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Title Page

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Abstract

Background

Bladder cancer (BLCA) is a highly heterogeneous malignancy, and accurate prognostic assessment remains challenging in clinical practice. Recent advances in multidimensional RNA sequencing, including bulk RNA-seq and single-cell RNA sequencing (scRNA-seq), have provided new opportunities to identify prognostic biomarkers. In this study, we integrated bulk RNA-seq and scRNA-seq data from BLCA to construct an immune microenvironment-related prognostic model for more accurate survival prediction.

Methods

Bulk RNA-seq and scRNA-seq datasets for BLCA were obtained from the GEO and TCGA databases to identify key genes and cell populations. After quality control of the scRNA-seq data, principal component analysis (PCA) and uniform manifold approximation and projection (UMAP) were used for dimensionality reduction and cell clustering. Cell types were annotated using previously reported markers and the CellMarker database. Core cell populations were identified by marker-gene analysis using Seurat and the MAST test, followed by single-sample gene set enrichment analysis (ssGSEA). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses

were performed using clusterProfiler. Differentially expressed genes (DEGs) were identified using DESeq2 and limma, and weighted gene co-expression network analysis (WGCNA) was used to identify BLCA-associated gene modules. Intersecting candidate genes were then evaluated by univariate Cox and LASSO-Cox regression analyses to establish a multigene prognostic risk model. Finally, gene set enrichment analysis (GSEA) and immune microenvironment analyses were performed to explore the model's potential association with immunotherapy response.

Results

scRNA-seq analysis identified 12 cell clusters and seven major cell types, of which five showed reduced enrichment in BLCA and were defined as core cell populations. Marker genes of these core cells were closely associated with immune responses, cell adhesion, and signal transduction. Differential analysis of bulk RNA-seq datasets identified 3,100 overlapping DEGs enriched in pathways related to the cell cycle, signal transduction, and immune regulation. WGCNA further identified key gene modules associated with BLCA progression. A five-gene prognostic model showed stable predictive performance for patient survival and was significantly associated with clinical features, including T stage, N stage, and M stage. GSEA and immune microenvironment analyses indicated that the high-risk group had a higher mutation frequency and was enriched for pathways related to immune cell infiltration and immune escape, suggesting a more immunosuppressive phenotype.

Conclusions

By integrating bulk RNA-seq and scRNA-seq data, we developed an immune microenvironment-related prognostic risk score model for BLCA. This model may serve as a useful tool for prognosis prediction and may help identify patients with BLCA who are more likely to benefit from immunotherapy.

Keywords: bladder cancer; scRNA-seq; bulk RNA-seq; tumor immune microenvironment; prognostic model

Introduction

Bladder cancer (BLCA), the predominant form of urothelial carcinoma, is among the most common malignancies of the urinary system. According to GLOBOCAN data, approximately 610,000 new BLCA cases are diagnosed worldwide each year, with more than 220,000 related deaths [1,2]. Pathologically, BLCA is classified as non-muscle-invasive bladder cancer (NMIBC) or muscle-invasive bladder cancer (MIBC). Approximately one-quarter of newly diagnosed patients present with MIBC or metastatic disease, and these patients generally have poor overall survival; the five-year mortality rate exceeds 77% among patients with lymph node-positive MIBC [3,4]. Current international guidelines recommend radical cystectomy with pelvic lymph node dissection as the standard treatment for high-risk NMIBC or MIBC [5,6]. Although recent studies have shown that neoadjuvant therapy can provide significant survival benefits [7-10], the overall survival of patients with BLCA remains unsatisfactory, and treatment responses are heterogeneous. Bladder tumor cells are characterized by marked invasiveness, biological heterogeneity, and diverse immune escape

mechanisms, which may limit responses to immunotherapy and adversely affect prognosis [11].

Therefore, identifying biomarkers associated with immunotherapy response and prognosis based on the BLCA immune microenvironment is essential for refining treatment strategies.

Bulk transcriptome sequencing is a key tool in transcriptomics and provides essential molecular information for tumor molecular subtyping, prognostic evaluation, and therapeutic target discovery.

Recent studies have shown that exosome-derived circTRPS1 promotes glutamine metabolism and induces CD8⁺ T-cell exhaustion in BLCA by sponging miR-141-3p and upregulating GLS1, thereby contributing to tumor progression and highlighting its potential therapeutic relevance [12]. In addition, circSTX6 promotes BLCA metastasis and cisplatin chemoresistance by binding miR-515-3p and interacting with PABPC1 to stabilize SUZ12 mRNA [13]. Single-cell RNA sequencing (scRNA-seq), a high-throughput technology, provides a more refined view of the cellular composition of the BLCA immune microenvironment than conventional bulk tissue sequencing. It enables classification and functional-state analysis of both tumor and non-tumor cells, thereby providing high-resolution biological insights [14,15]. Using this approach, researchers can characterize transcriptomic heterogeneity in BLCA cells and identify gene-expression signatures and molecular markers with potential prognostic value. Integrated single-cell omics analyses have identified cancer cell subpopulations associated with high-grade, highly invasive BLCA and poor prognosis, thereby deepening our understanding of BLCA heterogeneity [16]. Another scRNA-seq study identified a BLCA cell subpopulation with active lactate metabolism and substantial cisplatin resistance, suggesting a potential target for overcoming chemoresistance [17]. scRNA-seq also provides unique opportunities to study intercellular communication and to reveal complex interaction networks among

different cell types in the tumor microenvironment (TME). These findings underscore the value of single-cell sequencing for investigating prognosis and drug-resistance mechanisms in BLCA.

In this study, we integrated single-cell and bulk RNA-seq data from BLCA to identify prognostically relevant biomarkers and construct a risk score model. We systematically validated the model's predictive performance and further examined its association with immune infiltration, with the aim of providing a potentially useful perspective for the clinical management of BLCA.

Methods

Data sources and processing

Single-cell RNA sequencing (scRNA-seq) data from 11 BLCA tissues and five matched adjacent normal samples were obtained from the GEO database (GSE135337 and GSE222315). Bulk RNA-seq data with prognostic information, comprising 18 normal and 404 tumor tissue samples, were downloaded from TCGA. Quality control was performed on the scRNA-seq data by retaining cells with 500-6,000 detected genes and mitochondrial gene percentages below 10%. Genes were also required to be expressed in at least three cells, yielding 135,308 high-quality cells. The top 2,000 highly variable genes were selected using ANOVA for downstream analyses. After dimensionality reduction by principal component analysis (PCA), the first 50 principal components (PCs) were used for UMAP-based nonlinear dimensionality reduction and cell clustering [18]. Cell type annotation was performed using common marker genes and references from previous studies.

Core cell screening and functional enrichment analysis

Marker genes for each cluster were identified using the FindAllMarkers function in Seurat, and the MAST test was used to detect differentially expressed genes (DEGs). Based on the significant marker genes for each cell type, single-sample gene set enrichment analysis (ssGSEA) [19] was used to calculate enrichment scores for each cell type in the TCGA-BLCA tumor and normal datasets. Differences in ssGSEA scores between tumor and normal samples were assessed using the Wilcoxon test. Cell types whose marker gene sets showed significantly different ssGSEA scores between tumor and normal samples ($P < 0.05$) were defined as core cells. Functional enrichment analyses of Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways for core-cell marker genes were performed using the clusterProfiler R package.

DEG identification and functional enrichment analysis

Differential expression analysis was performed on 18 normal and 404 tumor tissue samples using both DESeq2 and limma [20,21]. Genes with an adjusted P value < 0.05 and $|\log_2FC| > 1$ were considered DEGs. Volcano plots and heatmaps of DEGs identified by each method were generated using ggplot2 and pheatmap. DEGs shared by both methods were subsequently subjected to KEGG pathway and GO enrichment analyses using clusterProfiler [22] to identify significantly enriched signaling pathways and biological processes.

WGCNA analysis

The WGCNA R package [23] was used to construct a co-expression network. Genes related to BLCA

were filtered using the goodSamplesGenes function, and an appropriate soft-thresholding power was selected. A co-expression network was constructed using a hybrid dynamic tree-cutting algorithm, with the minimum module size set to 300 genes. Pearson correlation analysis was used to assess associations between module eigengenes (MEs) and BLCA status.

Prognostic model construction

Candidate genes were obtained by intersecting core-cell marker genes, DEGs, and BLCA-related genes. TCGA-BLCA samples were randomly divided into training and validation sets at a 7:3 ratio. Univariate Cox regression analysis ($P < 0.05$) was performed in the training set to identify genes significantly associated with prognosis. LASSO-Cox regression implemented in the glmnet R package [24] was then used for variable selection and multigene prognostic model construction. The expression values of each signature gene were normalized before model construction. The risk score was calculated as follows: Risk score = sum(normalized gene expression x corresponding LASSO coefficient). Survival curves were generated using survminer, and time-dependent receiver operating characteristic (ROC) curves were generated using timeROC to evaluate predictive performance for 1-, 2-, 3-, 4-, and 5-year overall survival.

Clinical characteristics and independent prognostic analysis

Samples were stratified into high- and low-risk groups according to the risk score. Associations between risk groups and clinical characteristics, including survival status, age, sex, T stage, N stage, M stage, pathological stage, smoking status, and tumor mutational burden (TMB), were analyzed.

Differences in the distribution of clinicopathological features between risk subgroups were assessed using the Kruskal-Wallis test or Wilcoxon rank-sum test, as appropriate. Univariate Cox regression analysis was used to evaluate the prognostic value of the risk score and other clinical parameters, including TNM stage, sex, age, pathological stage, and smoking status. Multivariate Cox proportional hazards regression was performed to identify independent prognostic factors. A nomogram prediction model was constructed using the `cph` function in R [25] to visualize individualized prognostic predictions and estimate 1-, 3-, and 5-year overall survival (OS). Calibration curves were plotted to assess agreement between predicted and observed outcomes.

GSEA and immune microenvironment analysis

Gene set enrichment analysis (GSEA) of all genes in the high- and low-risk subgroups was performed using `clusterProfiler` to evaluate differences in biological functions and signaling pathways. A gene set comprising 50 Hallmark cancer pathways was obtained from the Molecular Signatures Database (MSigDB; <http://www.gsea-msigdb.org/gsea/index.jsp>). Infiltration levels of 28 immune cell types in the TCGA-BLCA cohort were quantified using `ssGSEA` from the `GSVA` package. Pearson correlation analysis was used to explore relationships between risk scores and immune cell infiltration. To assess the predictive potential of the risk score for immunotherapy response, an 18-gene T-cell-inflamed gene expression profile (GEP) associated with immune checkpoint blockade response was analyzed, and GO enrichment analysis was performed for the GEP genes. The top four significantly enriched pathways were visualized using Cytoscape, and protein-protein interaction (PPI) networks were constructed to investigate regulatory relationships. In addition, expression levels of four key immune

checkpoints (PD-1, PD-L1, CTLA-4, and TIGIT) were extracted, and Wilcoxon tests were used to compare their expression between the high- and low-risk groups.

Results

Identification of BLCA cell subpopulations

After quality control and filtering of the downloaded single-cell datasets, 135,308 high-quality cells were retained for downstream analyses. Read count data were log-normalized, and the top 2,000 highly variable genes were selected. The effects of unique molecular identifier (UMI) counts and mitochondrial gene percentage per cell were regressed out. Dimensionality reduction was then performed using principal component analysis (PCA). Cell clustering was conducted using the K-nearest neighbor (KNN) graph-based algorithm implemented in Seurat (first 50 PCs, resolution = 0.5). Based on canonical cell type-specific markers, cells were divided into 12 initial clusters (Fig. 1A). These clusters were systematically annotated by integrating the CellMarker database with previously reported marker genes (Fig. 1B), resulting in seven major cell types: endothelial cells, epithelial cells, fibroblasts, T/NK cells, B cells, myeloid cells, and mast cells (Fig. 1C,D). The upregulated expression of marker genes across these cellular populations is shown in Fig. 1E.

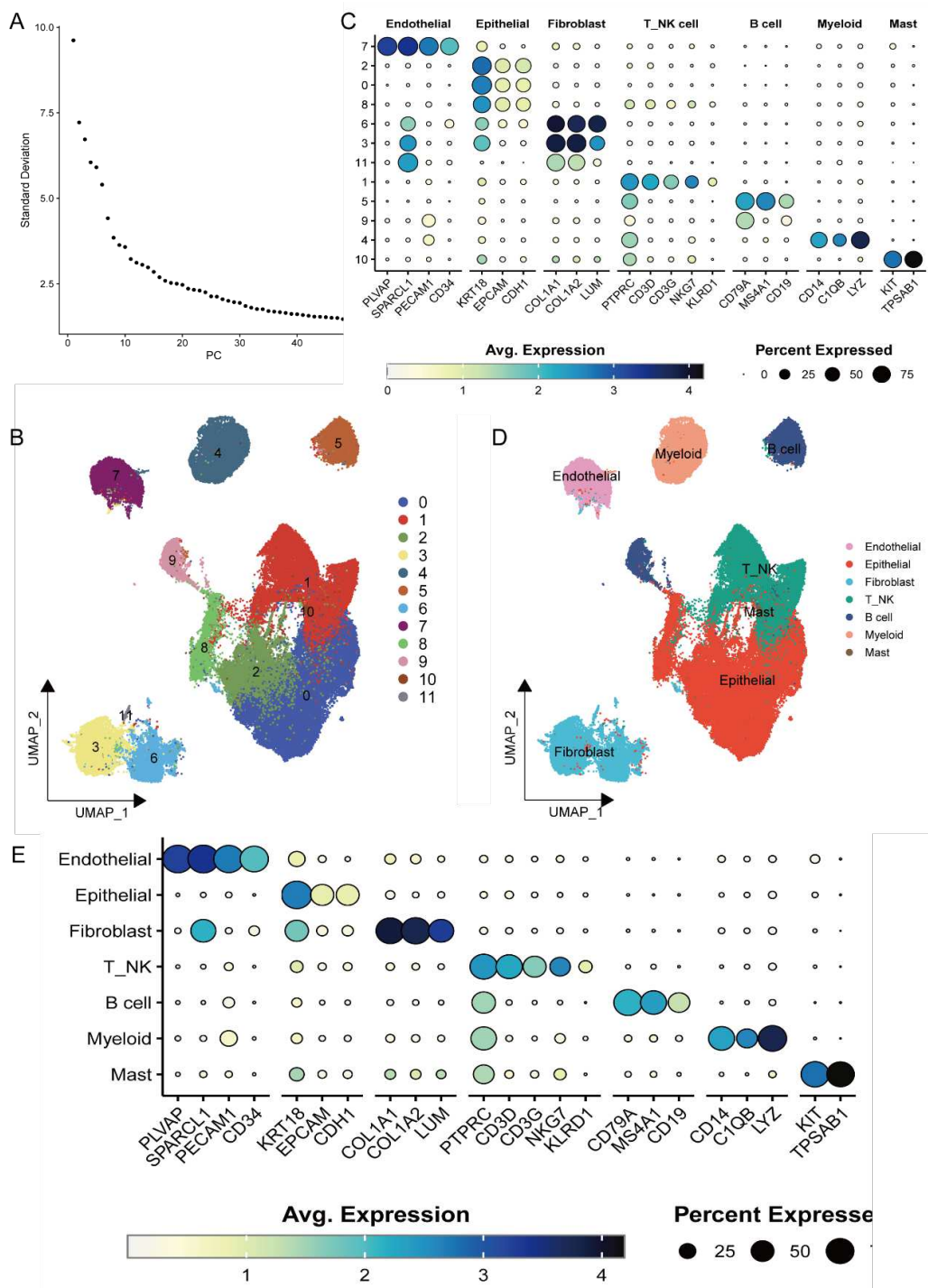


Fig. 1 Single-cell RNA sequencing analysis identifies distinct cellular subpopulations in bladder cancer. (A) UMAP visualization based on the top 50 principal components classified cells into 12 clusters. (B) The 12 cell clusters were annotated using established marker genes. (C,D) Bladder cancer

(BLCA) cells were classified into seven major subpopulations according to their marker gene expression profiles. (E) Heatmap showing the expression levels of representative marker genes across the seven BLCA cell subpopulations.

Marker gene functional enrichment analysis

Functional enrichment analysis was performed for marker genes of the seven identified cell types. Using the FindAllMarkers function combined with the MAST test, significantly differentially expressed genes were identified, and single-sample GSEA (ssGSEA) scores were calculated for each cell type (Fig. 2A). Among the seven major cell types, five (endothelial cells, fibroblasts, T/NK cells, B cells, and mast cells) showed significantly lower enrichment in BLCA and were therefore defined as core cell populations for subsequent analyses.

GO and KEGG enrichment analyses of marker genes from these core cells showed significant enrichment in immune- and inflammation-related pathways, as well as pathways involved in cell adhesion, signal transduction, and disease-specific processes (Fig. 2B). GO analysis further showed that these marker genes were highly enriched in biological processes such as immune response, cell adhesion and migration, and extracellular matrix organization, suggesting that core cells are closely associated with immune/inflammatory responses, the tumor immune microenvironment, and tissue repair processes (Fig. 2C-E).

Cell communication network analysis indicated that T/NK cells, myeloid cells, and fibroblasts had dominant roles in intercellular interactions, acting as major senders and receivers of signals and forming the backbone of the communication network (Fig. 3A,B). These cell populations may play

251 key roles in regulating the tumor microenvironment, including immune responses, inflammatory status,
252 and tissue remodeling (Fig. 3C,D). This finding is consistent with the enrichment of T-cell receptor
253 signaling and immune-response activation observed in the KEGG and GO analyses, further supporting
254 the functional relevance of these cell populations in the BLCA microenvironment.

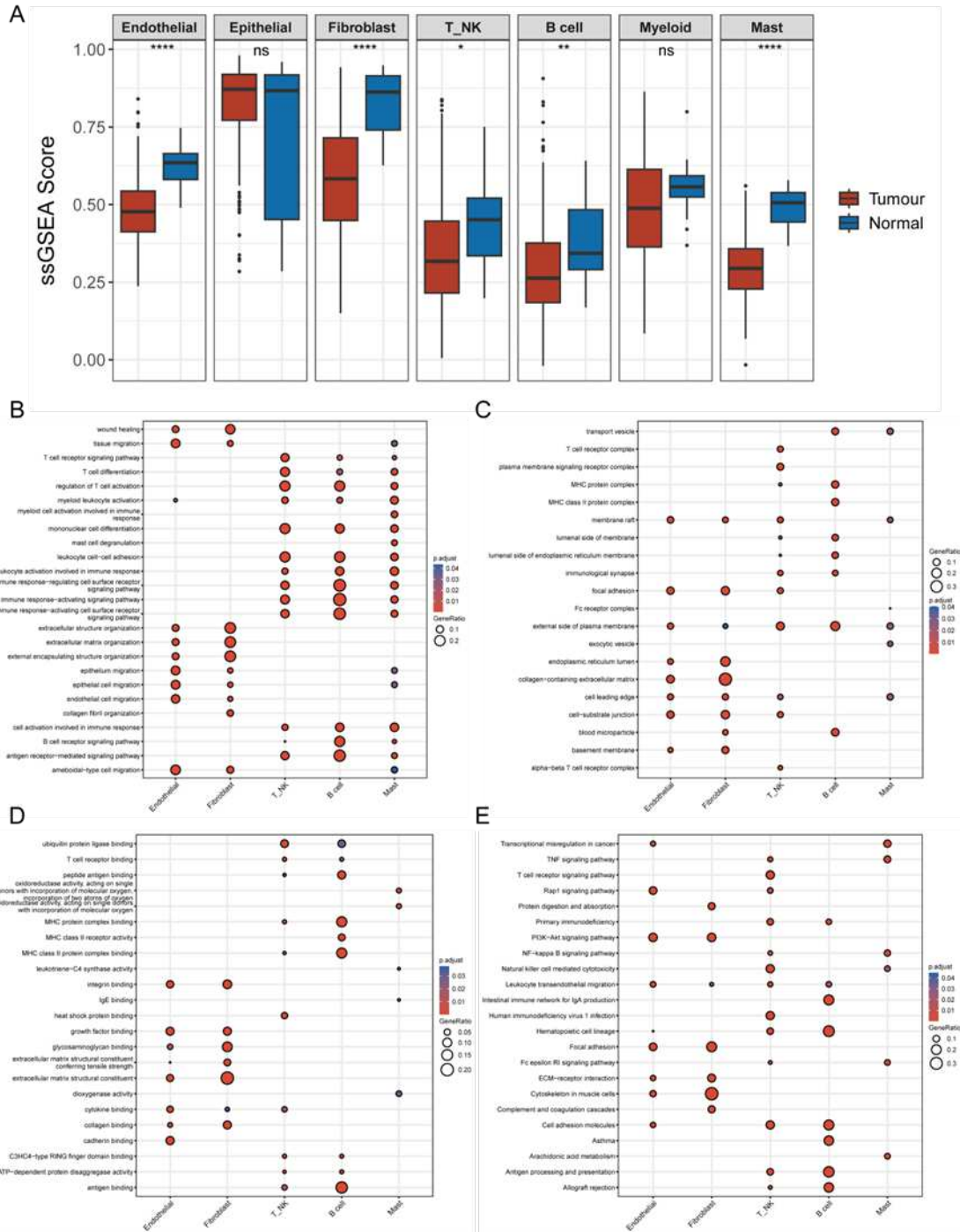


Fig. 2 Differential expression and functional enrichment analysis of marker genes across seven cell subpopulations. (A) ssGSEA scores were calculated based on marker genes of each cell population, and cell populations showing statistically significant differences were defined as core cells. (B-E) GO and KEGG functional enrichment analyses were performed using marker genes derived from

differentially enriched cell populations.

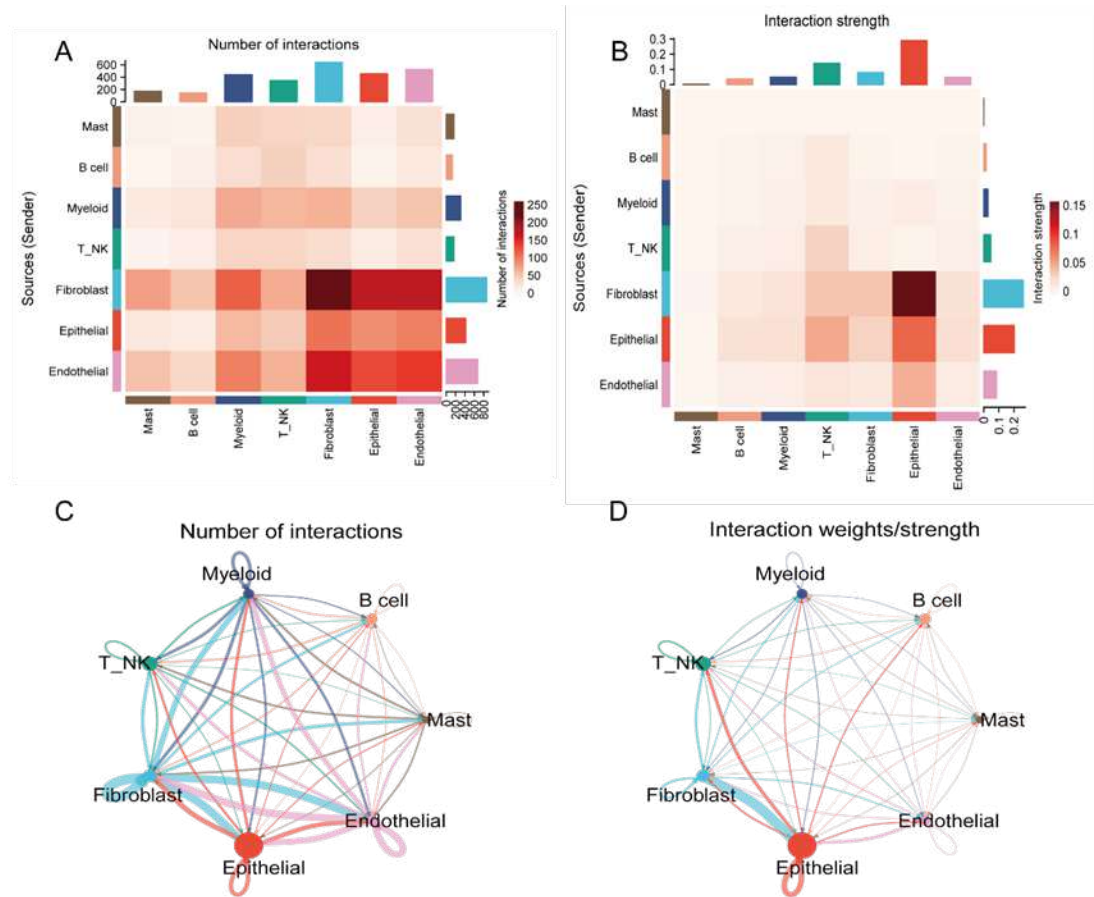


Fig. 3 Cell communication network analysis of core-cell marker genes. (A,B) Number of potential ligand-receptor pairs among key cell populations. (C,D) Interaction numbers and interaction strength between key cell populations.

Identification of differentially expressed genes and functional enrichment analysis in bulk RNA-seq data

Differential expression analysis was performed on bulk RNA-seq data from 18 normal and 404 tumor tissues using DESeq2 and limma (Fig. 4A-D). DESeq2 identified 3,837 DEGs, whereas limma identified 4,478 DEGs. Intersecting the two results yielded 3,100 significant DEGs, which were used

for subsequent analyses (Fig. 4E). KEGG and GO enrichment analyses were then performed for these overlapping DEGs. KEGG pathway analysis showed that the DEGs were mainly enriched in intracellular signaling, cell proliferation, and cytoskeletal regulation pathways. GO analysis indicated significant involvement in biological processes including the cell cycle, DNA replication, nuclear division, and extracellular matrix structural organization (Fig. 4F,G).

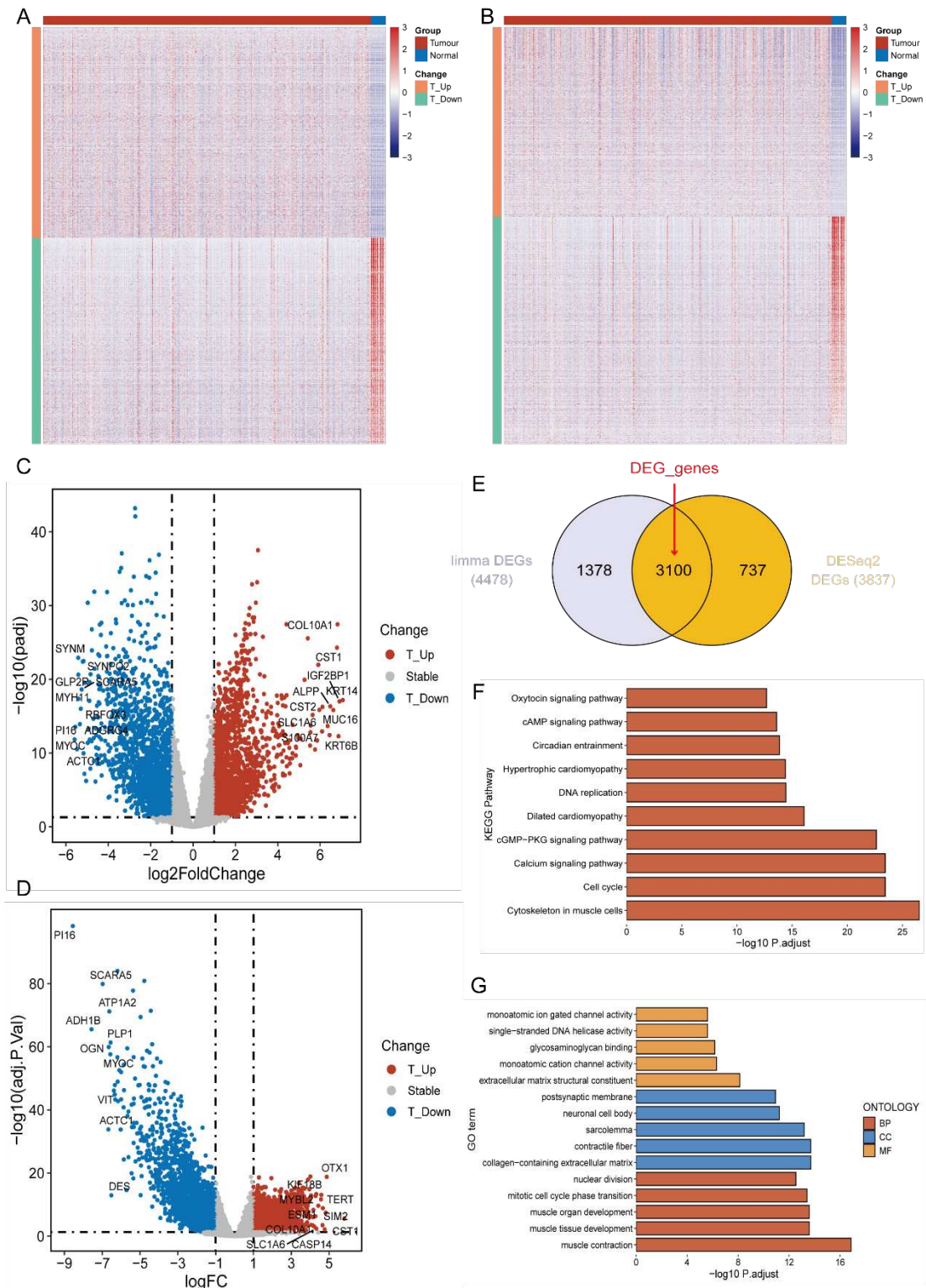


Fig. 4 Identification of differentially expressed genes and functional enrichment analysis between

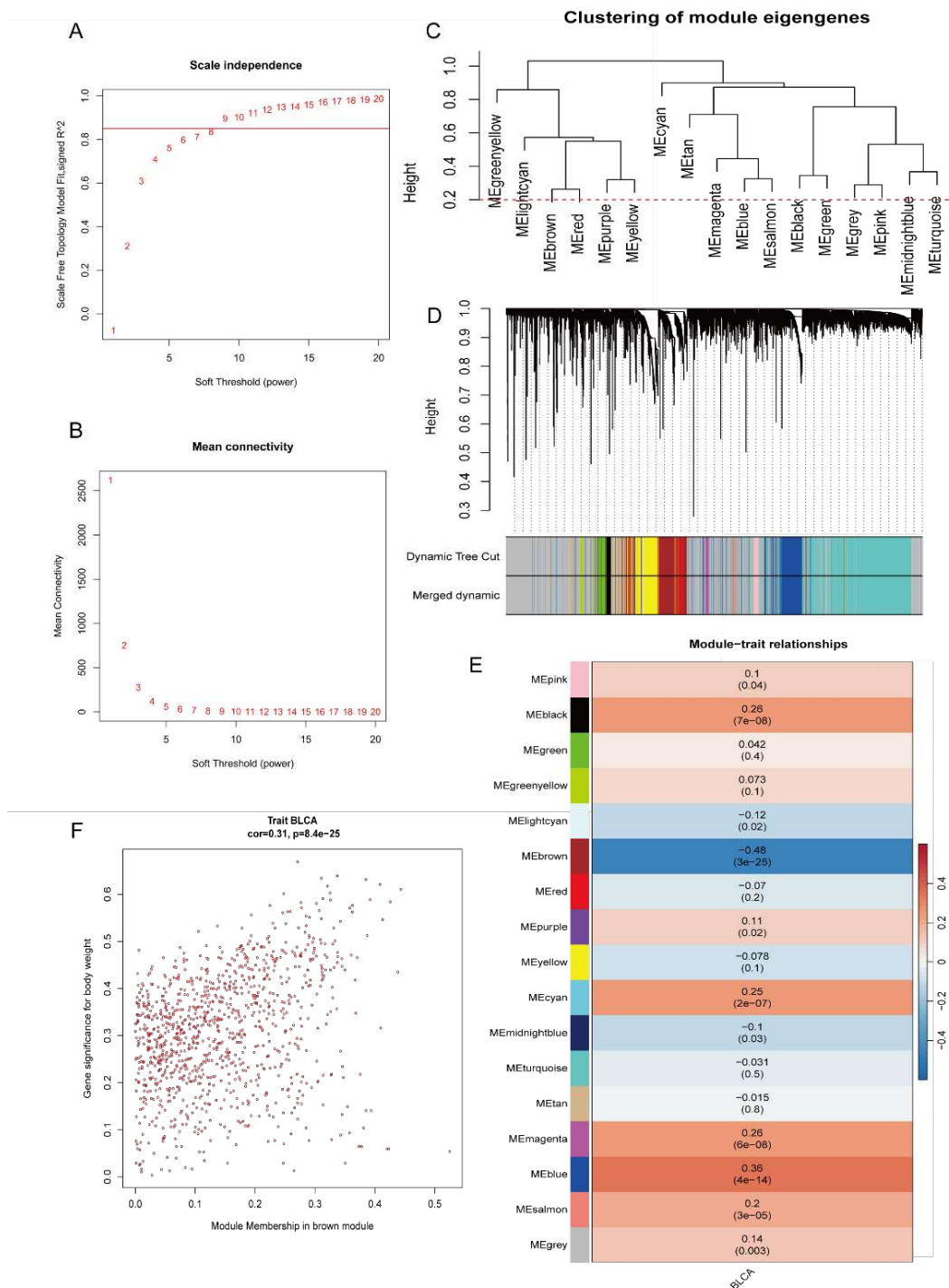
BLCA and normal control samples. (A,B) Heatmaps of differentially expressed genes generated using

280 DESeq2 and limma. (C,D) Genes with $P < 0.05$ and $|\log_2\text{FoldChange}| > 1$ were identified as
281 significantly differentially expressed by DESeq2 and limma. (E) Intersection of significant DEGs
282 identified by the two methods. (F,G) Bubble plots showing KEGG and GO pathway analyses of the
283 DEGs.

284

285 Identification of key modules associated with BLCA

286 Weighted gene co-expression network analysis (WGCNA) was used to identify genes associated with
287 BLCA development and progression. During co-expression network construction, the
288 soft-thresholding power beta was set to 5, yielding a scale-free topology fit index of 0.85 (Fig. 5A,B).
289 With a module merge threshold of 0.2, the dynamic tree-cutting algorithm was used to cluster and
290 merge initial modules, resulting in 17 distinct gene co-expression modules (Fig. 5C). Based on
291 correlation coefficients and significance levels between modules and clinical traits, the MEbrown
292 module was selected as the key module and contained 1,049 genes (Fig. 5D,E). Its association with
293 BLCA was further supported by clinical correlation scatter plot analysis (Fig. 5F).



294

295 Fig. 5 Identification of BLCA-associated genes using WGCNA. (A,B) Scale-free fit index under

296 different soft-thresholding powers (beta). (C) Co-expression modules identified when the minimum

297 module size was set to 300 and MEDissThres was set to 0.2. (D) Clustering dendrogram of

co-expression network modules (1-TOM). (E) Correlation analysis between the 17 gene co-expression modules and BLCA, with P values indicated.

(F) Scatter plot analysis of the brown module.

Construction and validation of a prognostic model based on five signature genes

A Venn diagram was used to identify the intersection of bulk DEGs, BLCA module genes, and cell subtype-associated DEGs, yielding 157 candidate genes (Fig. 6A). Univariate Cox regression analysis

of these candidates in the TCGA-BLCA training set identified 22 genes significantly associated with overall survival (OS) (Fig. 6B). LASSO regression was then applied for variable selection, and five

key genes were retained at the point of minimum cross-validation error. The prognostic risk score

formula was established as follows: RiskScore = 0.0004253 x MAP1B + 0.0009387 x EMP1 + 0.0002212 x CRTAP + 0.0088038 x TCF4 + 0.0003839 x LRP1 (Fig. 6C,D). Using a risk score cutoff

of -0.16, patients were stratified into high- and low-risk groups (Fig. 6E). Survival analysis showed significant differences in both overall survival (OS) and disease-free survival (DFS), with the

high-risk group having significantly worse OS and DFS than the low-risk group (Fig. 6F).

Time-dependent ROC curves showed that AUC values for 1- to 5-year OS all exceeded 0.61, indicating stable predictive performance (Fig. 6G).

Together, these results indicate that the five-gene signature-based prognostic model has robust prognostic value for predicting outcomes in patients with BLCA.

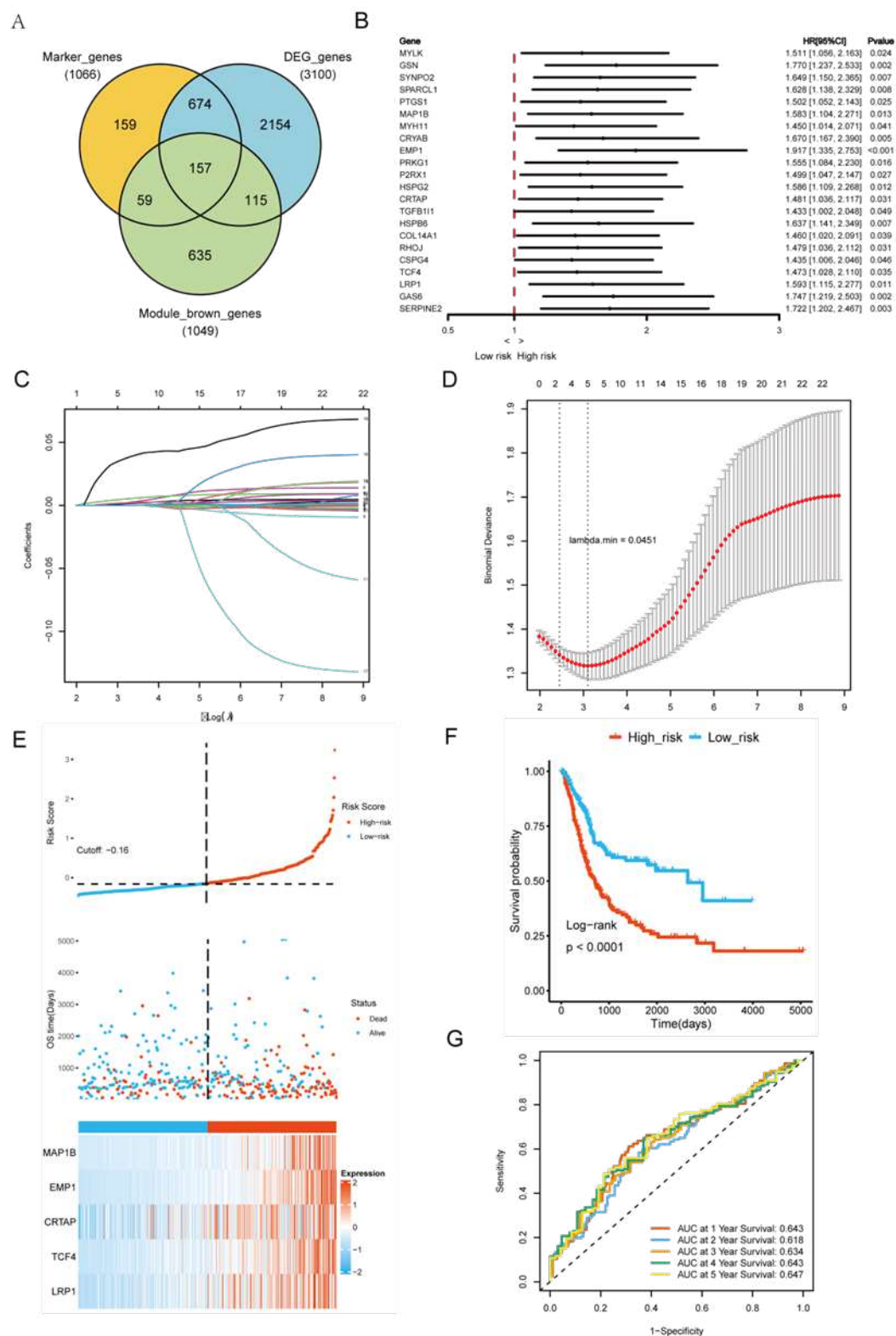


Fig. 6 Construction of a prognostic risk score model for patients with BLCA. (A) Venn diagram

showing the intersection of BLCA-associated genes, DEGs identified from core cells, and DEGs between BLCA and control groups. (B) Univariate Cox regression analysis of overall survival (OS). (C,D) LASSO regression analysis of genes significantly associated with OS. (E) Distribution of risk scores, survival status, and expression heatmap of signature genes in high- and low-risk subgroups. (F) Kaplan-Meier curves for the high- and low-risk subgroups. (G) Time-dependent ROC curves showing AUC values for predicting 1- to 5-year survival using the model.

Screening of independent prognostic factors and construction of a nomogram

A heatmap showing associations between risk scores and clinical characteristics for all patients is presented in Fig. 7A. Correlation analysis between risk model scores and clinical features showed that the risk score was associated with OS, pathological stage, T stage, N stage, and M stage (Fig. 7B-E). Subsequent univariate and multivariate analyses of clinical characteristics and the risk score identified both the risk score and distant metastasis status as independent prognostic factors (Fig. 7F,G). These two independent prognostic factors were incorporated into a nomogram model (Fig. 7H), and calibration curves were generated for 1-, 3-, and 5-year OS (Fig. 7I). These results indicate that the nomogram based on the risk score and distant metastasis status has strong predictive performance for estimating OS in patients with BLCA.

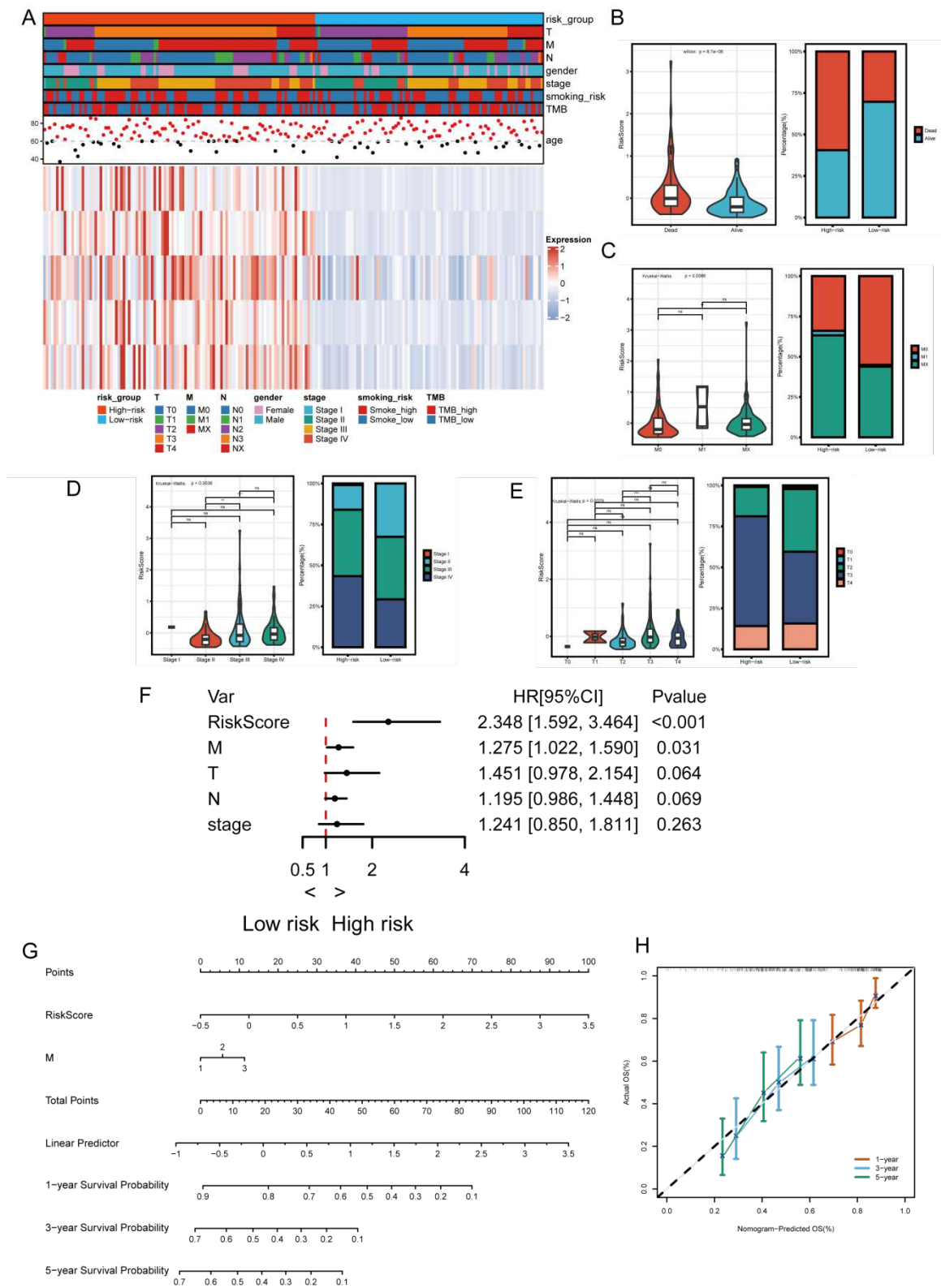


Fig. 7 Association analysis between the risk score model and clinical characteristics, with nomogram development. (A) Heatmap showing correlations between the risk score model and clinical features. (B-E) Clinical parameters significantly associated with the risk score model, including overall survival (OS), pathological stage, T stage, N stage, and M stage. (F) Univariate Cox regression analysis of the risk score model and clinical characteristics. (G) Multivariate Cox regression analysis of the risk score model and clinical characteristics. (H) Nomogram developed by incorporating independent risk factors. (I) Calibration curves evaluating nomogram accuracy for predicting 1-, 3-, and 5-year survival probabilities.

GSEA between high- and low-risk subgroups

GSEA showed that, compared with the low-risk subgroup, the high-risk subgroup was significantly enriched in multiple biological processes. These included KEGG pathways related to immune regulation, intercellular communication, and intracellular signal transduction, as well as GO biological processes associated with cell migration, adhesion, immune cell chemotaxis, and extracellular matrix organization (Fig. 8A,B). These findings indicate that the high-risk subgroup is closely linked to immune microenvironment regulation and cell-matrix interactions.

Further analysis of gene expression data from the high- and low-risk subgroups showed that pathways activated in the high-risk subgroup were mainly involved in tumor progression, immune responses, and metabolic reprogramming. These pathways included epithelial-mesenchymal transition (EMT), angiogenesis, hypoxia response, KRAS signaling, inflammatory response, and interferon response. Cell proliferation-related pathways, including E2F targets, MYC signaling, and the PI3K-AKT-mTOR

pathway, were also significantly enriched. By contrast, the low-risk subgroup showed significant downregulation of pathways related to UV response and KRAS signaling inhibition.

Overall, the high-risk subgroup displayed a distinct aggressive phenotype characterized by an immunosuppressive microenvironment, sustained proliferative capacity, and metabolic reprogramming (Fig. 8C,D).

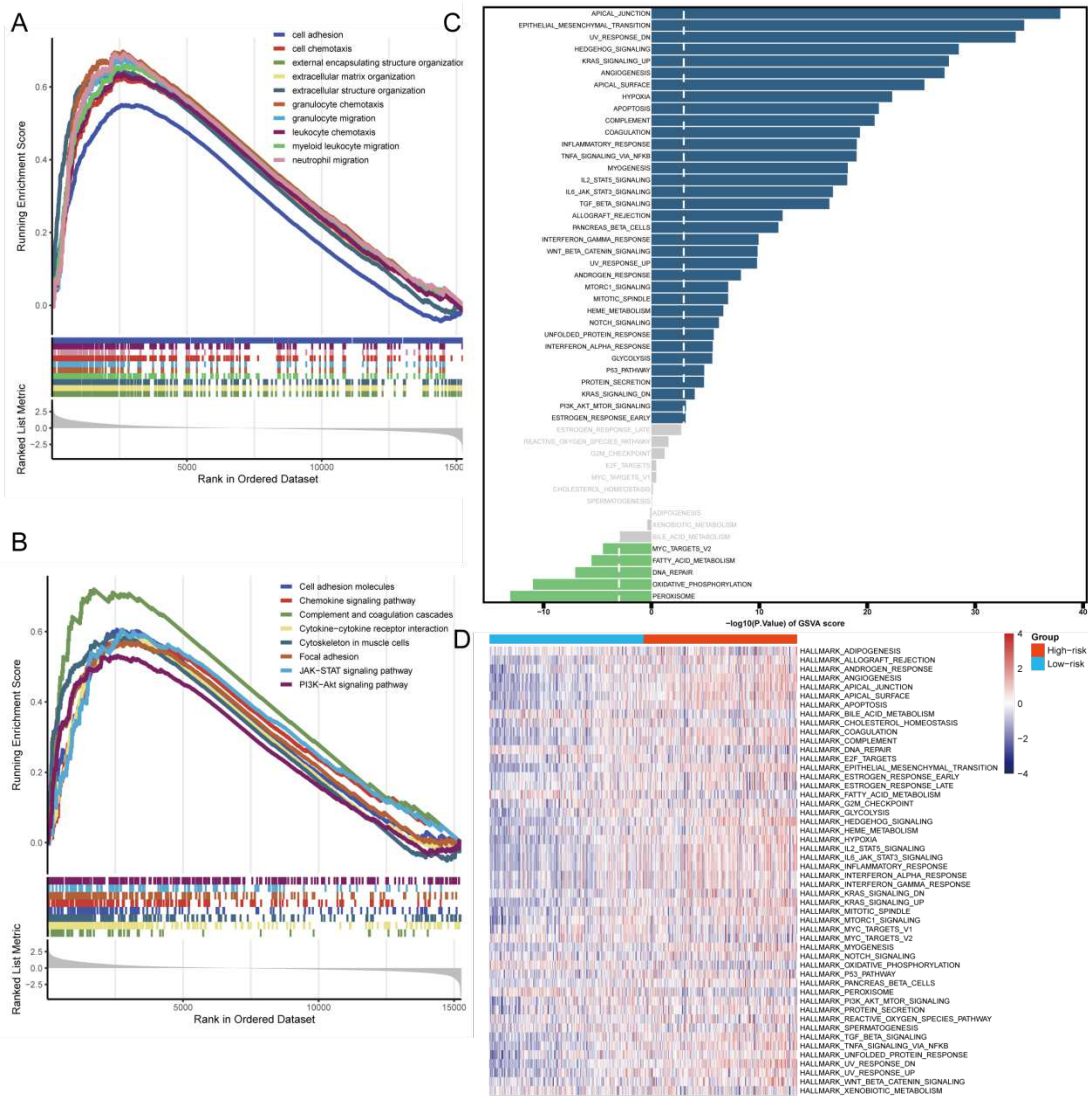


Fig. 8 Biological characteristics of high- and low-risk subgroups. (A,B) GSEA of GO terms and KEGG pathways between high- and low-risk subgroups. (C,D) GSVA of all genes in high- and

low-risk subgroups to identify enriched pathways.

Immune microenvironment analysis of high- and low-risk subgroups

ssGSEA was used to evaluate infiltration scores for 28 immune cell types in different risk groups.

Except for helper T cells, the infiltration levels of the remaining 27 immune cell types differed

significantly between the high- and low-risk groups (Fig. 9A). Pearson correlation analysis showed

that, except for activated CD8 T cells, both the prognostic genes and the risk score were significantly

correlated with infiltrating immune cells (Fig. 9C). Compared with the low-risk group, the high-risk

group generally showed higher abundances of infiltrating immune cells, including cells associated

with antitumor activity (e.g., activated CD8+ T cells, type 1 T helper cells, and activated dendritic

cells) and cells associated with protumor immunosuppression (e.g., MDSCs, regulatory T cells,

immature dendritic cells, and neutrophils) (Fig. 9D). Pearson correlation analysis further indicated that

the abundances of these two cell categories were positively correlated within the local

microenvironment (Fig. 9E). This finding suggests a potential feedback mechanism in which

antitumor inflammation may promote the recruitment or differentiation of specialized

immunosuppressive cells. Significant differences were also observed in the expression of the 18-gene

T-cell-inflamed GEP signature between the high- and low-risk groups (Fig. 9B). The PPI network of

differentially expressed GEP genes illustrates interactions among these genes (Fig. 9F). The

interaction network involving four significantly different infiltrating immune cell types and 11 GEP

genes is shown in Fig. 9G. Differential analysis of four immunotherapy targets - PD-1, PD-L1,

CTLA-4, and TIGIT - revealed significant expression differences between the high- and low-risk

(A) Differences in immune cell infiltration scores between high- and low-risk subgroups. (B) Differential analysis of the 18-gene T-cell-inflamed Gene Expression Profile (GEP) between high- and low-risk subgroups. (C) Correlation analysis between the five prognostic signature genes, risk score, and infiltrating immune cells. (D) Associations between risk subgroups and immune cell infiltration levels. (E) Positive correlations among infiltrating immune cells enriched in the high- and low-risk subgroups. (F) Protein-protein interaction (PPI) network of differentially expressed GEP genes. (G) Interaction network among four infiltrating immune cell types and 11 GEP genes. (H) Differential expression of four immune checkpoint targets (PD-1, PD-L1, CTLA-4, and TIGIT) between high- and low-risk subgroups.

Mutation profiles and candidate therapeutic agents in high- and low-risk subgroups

BLCA samples predominantly showed missense mutations and single-nucleotide polymorphisms (SNPs), with C>T transitions. Missense mutations were the most frequent mutation type in both high- and low-risk subgroups (Fig. S1A). Because high tumor mutational burden (TMB) is a strong predictor of response to immune checkpoint inhibitors, we compared TMB between risk groups and found that the high-risk subgroup had a higher mutation frequency and overall TMB than the low-risk subgroup. High-frequency mutation types included functionally impactful variants such as missense, nonsense, and frameshift mutations (Fig. S1B,C).

Screening of the GDSC database identified nine drugs whose half-maximal inhibitory concentration (IC50) values were significantly negatively correlated with the risk score ($R < -0.4$ and $P < 0.05$) (Fig.

10A). Sensitivity to these nine agents differed significantly between the high- and low-risk subgroups (Fig. 10B). Further screening of the CTRP database identified five compounds with the strongest negative correlations with the risk score: BRD-K34485477, NVP-BSK805, fluvastatin, tandutinib, and staurosporine (Fig. 10C). These five agents showed significantly lower AUC values in the high-risk subgroup than in the low-risk subgroup, suggesting potential therapeutic value in BLCA (Fig. 10D).

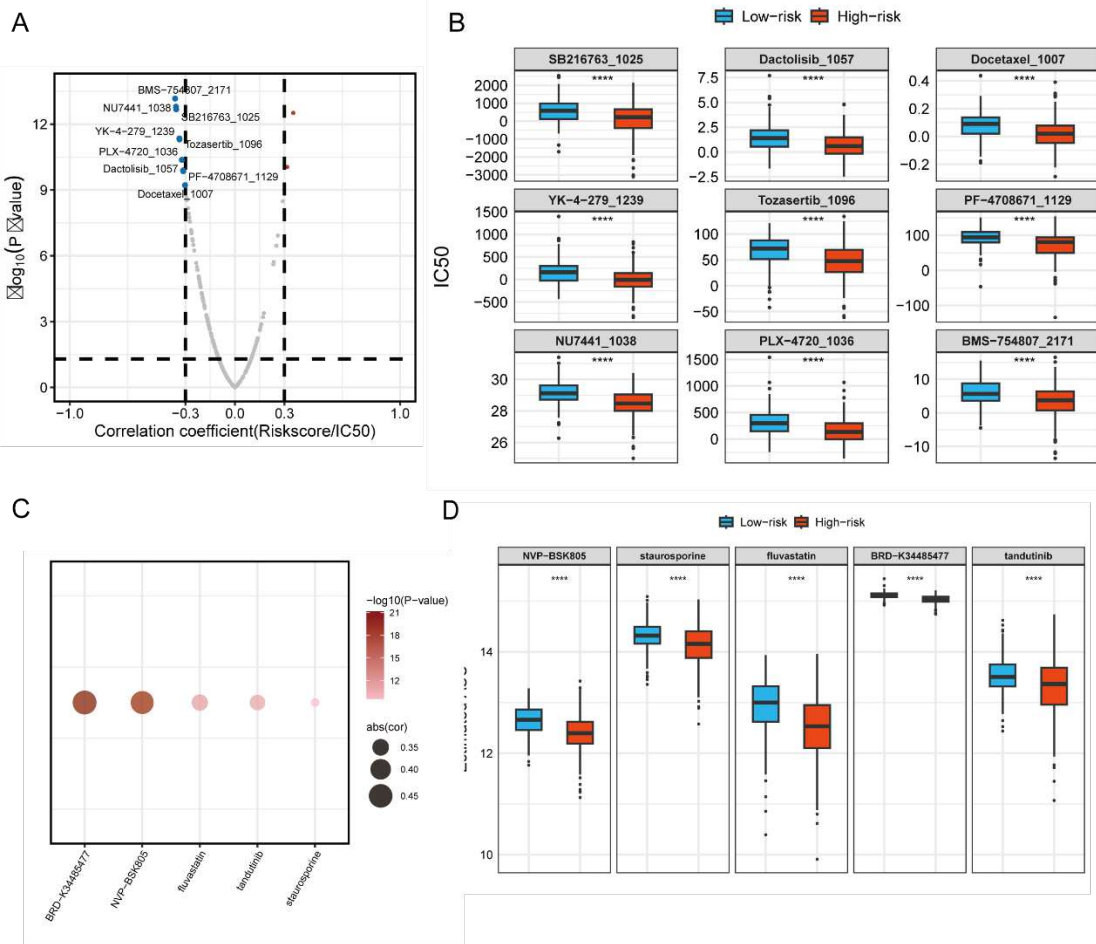
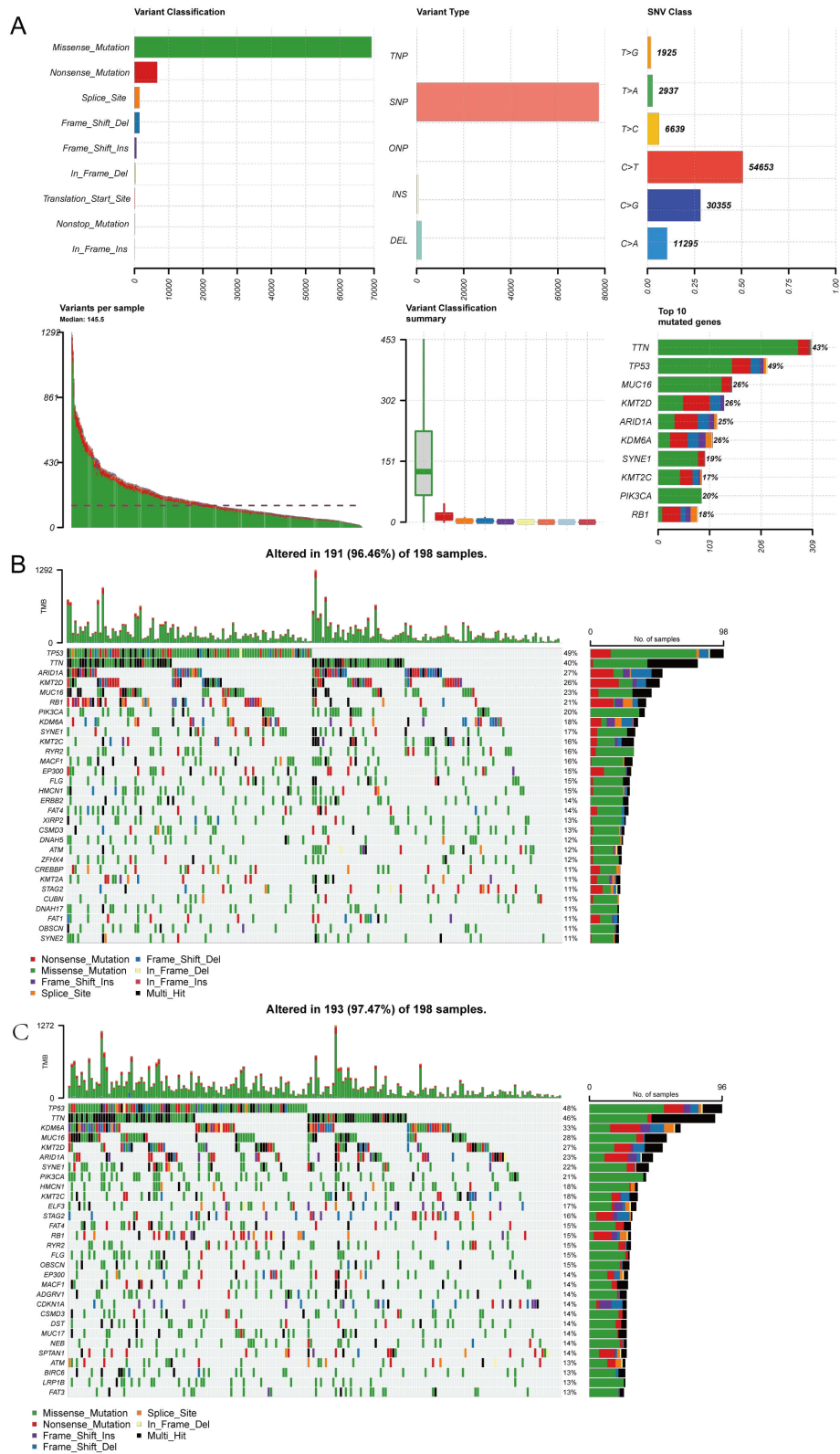


Fig. 10 Drug screening based on the risk score model. (A) Nine GDSC-derived drugs whose IC50 values correlated with the risk score model. (B) Differential drug sensitivity of the nine agents

421 between high- and low-risk subgroups. (C) Five CTRP-derived drugs showing strong negative
422 correlations with the risk score model. (D) Differences in AUC values of the five drugs between high-
423 and low-risk subgroups.



424

425 Fig. S1 (A) Mutation types in the high- and low-risk groups. (B,C) Main mutation types and mutation

sites in the high- and low-risk groups.

Discussion

BLCA is a common malignancy of the urinary system, and its incidence has increased in multiple countries [1,2]. Although substantial progress has been made in comprehensive BLCA treatment, prognostic assessment remains challenging because of the marked heterogeneity of bladder tumors [26]. Single-cell RNA sequencing (scRNA-seq) is a powerful transcriptomic tool that enables dissection of the tumor microenvironment (TME) at single-cell resolution and facilitates the discovery of more precise and dynamic biomarkers. It has been applied to identify specific biomarkers in various cancers, including BLCA [27,28]. In this study, we integrated scRNA-seq and bulk transcriptomic data from BLCA to construct a risk model with promising predictive performance for prognosis and immunotherapy response.

First, using the downloaded scRNA-seq datasets, we identified seven major cell types. Frequent cellular communication was observed primarily among T/NK cells, myeloid cells, and fibroblasts, suggesting that the BLCA TME is not dominated solely by cancer cells but instead comprises a complex regulatory network of immune and stromal cells. Previous studies have confirmed the high heterogeneity of BLCA and reported that T/NK cells, myeloid cells, and fibroblasts are closely associated with formation of an immunosuppressive microenvironment [29,30]. Although T/NK cells are key effectors of antitumor immunity, they often exhibit an exhausted state in BLCA. In parallel, high infiltration of immunosuppressive regulatory T cells (Tregs) in BLCA tissues further promotes

immunosuppression [31,32]. For example, Ting Liang et al. identified a subset of cancer-associated fibroblasts (CAFs) by scRNA-seq that significantly promoted formation of an immunosuppressive microenvironment through interactions with myeloid-derived suppressor cells and was closely associated with BLCA recurrence [33]. Thus, interactions among T/NK cells, myeloid cells, and fibroblasts constitute a complex regulatory network within the BLCA immune microenvironment.

We further identified marker genes from five cell types (endothelial cells, fibroblasts, T/NK cells, B cells, and mast cells) that differed significantly between normal and tumor tissues and defined them as the core gene set. KEGG and GO analyses indicated that these marker genes were enriched in immune- and inflammation-related pathways, including immune response, extracellular matrix (ECM) organization, cell migration, and inflammatory response. Previous studies have shown that activated CAFs in BLCA overproduce and remodel the ECM, forming dense fibrotic stroma that prevents cytotoxic T cells and other effector immune cells from infiltrating the tumor core. This leads to immune cell accumulation at the tumor margin and an immune-excluded phenotype, thereby contributing to immunosuppression [34-36]. Persistent inflammatory responses can also polarize macrophages toward the immunosuppressive M2 phenotype [37]. This immune response-associated marker gene set provided the basis for constructing a prognostic model related to the BLCA immune microenvironment.

We then used multiple analytical methods to identify DEGs from bulk RNA-seq data. WGCNA identified a module containing 1,049 BLCA-related genes. Intersecting these genes with the

previously identified marker genes and DEGs enabled construction of a five-gene prognostic signature using LASSO-Cox regression. This model showed good predictive performance in time-dependent ROC analysis, with AUC values all exceeding 0.61. All five genes included in the model are involved in intra- and extracellular signaling and functional regulation. MAP1B, a microtubule-associated protein, is upregulated in BLCA tissues and positively correlated with adverse prognostic factors such as pathological stage. MAP1B knockdown can reverse drug resistance by inducing cell-cycle arrest [38]. EMP1 is considered a tumor suppressor gene, and its loss promotes cell migration and enhances ferroptosis resistance by upregulating PPARG, thereby driving BLCA metastasis [39]. CRTAP, LRP1, and TCF4 collaboratively regulate the TGF-beta signaling pathway by mediating collagen barrier formation, TGF-beta activation, and myeloid cell M2 polarization, respectively, thereby contributing to an immunosuppressive tumor microenvironment [40-42]. To evaluate the clinical relevance of this scoring model, univariate and multivariate Cox regression analyses were performed, confirming that both the risk score and distant metastasis status were independent prognostic factors. A nomogram integrating these two factors showed excellent predictive accuracy and may provide a useful tool for individualized prognostic assessment and clinical decision-making in patients with BLCA.

To investigate the association between the risk score model and the BLCA immune microenvironment, patients were stratified into high- and low-risk groups according to their risk scores, followed by GSEA. The high-risk group was significantly enriched in TME-related biological processes, including immune regulation, inflammatory response, and EMT. Compared with the low-risk group, the high-risk group showed more pronounced immunosuppressive and metabolic reprogramming features.

Aberrantly activated immune regulation, cell-cell communication, and extracellular matrix interactions may collectively form the molecular basis of the aggressive phenotype in high-risk BLCA, promoting tumor invasion, metastasis, and therapy resistance. Further analysis of immune cell infiltration scores indicated a higher overall degree of immune cell infiltration in the high-risk group, reflecting the complexity of the BLCA immune microenvironment. Notably, immune infiltration in the high-risk group did not simply indicate immune activation or suppression; rather, it represented a state of immune dysregulation characterized by both antitumor effector cells (such as activated CD8⁺ T cells and Th1 cells) and abundant protumor immunosuppressive cells (such as Tregs and MDSCs). This immunosuppressive microenvironment may be an important mechanism underlying poor immunotherapy response in high-risk patients [43,44].

Finally, based on the risk score model, we screened the GDSC database and identified nine candidate drugs or compounds with potential therapeutic effects. These agents primarily target key biological processes, including cell-cycle progression, Wnt/beta-catenin signaling, mTOR signaling, and the PI3K pathway. These results are consistent with the pathway enrichment results from the DEG analysis and further suggest that these compounds may have potential translational value for BLCA treatment.

Conclusions

This study developed a novel prognostic prediction model for BLCA by integrating single-cell transcriptomic data with TCGA-BLCA bulk transcriptomic data using machine-learning algorithms.

The model was significantly associated with clinical characteristics and the tumor immune microenvironment in patients with BLCA. It may serve as a reliable indicator for assessing prognosis and has potential value for predicting therapeutic efficacy. These findings provide new directions for immunotherapy strategies in BLCA and warrant further clinical validation.

Declarations

Ethics approval and consent to participate

This study was based on publicly available datasets from TCGA and GEO; therefore, additional ethical approval and informed consent were not required.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. Public datasets were obtained from the GEO and TCGA databases.

Authors' contributions

YBZ, ZWW, ZHC, YXZ, and YHZ contributed equally to this work. KG, CT, and XY conceived and supervised the study. YBZ, ZWW, ZHC, YXZ, and YHZ performed data collection, bioinformatic analyses, model construction, and figure preparation. XWL, JGZ, XHD, and XWW assisted with data interpretation, literature review, and manuscript organization. YBZ and ZWW drafted the manuscript. ZHC, YXZ, YHZ, KG, CT, and XY critically revised the manuscript for important intellectual content. KG, CT, and XY provided administrative and technical support. All authors read and approved the

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532

533 Competing interests

534 The authors declare that they have no competing interests.

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546 References

547 [1] van Hoogstraten LMC, Vrieling A, van der Heijden AG, Kogevinas M, Richters A, Kiemeny LA.
548 Global trends in the epidemiology of bladder cancer: challenges for public health and clinical practice.
549 Nat Rev Clin Oncol. 2023 May;20(5):287-304.

550 [2] Bray F, Laversanne M, Sung H, Ferlay J, Siegel RL, Soerjomataram I, Jemal A. Global cancer
551 statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185

552 countries. *CA Cancer J Clin.* 2024 May-Jun;74(3):229-263.

553 [3] Roviello G, Santoni M, Sonpavde GP, Catalano M. The evolving treatment landscape of metastatic
554 urothelial cancer. *Nat Rev Urol.* 2024 Oct;21(10):580-592.

555 [4] Knowles M, Dyrskjöt L, Heath EI, Bellmunt J, Siefker-Radtke AO. Metastatic urothelial
556 carcinoma. *Cancer Cell.* 2021 May 10;39(5):583-585.

557 [5] Masson-Lecomte A, Birtle A, Pradere B, Capoun O, Compérat E, Domínguez-Escrig JL, Liedberg
558 F, Makaroff L, Mariappan P, Moschini M, Rai BP, van Rhijn BWG, Shariat SF, Smith EJ, Teoh JYC,
559 Soukup V, Wood R, Xylinas EN, Soria F, Seisen T, Gontero P. European Association of Urology
560 Guidelines on Upper Urinary Tract Urothelial Carcinoma: Summary of the 2025 Update. *Eur Urol.*
561 2025 Jun;87(6):697-716.

562 [6] Babjuk M, Burger M, Capoun O, Cohen D, Compérat EM, Dominguez Escrig JL, Gontero P,
563 Liedberg F, Masson-Lecomte A, Mostafid AH, Palou J, van Rhijn BWG, Rouprêt M, Shariat SF,
564 Seisen T, Soukup V, Sylvester RJ. European Association of Urology Guidelines on
565 Non-muscle-invasive Bladder Cancer (Ta, T1, and Carcinoma in Situ). *Eur Urol.* 2022
566 Jan;81(1):75-94.

567 [7] Powles T, Valderrama BP, Gupta S, Bedke J, Kikuchi E, Hoffman-Censits J, Iyer G, Vulsteke C,
568 Park SH, Shin SJ, Castellano D, Fornarini G, Li JR, Gümüş M, Mar N, Loriot Y, Fléchon A, Duran I,
569 Drakaki A, Narayanan S, Yu X, Gorla S, Homet Moreno B, van der Heijden MS; EV-302 Trial
570 Investigators. Enfortumab Vedotin and Pembrolizumab in Untreated Advanced Urothelial Cancer. *N*
571 *Engl J Med.* 2024 Mar 7;390(10):875-888.

572 [8] Gupta S, Loriot Y, Van der Heijden MS, Bedke J, Valderrama BP, Kikuchi E, Fléchon A, Petrylak

573 D, De Santis M, Galsky MD, Lee JL, Swami U, Sridhar SS, De Giorgi U, Wright P, Shih V, Lu YT,
 574 Guan X, Dillon R, Shetty A, Moreno BH, Beaumont JL, Purnajo I, McManus S, Powles T.
 575 Enfortumab vedotin plus pembrolizumab versus chemotherapy in patients with previously untreated
 576 locally advanced or metastatic urothelial cancer (EV-302): patient-reported outcomes from an
 577 open-label, randomised, controlled, phase 3 study. *Lancet Oncol.* 2025 Jun;26(6):795-805.
 578 [9] Yu EY, Petrylak DP, O'Donnell PH, Lee JL, van der Heijden MS, Loriot Y, Stein MN, Necchi A,
 579 Kojima T, Harrison MR, Hoon Park S, Quinn DI, Heath EI, Rosenberg JE, Steinberg J, Liang SY,
 580 Trowbridge J, Campbell M, McGregor B, Balar AV. Enfortumab vedotin after PD-1 or PD-L1
 581 inhibitors in cisplatin-ineligible patients with advanced urothelial carcinoma (EV-201): a multicentre,
 582 single-arm, phase 2 trial. *Lancet Oncol.* 2021 Jun;22(6):872-882.
 583 [10] Powles T, Rosenberg JE, Sonpavde GP, Loriot Y, Durán I, Lee JL, Matsubara N, Vulsteke C,
 584 Castellano D, Wu C, Campbell M, Matsangou M, Petrylak DP. Enfortumab Vedotin in Previously
 585 Treated Advanced Urothelial Carcinoma. *N Engl J Med.* 2021 Mar 25;384(12):1125-1135.
 586 [11] Kamat AM, Apolo AB, Babjuk M, Bivalacqua TJ, Black PC, Buckley R, Campbell MT,
 587 Compérat E, Efstathiou JA, Grivas P, Gupta S, Kurtz NJ, Lamm D, Lerner SP, Li R, McConkey DJ,
 588 Palou Redorta J, Powles T, Psutka SP, Shore N, Steinberg GD, Sylvester R, Witjes JA, Galsky MD.
 589 Definitions, End Points, and Clinical Trial Designs for Bladder Cancer: Recommendations From the
 590 Society for Immunotherapy of Cancer and the International Bladder Cancer Group. *J Clin Oncol.* 2023
 591 Dec 10;41(35):5437-5447.
 592 [12] Yang C, Wu S, Mou Z, Zhou Q, Dai X, Ou Y, Chen X, Chen Y, Xu C, Hu Y, Zhang L, Zou L, Jin
 593 S, Hu J, Mao S, Jiang H. Exosome-derived circTRPS1 promotes malignant phenotype and CD8+ T

cell exhaustion in bladder cancer microenvironments. *Mol Ther.* 2022 Mar 2;30(3):1054-1070.

[13] Wei W, Liu K, Huang X, Tian S, Wang H, Zhang C, Ye J, Dong Y, An Z, Ma X, Wang B, Huang Y, Zhang X. EIF4A3-mediated biogenesis of circSTX6 promotes bladder cancer metastasis and cisplatin resistance. *J Exp Clin Cancer Res.* 2024 Jan 2;43(1):2.

[14] Liu L, Hou Y, Deng C, Tao Z, Chen Z, Hu J, Chen K. Single cell sequencing reveals that CD39 inhibition mediates changes to the tumor microenvironment. *Nat Commun.* 2022 Nov 8;13(1):6740.

[15] Yang H, Song H, Yip E, Gilpatrick T, Chang K, Allegakoen P, Lu KL, Hui K, Pham JH, Kasap C, Kumar V, Gayle J, Stohr BA, Cornelia Ding CK, Wiita AP, Meng MV, Chou J, Porten SP, Huang FW. Bladder cancer variants share aggressive features including a CA125+ cell state and targetable TM4SF1 expression. *Nat Commun.* 2025 Jun 17;16(1):5312.

[16] Xiao Y, Jin W, Qian K, Ju L, Wang G, Wu K, Cao R, Chang L, Xu Z, Luo J, Shan L, Yu F, Chen X, Liu D, Cao H, Wang Y, Cao X, Zhou W, Cui D, Tian Y, Ji C, Luo Y, Hong X, Chen F, Peng M, Zhang Y, Wang X. Integrative Single Cell Atlas Revealed Intratumoral Heterogeneity Generation from an Adaptive Epigenetic Cell State in Human Bladder Urothelial Carcinoma. *Adv Sci (Weinh).* 2024 Jun;11(24):e2308438.

[17] Li F, Zhang H, Huang Y, Li D, Zheng Z, Xie K, Cao C, Wang Q, Zhao X, Huang Z, Chen S, Chen H, Fan Q, Deng F, Hou L, Deng X, Tan W. Single-cell transcriptome analysis reveals the association between histone lactylation and cisplatin resistance in bladder cancer. *Drug Resist Updat.* 2024 Mar;73:101059.

[18] Becht E, McInnes L, Healy J, Dutertre CA, Kwok IWH, Ng LG, Ginhoux F, Newell EW. Dimensionality reduction for visualizing single-cell data using UMAP. *Nat Biotechnol.* 2018 Dec 3.

615 [19] Yu Z, Qiu B, Zhou H, Li L, Niu T. Characterization and application of a lactate and branched
616 chain amino acid metabolism related gene signature in a prognosis risk model for multiple myeloma.
617 Cancer Cell Int. 2023 Aug 14;23(1):169.

618 [20] Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq
619 data with DESeq2. Genome Biol. 2014;15(12):550.

620 [21] Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK. limma powers differential
621 expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res. 2015 Apr
622 20;43(7):e47.

623 [22] Wu T, Hu E, Xu S, Chen M, Guo P, Dai Z, Feng T, Zhou L, Tang W, Zhan L, Fu X, Liu S, Bo X,
624 Yu G. clusterProfiler 4.0: A universal enrichment tool for interpreting omics data. Innovation (Camb).
625 2021 Jul 1;2(3):100141.

626 [23] Langfelder P, Horvath S. WGCNA: an R package for weighted correlation network analysis.
627 BMC Bioinformatics. 2008 Dec 29;9:559.

628 [24] Wu J, Lin K, Lu W, Zou W, Li X, Tan Y, Yang J, Zheng D, Liu X, Lam BY, Xu G, Wang K,
629 McIntyre RS, Wang F, So KF, Wang J. Enhancing Early Diagnosis of Bipolar Disorder in Adolescents
630 Through Multimodal Neuroimaging. Biol Psychiatry. 2025 Feb 1;97(3):313-322.

631 [25] Carreras-Dieguez N, Glickman A, Munmany M, Casanovas G, Agustí N, Díaz-Feijoo B, Saco A,
632 Sánchez B, Gaba L, Angeles MA, Pahisa J, Fernández-Galán E, Torné A, Fusté P. Comparison of HE4,
633 CA125, ROMA and CPH-I for Preoperative Assessment of Adnexal Tumors. Diagnostics (Basel).
634 2022 Jan 17;12(1):226.

635 [26] Hamidi H, Senbabaoglu Y, Beig N, Roels J, Manuel C, Guan X, Koeppen H, Assaf ZJ, Nabet BY,

636 Waddell A, Yuen K, Maund S, Sokol E, Giltane JM, Schedlbauer A, Fuentes E, Cowan JD, Kadel EE
 637 3rd, Degaonkar V, Andreev-Drakhlin A, Williams P, Carter C, Gupta S, Steinberg E, Lorient Y,
 638 Bellmunt J, Grivas P, Rosenberg J, van der Heijden MS, Galsky MD, Powles T, Mariathasan S,
 639 Banchereau R. Molecular heterogeneity in urothelial carcinoma and determinants of clinical benefit to
 640 PD-L1 blockade. *Cancer Cell*. 2024 Dec 9;42(12):2098-2112.e4.
 641 [27] Yu A, Hu J, Fu L, Huang G, Deng D, Zhang M, Wang Y, Shu G, Jing L, Li H, Chen X, Yang T,
 642 Wei J, Chen Z, Zu X, Luo J. Bladder cancer intrinsic LRFN2 drives anticancer immunotherapy
 643 resistance by attenuating CD8+ T cell infiltration and functional transition. *J Immunother Cancer*.
 644 2023 Oct;11(10):e007230.
 645 [28] Vandereyken K, Sifrim A, Thienpont B, Voet T. Methods and applications for single-cell and
 646 spatial multi-omics. *Nat Rev Genet*. 2023 Aug;24(8):494-515.
 647 [29] Tran L, Xiao JF, Agarwal N, Duex JE, Theodorescu D. Advances in bladder cancer biology and
 648 therapy. *Nat Rev Cancer*. 2021 Feb;21(2):104-121.
 649 [30] Lopez-Beltran A, Cookson MS, Guercio BJ, Cheng L. Advances in diagnosis and treatment of
 650 bladder cancer. *BMJ*. 2024 Feb 12;384:e076743.
 651 [31] Strandgaard T, Nordentoft I, Birkenkamp-Demtröder K, Salminen L, Prip F, Rasmussen J,
 652 Andreassen TG, Lindskrog SV, Christensen E, Lamy P, Knudsen M, Steiniche T, Jensen JB, Dyrskjöt L.
 653 Field Cancerization Is Associated with Tumor Development, T-cell Exhaustion, and Clinical
 654 Outcomes in Bladder Cancer. *Eur Urol*. 2024 Jan;85(1):82-92.
 655 [32] Liu L, Xu L, Wu D, Zhu Y, Li X, Xu C, Chen K, Lin Y, Lao J, Cai P, Li X, Luo Y, Li X, Huang J,
 656 Lin T, Zhong W. Impact of tumour stroma-immune interactions on survival prognosis and response to

neoadjuvant chemotherapy in bladder cancer. *EBioMedicine*. 2024 Jun;104:105152.

[33] Liang T, Tao T, Wu K, Liu L, Xu W, Zhou D, Fang H, Ding Q, Huang G, Wu S. Cancer-Associated Fibroblast-Induced Remodeling of Tumor Microenvironment in Recurrent Bladder Cancer. *Adv Sci (Weinh)*. 2023 Nov;10(31):e2303230.

[34] Caramelo B, Zagorac S, Corral S, Marqués M, Real FX. Cancer-associated Fibroblasts in Bladder Cancer: Origin, Biology, and Therapeutic Opportunities. *Eur Urol Oncol*. 2023 Aug;6(4):366-375.

[35] Li Y, Zheng H, Luo Y, Lin Y, An M, Kong Y, Zhao Y, Yin Y, Ai L, Huang J, Chen C. An HGF-dependent positive feedback loop between bladder cancer cells and fibroblasts mediates lymphangiogenesis and lymphatic metastasis. *Cancer Commun (Lond)*. 2023 Dec;43(12):1289-1311.

[36] Zhuang J, Shen L, Li M, Sun J, Hao J, Li J, Zhu Z, Ge S, Zhang D, Guo H, Huang R, Yan J. Cancer-Associated Fibroblast-Derived miR-146a-5p Generates a Niche That Promotes Bladder Cancer Stemness and Chemoresistance. *Cancer Res*. 2023 May 15;83(10):1611-1627.

[37] Yu Y, Liang Y, Xie F, Zhang Z, Zhang P, Zhao X, Zhang Z, Liang Z, Li D, Wang L, Chen Y, Sun L, Niu H, Wang Y. Tumor-associated macrophage enhances PD-L1-mediated immune escape of bladder cancer through PKM2 dimer-STAT3 complex nuclear translocation. *Cancer Lett*. 2024 Jul 1;593:216964.

[38] Chien TM, Chan TC, Huang SK, Yeh BW, Li WM, Huang CN, Li CC, Wu WJ, Li CF. Role of Microtubule-Associated Protein 1b in Urothelial Carcinoma: Overexpression Predicts Poor Prognosis. *Cancers (Basel)*. 2020 Mar 9;12(3):630.

[39] Liu S, Shi J, Wang L, Huang Y, Zhao B, Ding H, Liu Y, Wang W, Chen Z, Yang J. Loss of EMP1 promotes the metastasis of human bladder cancer cells by promoting migration and conferring

678 resistance to ferroptosis through activation of PPAR gamma signaling. *Free Radic Biol Med.* 2022
679 Aug 20;189:42-57.

680 [40] Li W, Peng J, Yao D, Rao B, Xia Y, Wang Q, Li S, Cao M, Shen Y, Ma P, Liao R, Qin A, Zhao J,
681 Cao Y. The structural basis for the collagen processing by human P3H1/CRTAP/PPIB ternary complex.
682 *Nat Commun.* 2024 Sep 8;15(1):7844.

683 [41] Lin H, Fu L, Zhou X, Yu A, Chen Y, Liao W, Shu G, Zhang L, Tan L, Liang H, Wang Z, Deng Q,
684 Wang J, Jin M, Chen Z, Wei J, Cao J, Chen W, Li X, Li P, Lu J, Luo J. LRP1 induces anti-PD-1
685 resistance by modulating the DLL4-NOTCH2-CCL2 axis and redirecting M2-like macrophage
686 polarisation in bladder cancer. *Cancer Lett.* 2024 Jul 1;593:216807.

687 [42] Tu W, Gong J, Zhou Z, Tian D, Wang Z. TCF4 enhances hepatic metastasis of colorectal cancer
688 by regulating tumor-associated macrophage via CCL2/CCR2 signaling. *Cell Death Dis.* 2021 Sep
689 27;12(10):882.

690 [43] Mao X, Xu J, Wang W, et al. Crosstalk between cancer-associated fibroblasts and immune cells
691 in the tumor microenvironment: new findings and future perspectives. *Mol Cancer.* 2021;20(1):131.

692 [44] Zhang F, Ma Y, Li D, et al. Cancer associated fibroblasts and metabolic reprogramming:
693 unraveling the intricate crosstalk in tumor evolution. *J Hematol Oncol.* 2024;17(1):80.

694 [45] TITAN Working Group. TITAN Guidelines 2025: governing declaration and use of AI. *Prem J*
695 *Surg.* 2025;3(1):1-8.

696