ORIGINAL RESEARCH

Therapeutic Benefits and Prognostic Value of a Model Based on 7 Immune-associated Genes in Bladder Cancer

Ming Cao, MD; Yang Cao, MD; Song Xue, MD; Qi Zhang, BS; Haiyan Zhang, MD; Wei Xue, MD

ABSTRACT

Objective • The emergence of immunotherapy has heralded a profound transformation in the therapeutic landscape of bladder cancer (BLAC). Immunotherapy, with its unique potential for "combination therapy", has brought about greater possibilities for treating BLCA. However, there is significant heterogeneity among bladder cancer patients, and a portion of those in advanced stages may not experience substantial benefits from chemotherapy. Immunotherapy offers a potential ray of hope for specific patient subsets. Thus, predicting the effectiveness of tumor immunotherapy and providing them with more precise treatment strategies hold paramount importance and clinical value in delivering personalized therapeutic interventions for advanced bladder cancer patients. This study is designed to establish a risk score model derived from immune-related genes that can effectively assess prognosis and immunotherapy outcomes in patients with bladder cancer.

Methods • The IMvigor210 dataset served as our training set for developing the prognostic model based on immune-related genes. Robust 7-gene expression patterns were investigated from the training set. A time-dependent receiver operating characteristic (ROC) curve and Kaplan-Meier (KM)analysis were employed to determine the prognostic relevance of these gene patterns. Independent datasets collected from the Cancer Genome Atlas Program (TCGA) and Gene Expression Omnibus (GEO) databases were additionally utilized for re-determination. The association between the 7-gene signature-based risk score and immunological subtypes, tumor mutational burden (TMB), immune checkpoint expressions, and the proportion of immune cell infiltration was assessed within training and test sets. Furthermore, the training set's predictive potential for immunotherapy response was assessed using the 7-gene signature, and its validity was externally verified on three datasets (GSE176307, GSE140901, and GSE91016). By validating the 7-gene signature externally, we eneralized the findings beyond the original training set, and assessed the model's performance in diverse contexts. Consistent performance across these datasets reinforces the robustness and clinical utility of our 7-gene signature.

Results • Employing the transcriptional and clinical information from the IMvigor210 for training, 348 patients were classified into two clusters with notable distinctions in prognostic stratification and immunotherapy efficacy. Seven immune-related genes Indoleamine 2,3-dioxygenase 1 (IDO1), TNF receptor superfamily member 17 (TNFRSF17), Killer Cell Lectin Like Receptor K1 (KLRK1), TNF receptor superfamily member 14 (TNFSF14), Lymphocyte-activation gene 3 (LAG3), Killer Cell Lectin Like Receptor C1 (KLRC1), and Ecto-5'-nucleotidase (NT5E) were screened based on different expression genes (DEGs) between the two clusters. The expression levels of these seven genes and the accompanying univariate component Cox regression coefficients, were computed to create a 7-gene signature-based risk score. The median value of the risk score was utilized to categorize the BLCA individuals into high-risk and low-risk groups. Researchers identified that in the low-risk group, individuals exhibited a noticeably improved chance of surviving. The external validation cohorts verified the risk score model's prognostic capacity. Furthermore, it was demonstrated that while low-risk individuals possessed higher TMB scores, higher expression of immune checkpoint genes, and lower levels of immunological infiltration, they responded more favorably to immunotherapy. The clinical relevance of the risk score model was validated in three immunotherapy groups.

Conclusion • The risk score model might be utilized to forecast the prognosis and efficacy of immunotherapy in BLCA patients, offering a novel course of treatment for these individuals. For patients undergoing immunotherapy, this gene signature can help predict treatment response. Low-risk patients may benefit from more tailored monitoring and personalized immunotherapy regimens. However, more investigations are required to validate its accuracy and effectiveness in a prospective cohort with larger sample sizes. (*Altern Ther Health Med.* 2024;30(4):130-138)

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INTRODUCTION

Bladder cancer (BLCA) ranks as a kind of prevalent tumor identified in the bladder cancer category.[1] It is expected to cause around 210 000 deaths worldwide in 2020 and roughly 574 000 new cases globally each year.[2] The conventional management of BLCA primarily encompasses surgical intervention and chemotherapy, both of which have shown enhanced survival outcomes. However, for some reasons, such as the tumor location and complexity, some BLCA patients cannot undergo these treatments.[3] Immunotherapy has become a popular cancer treatment strategy currently, and immune checkpoint inhibitors (ICBs) have been employed to treat BLCA. While ICB-based immunotherapy has shown promising outcomes in the BLCA, its long-term therapeutic effects are only observed in a limited proportion of patients. [4,5] Consequently, the exploration of

robust biomarkers for prognostic prediction and the evaluation of immunotherapy responses is crucial. This endeavor would enhance the precision and individualized approach to treatment strategies. Our study focuses on a 7-gene signature derived from immune-related genes. These genes hold the potential to revolutionize prognostic prediction and guide immunotherapy decisions for BLCA patients. By understanding the intricate interplay between gene expression patterns and treatment outcomes, we aim to enhance personalized care and ultimately impact survival rates.

The immune system acts as a tumor-suppressor, recognizing and generating immune responses to prevent tumor development and progression.[6] Still, malignant cells can evade immune elimination by expressing inhibitory receptor ligands.[7] Malignant tumors may prevent immune system assault and surveillance by using immunosuppressive proteins termed immune checkpoints.[8] Immune checkpoint inhibitors, e.g. CTLA-4, PD-1, and PD-L1, may strengthen the host's immune system against the growth of tumors in late-stage melanoma, renal cell carcinoma, and non-small cell lung cancer (NSCLC).[9,10] In BLCA, therapeutic medications that stop PD-L1 from engaging with the PD-1 receptor can revive repressed immune cells and increase anti-cancer T-cell activity.[11] Nevertheless, only a tiny proportion of individuals experience these side effects.[12] Recent advancements in immunotherapy prediction studies and the prognosis of BLCA have been noteworthy. Identifying biomarkers that predict patient response to ICIs remains a critical area of research. For instance, the PD-L1 protein has been studied as a potential biomarker for ICI response. Our study aims to enhance patient selection and treatment decisions by exploring the 7-gene signature's association with immunotherapy outcomes.

Tumor immune microenvironment (TIME) includes differentiated immune cell infiltration. It has been demonstrated that these immune cell types affect the prognosis and clinical outcomes of cancer patients undergoing immunotherapy.[13,14] For instance, the foundation of cancer immunotherapy is T cell-mediated anticancer immune responses, which are linked to a favorable prognosis.[15, 16] According to reports, T-cell tolerance and macrophage infiltration had an impact on BLCA patients' outcomes.[17,18] Furthermore, according to many findings, immune-associated indicators are linked to the prognosis and efficacy of immunotherapy in patients with brain lymphomas.[19-21] According to Wang et al., individuals with BLCA who had inhibitory immune cell infiltration had a worse prognosis and a reduced ability to respond to immune-based therapies.[22] Cao et al. have demonstrated that immune-associated lncRNAs can forecast the prognosis as well as clinical responsiveness to immunotherapy in BLCA.[23] Hence, the reliable approach for comprehensively evaluating immunotherapy and prognosis in BLCA may be derived from the immune-genes expression profiles. Approach for comprehensively evaluating immunotherapy and prognosis in BLCA may be derived from the immune-genes expression profiles.

In this study, we applied 'ConsensusClusterPlus' unsupervised clustering to categorize patients from the IMvigor210 cohort into two distinct clusters. These clusters exhibited notable differences in prognostic stratification and responsiveness to immunotherapy. Seven immune-related different expression genes (DEGs), including IDO1, TNFRSF17, KLRK1, TNFSF14, LAG3, KLRC1 and NT5E, between the two clusters were evaluated in conjunction with the immunomodulatory genes database. Next, we created a prediction model to forecast the effectiveness of immunotherapy for individuals with BLCA, regarding the seven genes' respective expression patterns. The ability to forecast treatment responses as well as survival outcomes was confirmed in many independent cohorts. The findings of this research will contribute to better prognostic prediction and immunotherapy response in BLCA patients, improving treatment outcomes and directing more targeted BLCA treatment. Our study aims to establish a predictive model for immunotherapy effectiveness based on these identified immune-related genes. By unraveling the molecular intricacies, we hope to provide clinicians with valuable tools for tailoring treatment strategies and improving patient outcomes.

MATERIALS AND METHODS

Patients

We applied the R package "IMvigor210CoreBiologies" as a training set to obtain all clinical and gene expression data (IMvigor210 cohort) for subjects with metastatic urothelial carcinoma with the treatment of anti-PD-L1 medications.[12] As a test set, the RNA-seq dataset and the matching clinical dataset of subjects with bladder cancer were collected from TCGA-BLCA using UCSC Xena (https://xenabrowser.net/datapages/). We also obtained two public bladder cancer cohorts (GSE48075 and GSE48276) from the GEO database to use as test sets (https://www.ncbi.nlm.nih.gov/geo/). GSE48075 encompasses 142 primary bladder tumors, including both superficial and invasive tumors. GSE48276 comprises 349 samples, which distincts basal and luminal subsets of human bladder cancers with different sensitivities to frontline chemotherapy. Additionally, the datasets for malignant melanoma with the therapy of anti-PD-1, as well as anti-CTLA4 (GSE91016), HCC immunotherapy with the therapy of anti-PD-L1 (GSE140901), and BLCA with the therapy of ICBs (GSE176307), were obtained. From TISIDB, 69 immune stimulators and inhibitors were obtained in total.[24] Furthermore, BLCA_TCGA, GSE48075, and GSE48276 were combined into a single dataset.539 samples with a survival duration of more than two months were chosen as the re-determination cohort after the ComBat function in the R package "sva"[25] eliminated any possible batch effects from the dataset. The pan-cancer cohorts with transcriptome profiles and prognostic data from the University of California (UC), Santa Cruz (https://xenabrowser.net/) Public Hub were also retrieved for additional research in the current work.

Consensus clustering analysis of the IMvigor210 cohort

The R package 'ConsensusClusterPlus' was utilized to perform unsupervised sample clustering and to recognize the ideal number of clusters in the training set.[26] The application of ConsensusClusterPlus allowed us to uncover hidden structures within the data without any preconceived notions or biases. This approach can lead to the discovery of novel insights and patterns that may not be evident with supervised methods.

To guarantee the classifier's resilience, the "hc" clustering technique with the distance function set to "Pearson" was used, and the number of repeat samplings, or "rep," was set at 1000. Additionally, the Kaplan-Meier curves were employed to evaluate the variations in survival between the two clusters.

Construction of immune-associated risk score model

1) Differential Expression Analysis: We performed a differential expression analysis to identify differentially expressed genes (DEGs) in both clusters. The threshold was set at FDR < 0.05 and | log2FC | > 1. The "limma" package in R was implemented for this procedure.[27] 2) Pathway Enrichment Analyses: The genes that disclosed variations in expression involved in both groups were utilized following that to complete the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) pathway enrichment analyses. 3) Univariate Cox Regression Analysis: We used the 'coxph' function from the R package survival to conduct a univariate Cox regression analysis to investigate the causal connection between DEGs and overall survivor (OS) in the training set. A subset of genes with a p-value of less than 0.05 was considered for prognosis. 4) Final Candidate Gene Set: The final candidate gene set was produced through the combination of this predictive gene set with 69 immunological genes. 5) Development of a Gene-Based Model: The regression coefficients from the multivariate Cox regression analysis of the seven genes in the training set were thereafter utilized for developing a gene-based model. The following formula was complied with for estimating the risk score:

$$\text{Risk score} = \sum_{i=1}^{n} (\text{Coefficient(i)} \times \text{Expression(i)})$$

Expression i refers to the expression value of gene (i) in each sample, and Coefficienti represents the multivariate Cox regression coefficient of gene i. 6) Risk Group Stratification: Regarding the median cut-off, all individuals were arranged into high-risk and low-risk groups. The BLCA cohort of IMvigor 210's survival difference was determined using Kaplan-Meier analysis. 7) Model Evaluation: ROC curve is extensively utilized to determine the precision of the Cox regression model. ROC analysis and AUC are adopted as indicators of the operation of the prognostic model, which was accomplished using the R packages "Proc" and "timeROC".[28] 8) Validation of the Model: The signatures' immunohistochemistry (IHC) analysis was subsequently verified using the Human Protein Atlas database (https://www.proteinatlas.org/). After that, the risk scores for each individual in the validation sets were determined. Similarly, the model's accuracy and robustness were determined using Kaplan-Meier analysis and AUC calculation. We also looked at the link between the TCGA-BLCA clinical characteristics and the gene-based risk score.

Tumor immune microenvironment and TMB analysis

The CIBERSORT program was performed to determine the rating distinctions of 22 immune cell types between the patients who exhibited high scores and low scores in the training and TCGA-BLCA dataset.[29] The immunological and stromal scores for the patients were determined using the ESTIMATE algorithm.[30] The Pearson correlation coefficient was employed to assess the links between immune cells and risk score. The R program "ggplot2" created the correlation scatter plot. Use the R package "map tools" to calculate TMB, and then set the capture size threshold to 50MB. The distribution of TMB in both subgroups was contrasted using the Wilcoxon rank sum test.[31]

Correlation analysis between risk scores and immunotherapy

We analyzed the distribution of risk scores across the three immunophenotypes (immune-excluded, immune-desert, and immune-inflamed) in the IMvigor210 cohort to provide insight into the relationship between risk scores and immunotherapy. Five recognized immunotherapy prognostic indicators were compared individually using correlation analysis. We examined the ratings of various immune response subtypes between two groups in the IMvigor 210_BLCA cohort, GSE176307, GSE140901, and GSE91016 cohorts, respectively, to further ascertain the forecasting ability of risk score for immunotherapy response.

Statistical Analysis

Statistical analyses were conducted using R software (4.2.1) and the related R packages. The Wilcoxon test is a non-parametric test that compares two paired groups to assess whether their population mean ranks differ. In this study, the Wilcoxon test was executed for the statistical analysis of the violin as well as box plots. The chi-square test is used to determine whether there is a significant association between two categorical variables. In this study, it was used to compare the responses to immunotherapy between different groups, with a threshold for significance of $P < .05$. The aim was to see if the response (a categorical variable: response vs. no response) was associated with the group (another categorical variable: high-risk vs. low-risk).

RESULTS

Recognition and analysis of subtypes based on the IMvigor 210 cohort

Figure 1 presents the schematic illustration of the investigation. First, to determine the likely molecular subgroups of the patients, we analyzed the transcriptome data from the IMvigor210 cohort using a consensus unsupervised clustering technique. To correctly categorize the group into clusters 1 and 2, cluster number k=2 was chosen based on the consensus matrix and cumulative

Figure 1. The study schematic.

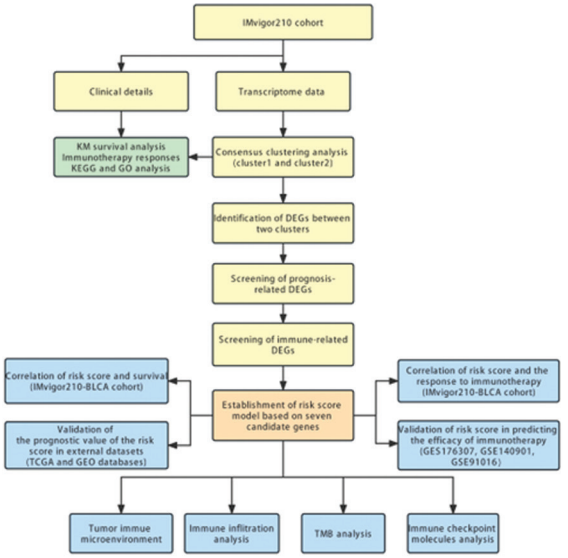
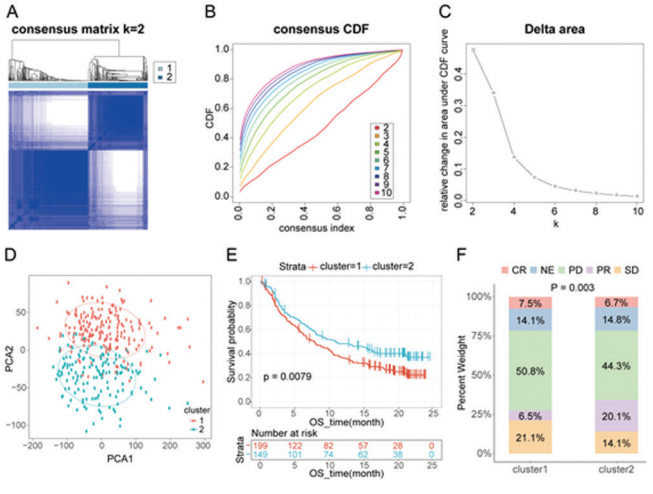
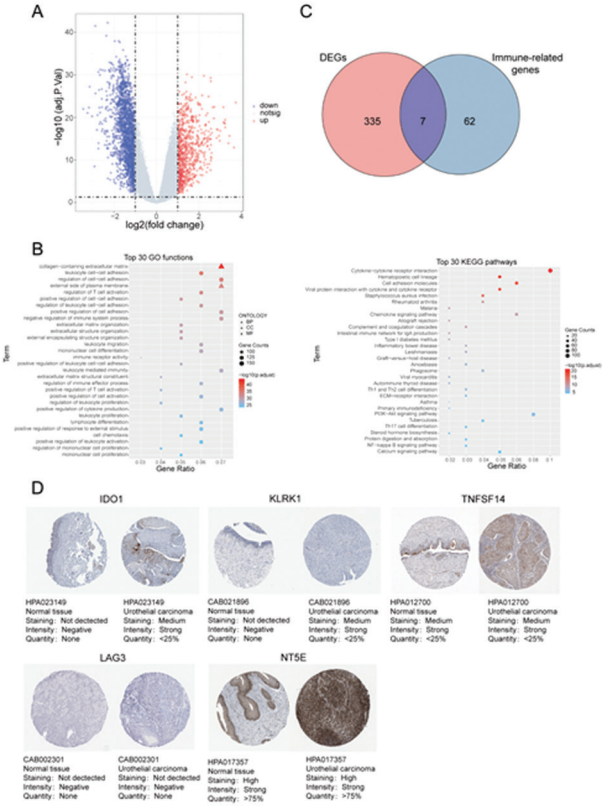


Figure 2. Identification and characteristics of two clusters based onIMvigor210 cohort. (A) Heatmap of the consensus matrix of two distinct clusters. (k =2). (B) Area under CDF curve when consensus index k from 2 to 10. (C) Changes in the relative area under the CDF curve when k=2-10. (D) PCA analysis of clusters in the IMvigor210 cohort. (E) K-M survival curves of OS between the two clusters. (F) The immunotherapy responses difference (CR, NE, PR, PD, SD) between the two clusters. Statistical significance at the level of $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$.



distribution function (Figure 2A-C). Two separate portions can be identified in all patients based on the Principal Component Analysis (PCA) analysis, further validating the existence of two clusters (Figure 2D). According to the Kaplan-Meier survival statistics, cluster 2 disclosed a longer OS than cluster 1 (P = .0079; Figure 2E). Additionally, a notable distinction in the effectiveness of immunotherapy between the two groups was seen. In cluster 2, the CR and PR rates were 6.7% and 20.1%, respectively, whereas in cluster 1, they were 7.5% and 6.5%, respectively. (Figure 2F, P = .003).

Figure 3. Screening of differentially expressed genes (DEGs) between two clusters. (A) The volcano plot of DEGs between two clusters. (B) GO and KEGG analysis of the DEGs between the two clusters. (C) Venn diagrams showing the intersection of DEGs and immune-related genes. (D) The IHC analysis of 7 immune-associated genes by HPA database.



These suggested that one or more of the possible pathways by which differently expressed genes in two subtypes can influence tumor progression and therapeutic responses.

Examining immunological genes associated with BLCA Prognosis

Different gene expression analyses were conducted, comparing the two clusters to pinpoint important genes associated with BLCA patients' immunotherapy response. A total of 2533 substantially downregulated and 739 upregulated genes were among the 2533 DEGs that we found (Figure 3A). The GO and KEGG analyses exhibit that the DEGs were considerably enriched in the processes linked to the immune system and cell-cell adhesion (Figure 3B). 342 genes with a statistically significant connection ($P < .05$) with OS were found using univariate Cox regression (supplementary table 1). *IDO1*, *TNFRSF17*, *KLRK1*, *TNFSF14*, *LAG3*, *KLRC1*, and *NT5E* are the seven candidate genes that we ultimately acquired by taking the intersection of the prognosis-related gene set and the immunomodulatory genes.[32] These are disclosed in Figure 3C. The HPA database carried out IHC analysis of the genes in normal as well as tumor tissues to ascertain the expression level of these clinically significant genes in bladder tissues (Figure 3D). IDO1 and KLRK1 showed considerable staining in tumor

Figure 4. Construction of an immune-related risk score. K-M plotter analysis of the seven candidate genes in the IMvigor210 database (A) and the TCGA-BLCA cohort (B). (C) Heatmap of gene expression distinction between the different risk groups. (D-E) Survival curve for different risk groups and time-dependent ROC curves predicting the prognosis in the IMvigor 210-BLCA cohort. (F) The correlation between the risk score and survival outcomes of the BLCA.

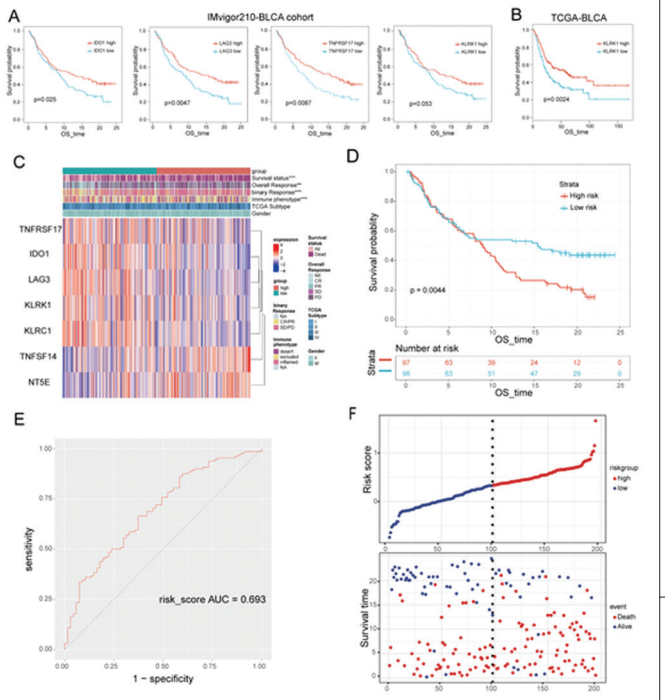
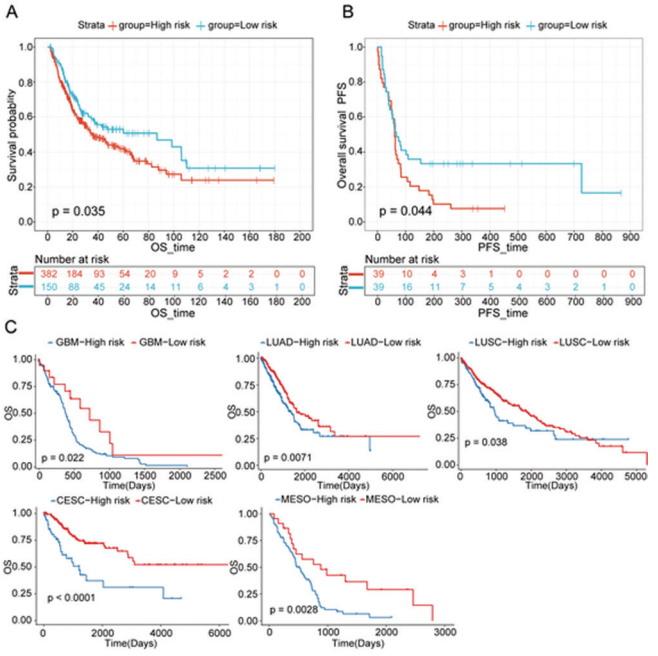


Figure 5. Validation of the risk scorein the testing cohort of BLCA. (A) K-M curves of OS for two groups in BLCA patients from the TCGA and GEO database. (B) K-M curves of PFS inBLCA patients treated with immunotherapy from the GEO database. (C) Survival analysis for a different risk scores in GBM, LUAD, LUSC, CESC, and MESO cohorts of TCGA.



tissues, but in normal tissues, they were not visible. In both normal and malignant tissues, LAG3 was negative. In both the tumor and normal tissues, TNFSF14 and NT5E were highly expressed. There are no entries for TNFRSF17 and KLRC1 in the HPA database. Moreover, it is identified that NT5E was more expressed in tumor tissues than IDO1, which was substantially less expressed in normal tissues. The TCGA database revealed no discernible variation in the expression of additional genes between normal tissues and the BLCA tumor.

The predicted function of immune-associated risk score of BLCA patients

The relationship between these candidate immune-related gene expressions and the prognosis of individuals with BLCA using 195 BLCA samples from the immunotherapy cohort Mvigor210 database is further explored. The Kaplan-Meier survival analysis revealed that the up-expression of *IDO1*, *LAG3*, *TNFRSF17*, and as*KLKR1* was associated with increased OS (Figure 4A). However, only KLRK1 was substantially correlated with the favorable OS in the TCGA-BLCA cohort (Figure 4B). It is hypothesized that in BLCA patients receiving immunotherapy, these potential genes may have a stronger prognostic correlation. After calculating a risk score determined by the expression of the seven genes, the multivariate Cox regression coefficients were employed to weight the score to predict survival. The median risk score was established as a criterion for sorting the bladder cancer individuals into high-risk and low-risk groups. The variation in gene expression between the two risk score groups appears in Figure 4C. After that, the link between risk score and OS in the IMvigor 210-BLCA group was looked at using the K-M survival curve. The high-risk group's prognosis was discovered to be poor (P = .004, Figure 4D). The risk score could accurately forecast OS, according to the time-dependent ROC curve data (Figure 4E). Individuals with low-risk scores remained longer, while others with high-risk scores died quicker, according to our analysis of the survival status in the various risk categories (Figure 4F).

Assessment of the predictive effectiveness of various risk score in external validation cohorts

We constructed a proof-of-concept cohort of 539 BLCA patients using clinical data collected from the TCGA and GEO datasets (GSE48075 and GSE48276) in order to measure the forecast value of the risk score model. Using their median risk score as a guide, we arranged the patients as either high-risk or low-risk. Individuals in the low-risk group did considerably better in BLCA, as reported by OS prognostic data (Figure 5A, P = .035). Concurrently, RNAseq data from 79 BLCA patients treated with immunotherapy were collected from the GEO database (GSE176307) for another validation cohort as further confirmation. PFS was longer for those in the high-risk group in contrast to the low-risk group (P=0.044, Figure 5B). In order to explore the prognostic relevance of the risk score, we also finished a KM survival study of pan-cancer patients in the

Figure 6. Contrasting characteristics of the tumor immune microenvironment in different risk patients. (A) Boxplots showing the difference of tumor-infiltrating immune cells in IMvigor210 (left) and TCGA-BLAC (right) between different risk groups. (B) The relationship between immune cell infiltration and risk score. (C) Violin plots showing immune, stromal, and ESTIMATE scores between the different risk groups in the IMvigor210 cohort. (D) The expression of immunostimulator genes between different risk groups in iIMvigor210 (left) and TCGA-BLAC (right).

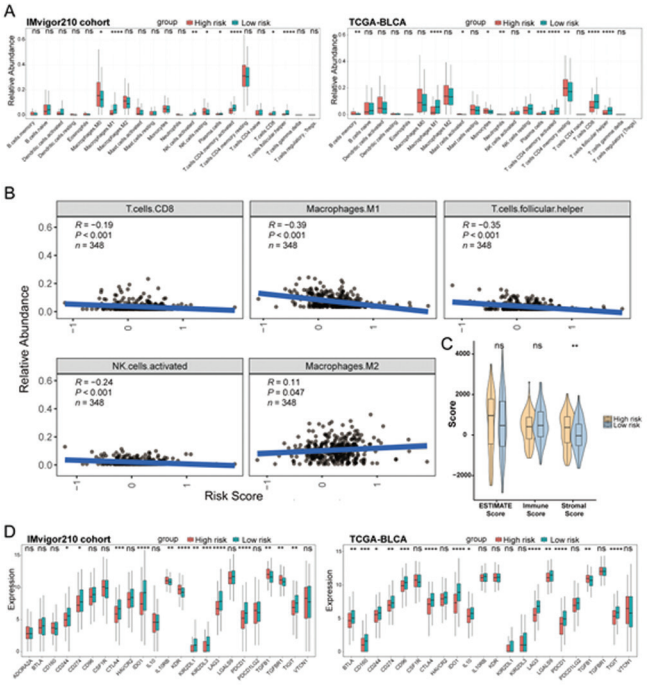
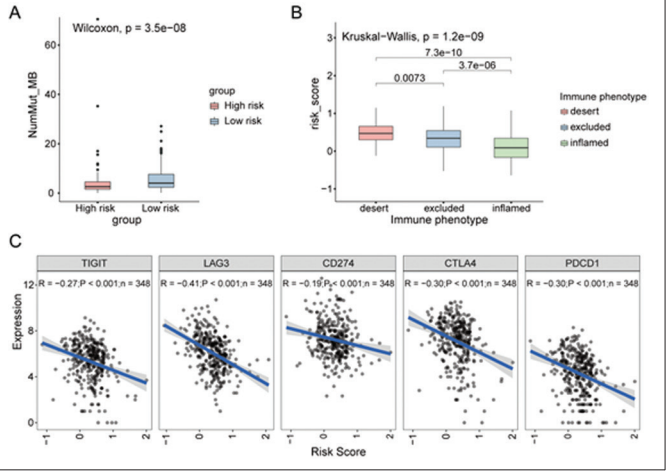


Figure 7. Correlation of risk score and immunotherapy response. (A) The difference in TMB score between different risk groups. (B) The difference in risk score among three immune phenotypes of BLCA. (C) The expression of immune checkpoint in different risk scores.



TCGA database. For glioblastoma multiforme (GBM, P = .022), lung adenocarcinoma (LUAD, P = .007), lung squamous cell carcinoma (LUSC, P = .038), esophageal carcinoma (CESC, P = .001), as well as mesothelioma (MESO, P = .0028), individuals in the low-risk group displayed more favorable diagnoses (Figure 5C). The risk score is a strong predictor of the prognosis of BLCA patients, according to the previously cited research. While the risk score model has potential applicability to other cancers, it's important to validate it in each specific cancer type.

The immune landscape analysis of different risk groups in BLCA

Utilizing the CIBERSORT approach, we compute the 22 distinct immune cell types ratios to elucidate the temporal differences in both risk groups. We noticed that the high-risk group in the IMvigor210-BLCA cohort included fewer M1 macrophages, CD8+ T cells, activated CD4 memory cells, active NK cells, and T follicular helper cells. Conversely, the high-risk group reported increased M2 macrophages (Figure 6A). A similar pattern occurred in the TCGA-BLCA cohort (Figure 6A). The quantity of CD8+ T cells (r = -0.19, P < .001), M1 macrophages (r = -0.39, P < .001), T follicular helper cells (r = -0.35, P < .001), as well as activated NK cells (r = -0.24, P < .001) was observed with a severe negative influence on the risk score. The risk scores of BLCA and immune cell infiltration appear to be strongly linked. Similarly, considerable evidence of association (r = 0.11, P < .047) has been perceived between the risk score and M2 macrophage infiltration in Figure 6B. We also assessed how immune and stromal ratings varied amongst various risk categories using the ESTIMATE approach. Immune ratings were greater in the low-risk group, but stromal values were raised in the high-risk group (Figure 6C). Moreover, we examined the distinctions in immunostimulator gene expression between the TCGA-BLCA and IMvigor210-BLCA cohort risk groups. The analysis confirmed that the majority of immune checkpoint genes involving *CD244*,*CD274*, *IDO1*, *TIGIT*, *CTLA-4*, PDCD1,as well as*LAG3*, were more abundant in the low-risk group (Figure 6D). Owing to these outcomes, individuals in the low-risk group may be arranged with a hot immunological profile with higher immune infiltration.

The association of risk score, TMB, immune checkpoints, and immune subtypes

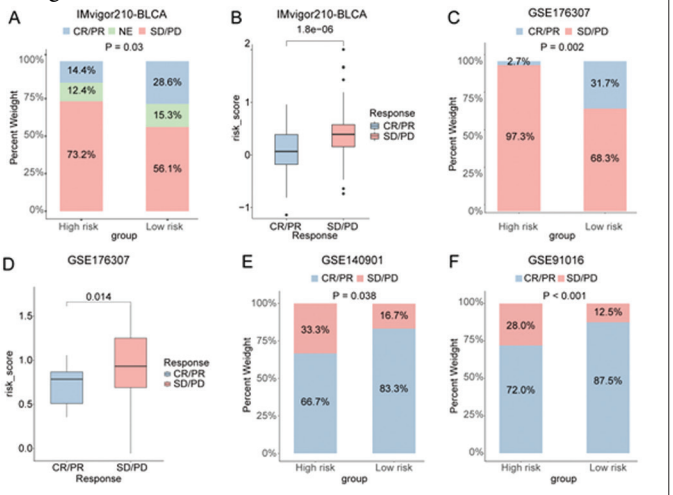
In the IMvigor210-BLCA and TCGA-BLCA datasets, the correlation between immune subtypes, TMB, immunological checkpoints, and risk score was further examined to find out the potential use of the risk score in BLCA immunotherapy. First, we examined how TMB scores were distributed throughout the TCGA-BLCA cohort's various risk categories. The low-risk group showed considerably higher TMB scores (P < .001, Figure 7A), as we found. Furthermore, in the IMvigor210-BLCA cohort, the risk score for every immunotype of bladder cancer was explored. According to the findings, the immune-excluded phenotype had risk scores that were considerably greater than those of the immune-inflamed and immune-desert phenotypes (Figure 7B). We next analyze the link between the risk score and five well-known immune checkpoint

molecules to better understand the risk score's predictive power for immunotherapy effectiveness. With regard to LAG3 (r = 0.41, *P* = .049), TIGIT (r = 0.27, *P* = .049), PD-L1 (r = - 0.19, *P* < .001), CTLA (r = - 0.30, *P* < .001), and PD1 (r = 0.30, *P* < .001), the risk score revealed a strongly negative link (Figure 7C). These outcomes suggest that low-risk individuals could gain more from enhanced immunotherapy.

The risk score of immunotherapy response prediction in BLCA

Following that, we looked at the function of the risk score in forecasting immunotherapy reaction. The therapy outcome evaluation indices varied significantly between the various risk categories. greater percentages of CR/PR were seen in low-risk individuals, while greater rates of SD/PD were found in high-risk patients (*P* = .03, Figure 8A).The analysis revealed that patients who achieved PR/CR had significantly lower risk scores compared to those who had SD/PD, indicating the lower risk scores had significantly better treatment effectiveness (*P* < .001, Figure 8B). We verified our observations using 87 BLCA patients who received ICI therapies from the GEO database (GSE176307). The individuals were arranged into two groups depending on their high or low median risk scores. In contrast to the high-risk group, the other group disclosed a much higher performance of CR/PR and a lower proportion of SD/PD (Figure 8C). Individuals with SD/PD, on the other hand, exhibited much higher risk ratings (*P* = .014, Figure 8D). Furthermore, two GEO databases of HCC immunotherapy (GSE140901) and malignant melanoma (GSE91016) where patients underwent ICIs therapy were chosen for further validation due to the limited instances in the BLCA immunotherapy database. The low-risk group had a lot higher CR/PR ratio, whereas the other group had a considerably greater percentage of SD/PD (Figures 8E, F). These findings proved that, as compared to high-risk individuals, low-risk individuals responded more favorably to immunotherapy. It might explain why BLCA patients with low-risk ratings possess a substantially higher survival rate.

Figure 8. Prediction of immunotherapy efficacy by the risk score. Boxplot (A) and Bar graph (B) illustrated the treatment response to immunotherapy between different risk groups in the IMvigor210-BLCA cohort. Boxplot(C) and Bar graph (D) illustrated the treatment response to immunotherapy between different risk groups of BLCA patients in geo database (GSE176307). Immunotherapy response between different risk groups in HCC (GSE140901) (E) and (F) malignant melanoma cohorts (GSE91016).



DISCUSSION

Bladder cancer serves as a frequently occurring malignant tumor of the urinary system that poses a significant public health concern.[33] The emergence of immunotherapy in recent years has brought about a profound transformation in the treatment landscape of BLCA.[34] However, immunotherapy only produces long-lasting clinical improvements in a small percentage of patients.[35] Numerous studies have indicated that PD-L1 is a candidate biomarker with predictive potential for assessing the effectiveness of immunotherapy in various cancer types.[36] However, the results obtained in different studies are inconsistent.[16,37] Besides, due to intra/intratumor heterogeneity, non-standardized cut-off value, and relatively high cost-effectiveness, TMB also has some limitations.[38] Therefore, exploring more reliable clinically predictive biomarkers of prognosis and immunotherapy for BLCA patients is crucial. Using risk profiles derived from gene transcriptome characteristics to forecast immunotherapy effectiveness and prognosis has shown promise in a number of cancer cases in recent years.[39-41] Here, utilizing seven immune-related genes, we designed a risk model that may be attempted to forecast prognosis and recognize individuals more inclined to benefit therapeutically from immunotherapy in BLCA.

Given RNAseq data from the IMvigor210 database, the patients may be categorized into two groups. Notable variations were found when comparing the prognosis and immunotherapy effectiveness of the two discovered clusters. Seven potential genes were evaluated after completing a thorough examination of the genes that differed between the two clusters. Among them, via controlling many immune cells, Indoleamine 2,3-dioxygenase 1 (IDO1) serves as an essential gene for tumor immune escape.[42,43] IDO1 was shown to be substantially expressed in BLCA and to be strongly correlated with unfavorable clinical outcomes.According to data, 44 Lymphocyte-activation gene 3 (LAG3) is linked to immune cell infiltration and functions as an immunological inhibitory checkpoint.[45] According to Jiang et al., *NT5E* is crucial for improving cancer cells' invasive and metastatic capabilities.[46] Notably, there were also many reports in the literature showing that up-regulation of*NT5E*resulted in poor outcomes in several types of cancers, including gastric carcinoma (GC), renal cell carcinoma (RCC), B-cell chronic lymphocytic leukemia (B-CLL), urothelial carcinoma (UC), and papillary thyroid carcinoma (PTC).[47-50] Sun et al. also demonstrated that better prognosis can be found in high expression levels of KLRK1 in BLCA patients.[51] There are few studies about *TNFSF14* and *TNFRSF17* in BLCA, while the interactions between these genes and immunity have been

supported and verified in other research.[52] Accumulating investigations exhibit that these genes critically impact regulating innate cellular immunity and are correlated with tumor progression.[53] We created a risk score with seven genes to examine the association between risk score, immunotherapy, and prognosis in BLCA, taking into consideration the mechanism of these genes on the clinical detected findings. For individuals in the low-risk group, the OS was superior. Along with the validation BLCA cohort, the observation was validated in several cancer cohorts. These findings demonstrated the risk score's predictive efficacy in BLCA patients.

While PD-L1 expression and TMB give insights into the tumor's potential response to immunotherapy, the risk score model provides a more comprehensive view of the tumor's immune landscape by taking into account the expression of multiple immune-related genes. The risk score model might be more robust than single biomarkers like PD-L1 or TMB. This is because it integrates information from multiple genes, which can capture more complexity and heterogeneity of the tumor immune environment. The risk score in our study can serve as a prognostic tool to predict overall survival and progression-free survival in bladder cancer (BLCA) patients. By stratifying patients into high-risk and low-risk groups, clinicians can have a better understanding of a patient's prognosis, which can guide treatment decisions. Also, the risk score can help in the development of personalized treatment strategies. For instance, patients in the high-risk group might benefit from more aggressive treatments or novel therapies, while those in the low-risk group might be candidates for standard treatments or immunotherapy. The risk score could be calculated for each patient at the time of diagnosis or prior to treatment initiation. This information could then be incorporated into the patient's medical record and used to inform treatment decisions.

There is mounting evidence that the prognosis and treatment for many malignancies are closely correlated with immune cells.[54] Tumor-associated macrophages (TAMs) are classified into two primary subtypes: M1 macrophages, which are engaged in anti-tumor immunity, and M2 macrophages, which decrease tumor immunity and promote the growth of tumors.[55] T-follicular helpers stimulate tumor immune responses and have elevated PD-1 expression.[56] Natural killer (NK) cells have a strong antitumor effect and are essential in initiating the immune response against abnormal cells.[57] Experiments suggest the relevance of the reactions of CD8+ T cells in the adaptive immune system and their crucial role in immune responses against tumors.[58] Our findings disclose that the risk-high group featured a higher population of M2 macrophages. In contrast, the low-risk group had substantially larger numbers of CD8+ T cells, active NK cells, T follicular helper cells, as well as M1 macrophages. Moreover, an opposite association was seen between the risk score value and the proportion of these immune cells. These findings therefore showed that an immunosuppressive environment may be linked to the poor outcomes seen in individuals with high-risk traits. Conversely, individuals with low-risk scores showed more infiltration of immune-related cells that were activated, indicating "hot" tumors and perhaps being candidates for immunotherapy.

Numerous cancer types have demonstrated the effectiveness of immunotherapy.[59] Nevertheless, a relatively tiny ratio of those receiving treatment respond to immunotherapy in a long-term manner[60], despite the fact that ICIs have been clinically beneficial in treating cancer. This highlights the critical importance of those patients who may benefit from immunotherapy. Our analysis stated a considerable opposite association between the risk score and multiple crucial immune checkpoint molecules. This implies that the risk score might be a major factor in determining the effectiveness of immunotherapy prediction. Depending on the immunological milieu, the majority of solid tumors may be defined as immune excluded, immune inflamed, or immune desert.[61] Immune-desertphenotypes and Immune-excluded were initially described as non-inflammatory microenvironments and might be less sensitive to immunotherapy than inflamedphenotypes.[62] According to our research, the desert phenotype had a greater risk score than the other two phenotypes. In terms of the association between TMB and the quantity of neoantigens, a larger TMB is linked to a more robust immune response to immunotherapy.[63,64] It was claimed that a lower-risk score is correlated with a greater TMB and a more positive response to immunotherapy because the individuals with lower-risk scores had stronger immunogenicity, which showed up as higher TMB. Additionally, the patients with low-risk scores had more improved objective responses in immunotherapy-treated melanoma and HCC cohorts. However, further research is needed to fully understand the biological mechanisms underlying these observations and to validate the risk score model in larger, prospective cohorts of different cancer types, due to differences in the tumor microenvironment, genetic mutations, and other factors. This will help to ensure the robustness and generalizability of the model, and to optimize its use in clinical practice.

These findings support the notion that BLCA individuals with high-risk scores receive a poor prognosis but would benefit more from ICI treatment. Our study does have some limitations, though. Retrospective data from public databases were employed in the study. The use of retrospective data from public databases, while valuable for initial model development, may not fully capture the diversity and complexity of real-world patient populations. Therefore, further validation in prospective cohorts with larger sample sizes is needed to confirm the accuracy and effectiveness of the risk score model. Moreover, it's important to note that while the risk score model shows predictive capacity, it's just one piece of the puzzle. Other factors such as the patient's overall health, genetic profile, tumor heterogeneity, and treatment history also play crucial roles in determining their prognosis and response to treatment. Therefore, the risk score model should be used in conjunction with other clinical information to guide treatment decision-making, and more research is needed to fully realize its potential in clinical settings. In future, for the identified immune-related genes in BLCA, we could conduct experimental studies using cell lines or animal models to elucidate the roles of these genes in bladder cancer

and explore how they modulate immune responses, tumor growth, and metastasis.

In conclusion, given the seven immune genes, the risk score model presents an insightful approach to figuring out those who might gain value from immunotherapy; however, more investigations are required to validate its accuracy and effectiveness in a prospective cohort with larger sample sizes. The risk score model derived from the seven immune-related genes shows an excellent predictive capacity for both immunotherapy responses and survival. These results may enhance the prediction of a patient's personalized prognosis and offer fresh approaches to treating patients with BLCA.

ETHICAL COMPLIANCE

Not applicable.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to report relevant to this article.

AUTHOR CONTRIBUTIONS

Ming Cao and Yang Cao contributed equally. Ming Cao, Yang Cao and Song Xue: Conceptualization, methodology, writing original draft preparation. Qi Zhang and Haiyan Zhang: Investigation, software, statistical analysis. Wei Xue: Reviewing and editing, funding acquisition, supervision.

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