Original Article

Identification and validation of a 9-RBPs-related gene signature associated with prognosis and immune infiltration in bladder cancer based on bioinformatics analysis and machine learning

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Background: Bladder cancer (BLCA) is the most common type of malignancy affecting the urinary tract, characterized by high recurrence rates, propensity for progression, metastatic potential, and multidrug resistance, all of which ultimately contribute to an unfavorable prognosis. RNA-binding proteins (RBPs) play a critical role in cancer development and have been associated with the progression and prognosis of the disease. However, comprehensive investigations into the biological functions and molecular mechanisms of RBPs in BLCA remain limited. The study aims to explore the relationship between RBPs and prognosis in BLCA, and to develop and validate an RBPs-based prognostic signature, providing new insights for the diagnosis and treatment of BLCA.

Methods: Clinical data and RBPs expression profiles of BLCA patients were sourced from The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO). A systematic bioinformatics analysis was conducted to identify differentially expressed RBPs and assess their prognostic significance. The optimal predictive model was selected by integrating multiple machine learning algorithms, enabling the identification of hub genes associated with BLCA prognosis and developing an RBP-related gene signature. To evaluate the prognostic signature's efficacy, survival curves and receiver operating characteristic (ROC) curves were generated. A nomogram was constructed and validated to predict the survival of BLCA patients at 1, 3, and 5 years. Furthermore, analyses of immune infiltration and gene set enrichment analysis (GSEA) were conducted to explore the roles of RBPs in immune cell interactions and elucidate underlying biological pathways.

Results: A prognostic signature was effectively developed using nine RBPs (OAS1, MTG1, DUS4L, IGF2BP3, NOL12, PABPC1L, ZC3HAV1L, TRMT2A and TRMU), represented as risk score, through the integration of 13 combinatorial machine learning algorithms. Kaplan-Meier analysis revealed that the high-risk group exhibited a significantly poorer overall survival (OS) probability compared to the low-risk group. The areas under the ROC curves for the risk score model at 1, 3, and 5 years were 0.661, 0.655, and 0.676, respectively. The nomogram, which integrated clinical characteristics and risk scores, demonstrated robust prognostic accuracy. Furthermore, single-sample gene set enrichment analysis (ssGSEA) demonstrated significant correlations between both the risk score model and hub RBPs with the immune status of BLCA patients. GSEA indicated that major signaling pathways enriched in the high-risk group included

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extracellular matrix (ECM) components and interaction, as well as cytokine and receptor interaction.

Conclusions: This study successfully identified and developed a prognostic signature based on nine RBPs, accompanied by a nomogram for predicting survival probability in BLCA patients. Our findings demonstrate that these nine RBPs function as significant biomarkers for forecasting the prognosis and immune status in BLCA, suggesting their potential as therapeutic targets for BLCA.

Keywords: Bladder cancer (BLCA); RNA-binding proteins (RBPs); prognosis signature; immune infiltration; machine learning

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Introduction

Bladder cancer (BLCA) ranks among the most prevalent malignant tumors of the urinary system (1). According to statistical data, approximately 613,000 new cases and 220,000 deaths occurred globally in 2022, positioning BLCA as the ninth most common cancer in terms of incidence and thirteenth in mortality among malignant tumors (1). BLCA is classified into two categories—non-muscle-invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC)—based on clinical symptoms and the extent of tumor infiltration, with NMIBC constituting 75% to 85% of cases, while MIBC accounts for 15% to 25% (2). Currently, the treatment of BLCA primarily involves surgical intervention, complemented by radiotherapy and chemotherapy (3). Most patients present with NMIBC upon initial diagnosis (4). Although NMIBC is not life-threatening, up to 70% of these patients experience tumor recurrence and postoperative deterioration (5), with 10% to 25% progressing to MIBC based on staging and grading (6,7). MIBC often leads to a poor prognosis, characterized by the invasion of blood vessels and lymph nodes, as well as the development of distant metastases. Both MIBC and metastatic BLCA are significant contributors to mortality and poor prognosis in BLCA patients, with 5-year survival rates estimated at only 36% to 48% and 5% to 36%, respectively (8). Furthermore, BLCA patients require repeated medical evaluations and treatments, rendering it one of the most costly malignancies to address (9). Despite advancements in the diagnosis and treatment of BLCA, the prognosis remains variable, underscoring the necessity for more accurate prognostic biomarkers and therapeutic targets. Improving long-term survival rates for BLCA patients relies on early detection, accurate risk assessment, timely interventions, and comprehensive monitoring (10). Identifying biomarkers specific to BLCA is essential not only for facilitating early and accurate diagnosis but also for effective tumor classification. Therefore, it is crucial to conduct systematic research and clinical studies focused on the accurate classification of BLCA and the discovery of novel potential biomarkers. Such efforts are vital for

Highlight box

Key findings

- A novel gene signature was developed and validated based on nine RNA-binding proteins (RBPs), which may enhance prognostic evaluation and prediction for patients with bladder cancer (BLCA).

What is known and what is new?

- RBPs are essential in the developmental processes of cancer and have been linked to disease progression and prognosis. RBPs are implicated in the initiation, progression, and metastasis of BLCA, as well as in predicting patient survival.
- In this study, we identified nine RBPs associated with the prognosis of BLCA and constructed a signature for predicting the clinical prognosis of BLCA patients. These results may provide new insights into the mechanisms underlying the occurrence and progression of BLCA and contribute to developing new clinical therapeutic targets and prognostic biomarkers.

What is the implication, and what should change now?

- These differentially expressed RBPs are likely to play a significant role in the tumorigenesis, progression, invasion, and metastasis of BLCA. Identifying the RBP signature could enhance our understanding of the molecular mechanisms involved in BLCA progression.
- DERBPs should be incorporated into the prognostic assessment of BLCA patients. Additionally, the effectiveness of the RBP signature should be validated in larger, prospective, multicenter cohorts to support its further application.

developing personalized, effective, and reliable therapeutic strategies aiming at enhancing the quality of life and improving the long-term survival of patients with BLCA.

RNA-binding proteins (RBPs) are proteins capable of binding to various types of RNA molecules, forming ribonucleoprotein complexes (11). These complexes are essential for maintaining gene expression homeostasis and regulating post-transcriptional RNA processes (12). RBPs are integral to numerous biological processes, and their regulatory mechanisms in tumor cells have been elucidated, encompassing alternative splicing, polyadenylation, stability, subcellular localization, and translation (13). As components of ribonucleoprotein complexes, RBPs can be affected by genomic and RNA editing-induced amino acid substitutions, which may affect their functional interactions (14). Consequently, any alterations in their expression levels or functional states may contribute to the pathophysiological changes associated with malignant tumors. Research has shown that RBPs are vital in the initiation and progression of several malignancies, including BLCA (14-16). Specifically, RBPs are implicated in the initiation, progression, and metastasis of BLCA (17), as well as in predicting patient survival (18). Thus, this study aims to explore the relationship between RBPs and BLCA prognosis to identify accurate and reliable prognostic biomarkers.

The biological process of malignant tumor development disrupts gene expression (19), leaving behind features that may contain valuable information. In recent years, bioinformatics and machine learning techniques have been extensively utilized to analyze gene and protein expression profiles, facilitating rapid and precise biomarker screening and the construction of prognostic models. The available dataset contains vast amounts of information related to prognostic genes in BLCA, in which the features to be analyzed consist of complex, nonlinear gene combinations embedded within multiple gene expressions, and machine learning has become the primary method for uncovering these patterns and building predictive models based on gene expression data (20). Several classical machine learning algorithms such as linear regression analysis (17) and logistic regression analysis (15,21), have been employed in studies involving RBPs. While these approaches have yielded promising results, challenges remain in constructing models that are both sufficiently accurate and robust for practical clinical applications. Therefore, this study aims to apply bioinformatics and machine learning techniques to identify RBPs that are strongly associated with BLCA prognosis, as well as investigating their roles in biological processes. Moreover, the accuracy of the models will be validated through multiple machine learning algorithms, aiming to identify prognostic markers capable of effectively predicting BLCA prognosis. These findings may provide novel insights into the diagnosis, prognostic assessment, and therapeutic targets of BLCA, ultimately improving clinical decision-making, facilitating early diagnosis, enhancing disease monitoring, and optimizing treatment strategies for BLCA. We present this article in accordance with the TRIPOD reporting checklist (available at https://tau.amegroups.com/article/view/10.21037/tau-2024-688/rc).

Methods

Data acquisition and preprocessing

The "TCGAbioLinks" package of R (https://www.r-project.org/) was utilized to obtain the BLCA dataset (TCGA-BLCA) from the TCGA database (https://portal.gdc.cancer.gov/), and GSE31684 of BLCA patients from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) through the "GEOquery" package to obtain raw gene expression profiling data and clinical characterization of BLCA patients. The TCGA contained 412 BLCA samples and 19 normal tissue samples, serving as the training cohort. The GEO contained 93 BLCA samples and was used as the validation cohort for follow-up analysis and model validation. The "limma" package in R was employed for background correction, median normalization and gene symbol conversion for both datasets. The study was conducted in accordance with the Declaration of Helsinki and its subsequent amendments.

Identification of differentially expressed RBPs (DERBPs)

Differential expression analysis was performed using the "limma" package in R (version 4.4.1) to identify and screen for DERBPs between BLCA and normal tissue samples. DERBPs were defined by an absolute $\log_2$ fold change [$\log_2$(fold change)] greater than 1 and a false discovery rate (FDR)-adjusted P value below 0.05. To visualize the results of the differential expression analysis, heatmaps of genes associated with DERBPs were generated using the "pheatmap" package in R (version 4.4.1).

Screening hub RBPs-related genes through machine learning and determining their predictive ability

The TCGA dataset served as the training cohort for

identifying RBPs-related genes significantly associated with BLCA prognosis. This was accomplished through univariate Cox regression analysis, utilizing a threshold value of 0.05 to select candidate genes to develop a prognostic model. Risk models were created based on gene expression values and corresponding correlation coefficients. Within the training cohort, we constructed consistent models by employing a combination of multiple machine learning algorithms and calculated the average concordance index (C-index) for each model to assess the consistency and predictive power of the identified signature genes across different algorithms. Ultimately, a risk score prediction model was established. The risk value for each patient was determined using the following formula:

$$Risk\ score = \sum_{i=1}^{n}(Coef_i * x_i) \quad [1]$$

In this equation, $Coef_i$ denotes the RBPs coefficient, and x_i represents the expression level of the RBPs.

Each patient's risk score was calculated accordingly, and the cohort was subsequently categorized into high-risk and low-risk groups based on the best cut-off risk score. Kaplan-Meier survival curves were generated using the "survival" and "survminer" packages in R, with the log-rank test employed to compare overall survival (OS) between the high- and low-risk groups, where a P value of less than 0.05 indicated statistical significance.

Independent analysis of the prognostic model

To assess whether the prognostic signature developed in this study could function as an independent predictor of BLCA patient outcomes, we performed both univariate and multivariate Cox regression analyses, involving integrating clinical characteristics such as age, gender, and cancer stage, applying a log-rank threshold of P<0.05 for significant correlations. The analysis was conducted using the "survival" package in R. Additionally, the "timeROC" and "pROC" packages were utilized to generate receiver operating characteristic (ROC) curves over time, along with calibration curves for 1-, 3-, and 5-year intervals to evaluate the prognostic model's sensitivity and specificity.

Construction and verification of prognostic nomogram

To further the prediction of prognosis for BLCA patients, a nomogram was developed. Using the "rms" package in R (version 4.4.1), we constructed a nomogram that integrates the risk score with clinical characteristics, including gender, age, and stage, to forecast 1-, 3-, and 5-year survival for BLCA patients. The accuracy and reliability of the nomogram were confirmed by plotting a calibration curve with the "calibration" function.

Analysis of immune features and immune subtypes in high- and low-risk groups

To begin, the "corcorplot" package was utilized to assess the correlation between immune cell infiltration and RiskScore. Next, we applied single-sample gene set enrichment analysis (ssGSEA) within the "GSVA" package of R software (version 4.4.1) to explore the levels of immune cell infiltration, along with the expression of immune checkpoints and immune-related pathways, between tumor samples from the high- and low-risk groups. A boxplot was generated to visually represent the differences in immune cell infiltration between these two groups. Furthermore, the "TCGAbiolinks" package in R (version 4.4.1) was used to retrieve immune subtyping data for BLCA from the TCGA database. Immune subtyping analysis was then conducted on tumor samples from both the high-risk and low-risk groups.

Gene set enrichment analysis (GSEA) of DERBPs

GSEA is a method for identifying significant biological functions and signaling pathways that are enriched within a predefined set of genes, typically based on differences observed between two biological conditions (22). In this study, GSEA was performed to generate an ordered list of genes based on the expression correlation of DERBPs. This analysis was performed to explore the survival differences between the high-expression and low-expression groups of DERBPs. GSEA was also employed to examine the potential biological functions and signaling pathways associated with the hub DERBPs in the context of BLCA pathogenesis. A FDR threshold of less than 0.25 and a P value threshold of less than 0.05 were applied for statistical significance. The results were visualized using the "GOplot" package.

Statistical analysis

All data processing and statistical analyses were conducted using R software version 4.4.1. For comparisons between two sets of continuous data, independent Student's *t*-tests were used to assess statistical significance for regularly distributed variables. For non-normally distributed data, the

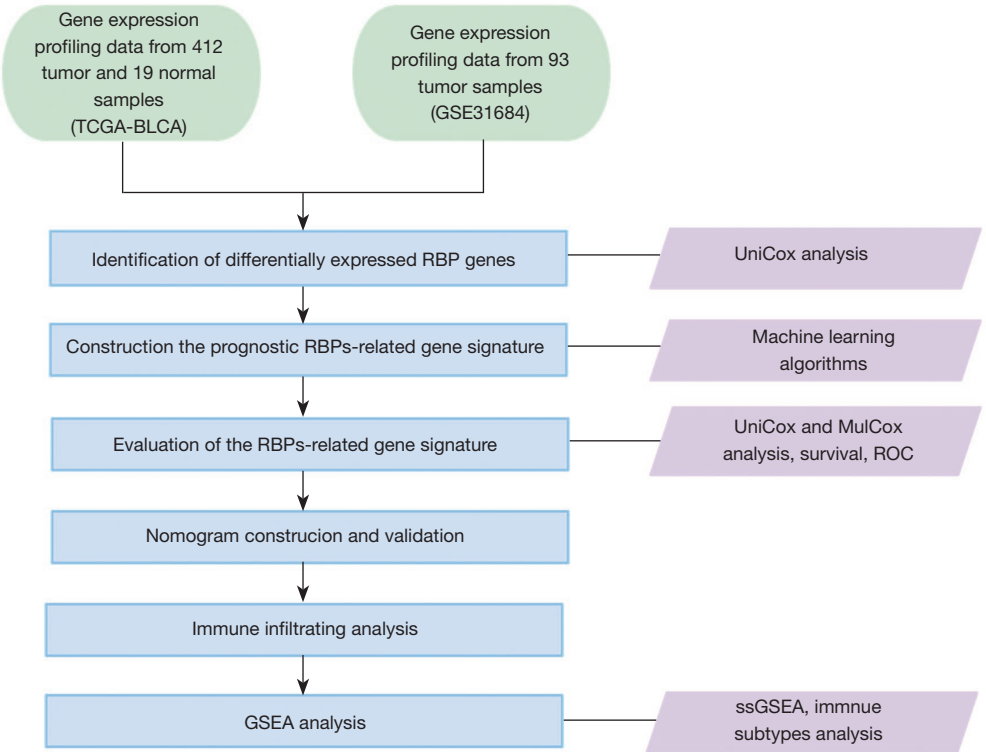

Figure 1 Flowchart showing the scheme of this study in the identification of a RBPs-related gene signature predictive of prognosis in BLCA. BLCA, bladder cancer; GSEA, gene set enrichment analysis; RBP, RNA-binding protein; ROC, receiver operating characteristic; ssGSEA, single-sample gene set enrichment analysis; TCGA, The Cancer Genome Atlas.

Mann-Whitney *U* test was applied. Spearman's correlation analysis was performed to calculate correlation coefficients between genes, as well as between genes and immune cells, and to assess module-trait associations. Statistical significance was set at a P value of less than 0.05.

Results

Identification of differentially expressed genes

The bioinformatics analysis process of this study is summarized in *Figure 1*. The heatmap shows the expression of RBPs in BLCA samples and normal tissues, with a total of 116 genes obtained (*Figure 2A*). The 116 DERBPs associated with prognosis in the sample were then analyzed through univariate cox regression analysis, yielding 34 RBPs associated with prognosis (P<0.05). Twenty-three of the DERBPs (PABPN1, TRMU, MRPL38, OAS1, CLK2, DUS4L, MTG1, MOV10, C2orf15, FASTKD3, GEMIN6, MRPS26, HEXIM2, SNRPF, PPARGC1B, RPS10, NOL12, SNRPA1, TRMT2A, EEF1E1, PABPC1L, POLR2H, GTPBP2) were identified as protective genes with hazard ratio (HR) less than 1, and another eleven DERBPs (DARS2, CALR, IGF2BP3, ZC3HAV1L, ATXN1, XPOT, CSDC2, RBM24, ENOX1, RBPMS2, ZNF106) were identified as risk genes with HR greater than 1. To visualize these findings, we generated a Forest (*Figure 2B*).

Constructing the RBPs-related gene signature using machine learning methods

A hybrid approach incorporating 13 machine learning algorithms was utilized to analyze the 34 DERBPs associated with the prognosis of BLCA and to develop a prognostic signature. In the training set, 101 predictive models were generated by combining different algorithms and hyperparameter configurations. Model performance was evaluated using the C-index for both training and validation sets, which quantifies the ability to rank survival outcomes accurately. While initial model (plsRcox) achieved marginally higher C-index, it identified over 30 genes,

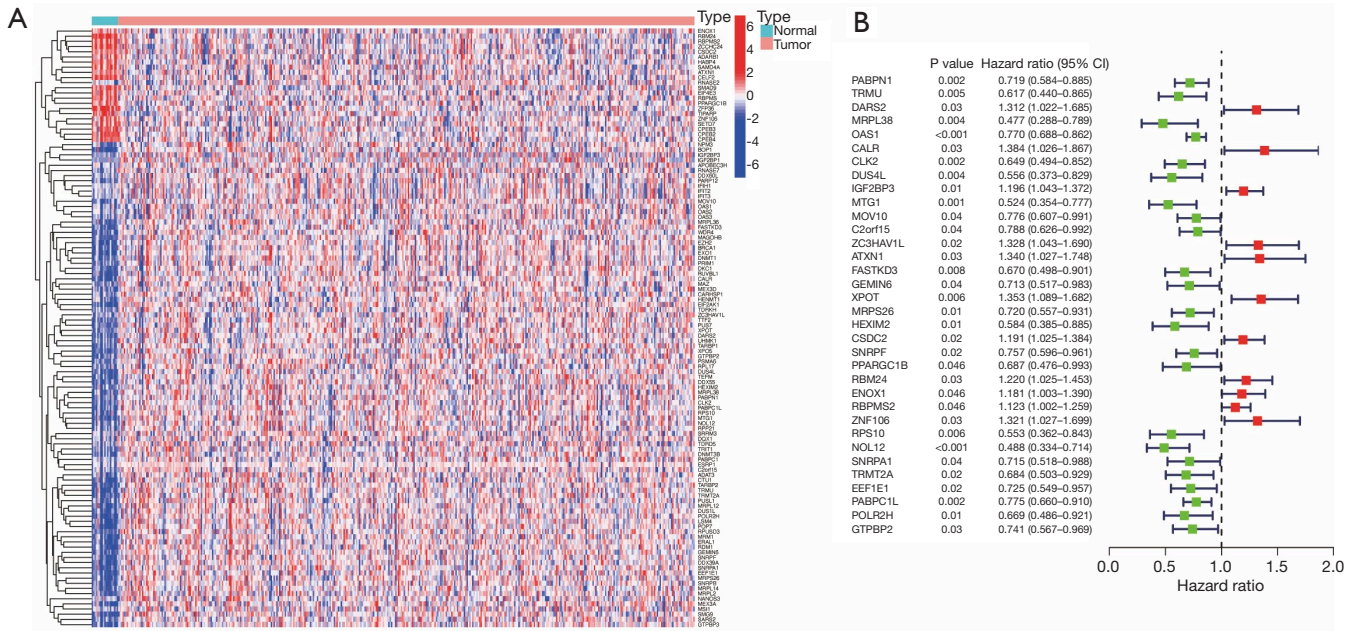

Figure 2 Identification of differentially expressed RBPs. (A) DERBPs between tumor and normal bladder tissues were plotted on a heat map. Color scale represents gene expression levels (red: high expression; blue: low expression). (B) The univariate Cox regression analysis of the 34 DERBPs was performed. DERBPs, differentially expressed RBPs; RBPs, RNA-binding proteins.

introducing high complexity and susceptibility to overfitting. In contrast, RSF + Enet [alpha=0.2] prioritized sparsity via L1 regularization (alpha =0.2), yielding a concise 9-gene signature. This alignment with the principle of parsimony enhances interpretability and generalizability by minimizing redundant variables while retaining predictive power. A smaller gene set reduces noise from redundant signals and facilitates functional validation. The alpha parameter in Elastic Net balances L1 (sparsity) and L2 (multicollinearity control) penalties. Setting alpha =0.2 emphasized L1 regularization, enabling stringent variable selection without sacrificing predictive accuracy. RSF + Enet [alpha=0.2] demonstrated superior stability and clinical utility (*Figure 3A*). By adhering to the bias-variance tradeoff, this model minimized overfitting risks while maintaining competitive discrimination. Based on this model, nine genes (*OAS1*, *MTG1*, *DUS4L*, *IGF2BP3*, *NOL12*, *PABPC1L*, *ZC3HAV1L*, *TRMT2A*, and *TRMU*) were identified as key prognostic markers, and BLCA patients in the dataset were classified into high- and low-risk group. Kaplan-Meier survival curve analysis demonstrated that the OS was significantly worse in the high-risk group than low-risk group in the GEO cohort (P=0.06; *Figure 3B*), and TCGA cohort (P<0.001; *Figure 3C*).

Performance evaluation of the prognostic signature

To assess whether the RiskScore could serve as an independent prognostic factor for BLCA, both univariate and multivariate Cox regression analyses were performed, incorporating clinical characteristics such as stage, gender, and age. The results indicated that RiskScore, stage, and age were all significantly associated with survival (P<0.001) (*Figure 4A*,*4B*). Specifically, univariate analysis revealed the following risk ratios: staging hazard ratio =1.696, age hazard ratio =1.035, riskscore hazard ratio =2.438 (*Figure 4A*). Multivariate analysis of the data set showed stage hazard ratio =1.534, age hazard ratio =1.029, and risk score hazard ratio =2.073 (*Figure 4B*). In the 5-year ROC of the model, the AUCs of risk score, stage, gender, and age were 0.661, 0.629, 0.479, and 0.674, respectively, demonstrating the satisfactory predictive performance of the RiskScore (*Figure 4C*). Moreover, the model's ability to predict survival at 1-, 3-, and 5-year intervals was confirmed, with RiskScore demonstrating an AUC of 0.661, 0.655 and 0.676 for each time point (*Figure 4D*). These findings support the use of the RiskScore, derived from the RBPs-related gene signature, as an independent prognostic factor for BLCA, highlighting the potential clinical value of DERBPs in

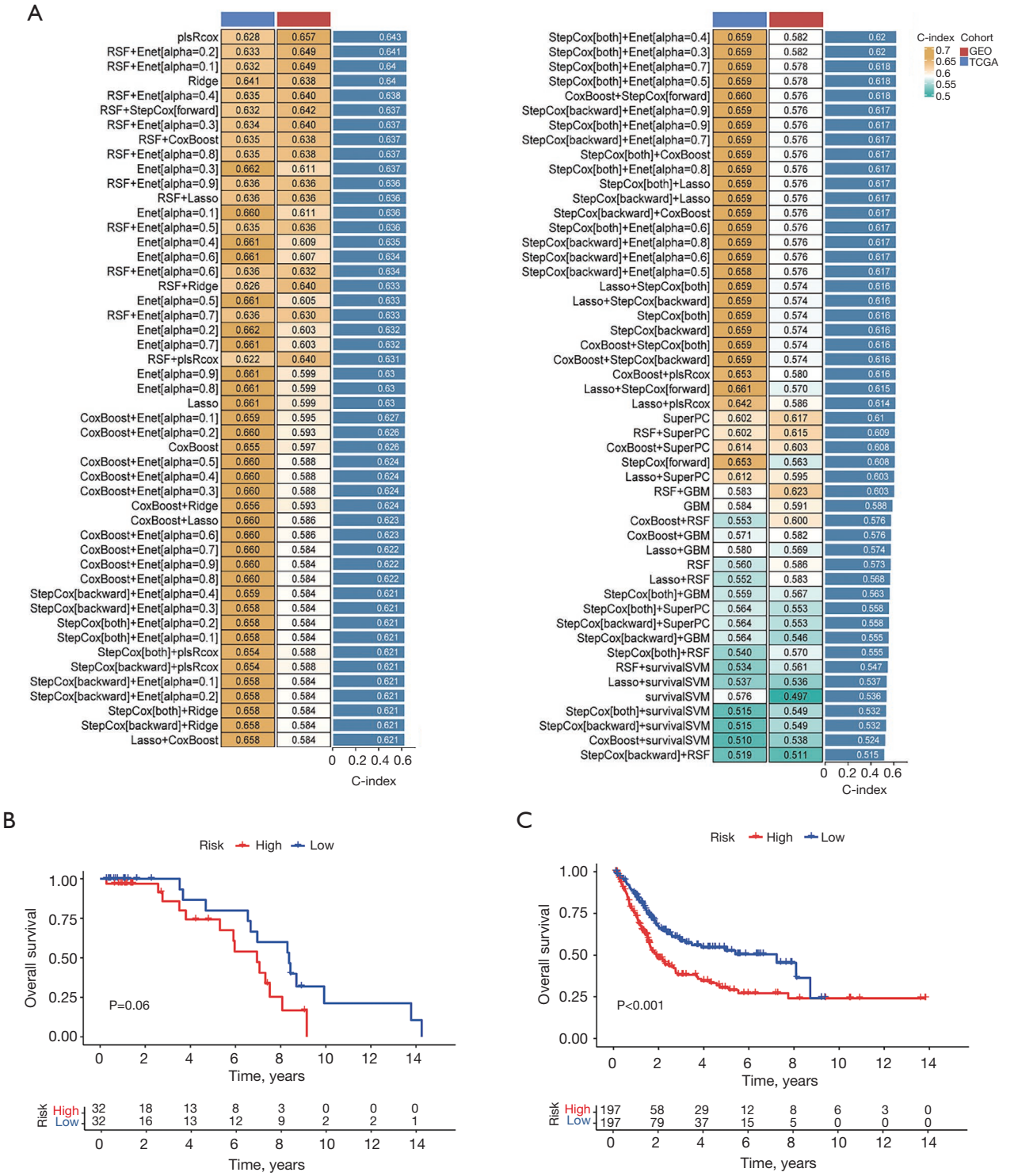

Figure 3 Machine learning constructs prognostic RBPs-related gene signature. (A) A combination of 101 machine learning algorithms was generated through a comprehensive computational framework. Each model's C-index was calculated through the TCGA and GEO cohorts. (B) Kaplan-Meier survival curve in GEO cohort; (C) Kaplan-Meier survival curve in TCGA cohort. GEO, Gene Expression Omnibus; RBPs, RNA-binding proteins; TCGA, The Cancer Genome Atlas.

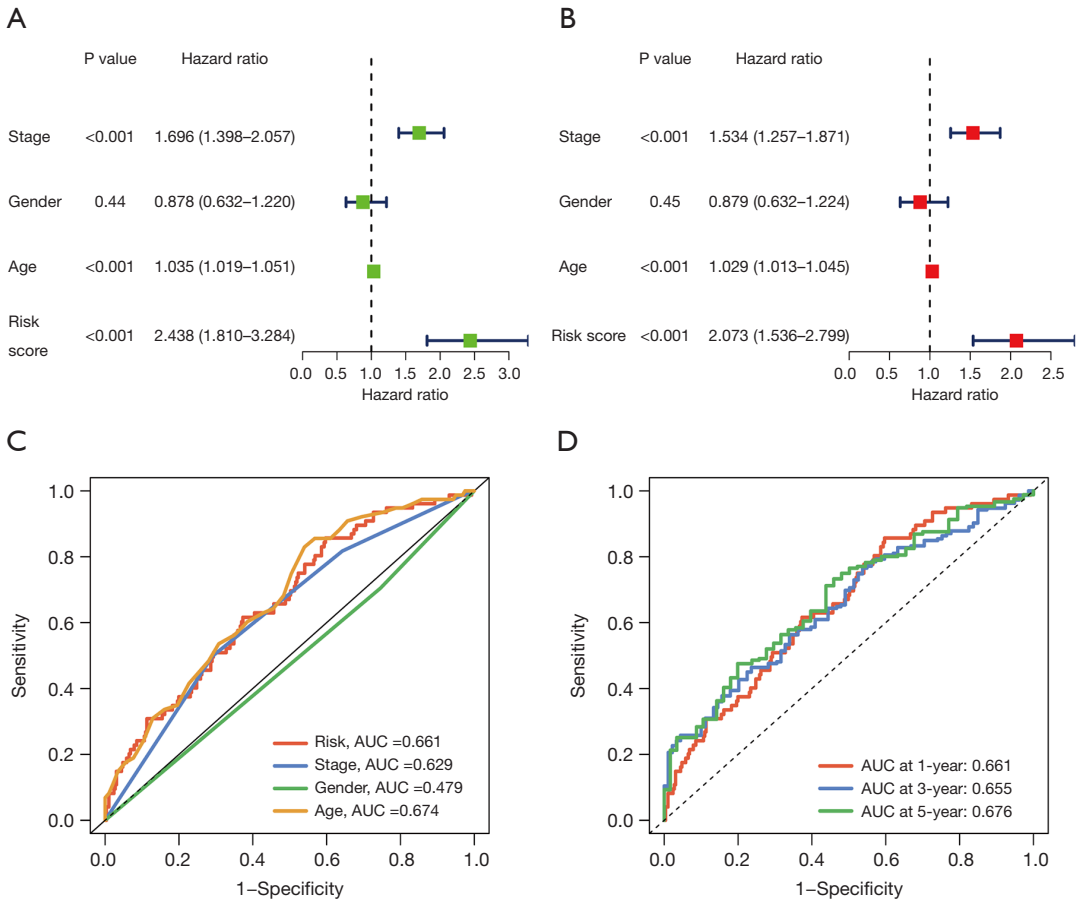

Figure 4 Evaluation of the RBPs-related gene signature. (A) Univariate analysis of the risk score and other clinical characteristics. (B) Multivariate analysis of the risk score and other clinical characteristics. (C) ROC curve of the risk score and other clinical characteristics at 5 years. (D) ROC curves show the potential of the prognostic signature for predicting 1-, 3-, and 5-year OS in the training and validation cohorts. AUC, area under the curve; OS, overall survival; RBPs, RNA-binding proteins; ROC, receiver operating characteristic.

BLCA prognosis.

Construction and validation of the nomogram model

A nomogram integrating clinical characteristics (gender, age, BLCA stage) and the RiskScore was developed for predicting 1-, 3-, and 5-year OS in BLCA patients (*Figure 5A*). The nomogram assigns weighted points to each variable based on their prognostic contributions. As shown in *Figure 5A*, BLCA stage contributed the highest points, reflecting its strong association with poor survival. To validate the RBPs-related gene signature and assess the accuracy of the nomogram, a prognostic calibration analysis was conducted. The calibration curves showed that the model-predicted survival (nomogram-predicted OS) was highly consistent with the observed outcomes (observed OS), especially at the 1- and 3-year time points (curves close to the diagonal line). The 5-year predicted values were slightly biased, which might be related to the missing data from long-term follow-up. The model achieved a C-index of 0.680 [95% confidence interval (CI): 0.639–0.721], suggesting that the nomogram is a reliable tool for predicting survival in BLCA patients (*Figure 5B*).

Immune status varies among patients in different risk groups

The association between risk scores and immune status was examined using various algorithms. The immune cell bubble plots indicated a significant correlation between risk scores and the majority of immune cells, predominantly exhibiting a positive relationship (P<0.05) (*Figure 6A*).

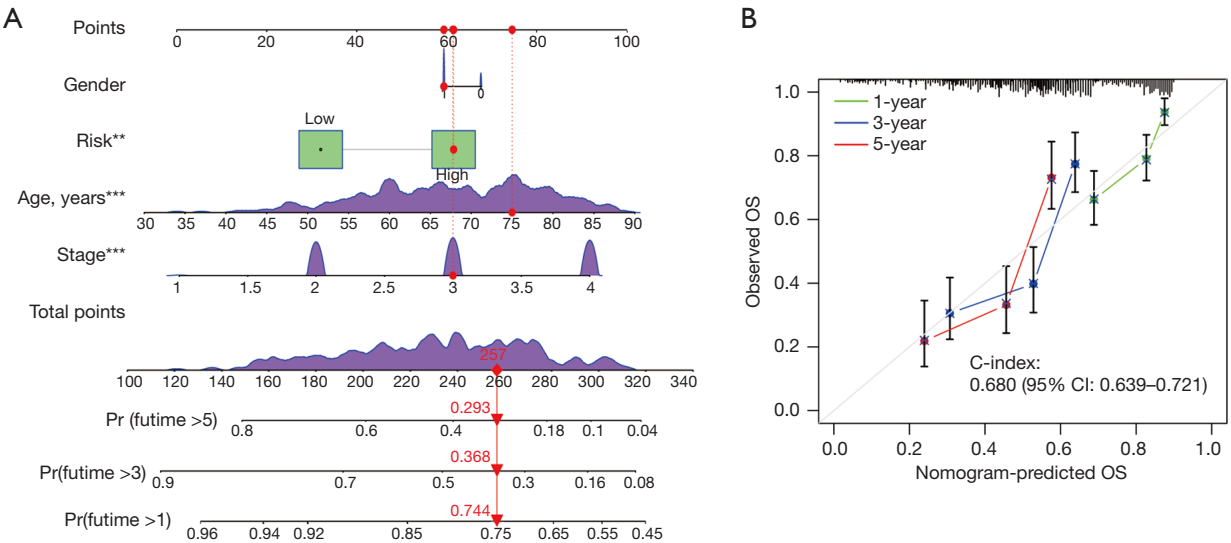

Figure 5 Construction and validation of the nomogram. (A) The nomogram of 1-, 3-, and 5-year OS was constructed by integrating clinical characteristics and risk scores. Baseline clinical characteristic scales (beneath the blue line): age, 30–90 years (5-year intervals); stage, I–IV (0.5-point increments); risk score, 100–340 points (20-point intervals). (B) Nomogram calibration plots for the C-index. **, P<0.01; ***, P<0.001. CI, confidence interval; OS, overall survival.

Findings revealed that patients classified in the high-risk group exhibited higher expression levels of numerous immune checkpoints, suggesting a potential link to poorer survival outcomes in this group (*Figure 6B*). Additionally, ssGSEA was employed to assess immune-related pathways and cell types across low- and high-risk groups. Results indicated that the high-risk group was associated with several immune-regulatory signaling pathways. Moreover, infiltration levels of various immune cells, including activated dendritic cells (aDCs), dendritic cells (DCs), and plasmacytoid dendritic cells (pDCs), were notably higher in the high-risk group compared to the low-risk group. In terms of immune functionality, metrics such as antigen-presenting cell (APC)_co_inhibition, APC_co_stimulation, chemokine receptor (CCR), check-point and cytolytic_activity were all elevated in the high-risk cohort relative to the low-risk group (*Figure 6C*). Previous research has categorized tumors in the TCGA database into six distinct subtypes based on their immune status: C1 (wound healing), C2 (IFN-g dominant), C3 (inflammatory), C4 (lymphocyte depleted), C5 (immunologically quiet), and C6 (TGF-β dominant) (23). The subtype distribution observed in this study indicated that the proportions of C1 (43%), C3 (4%), and C4 (3%) were lower than the corresponding proportions of C1 (44%), C3 (7%), and C4 (15%) found in the high-risk group. Conversely, the proportion of subtype C2 (50%) was greater in the high-risk group compared to subtype C2 (34%) in the low-risk group (*Figure 6D*).

Signal pathway enrichment analysis of characteristic gene sets

To investigate the biological functions and pathways linked to DERBPs associated with poor survival outcomes, GSEA was conducted to elucidate the functional differences between the high- and low-risk groups identified through the prognostic signature (*Figure 7A-7D*). In the TCGA-BLCA cohort, GSEA results demonstrated that the high-risk group exhibited significant enrichment in pathways related to extracellular matrix (ECM) components and interactions, as well as pathways involving cytokine and receptor interactions.

Discussion

Currently, there are several clinical therapies for BLCA, including surgical resection, radiotherapy, chemotherapy and immunotherapy. Although each treatment modality has its own characteristics, due to the significant rates of recurrence, propensity for progression, potential for metastasis, and resistance to multiple drugs of BLCA, patients often have a poor prognosis (3,24,25). With

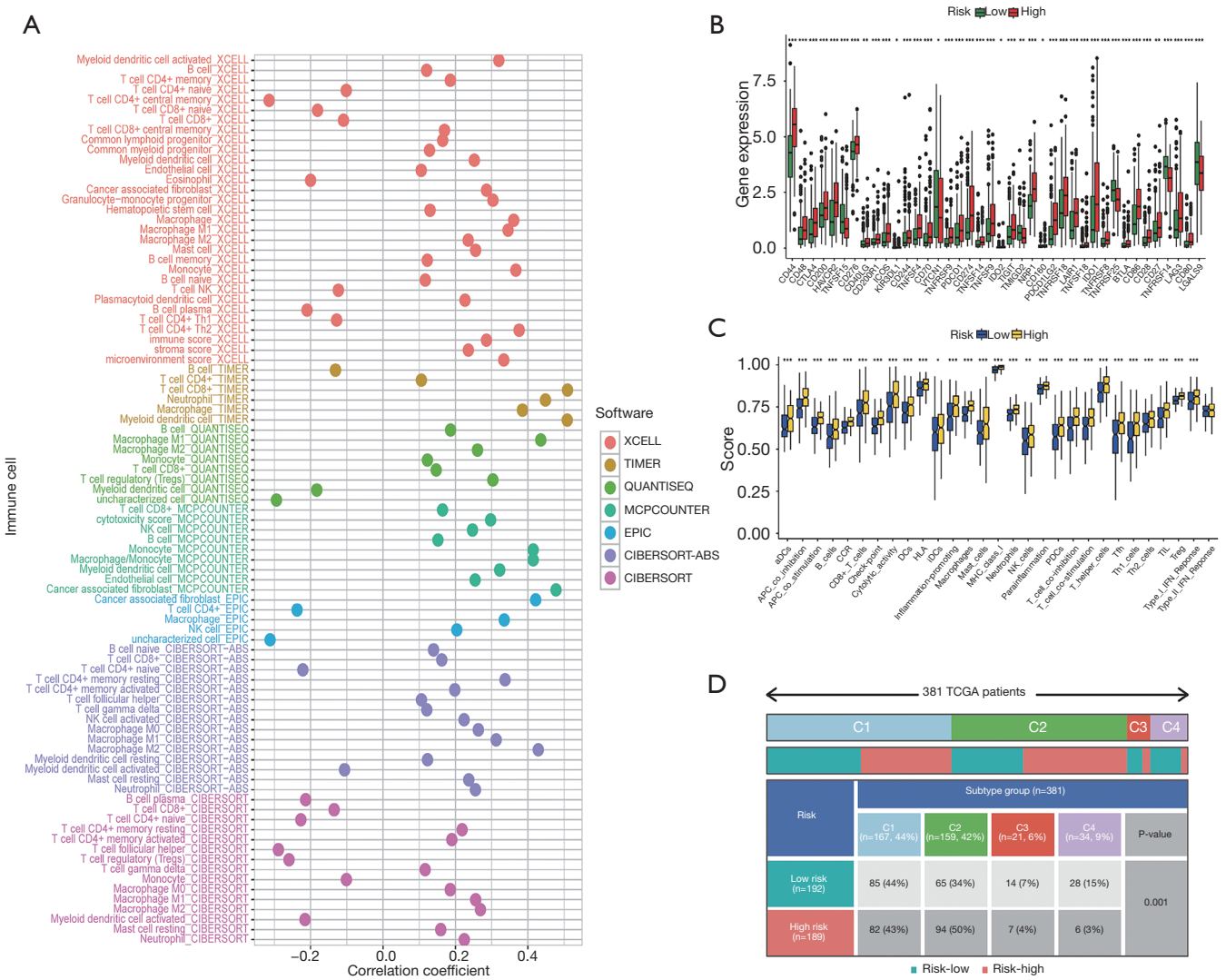

Figure 6 Correlation between DERBPs, immune cells and subtypes. (A) The relationship between Riskscore and immune cell infiltrating was analyzed using seven different softwares. A positive correlation was indicated by a correlation coefficient greater than 0, while a negative correlation was represented by a coefficient less than 0. (B) The expression of immune checkpoint genes was compared between the high- and low-risk groups, revealing notable differences. (C) The ssGSEA analysis was employed to assess immune cells and immune-related functions. (D) Among the 381 patients in the TCGA cohort with immune subtype data, no patients were classified into subtypes C5 or C6. Notably, subtype C1 was the most prevalent in both high- and low-risk groups. *, P<0.05; **, P<0.01; ***, P<0.001. aDCs, activated dendritic cells; APC, antigen-presenting cell; CCR, chemokine receptor; DCs, dendritic cells; DERBPs, differentially expressed RBPs; HLA, human leukocyte antigen; iDCs, immature dendritic cells; IFN, interferon; MHC, major histocompatibility complex; NK, natural killer; pDCs, plasmacytoid dendritic cells; RBPs, RNA-binding proteins; ssGSEA, single-sample gene set enrichment analysis; TCGA, The Cancer Genome Atlas.

the development of precision medicine for cancer and advances in biotechnology and bioinformatics, a diverse set of markers or biomarkers has been established to predict disease prognosis and treatment response through genomic analysis and various bioinformatics tools (26-28). Therefore, the identification of reliable biomarkers is important for the improvement of survival prediction in patients with BLCA.

RBPs are essential in the regulation of RNA. Dysregulation of RBPs has been implicated in the progression of various malignancies (29-31). However, the

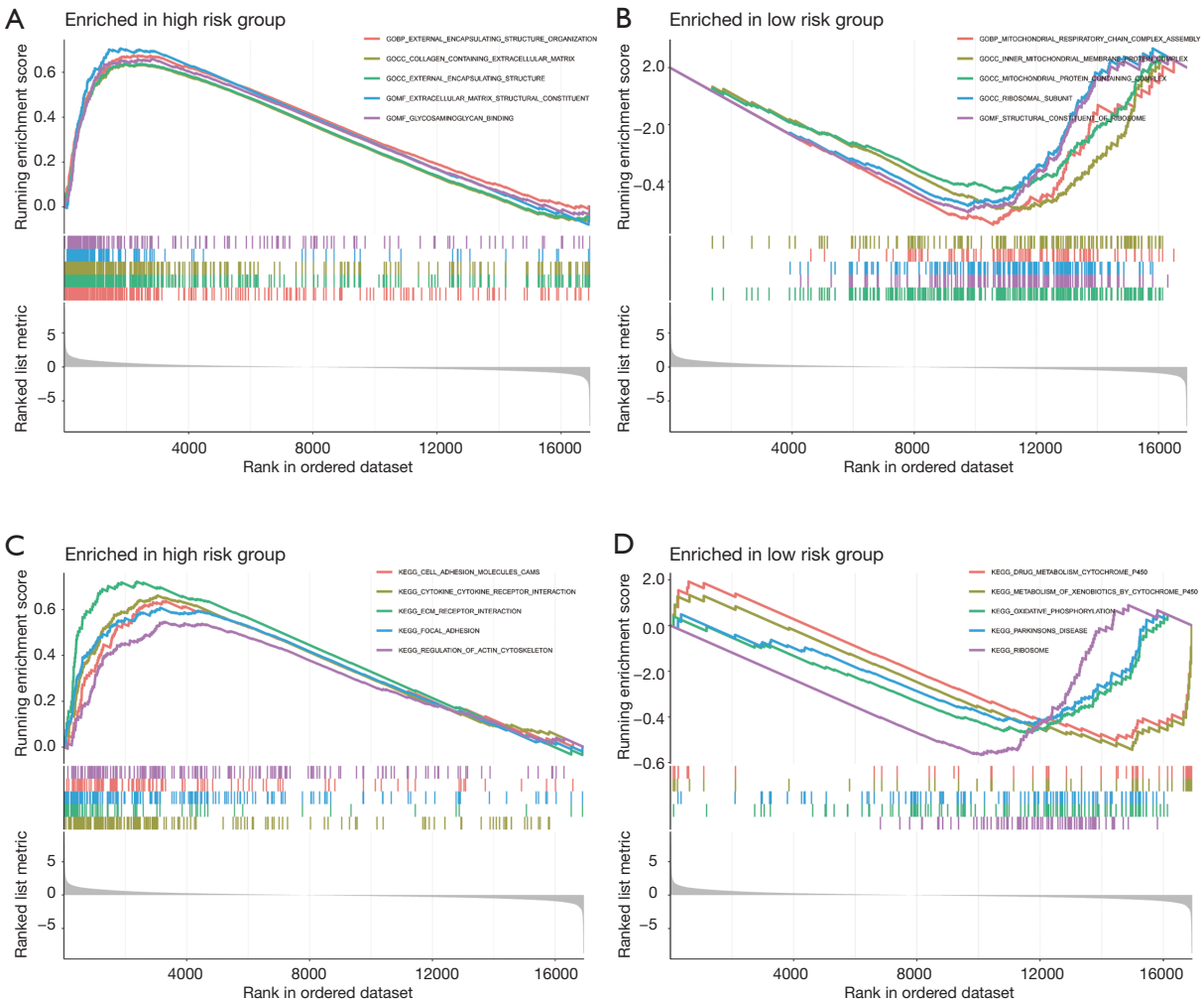

Figure 7 GSEA analysis in the high-risk and low-risk cohorts based on the prognosis signature. (A,C) GSEA enrichment plots for the high-risk group. (B,D) GSEA enrichment plots for the low-risk group. GSEA, gene set enrichment analysis.

precise functional roles and underlying mechanisms of many RBPs in human cancers remain unclear (13,32). While several prognostic signatures for BLCA have recently been developed (15,21,28,33-35), limited research has focused on the specific role of RBPs in predicting BLCA survival, leaving a significant gap in understanding their relevance in this context. Consequently, the clinical value and potential molecular mechanisms of RBP-associated genes in BLCA warrant further investigation.

In this study, 116 differentially expressed RBPs between normal and BLCA tissues were identified through bioinformatics analysis using TCGA and GEO datasets. Bioinformatics methods combined with machine learning algorithms led to the identification of nine key RBP-related genes, and a prognostic signature was successfully developed. The signature demonstrated diagnostic utility for predicting OS in BLCA patients, as shown by ROC analysis. Additionally, nomograms were constructed to predict OS at 1, 3, and 5 years, showing robust predictive performance. These findings suggest that the prognostic signature established in this study holds significant potential for predicting BLCA patient prognosis.

By combining machine learning algorithms, we screened nine RBPs-related genes that are closely associated with BLCA prognosis (*OAS1*, *MTG1*, *DUS4L*, *IGF2BP3*, *NOL12*, *PABPC1L*, *ZC3HAV1L*, *TRMT2A* and *TRMU*). Previous studies have highlighted the significant role of RBPs in tumorigenesis, yet the molecular mechanisms underlying their involvement in cancer pathogenesis remain poorly understood. Oligoadenylate synthetase 1 (OAS1), is

a key interferon-stimulated gene effector protein essential in antiviral defense (36). OAS1 has been shown to be differentially expressed in pan-cancer and correlates with patient prognosis, suggesting its potential as a prognostic marker (37). Additionally, OAS1 may regulate the anti-tumor immune response and has been identified as a valuable predictor for immunotherapy outcomes in BLCA, offering insights into BLCA development and potential individualized treatment protocols (33,38). MTG1, a conserved ribosomal guanosine triphosphatase, is a critical cofactor for mitochondrial translation. Studies have demonstrated that MTG1 is significantly overexpressed in BLCA tumor tissues, positioning it as a potential prognostic biomarker for BLCA (15). MTG1 plays an important role in tumor induction or progression. Although its involvement in tumor initiation and progression is acknowledged (39), the exact mechanisms by which MTG1 contributes to BLCA development remain unclear. Dihydrouridine synthase 4-like (DUS4L) has been implicated in transcriptional and genetic alterations in several cancers, including gastric and prostate cancers (40,41). Moreover, Li *et al.* reported that DUS4L is significantly upregulated in lung adenocarcinoma (LUAD) tissues, where it inhibits cell proliferation and promotes apoptosis in LUAD A549 cells (42). Further research has shown that DUS4L interacts with the signaling molecule GRB2, triggering epithelial-mesenchymal transition through the PI3K/AKT and ERK/MAPK pathways, thereby enhancing LUAD progression and metastasis (43). Insulin-like growth factor II mRNA binding protein (3IGF2BP3) has been identified as a driver of BLCA progression, with studies revealing that PM2.5-induced m6A modifications regulate the stability of BIRC5 mRNA through METTL3/IGF2BP3, promoting BLCA proliferation and metastasis (44). Furthermore, IGF2BP3 has been shown to regulate programmed death ligand 1 (PD-L1) expression in BLCA, suggesting its potential as a prognostic marker for BLCA (45). NOL12 is a multifunctional RBP that acts as a link between RNA and DNA metabolism (46). NOL12 may serve as an independent prognostic predictor in renal clear cell carcinoma and HBV-related hepatocellular carcinoma (47,48), with significant overexpression reported in HCC tissues (49). Its upregulation correlates with patient prognosis, making it a potential therapeutic target. PABPC1-like (PABPC1L), an important paralog of PABPC1, regulates mRNA translation and stability. Overexpression of PABPC1L has been linked to multiple cancers, including prostate cancer, renal cell carcinoma, and colorectal cancer (47,50-52). In addition, PABPC1L was overexpressed in colorectal cancer tissues and correlated with the prognosis of colorectal cancer patients. In colorectal cancer, PABPC1L overexpression correlates with poor prognosis, and its reduction inhibits the activation of p-AKT and p-PI3K, leading to suppressed cell proliferation and migration. These findings suggest PABPC1L as a potential therapeutic target for colorectal cancer (53). Zinc finger CCCH-type containing, antiviral 1 (ZC3HAV1L) has been shown to limit the replication of specific viruses, playing a protective role against virus-associated cancers such as liver cancer and leukemia (54,55). Previous studies reported that TRMT2A has been linked to the risk of recurrence in breast cancer and can serve as a predictor for treatment response (56). TRMU, a nuclear gene encoding a mitochondrial protein involved in tRNA modifications, has been found to be upregulated in BLCA tumor tissues and is significantly associated with the tumor immune microenvironment and immune status (57,58). Therefore, TRMU may serve as a potential biomarker for BLCA prognosis. Overall, our findings provide new insights into the role of RBPs in BLCA development and could offer valuable perspectives for future cancer diagnosis and therapy. However, the precise biological roles and mechanisms of these biomarkers in BLCA require further investigation to fully elucidate their potential clinical applications.

Recent studies have increasingly highlighted the crucial role of tumor immune infiltration in both tumorigenesis and cancer progression, with significant implications for immunotherapy (59-61). In this study, we aimed to explore the relationship between the constructed signature and tumor immune infiltration. Our findings revealed that the high-risk group exhibited a notably higher number of immune checkpoint genes, suggesting a worse prognosis. Furthermore, ssGSEA immune infiltration analysis identified significant differences between the two subgroups in terms of immune-related pathways and cell types. Specifically, immune cells such as aDCs, DCs, and pDCs were more abundant in the high-risk group compared to the low-risk group. Additionally, immune functions, including APC co-inhibition, APC co-stimulation, CCR, checkpoint, and cellular cytolytic activity, were elevated in the high-risk group, indicating that increased immune checkpoint activity may contribute to tumor immune evasion. High expression of immune checkpoints is typically associated with poorer patient prognosis in cancer (62,63). These findings suggest that DERBPs may play a significant role in immunotherapy for BLCA patients. Immune subtypes are closely linked to tumor prognosis, with previous research showing that

the C3 subtype has the best prognosis, while C6 and C4 subtypes correlate with worse outcomes (64). Compared to the low-risk group, the high-risk group had higher proportions of subtypes C1, C3, and C4, and a lower proportion of subtype C2, indicating a poorer prognosis for high-risk patients. These results imply that the disparity in prognosis between the high- and low-risk groups may partially arise from differences in the patients' immune status.

Finally, GSEA analysis was conducted to examine the expression differences of DERBPs-associated biological functions and signaling pathways between BLCA and normal tissues. The results revealed that DERBPs-related genes were predominantly enriched in pathways involved in the ECM composition and interaction pathways. Consequently, the poor survival outcomes observed in the high-risk group appear to be primarily driven by dysregulated ECM signaling. During cancer development, tumor cells tend to alter the surrounding microenvironment by secreting specific ECM components. Oncogenes can drive cancer progression by remodeling the ECM to enhance tumor cell viability, migration, and invasiveness (65). Alterations in the ECM can lead to remodeling of the tumor microenvironment, affecting cell signaling and promoting tumor growth and metastasis. For example, changes in ECM composition may affect tumor angiogenesis and enhance the supply of nutrients to the tumor (66). Moreover, oncogenes can modify the interaction between cells and the ECM by influencing the expression of ECM components and their receptors. Such modifications can enhance the interactions between tumor cells and the surrounding microenvironment, thereby facilitating the migration and invasiveness of these cells (65). Research has demonstrated a significant correlation between tumor-associated pathways and poor prognosis (27), indicating that the 9-RBPs-related gene signature warrants further investigation to uncover its potential mechanisms in BLCA. Overall, our comprehensive analysis highlights that the 9-RBPs-related gene signature may serve as a valuable predictive biomarker for immunotherapy response and could assist in tailoring personalized therapeutic strategies for patients with BLCA.

In conclusion, this study successfully developed a robust RBPs-related signature by systematically integrating data from TCGA and GEO, employing various widely recognized machine learning algorithms instead of relying on a single method. This approach allowed us to identify the model with the best average C-index performance, thereby minimizing the potential influence of overfitting among features on our results. Our predictive signature demonstrated commendable efficacy in forecasting the survival of patients with BLCA. While our research offers valuable insights into the role of RBPs in BLCA and their potential prognostic and therapeutic implications, several limitations must be acknowledged. First, our analysis is primarily based on publicly available datasets, which, although they provide a substantial amount of data, may carry inherent biases and limitations. The samples within these datasets might not fully represent the entire BLCA population, and variations in data quality and experimental methodologies across different studies could affect the results. Consequently, caution is warranted when generalizing our findings to the wider BLCA patient population. Second, due to the retrospective nature of this study, larger sample sizes and more comprehensive mechanistic investigations are necessary to validate the 9-RBPs-related gene signature. Future research should focus on evaluating these effects in larger, prospective, multicenter cohorts. Lastly, while our study identified nine prognostic RBPs significantly associated with OS in BLCA, the analysis was conducted solely through data mining. We primarily concentrated on the prognostic and therapeutic implications of DERBPs in BLCA; however, the specific molecular mechanisms and pathways through which these genes exert their effects remain inadequately explored. Further functional studies—including gene knockdown or overexpression experiments, pathway analyses, and mechanistic investigations—are crucial to fully elucidate the biological significance of DERBPs in BLCA and to examine the impact of other potential biological processes and environmental factors on this association. Despite these limitations, the predictive value of this signature in BLCA patients is noteworthy. Future well-designed, multi-institutional studies will be essential to further validate our findings.

Conclusions

In conclusion, this study conducted a systematic analysis of the biological functions and prognostic significance of differentially expressed RBPs in BLCA using bioinformatics techniques and machine learning algorithms. We identified nine DERBPs that are associated with the prognosis of BLCA. These RBPs are likely to play a critical role in the tumorigenesis, progression, invasion, and metastasis of BLCA. A novel prognostic signature was developed and validated based on these nine DERBPs, demonstrating its potential as an independent prognostic factor for

BLCA. Furthermore, investigations into immune cell infiltration and immune checkpoint expression highlighted the prognostic signature's utility in forecasting responses to immunotherapy, along with enrichment analyses that indicated high-risk groups were associated with tumor-related pathways. Collectively, these findings offer valuable insights into the mechanisms that underlie the development and progression of BLCA. They may also aid in the identification of new clinical therapeutic targets or prognostic markers, which are essential for tailoring personalized treatment strategies for BLCA patients.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki and its subsequent amendments.

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