive value for immunotherapy of the IPSP model, we collected two independent immunotherapy cohorts of metastatic melanoma patients. The first validation cohort was from the Miao et al. study,[20] including 145 metastatic melanoma patients treated with CTLA-4 inhibitors. To test if the IPSP could predict the prognosis of patients treated with other inhibitors, we also collected data from a second validation cohort from the Hugo et al. study[21] including 37 metastatic melanoma patients treated with PD-1 inhibitors. Using the IPSP cutoff value obtained from the training cohort, the patients in both cohorts were divided into an IPSP-high group and an IPSP-low group. For the Miao et al. cohort, 99 of 145 patients were classified as the IPSP-high group, and their OS was significantly longer than that of the IPSP-low group (median OS, 20.66 months vs. 8.01 months; log rank test, p = 0.0041; Figure 4A). For the Hugo et al. cohort, IPSP-high patients (n = 28) had a better OS compared with IPSP-low patients (n = 9) (median OS, 20.8 months vs. 10.7 months; log rank test, p = 0.011; Figure 4B). However, no significant association was found between the TMB level and the OS in both validation cohorts (log rank test, p = 0.10 for the Miao et al. cohort; p = 0.17 for the Hugo et al. cohort; Figures S1C and S1D). Moreover, through multivariable adjustment by clinicopathological variables, the IPSP score was shown to be an independent prognostic factor for OS in these two patient cohorts (Table S3). These results validated the predictive efficacy of the IPSP for OS in the independent patient cohorts treated with CTLA-4 or PD-1 inhibitors.

We next tested if the IPSP could predict the response to immunotherapy of patients in the two independent validation sets. In the Miao et al. cohort, IPSP-high patients displayed higher response rates than IPSP-low patients (28.3% vs. 9.5%, chi-squared test, p = 0.03; Figure 4D), which also showed a better prediction result than TMB (TMB-high vs. TMB-low, 28.2% vs. 17.1%, chi-squared test, p = 0.17; Figure S1E). Similarly, in the Hugo et al. cohort, the IPSP-high patient group also showed a remarkably higher response rate than the IPSP-low patient group (60.7% vs. 33.3%, chi-squared test, p = 0.29; Figure 4E). This result was not statistically significant, possibly because of the small sample size of this dataset. In spite of this, the IPSP showed a better prediction result of the immunotherapy response than TMB (TMB-high vs. TMB-low, 57.9% vs. 50.0%, chi-squared test, p = 0.88; Figure S1F). Finally, through the ROC curve analysis, the predictive power of the response to immunotherapy of IPSP was demonstrated to be superior to that of TMB in the Miao

Table 1. Univariable and multivariable Cox regression analyses of clinicopathological factors in melanoma training cohorts for overall survival

	Univariate analysis			Multivariate analysis		
Variable	HR	95% CI	p value	HR	95% CI	p value
IPSP (high vs. low)	0.37	0.23–0.59	**<0.01**	0.29	0.18–0.49	**<0.01**
TMB (high vs. low)	0.91	0.58–1.42	0.67	1.12	0.69–1.81	0.65
Sex (female vs. male)	1.20	0.73–1.95	0.47	1.31	0.76–2.28	0.33
Age (≥65 vs. < 65)	1.16	0.75–1.82	0.37	1.43	0.85–2.40	0.18
M_stage (M1 vs. M0)	4.53	1.42–14.40	**<0.01**	5.25	1.62–16.99	**<0.01**

HR is the hazard ratio of cox proportional-hazards regression model; 95% CI is the 95% confidence interval of cox proportional-hazards regression model; p value is the statistic significance p vale of cox proportional-hazards regression model. The p value in bold indicates that the p value is statistically significant.

et al. cohort (AUROC = 0.66 for IPSP vs. AUROC = 0.63 for TMB) and the Hugo et al. cohort (AUROC = 0.64 for IPSP vs. AUROC = 0.58 for TMB) (Figure S2).

Moreover, in order to explore whether the IPSP model was cancer-specific, we applied the IPSP model trained by the melanoma cohort to the NSCLC cohort, which was obtained from the Hellmann et al. study.[22] Our analysis showed a significant difference in patient prognosis and immunotherapy response between IPSP-high and IPSP-low groups in the Hellmann et al. cohort (median OS, 13.34 months vs. 4.11 months, log rank test, p = 0.033; ORR, 47.2% vs. 21.9%, chi-squared test, p = 0.05; Figure S3), indicating that the IPSP model was not cancer-specific.

To further test if IPSP could serve as a predictive or prognostic factor for melanoma patients who do not receive immunotherapy, we performed survival analysis in the skin cutaneous melanoma (SKCM) cohort of The Cancer Genome Atlas (TCGA). According to the IPSP cutoff value obtained from the training cohort, significant differences in OS were observed between the IPSP-high and IPSP-low groups (log rank test, p = 0.014; Figure 4C), suggesting that the IPSP is not only a predictor of the response to immunotherapy but also a prognostic marker.

Comparison of the IPSP signature with other signatures

To further verify the performance of our PathwayTMB approach in predicting immunotherapy benefits, we compared our PathwayTMB method with the Long et al. method[23] and the Wang et al. method.[10] Long et al. developed a mutational signature to predict the prognosis of patients treated with ICIs, while Wang et al. found that co-mutations in the DDR pathways could serve as potential biomarkers for ICI treatment.

We first applied the mutational signature developed by Long et al. in our melanoma training cohort and two independent validation cohorts, and then patients in each cohort were defined as High-risk and Low-risk groups. In the melanoma training cohort (Van Allen et al.[15] cohort), there was no significant difference found in the patients' OS and immunotherapy response between High-risk and Low-risk groups (median OS, 10.55 months vs. 8.33 months, log rank test, p = 0.078; ORR, 16.67% vs. 15.38%, chi-squared test, p = 1; Figures S4A and S4B). As for the Miao et al. cohort, patients with low-risk scores (Low-risk) had a significantly better prognosis than those with high-risk scores (High-risk), but no significant difference was found in immunotherapy response (median OS, 26.70 months vs. 14.50 months, log rank test, p = 0.04; ORR, 23.89% vs. 26.47%, chi-squared test, p = 0.82; Figures S4C and S4D). As we excepted, in the Hugo et al. cohort, the patients' prognosis and immunotherapy response also did not show significant differences between High-risk and Low-risk groups (median OS, 31.20 months vs. 27.40 months, log rank test, p = 0.38; immunotherapy response, 59.30% vs. 40%, chi-squared test, p = 0.46; Figures S4E and S4F). However, our IPSP signature displayed excellent prognostic and predictive power in all these three melanoma cohorts (Figures 2D, 3, and 4). Moreover, we also compared the performance of our PathwayTMB method with the Wang et al. method. We then applied the Wang et al. method to the above three melanoma cohorts, and our results showed that co-mutations in the DDR pathways failed to predict the clinical benefits of ICI treatment in the melanoma training cohort and two independent validation cohorts (Figures S4G–S4L). Altogether, these findings suggested that the PathwayTMB outperforms the Long et al. and Wang et al. methods in predicting the clinical outcomes of immunotherapy.

Association of IPSP with tumor immune-related features

According to the above results, we hypothesized that the IPSP may be an indicator of tumor immune-related features. We first investigated the correlations between the IPSP and immune-related pathways in SKCM samples from TCGA. The SKCM samples were stratified into an IPSP-high group and an IPSP-low group based on the IPSP. The gene differential expression levels between the IPSP-high and IPSP-low groups were calculated with the Student's t test, and a ranked gene list was constructed. Then, the gene set enrichment analysis (GSEA) method was applied to identify the significantly enriched pathways in IPSP-high samples (Figure S5). With false discovery rate <0.05, the results showed that several DDR pathways, such as base excision repair, homologous recombination, mismatch repair, and nucleotide excision repair, were significantly enriched in the IPSP-high group (Figure 5A). The DDR pathways are known to be important determinants of tumor immunogenicity, whose deficiencies may result in a durable clinical benefit from ICIs.[10] These findings indicate that the IPSP is associated with the DDR pathways in melanoma and partially explain the correlation between the IPSP and immunotherapy benefits.

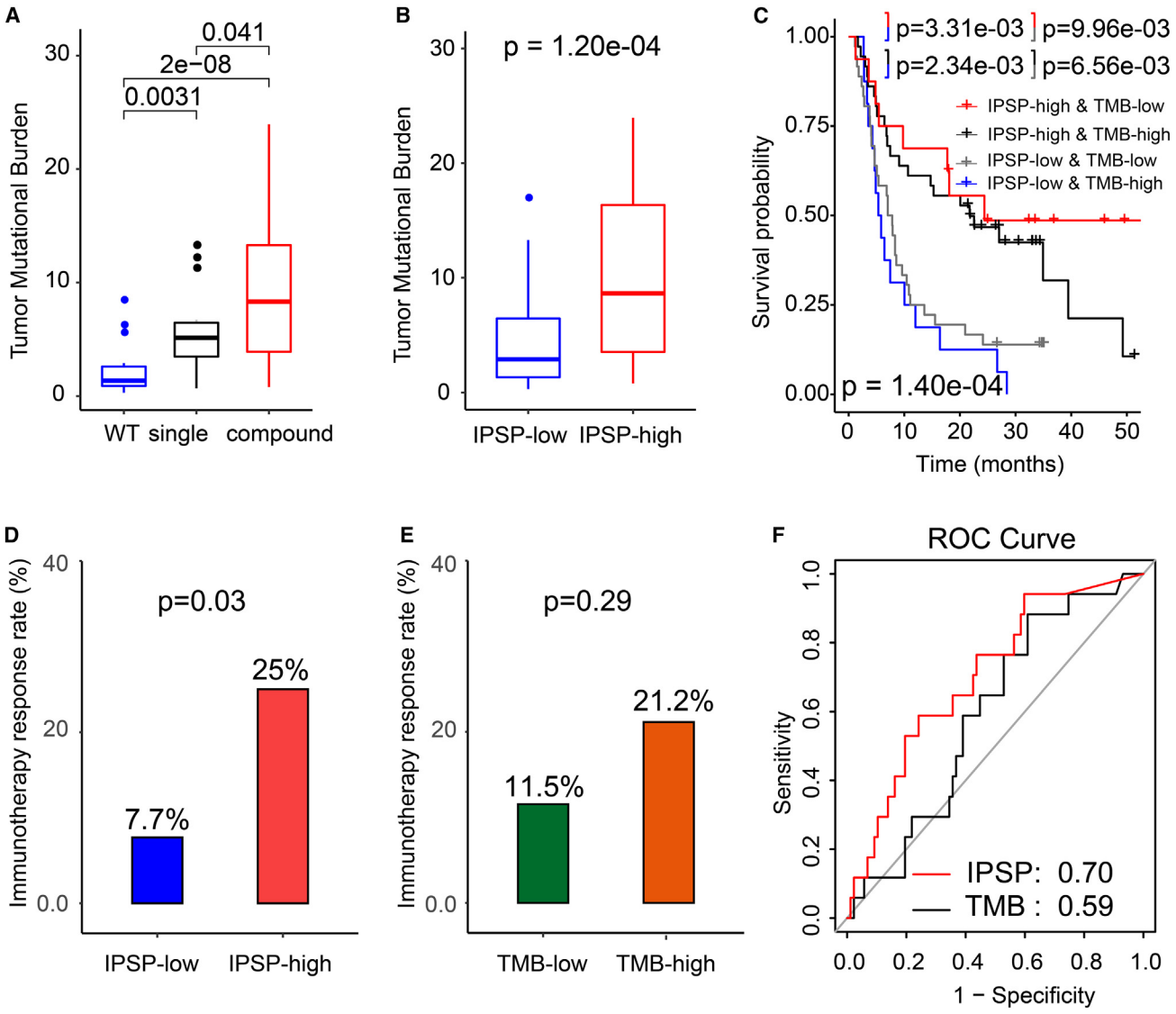

Figure 3. Prediction of clinical benefit with IPSP in the melanoma training cohort

(A) Comparison of TMB among the three subgroups with different pathway mutation signature components. (B) Comparison of TMB between IPSP-high and IPSP-low groups. Statistical significance was tested by rank-sum Wilcoxon test. (C) Kaplan-Meier survival analysis of OS among patients within each of the four indicated subgroups (IPSP-high and TMB-low, IPSP-high and TMB-high, IPSP-low and TMB-low, IPSP-low and TMB-high) from the melanoma training cohort. (D) Comparison of the objective response rate between the IPSP-high and IPSP-low groups from the melanoma training cohort. (E) Comparison of the objective response rate between the TMB-high and TMB-low groups from the melanoma training cohort. Statistical significance was tested by chi-squared test. (F) ROC curves of the IPSP and TMB to predict immunotherapy response from the melanoma training cohort.

A previous study suggested that gene mutations not only generate neoantigens that might be recognized by the immune system but may also influence the biology of the tumors such as altered tumor immune microenvironment.[12] We thus examined the relationships between the IPSP and tumor immune microenvironment features in metastatic melanoma patients. The CIBERSORT algorithm[24] was used to estimate the abundances of TIICs with gene transcriptomic data from the TCGA-SKCM samples. Compared with IPSP-low tumors, IPSP-high tumors were more infiltrated by M1 Macrophages, plasma cells, CD4+ memory-activated T cells, and follicular helper T (Tfh) cells, but had a low number of regulatory T cells (Tregs) (Figure 5B). Previous research confirmed that M1 Macrophages can kill tumor cells and inhibit tumor cell growth through phagocytosis[25] and that Tfh cells play key roles in enhancing the immune response.[26] Conversely, Tregs were proposed to be a barrier to anti-tumor immunity, which has been confirmed to suppress immune activity and whose malfunction has been associated with cancer progression and immunological disorders.[27,28] These observations indicate that the IPSP is positively

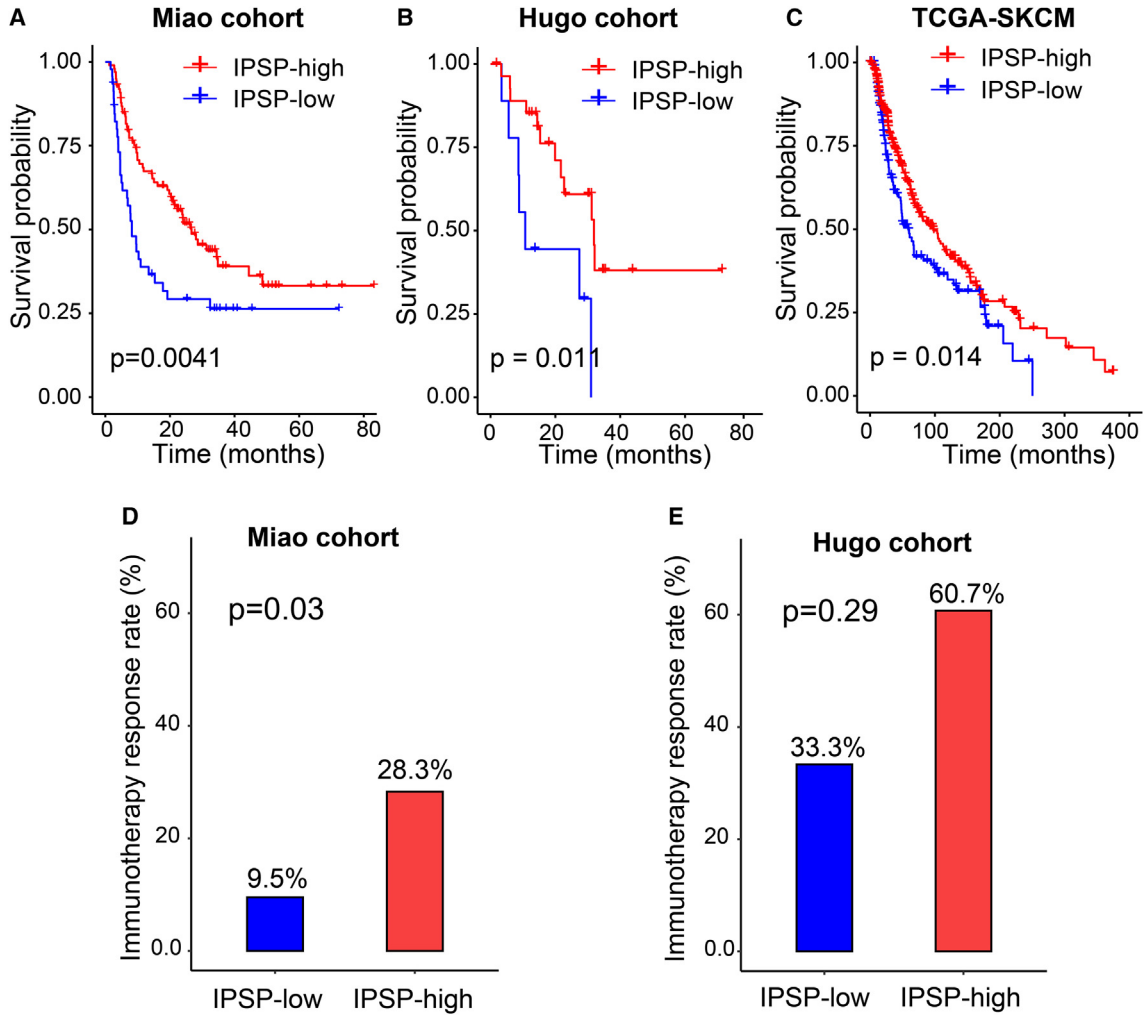

Figure 4. Validation of the predictive value of IPSP in the independent cohorts

(A) Kaplan-Meier survival analysis of OS comparing the IPSP-high and IPSP-low groups from the Miao cohort. (B) Kaplan-Meier survival analysis of OS comparing the IPSP-high and IPSP-low groups from the Hugo cohort. (C) Kaplan-Meier survival analysis of OS comparing the IPSP-high and IPSP-low groups from TCGA-SKCM cohort. (D) Comparison of the objective response rate between the IPSP-high and IPSP-low groups from the Miao cohort. (E) Comparison of the objective response rate between the IPSP-high and IPSP-low groups from the Hugo cohort.

correlated with some immunopromoting cells and negatively correlated with immunosuppressive cells, and thus IPSP-high patients are more inclined to respond to immunotherapy.

Analysis of the pathway signatures in IPSP

In the melanoma training cohort, IPSP included three pathway signatures: the JAK-STAT signaling pathway, the adipocytokine signaling pathway, and signaling pathways regulating PSC. The JAK-STAT signaling pathway is a critical cytokine pathway involved in proliferation, and the immune and inflammatory responses, and is thus closely related to cancer.[29] The adipocytokine signaling pathway refers to a cascade of events involving the adipocytokines in the body, which may participate in tumor growth and metastasis by secreting signaling factors (such as adipokines, proinflammatory cytokines, and extracellular matrix constituents) and acting as energy storage for embedded cancer cells.[30] Meanwhile, signaling pathways regulating PSC plays a critical role in maintaining the stemness and undifferentiated state of cancer stem cells, such as epithelial-to-mesenchymal transition linked to cancer progression and metastasis.[31] These studies showed that the three pathways may play an important role in tumorigenesis and development either individually or by interacting. Besides, our previous results suggested that IPSP-high patients were significantly enriched in DDR pathways, which are intimately connected to the emission of immunomodulatory signals.[32] Altogether, these findings indicated that the candidate pathways and the dysregulated pathways may influence tumorigenesis and development by co-regulating anti-tumor immune responses in patients.

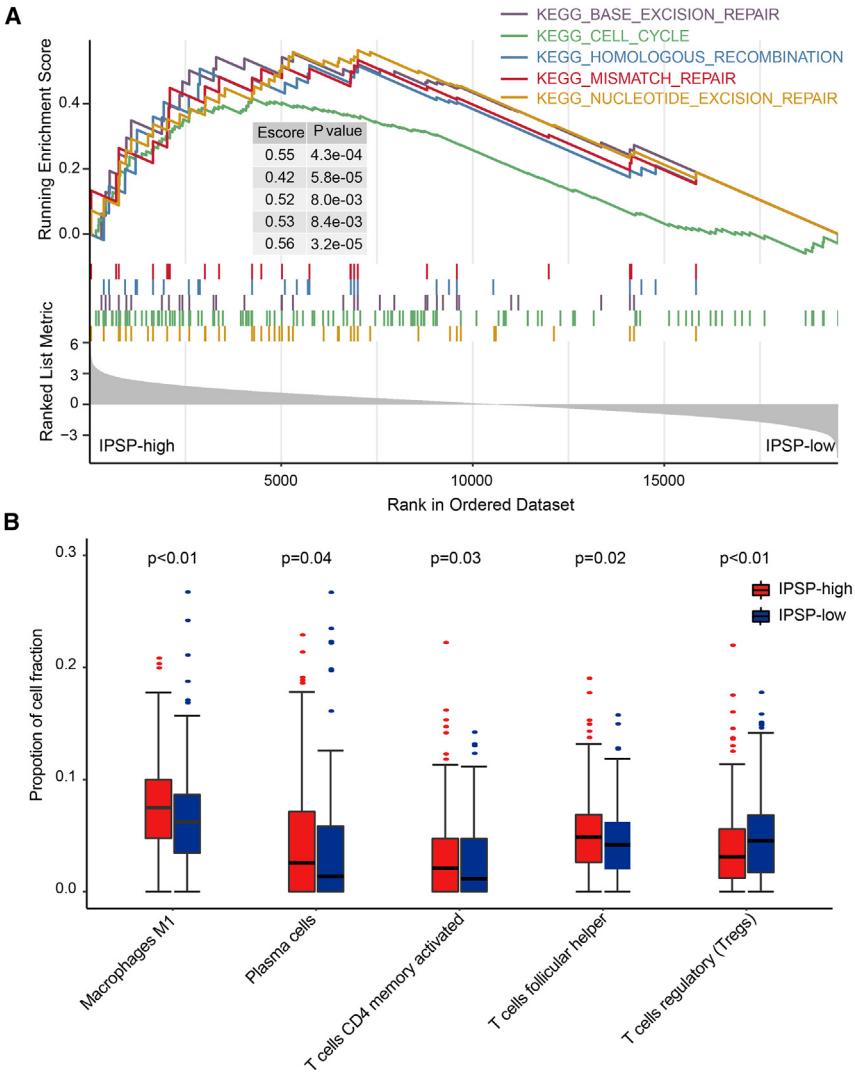

Figure 5. Correlation between IPSP score and immune-related features

(A) GSEA plot of important pathways in comparison between the IPSP-high and IPSP-low groups. (B) Comparison of TIICs relative infiltrated abundance between the IPSP-high and IPSP-low groups. Statistical significance was tested by rank-sum Wilcoxon test.

To further explore the correlation among the three pathways, a pairwise analysis of co-occurrence or mutual exclusion was performed according to the pathway mutation (at least one gene mutated in the pathway) or WT status. We applied the "maftools" package to implement this process.[33] The results showed that mutations in these pathways generally co-occurred (Figure 6A), highlighting that the three pathways in the IPSP may interact or influence each other to play a role in tumorigenesis and development. We next tested the gene mutations in the three pathways. For each pathway, the top 10 genes in terms of mutation rate were involved, and the waterfall plot showed that IPSP-high patients possess more mutations in both genes and pathways compared with IPSP-low patients (Figure 6B). As expected, more IPSP-high patients respond to immunotherapy. Moreover, similar results were observed in both the Miao et al. and Hugo et al. cohorts (Figure S6).

We further mapped the mutated genes to the pathways. We took the JAK-STAT signaling pathway as an example, which participates in various signal transduction processes. We observed that a number of genes are mutated (Figure 6C). *PTPN11* was the most frequently altered gene (10.5% across all samples), followed by *OSMR* (9.5%), *mTOR* (8.6%), and *IL2RB* (8.6%) (Figure 6C). It was observed that *PTPN11* mutations are enriched in tumors responsive to PD-1 inhibitor therapy in multiple cancers.[34] *mTOR* is often activated in tumors, promoting tumor growth by regulating the differentiation and function of immune cells; its mutation plays a positive role in the exploration of new immunotherapeutic strategies.[35] Although their mutation frequencies were modest, the combination of some of these mutations may facilitate the response to immunotherapy.

Application of the PathwayTMB method to the multi-cancer cohort

To further test if the PathwayTMB method could be used in other cancer types, we applied it to a multi-cancer cohort obtained from the cBioPortal database (https://www.cbioportal.org/).[20] Patients in the cohort included melanoma, NSCLC, head and neck cancer, and bladder cancer patients, who were all treated with ICIs (Table S1). We first calculated the patient-specific PTMB by mapping the somatic mutation data to the pathway. Based on the PTMB, nine survival benefit-related pathways were identified (Table S4), including the mTOR signaling pathway, the Hippo signaling pathway, and the cytokine-cytokine receptor interaction pathway, which are all associated with cancer immunity. For example, the mTOR signaling pathway is often activated in tumors and regulates cell proliferation and immune cell differentiation, which is a hot target in tumor therapy research.[35] The Hippo signaling pathway has critical functions in cancer immunity, innate immune responses against pathogens, and autoimmune diseases.[36] The cytokine-cytokine receptor interaction pathway is the major regulator of innate and adaptive immunity.[37] With these pathways, an IPSP was finally constructed. The results showed that the IPSP-high patient group had a better OS than the IPSP-low patient group (median OS, 16.71 months vs. 6.94 months, log rank test, $p < 0.001$; Figure 7A). It also showed that the IPSP had a superior prognostic value compared with TMB in the cohort (median OS, 14.61 months vs. 9.61 months, log rank test, $p = 0.18$; Figure S7A). We then stratified patients with

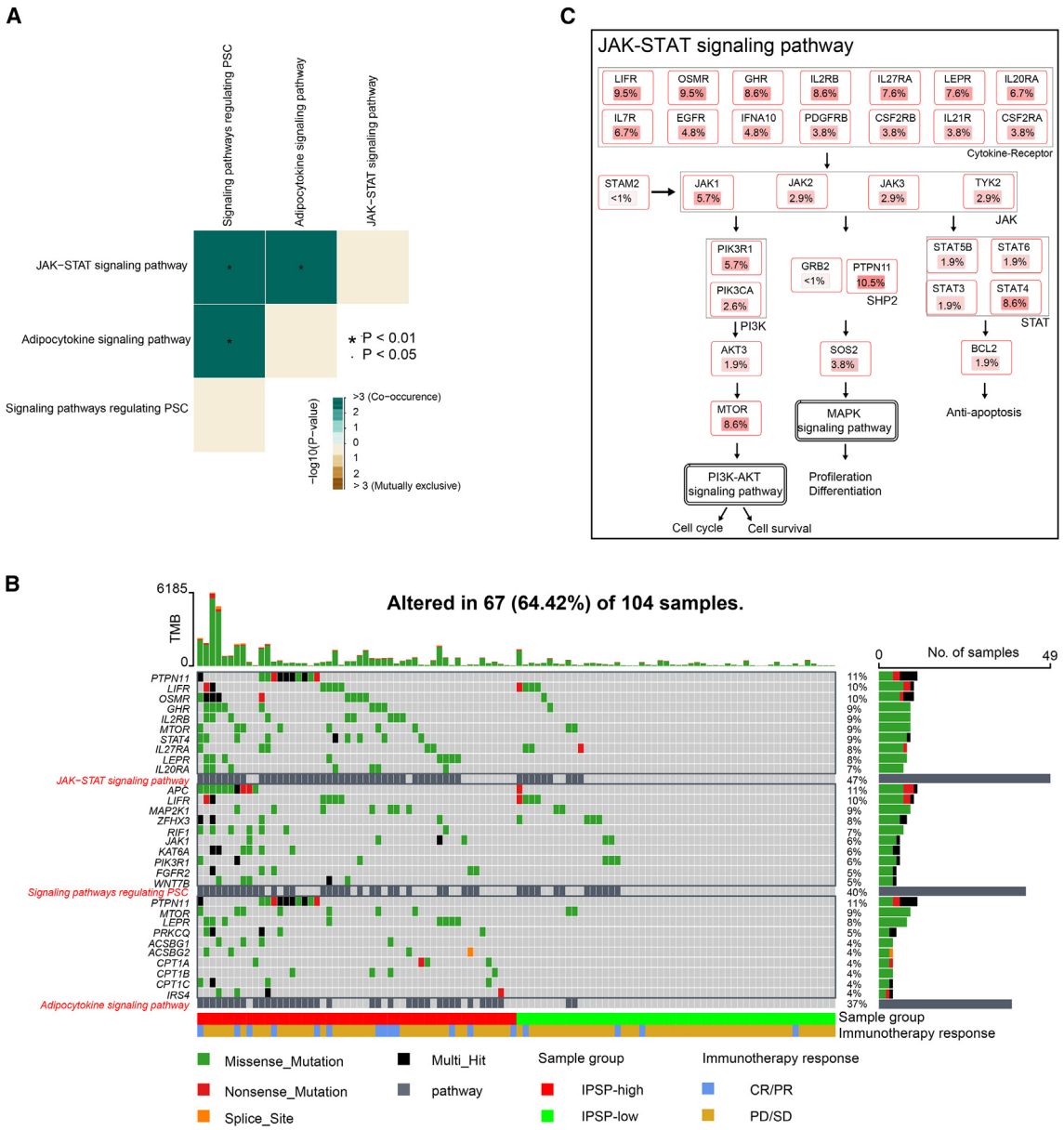

Figure 6. Analysis of the pathway signatures in IPSP

(A) Co-occurrence and mutual exclusivity plots among the three candidate mutation pathways in the IPSP signature. Statistical significance was tested by Fisher's exact test, '.' $p < 0.05$, '*' $p < 0.01$. (B) Waterfall plot of the top 10 genes in terms of mutation rate were involved in each candidate mutation pathway. (C) Altered genes and their functional relationship in the JAK-STAT pathway. Shades of red indicate gene mutation frequency.

high or low TMB based on the IPSP and compared their OS levels (Figure 7B), and observed that IPSP-high patients with low TMB had a significant survival advantage compared with IPSP-low patients with either high TMB or low TMB (log rank test, IPSP-high and TMB-low vs. IPSP-low and TMB-high, p = 1.25e−05, IPSP-high and TMB-low vs. IPSP-low and TMB-low, p = 8.80e−04).

Moreover, a higher response rate to immunotherapy was observed in the IPSP-high patient group than in the IPSP-low patient group (43% vs. 13.2%, chi-squared test p = 5.57e−07; Figure 7C). This result was superior to the response rate to immunotherapy of the TMB-high groups compared with the TMB-low groups (39.7% vs. 16.5%, chi-squared test, p = 1.13e−04; Figure 7D). Finally, we compared the predictive power of the response to immunotherapy of IPSP scores and TMB through ROC curve analysis, and PSP scores showed a better predictive effect (AUROC of IPSP = 0.74 vs. AUROC of TMB = 0.66; Figure 7E). These results indicate that our method could find genomic mutation pathways that may serve as potential biomarkers for immunotherapy.

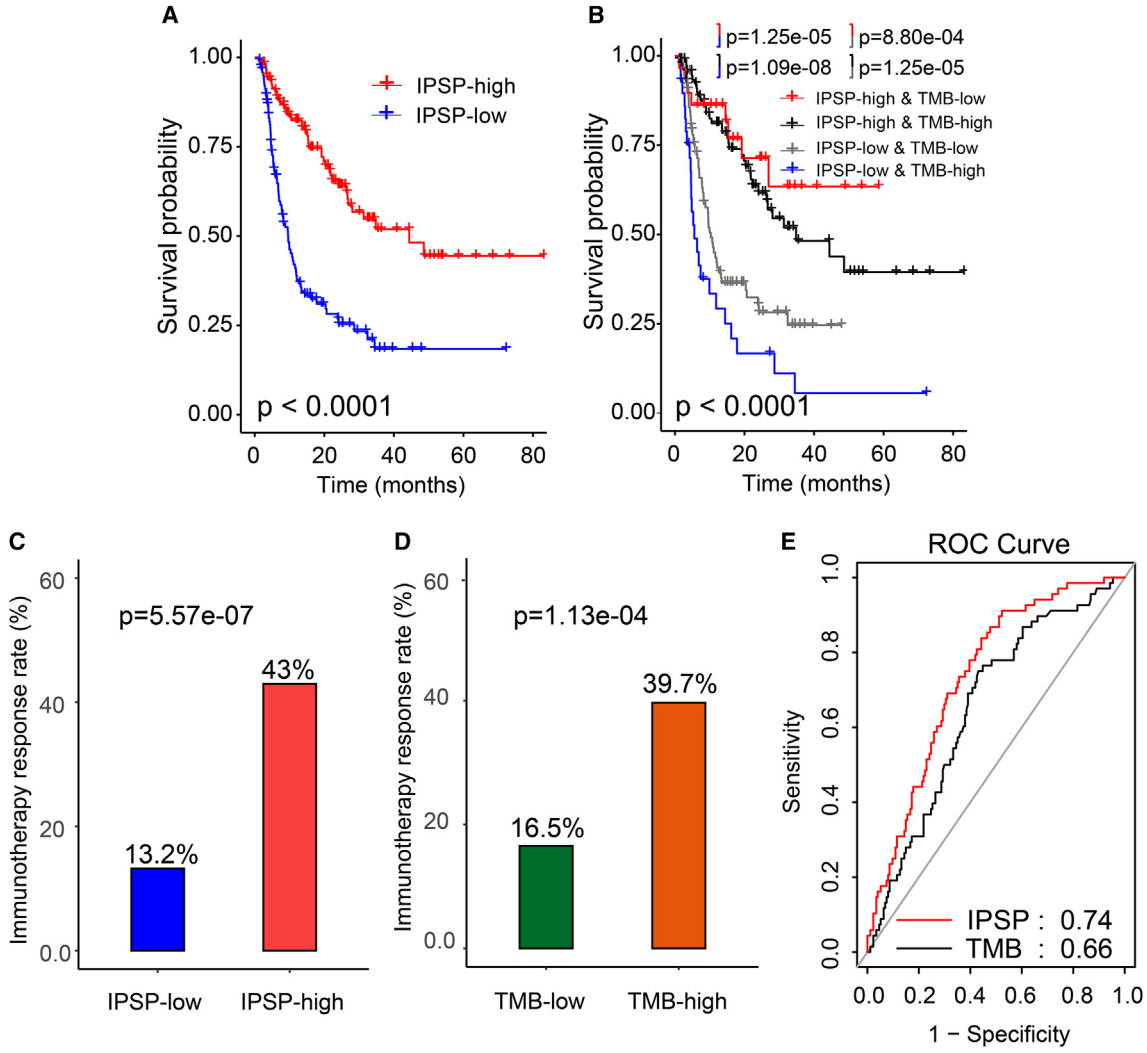

Figure 7. Application of the PathwayTMB method to the multi-cancers cohort

(A) Kaplan-Meier survival analysis of OS comparing the IPSP-high and IPSP-low groups from the multi-cancers cohort. (B) Kaplan-Meier survival analysis of OS among patients within each of the four indicated subgroups (IPSP-high and TMB-low, IPSP-high and TMB-high, IPSP-low and TMB-low, IPSP-low and TMB-high) from the multi-cancers cohort. (C) Comparison of the objective response rate between the IPSP-high and IPSP-low groups from the multi-cancers cohort. (D) Comparison of the objective response rate between the TMB-high and TMB-low groups from the multi-cancers cohort. (E) ROC curves of the IPSP and TMB to predict immunotherapy response from the multi-cancers cohort.

DISCUSSION

Immunotherapy with ICIs has become one of the most promising therapy methods for a range of tumors. However, only a small proportion of patients respond to ICIs, and thus there is an unmet need to discover effective biomarkers for the precise identification of patients who can benefit from ICI treatment. Genomic mutations in cancer have been proposed to be associated with the clinical outcome of immunotherapy. However, Boca et al. revealed that genomic mutation landscapes in human cancers are complex and heterogeneous, but mutations tend to occur within a core group of pathways.[13] Biological pathways were reported to have key intracellular roles and to be involved in mechanisms that dictate disease states,[38] drug responses,[39,40] and altered cellular function.[41,42] We acknowledge that gene mutations in the pathway can not only produce neoantigens but may also functionally affect the outcome of immunotherapy.[12,43] Therefore, investigation of genomic mutation pathways may lead to the discovery of effective biomarkers for immunotherapy.

In this study, we developed a novel method, named PathwayTMB, to identify genomic mutation pathways that may serve as potential biomarkers for predicting the clinical outcome of immunotherapy. In PathwayTMB, we innovatively proposed a novel definition, PTMB, to reflect the cumulative extent of mutations in the pathways. For each pathway, PTMB considers not only the number of mutations

but also the length and number of genes in the pathway. PTMB is calculated by summing up the gene mutation density across all the genes belonging to the pathway and then dividing by the number of genes in the pathway. Larger PTMB values indicate that a pathway is enriched in mutant genes to a greater extent, which may correspond to more T cell-recognized tumor neoantigens and lead to stronger anti-tumor immune responses. Thus, PTMB may provide new insights to improve cancer immunotherapies.

In the metastatic melanoma training cohort, we mapped the mutations to the genes in the pathways and calculated the patient-specific PTMB for each pathway. Based on the PTMB, we screened three survival benefit-related pathways, and their combination could serve as a strong prognostic factor for metastatic melanoma patients treated with ICIs (Figure 2C). Then, we constructed an IPSP with these pathways, and IPSP-high patients presented a higher ORR and longer OS than IPSP-low patients in the training cohort. The prognostic and predictive value of IPSP was validated in two independent immunotherapy cohorts. The IPSP was also shown to be superior to TMB in predicting the clinical benefits of therapy with ICIs in these cohorts. To test if PathwayTMB could identify potential mutation pathways in other cancer types treated with ICIs, we applied it to a multi-cancer cohort and obtained nine survival benefit-related pathways. An IPSP constructed with these pathways also showed good performance in predicting the clinical outcome of immunotherapy. To facilitate the use of the PathwayTMB method, we implemented it as a freely available R-based package (https://CRAN.R-project.org/package=pathwayTMB).

The advantage of the IPSP is that it increases cost-effectiveness by offering a smaller panel of genes in the pathways that can be easily translated into an easy-to-use clinical assay. As ICI treatment datasets including both genome sequencing data and survival data are rare, our method should be further validated in more cancer patient cohorts treated with immunotherapy in the future.

To sum up, PathwayTMB, which can be used to identify mutation pathways that serve as predictors of the response to ICIs, provides a convenient approach for future clinical practice of immunotherapy.

MATERIALS AND METHODS

Data source

We collated data from previously published clinical cohorts of cancer patients who were treated with ICIs. Detailed information regarding all of the cohorts included in this study is summarized in Table S1. The training cohort was composed of 104 metastatic melanoma patients treated with cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) inhibitors, which was obtained from the Van Allen et al. study.[15] Moreover, two independent cohorts were collected to validate the model derived from the training cohort. The first validation cohort was from the Miao et al. study,[20] and we extracted data from 145 metastatic melanoma patients treated with CTLA-4 inhibitors. The second validation cohort, extracted from the Hugo et al. study,[21] included 37 metastatic melanoma patients treated with PD-1 inhibitors. To further test if our PathwayTMB method could be applied to other cancer types, we collected a multi-cancer cohort from the Miao et al. study, including 249 NSCLC, melanoma, head and neck cancer, and bladder cancer patients treated with CTLA-4 or PD-1/PD-L1 inhibitors.[20] Patients from the above cohorts were available for whole-exome sequencing (WES), OS, and ORR for immunotherapy. The ORR was assessed by the Response Evaluation Criteria In Solid Tumors (RECIST) v1.1. Patient outcome was characterized as response (complete response [CR]/partial response[4]) or nonresponse (stable disease [SD]/progressive disease [PD]).

To investigate whether our pathway-based predictive model developed using immunotherapy data can also be employed to predict prognosis for non-immunotherapy patients, we downloaded somatic mutation and survival data from TCGA database for SKCM.

We downloaded the pathway data from the Kyoto Encyclopedia of Genes and Genomes database (KEGG; https://www.kegg.jp/).[44] The KEGG database is composed of metabolic and non-metabolic pathways. Non-metabolic pathways include genetic information processing, signaling pathways, and cellular processes, which control important biological processes such as cell proliferation, growth, and apoptosis. Compared with metabolic pathways, non-metabolic pathways may tend to be stimulated by the external environment, such as immunotherapy with ICIs. Thus, we only analyzed the non-metabolic pathways in our studies. We downloaded the XML format files of 157 non-metabolic pathways from the KEGG database and extracted gene sets from these pathways.

Identification of mutated genes correlated with survival benefits

For somatic mutation data, we converted MAF format data into a binary mutation matrix, in which each row represents a gene and each column represents a sample. For a given sample, a gene with one or more mutations was assigned a 1; otherwise, it was assigned a 0. In our study, we only extracted the non-silent somatic mutations in gene-coding regions, including missense, nonsense, insertion, deletion, and splice mutations. Non-silent mutations are coding sequencing mutations that cause a change in the amino acid sequence of proteins, which are then processed to neoantigens and presented into T cells by the major histocompatibility complex (MHC) protein on the surface of cancer cells, thereby destroying the cancer cells.[45] The genes with a mutation frequency of at least 1% in all of the samples were retained. Then Fisher's exact test was performed to identify genes correlated with survival benefits by comparing the gene mutation status (mutation and WT) with survival status (alive and deceased). We selected genes with odds ratio >1 where the gene mutation frequency was higher among the surviving patients than that among the deceased patients (at the end of the clinical observation period).

Calculation of the PTMB for each patient

Recent research has shown that mutations tend to occur within a core group of pathways, which led to the idea that an analysis of pathways or gene sets may provide more information about the pathways altered in cancers than an analysis of individual genes.[13] Thus, we

defined a PTMB to reflect the patient-specific cumulative extent of mutations for each pathway. For pathway *i* in patient *j*, we calculated $PTMB_{ij}$ by summing up the gene mutation density across all of the survival-benefit genes belonging to the pathway and then dividing by the number of genes in the pathway, even the survival-benefit genes were involved in multiple pathways. The formula is as follows:

$$PTMB_{ij} = \frac{\sum_{k \in I} \frac{mut_{kj}}{L_k}}{N_i} \quad \text{(Equation 1)}$$

Where I is a gene list of survival-benefit genes involved in pathway i; mut_{kj} is the number of mutations in gene k for sample j, L_k is the length of gene k, and N_i represents the total number of genes involved in pathway i. Thus, PTMB takes into account not only the number of mutations but also the length and number of genes in the pathways, which reflects the cumulative extent of TMB at the pathway level. As PTMB was calculated in the context of mutated genes related to survival benefit, it may be used to predict the response to immunotherapy or survival. Through the above process, we obtained PTMB profiles with rows as pathways and columns as samples.

Construction and validation of the pathway risk model based on PTMB

To test whether the PTMB could predict the immunotherapy response and clinical outcome of patients, we first performed the differential analysis of PTMB between the alive and deceased samples. The Wilcoxon rank-sum test and FC values were used to identify the pathways with differential PTMB. P value <0.01 and $|\log_2(FC)| > 1$were considered as the cutoff values for determining the significant differential PTMB pathways. Next, random forest analysis with nested cross-validation was performed to select the most important survival-related pathways based on PTMB by calculating the importance score for each pathway via the "caret" package in R. The pathways of differential PTMB were selected as the input variable, and the patients' survival status was selected as the outcome (binary variables, alive or deceased [0 or 1]). Then, for the pathways obtained from the random forest algorithm, a LASSO-Cox regression model was constructed and the log rank test was performed to find the most critical prognosis-related pathways. Finally, a risk model defined as IPSP was calculated for each patient using a formula derived from the PTMB of the prognosis-related pathways. The IPSP formula is as follows:

$$\text{IPSP score} = \sum_{i \in S} \beta_i PTMB_i \quad \text{(Equation 2)}$$

where S is the set of prognosis-related pathways, $PTMB_i$ is the PTMB of pathway i, and β_i is the regression coefficient of a multivariate Cox proportional hazards regression model estimated on $PTMB_i$ and the OS data. All of the patients in the training cohort were then divided into an IPSP-high group and an IPSP-low group based on the median of the IPSP scores. Kaplan-Meier survival analysis and the log rank test were further performed to evaluate the power of prognostic classification. Furthermore, the performance of the model to predict the response to immunotherapy and the clinical outcome was quantified by the AUROC. We developed an R-based package to implement the above flow (https://CRAN.R-project.org/package=pathwayTMB).

Correlation analysis of IPSP with immune-related features

To investigate the correlations between IPSP score and immune-related features, we downloaded 447 SKCM samples from TCGA with both RNA-sequencing and WES data. For gene expression data, FPKM-normalized profiles were used. We first applied a recently described deconvolution algorithm (CIBERSORT)[24] to infer the relative infiltration abundance of 22 tumor-infiltrating immune cells (TIICs) using gene expression profiles. The Wilcoxon rank-sum test was performed to investigate the significantly differentially infiltrated TIICs between the IPSP-high and IPSP-low groups. To further test the immune function associated with the IPSP score, we used GSEA to identify the significant immune-related dysregulated pathways. Specifically, a ranked list of genes was constructed based on the gene differential expression levels (T-score) between the IPSP-high and IPSP-low patient groups. The KEGG pathways were then mapped to the ranked list, and the R package "clusterProfiler"[46] was applied to implement the GSEA method.

Statistical analysis

Statistical analysis was performed with R version 4.1.0 software. Categorical variables were compared using the chi-squared test and continuous variables were compared using the Wilcoxon rank-sum test. All tests were two-sided and a p value of less than 0.05 was considered as the threshold for significance.

DATA AND CODE AVAILABILITY

Data are available in a public, open access repository. Three cohorts of patients with melanoma treated with immunotherapy are available from the cBioPortal database (https://www.cbioportal.org/). TCGA melanoma patients with somatic mutation and survival data are available from the GDC TCGA data portal (https://portal.gdc.cancer.gov/).

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2023.09.003.

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AUTHOR CONTRIBUTIONS

J.H. and X.L. conceived and designed the study. X.L., Y.H., and Y.J. developed the method. J.W., X.Z., B.P., and J.H. analyzed the data and implemented the methodology. Y.J. and L.C. provided constructive discussions. J.H. and X.L. drafted the manuscript. All the authors read and agreed to the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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