

A novel tumor recurrence–associated gene signature predicts prognosis in bladder cancer and identifies EIF1 as a therapeutic target

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Abstract

Background

Bladder cancer (BLCA) is a malignant tumor characterized by a high progression rate and a high incidence of muscle-invasive disease, with over 50% of patients experiencing recurrence or progression following treatment. Given that recurrence poses a major threat to the prognosis of BLCA, improving the precision of clinical management and identifying effective therapeutic targets is crucial. To address this, we construct a molecular framework based on tumor recurrence-related genes (TRGs) to achieve precise molecular subtyping and prognostic prediction for BLCA patients, while providing potential targets for clinical therapy.

Methods

Transcriptomic and clinical data were obtained from the TCGA and GEO databases. Differential expression analysis and weighted gene co-expression network analysis (WGCNA) were used to identify specific tumor recurrence-related genes (TRGs) between primary and recurrent BLCA samples. Potential BLCA molecular subtypes were determined through clustering analysis based on these genes, and the associations between different subtypes, patient prognosis, and immune cell infiltration were evaluated. Subsequently, multiple combined machine learning algorithms and multivariate Cox regression analysis were applied to construct a prognostic model (tumor recurrence index, TRI), followed by an assessment of the relationships between TRI and clinicopathological features, immune microenvironment, and immunotherapy response. Finally, the function of the key gene EIF1 in the model was validated through both in vivo and in vitro experiments.

Results

We initially identified 280 key genes associated with bladder cancer recurrence. Clustering analysis divided BLCA into two TRG-based molecular subtypes, among which the R2 subtype exhibited poorer overall prognosis, a more immunosuppressive tumor microenvironment, and enhanced oncogenic pathway activation. The prognostic model TRI was confirmed as an independent prognostic indicator for BLCA, demonstrating superior predictive accuracy compared with other clinical features and previously published prognostic models, and showing stable performance across multiple independent cohorts. Patients with high TRI scores displayed worse overall survival, a more immunosuppressive microenvironment, and unfavorable responses to immunotherapy. Functional experiments further validated that EIF1 was upregulated in recurrent BLCA tissues and cancer cell lines. Moreover, silencing EIF1 markedly inhibited BLCA cell proliferation and migration, as well as tumor growth in vivo.

Conclusion

This study developed and validated a novel prognostic model that integrates tumor recurrence–related processes and offers significant prognostic value in BLCA management. Moreover, the identification of EIF1 as a novel biomarker associated with bladder cancer recurrence offers a potential therapeutic target for managing recurrence.

Introduction

BLCA is the second most prevalent malignancy of the urinary system globally, with an annual incidence exceeding 500,000 new cases and causing over 200,000 deaths annually [1, 2]. According to statistics from the American Cancer Society, in 2024 the United States is expected to have 83,190 new cases and 16,840 related deaths [3]. The vast majority of cases originate from the urothelium and progress to urothelial carcinoma, and are classified according to the depth of tumor invasion into non–muscle-invasive bladder cancer (NMIBC, accounting for approximately 75%) and muscle-invasive bladder cancer (MIBC) [4]. Although the two types show significant differences in molecular characteristics and clinical behavior, about half of MIBC is considered to originate from recurrent NMIBC [5]. Currently, the standard treatment for NMIBC includes transurethral resection of bladder tumor (TURBT) combined with intravesical instillation therapy[6], while MIBC is mainly treated with radical cystectomy combined with pelvic lymph node dissection and cisplatin-based chemotherapy regimens [7].

Postoperative recurrence is a major challenge affecting the prognosis of BLCA patients [8]. Even after standardized treatment, BLCA patients still exhibit a high risk of recurrence and progression. The 5-year recurrence and progression rates of NMIBC can reach 78% and 45%, respectively [9], while the risk of recurrence or progression in MIBC within the same time window approaches 50%. Distant metastasis may still occur despite salvage cystectomy [10, 11]. Therefore, elucidating the molecular mechanisms underlying BLCA recurrence is of great importance. Current studies suggest that clinical factors contributing to BLCA recurrence include undetected lesions during cystoscopy, residual disease after transurethral resection, tumor re-implantation, and the spread of urothelial carcinoma cells from the upper urinary tract [12, 13]. However, at the microscopic level, the molecular mechanisms driving BLCA recurrence remain unclear. Several studies have indicated that certain genes are associated with postoperative recurrence of bladder cancer, including TERT, FGFR3, and the long non-coding RNA PCA-1 [14–16]. Therefore, identifying novel biomarkers is crucial for the early detection of bladder cancer recurrence and for more precise clinical management.

This study utilized multiple bioinformatics datasets from public databases to analyze transcriptomic alterations during bladder cancer recurrence, identify core tumor recurrence-related genes (TRGs), and construct molecular subtypes and a prognostic prediction model. Using various immune infiltration algorithms, we further evaluated the associations between TRG-related subtypes and the tumor immune microenvironment. By integrating multiple independent cohorts and applying machine learning approaches, we developed a new TRG-based prognostic signature and systematically assessed its value in patient risk stratification and immunotherapy response. In addition, we investigated the biological function of the core gene EIF1 in BLCA. Overall, this study offers important insights for recurrence risk

assessment, therapeutic optimization, and novel immunotherapeutic targets development. The workflow of this study is illustrated in Figure S1.

Materials and methods

Data collection

All data used in this study were obtained from publicly accessible databases. The TCGA-BLCA cohort was retrieved from The Cancer Genome Atlas (TCGA, <https://portal.gdc.cancer.gov/>). The single-cell sequencing dataset GSE277524 was obtained from the Gene Expression Omnibus (GEO, <https://www.ncbi.nlm.nih.gov/geo/>). To validate the predictive value of the model, three independent BLCA cohorts (GSE13507, GSE32548, and GSE32894) were included. In addition, multiple immunotherapy-related cohorts, including GSE78220, GSE126044, and IMvigor210, were incorporated to evaluate the model's predictive capacity for immunotherapy response.

Identification of tumor recurrence-related genes in BLCA

Using the transcriptome sequencing dataset GSE128959, which contains primary and recurrent bladder cancer samples, we constructed a gene co-expression network with the “WGCNA” package (version 1.73) to identify gene modules associated with bladder cancer recurrence. Subsequently, differentially expressed genes involved in the recurrence process were screened using the “limma” package (version 3.60.6) based on the criteria of $|\log_2FC| > 0.5$ and $\text{adj.P.Val} < 0.05$, and volcano plots were generated using ggplot2 package for visualization. Finally, we performed an intersection analysis between the WGCNA-identified module genes and the differentially expressed genes, yielding a set of tumor recurrence-related genes (TRGs) for subsequent analyses.

Construction and validation of a TRG-based prognostic model

We first performed univariate Cox regression analysis on TRGs across all cohorts to identify prognosis-related genes ($p < 0.05$). To develop a novel TRG-based prognostic model, we integrated 10 machine learning algorithms (CoxBoost, Lasso, stepwise Cox, plsRcox, Ridge, Enet, SurvivalSVM, GBMs, SuperPC, and random forest) and generated 101 algorithmic combinations to screen for the optimal prognostic model[17]. The TCGA-BLCA dataset was used as the training cohort, while three independent cohorts (GSE13507, GSE32548, and GSE32894) served as validation cohorts. Subsequently, multivariate Cox regression analysis was applied to calculate the coefficients of the model genes. The TRI score for each patient was computed using the following formula: $\text{TRI} = \text{EIF1} \times 0.5475691 + \text{ANAPC5} \times 0.6886301 + \text{EGR1} \times 0.2506998 + \text{PFDN5} \times -0.6367359 + \text{PPP2R2B} \times -0.2465871 + \text{DEXI} \times -0.45121$. Patients were then stratified into high- and low-TRI groups based on the median TRI score. Kaplan–Meier (KM) analysis, ROC curves, DCA curves, and calibration curves were generated using the “Survival” package (version 3.8-3), “survminer” package (version 0.5), “timeROC” package (version 0.4), and “ggDCA”

package (version 1.2) to evaluate the predictive accuracy and consistency of the model across different cohorts.

Pathways and functional enrichment analysis

To analyze the potential molecular mechanisms associated with the TRI, functional enrichment analyses were performed between the high- and low- TRI groups, including Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis via the “clusterProfiler” package (version 4.12.6) and “org.Hs.eg.db” package (version 3.19.1), respectively.

Immune microenvironment and immunotherapy response analysis

We employed multiple approaches to comprehensively evaluate the characteristics of the tumor microenvironment (TME). The “estimate” package (version 1.0.13) was used to calculate the stromal score, immune score, and ESTIMATE score for each BLCA sample. Subsequently, several algorithms, including TIMER, CIBERSORT, CIBERSORT-ABS, QUANTISEQ, MCPOUNTER, XCELL, and EPIC, were applied to quantify the abundance of immune cell populations within the samples. In addition, single-sample gene set enrichment analysis (ssGSEA) was performed to assess differences in immune cell infiltration and immune-related functions between TRI-defined groups. Furthermore, three independent immune checkpoint inhibitor treatment cohorts (IMvigort210, GSE78220, and GSE126044) were utilized to evaluate the potential predictive value of TRI in immunotherapy response.

Single-cell analysis

Single-cell transcriptomic data of recurrent bladder cancer were downloaded from the GEO database (GSE277524), which contains nine samples. The “Seurat” package (version 5.4) was used for quality control and cell clustering analysis. Using UMAP, we identified seven major cell types, including epithelial cells, endothelial cells, T cells, B cells, fibroblasts, myeloid cells, and mast cells. We then applied the “Copykat” package (version 1.1.0) to identify malignant cells within the epithelial cell population and used the “AUCell” package (version 1.32.0) to calculate the TRI score for each malignant cell. To further explore the cellular characteristics associated with different TRI scores, the “CellChat” package (version 2.2.0) and the “monocle” package (version 2.36.9) were employed to evaluate intercellular communication and perform pseudotime analysis, respectively.

Clinical samples

Primary and recurrent bladder cancer tissues were obtained from patients pathologically diagnosed with bladder cancer at Shenzhen Second People’s Hospital. The collection and use of human tissue samples were approved by the Medical Ethics Committee of Shenzhen Second People’s Hospital. All procedures were conducted with full informed consent from the patients (Approval No. KS201900604004).

Cell culture

The following bladder cell lines, including SV-HUC-1, UMUC-3, SW780, T24 and TCC-SUP were obtained from the American Type Culture Collection (Virginia, USA). Human bladder epithelial cells SV-HUC-1 were cultured in Ham's F-12K medium (HyClone, USA). BLCA cell lines T24 and SW780 cells were cultured in RPMI-1640 (Gibco, USA) medium; UMUC-3 and TCC-SUP cells were cultured in Eagle's Minimum Essential Medium (Gibco, USA). All these mediums were supplemented with 10% fetal bovine serum (Excell, China) and 1% penicillin-streptomycin (Solarbio, China). Cells were maintained in a cell incubator (Thermofisher, USA) at 37°C with 5% CO₂. The siRNAs targeting EIF1 was purchased from IGE Biotech (Guangzhou, China). The transfection reagent Lipofectamine RNAi MAX (Thermofisher, USA) was used according to the manufacturer's protocol. The sequences of siRNA targeting EIF1 were as follows: siEIF1-1: GACCCUUACUACUGUCCAA, siEIF1-2: GCCCUAAGAUUACAAACAA.

CCK-8 assay

The cell proliferation assay was performed as described in our previous paper[18]. Briefly, 3×10^3 treated BLCA cells were seeded into 96-well plates. After incubated with 10 μ L of CCK-8 reagent (Yesen, Shanghai, China) for 2 hours, absorbance was measured by a microplate reader at wavelength of 450 nm. For the colony formation assay, 1×10^3 cells were seeded into 6-well plates and cultured continuously for 7–10 days and the colonies were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet and counted.

Wound healing assay

For wound-healing assay, cells were seeded into 12-well plate. The wound was created by scratching the cell monolayer using a sterile 200 μ L pipette tip. Subsequently, cells were cultured with FBS free medium. Scratch images were captured at 0, 24, and 48 hours under the optical microscope (Discover Echo, USA). The cell migration ability was assessed by measuring the change in the wound area over time.

Migration and invasion assay

Migration and invasion assay were conducted using trans-well membrane (Corning 3422, 8 μ m) with or without Matrigel. Briefly, 3×10^4 cells were incubated into the upper chamber containing 200 μ L FBS free medium. Meanwhile, medium containing 10% FBS was added into the lower chamber. After 24h incubation, the upper chambers were washed with PBS and fixed by 4% paraformaldehyde for 30 min. The non-migrated cells were gently scraped off, and the membranes were stained with crystal violet for 30 min at room temperature. After washing and air-drying the chambers, images were captured for further analysis.

Quantitative real-time PCR (qRT-PCR) analysis

Total RNA from BLCA cell lines was extracted using the RNA-easy Isolation Kit (Vazyme, Nanjing, China), followed by reverse transcription to synthesize cDNA using the HiScript II 1st Strand cDNA Synthesis Kit

(Vazyme, Nanjing, China). qRT-PCR was performed using NovoStart® SYBR qPCR SuperMix Plus (Novoprotein, Shanghai, China), with EIF1 transcript levels normalized to the reference gene β -actin. The primer sequences used were as follows: β -actin forward 5'-AAGATCAAGATCATTGCTCCTCCTG-3' and reverse 5'-GCAACTAAGTCATAGTCCGCCT-3', EIF1 forward 5'-AACCTCCACTCTTTCGACCC-3' and reverse 5'-CATCAGCGATCCCTTGGACA-3'. Relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method.

Western blot

The total proteins were extracted using RIPA buffer (Beyotime, China) and denatured by 5 x loading buffer at 100°C for 10 minutes. Proteins were then separated by 10% SDS-PAGE and transferred onto PVDF membranes (Millipore, USA). After blocking with 5% non-fat milk at room temperature for 1 hour, the membrane was incubated overnight at 4°C with primary antibodies EIF1 (1:1000, Cat# 15276-1-AP, Proteintech, USA), N-Cadherin (1:1000, Cat# 13116S, CST, USA), Vimentin (1:1000, Cat# 10366-1-AP, Proteintech, USA), Slug (1:1000, Cat# 9585S, CST, USA), Snail1 (1:1000, Cat# BS8479, Bioworld, USA), and β -actin (1:5000, Cat# sc-47778, Santa Cruz, USA). Following primary antibody incubation, the membranes were incubated with HRP-conjugated secondary antibodies (anti-mouse/anti-rabbit, 1:5000, Cat# 7076s/7074s, CST, USA) at room temperature for 1 hour. Protein bands were visualized using an ECL kit (Millipore, USA) and imaging system.

Subcutaneous Xenograft model

Four-week-old male BALB/c nude mice, purchased from Guangdong Medical Animal Experimental Center (Guangdong, China), were housed under specific pathogen-free conditions. Each mouse received a subcutaneous injection of 5×10^6 SW780 cells, which were infected with either a control lentivirus or an EIF1-targeting lentivirus, into the right axillary region. Tumor dimensions were measured and recorded every two days, and tumor volumes were calculated using the formula: tumor volume = $0.5 \times (\text{length} \times \text{width}^2)$. After 28 days, mice were sacrificed, and xenograft tumors were collected for subsequent analysis.

Statistical Analysis

All data processing and visualization were performed using R Studio (version 4.3.2) and GraphPad Prism 8. Data conforming to a normal distribution were analyzed using the Student's t-test, while non-normally distributed data were analyzed using the Wilcoxon rank-sum test. Pearson or Spearman correlation tests were used to assess the correlation between variables. All experiments were independently repeated three times, and results are presented as mean $\pm$ standard deviation (SD). A two-tailed p-value less than 0.05 was considered statistically significant.

Results

Identification of tumor recurrence-related genes in BLCA

We analyzed the transcriptomic differences between primary and recurrent bladder cancer tissues in the GSE128959 dataset and identified 445 differentially expressed genes (DEGs) with adjusted p-value < 0.05 and $|\log FC| > 0.5$, including 370 upregulated genes and 75 downregulated genes. The expression patterns of the top 50 upregulated genes and top 50 downregulated genes were visualized using heatmaps (Fig. 1A–B). To further identify key genes most strongly associated with bladder cancer recurrence, we constructed a gene co-expression network. When the soft-thresholding power $\beta = 6$, the resulting approximated a scale-free topology. Subsequently, recurrence-related gene modules were identified using the topological overlap matrix (TOM), and a total of 13 modules were detected (Fig. 1C–D). Among these modules, the lightcyan and blue modules showed significant correlations with bladder cancer recurrence (Fig. 1E). By intersecting the DEGs with the WGCNA module genes, 280 overlapping genes were identified for further analyses (Fig. 1F). To elucidate the biological functions associated with TRGs, we performed GO and KEGG enrichment analyses on these genes. GO enrichment revealed that these genes were mainly involved in cytoplasmic translation, cadherin binding, and pathways related to cytoskeletal remodeling and cell adhesion, and this trend was further supported by the KEGG analysis (Fig. S2A–B).

Identification of TRGs-based consensus clustering subgroups

To investigate the role of TRGs in BLCA, we performed consensus clustering analysis on the TCGA BLCA cohort based on TRG expression profiles. When $k = 2$, the intra-cluster variation was minimal and the inter-cluster variation was maximal. Under this condition, all samples were divided into two clusters: the R1 cluster ($N = 193$) and the R2 cluster ($N = 212$) (Fig. 2A–C). PCA and t-SNE analysis indicated distinct expression patterns (Fig. 2D, E). Survival analysis showed that patients in the R2 cluster exhibited significantly poorer prognosis compared with those in the R1 cluster (Fig. 2E, F). Meanwhile, the two clusters exhibited marked differences in clinical characteristics, including tumor stage and T stage (Supplementary Fig. S3A). We further evaluated the TME differences between the two clusters. Using the ESTIMATE algorithm, we found that the R2 cluster had higher stromal scores, immune scores, and ESTIMATE scores, along with lower tumor purity (Fig. 2G). The ssGSEA analysis revealed that the R2 cluster exhibited higher abundance of immune effector cells, such as NK cells, CD8⁺ T cells, and CD4⁺ T cells, compared with the R1 cluster; meanwhile, immunosuppressive cells, including regulatory T cells (Tregs), were also markedly increased (Fig. 2H). We further analyzed the expression differences of HLA family genes and immune checkpoint genes between the two clusters (Fig. 2I, J). Several immune checkpoint genes (e.g., CD274/PD-L1, CTLA4, PDCD1/PD-1, LAG3) were significantly upregulated in the R2 cluster, whereas only VTCN1 showed higher expression in the R1 cluster (Fig. 2J). Moreover, the two clusters exhibited marked differences in TIDE scores, and the R1 cluster showed significantly higher predicted immunotherapy response rates than the R2 cluster (Fig. 2K, L). GSVA analysis (Supplementary Fig. 3B, C) further revealed pronounced activation of pathways related to cell proliferation, oncogenic signaling, and metabolism in the R2 cluster. Collectively, the R2 cluster displayed stronger immunosuppressive characteristics, including increased infiltration of immunosuppressive cells and

upregulation of immune checkpoint genes, thus establishing a pronounced immunosuppressive microenvironment, enhancing tumor immune evasion, ultimately resulting in poorer patient prognosis.

Construction of machine learning–based prognostic model for BLCA

The above analyses preliminarily revealed the significance of TRGs in BLCA and their association with immunotherapy. We further developed and validated a TRG-based prognostic model, termed TRI, to elucidate the critical role of TRGs in BLCA prognosis and immunotherapy. First, 81 prognosis-related genes were screened from the core TRGs using univariate Cox regression analysis (Supplementary Fig. S4A). Subsequently, 101 prognostic models were fitted using LOOCV and applied to the TCGA-BLCA cohort (training set), while GSE13507, GSE32548, and GSE327894 served as test sets. Based on the ranking of C-index values across all models, the combination of Lasso and plsRcox was selected to further identify core prognostic genes, yielding 16 prognostic genes (Fig. 3A). Further multivariate Cox regression analysis was performed to construct the prognostic model based on these genes, and six significant genes (P -value < 0.05) were ultimately selected. The TRI value for each BLCA patient was calculated using the following formula: $TRI = (0.5475681 \times EIF1) + (0.6886301 \times ANAPC5) + (0.2506998 \times EGR1) + (-0.6367359 \times PFDN5) + (-0.2465871 \times PPP2R2B) + (-0.4512100 \times DEXI)$ (Fig. 3B–C). Based on the median TRI value, BLCA patients were classified into high-TRI and low-TRI groups. In the TCGA-BLCA cohort and all three test cohorts, patients in the high-TRI group exhibited significantly worse prognosis (Fig. 3D–G). ROC analysis demonstrated that TRI showed robust and consistent discriminative performance across multiple independent cohorts. In the TCGA cohort, the AUCs for 1-year, 3-year, and 5-year survival were 0.702, 0.676, and 0.694, respectively. In the GSE13507 cohort, the corresponding AUCs were 0.696, 0.682, and 0.734; in the GSE32548 cohort, 0.614, 0.599, and 0.590; and in the GSE32894 cohort, 0.675, 0.684, and 0.634 (Fig. 3H–K). Moreover, we compared the predictive performance of TRI with previously published prognostic models for BLCA, and TRI exhibited superior predictive accuracy in different cohorts, with higher C-index values (Fig. 3L, M) and improved ROC results (Supplementary Fig. S4B, C). These findings indicate that the TRI model, composed of six genes, possesses robust survival prediction capability.

Establishment and evaluation of the nomogram survival model

TRI was identified as a significant risk factor in the univariate Cox regression analysis and further confirmed as an independent prognostic factor in the multivariate Cox regression analysis ($p < 0.001$) (Fig. 4A–B). Furthermore, we constructed a prognostic nomogram model in the TCGA training cohort to predict the 1-, 3-, and 5-year overall survival of BLCA patients (Fig. 4C). Calibration curves demonstrated good accuracy of the nomogram in predicting 1-, 3-, and 5-year overall survival (Fig. 4D). Similar results were observed in the three external validation cohorts (Supplementary Fig. S5A–C). DCA analysis further confirmed that the nomogram outperformed other predictive indicators used in this study, exhibiting

higher clinical net benefit and potential utility for guiding individualized treatment (Fig. 4E–G). In addition, patients stratified into high- and low-score groups based on the nomogram showed significantly different overall survival outcomes (Fig. 4H). Prognostic ROC analysis revealed high AUC values for predicting 1-, 3-, and 5-year survival in the TCGA training cohort, with similar accuracy observed in the independent validation cohorts (Fig. 4I–K). These results indicate that the TRI-based nomogram provides robust survival prediction performance and precise risk stratification.

Gene set enrichment analysis between two risk subgroups

To further investigate the biological processes associated with the TRI model, we identified significantly differentially expressed genes between the two groups and performed comprehensive enrichment analyses. GO analysis revealed enrichment of DEGs in biological processes such as epidermis development, extracellular matrix organization, extracellular matrix, and external encapsulating structure, all closely related to tumor cell invasion and metastasis (Fig. 5A). KEGG analysis further indicated that the DEGs were involved in multiple tumor-related biological processes, including the PI3K–AKT pathway, cadherin signaling pathway, and muscle cell cytoskeleton (Fig. 5D). In addition, GSEA showed that the high-TRI group was significantly associated with extracellular matrix remodeling, focal adhesion, and cytokine–receptor interaction, whereas the low-TRI group was enriched in cytochrome P450-related drug metabolism and xenobiotic metabolism (Fig. 5B–C). Finally, GSVA revealed pronounced differences in pathway activation between the TRI subgroups, with the majority of pathways being prominently activated in the high-TRI group (Fig. 5E).

Immune infiltration and immunotherapy response between TRI-defined subgroups

To comprehensively evaluate the immune infiltration patterns across different risk groups, we applied multiple algorithms, including MCP-counter, EPIC, xCELL, CIBERSORT, Quantiseq, and TIMER, to systematically assess the distribution of immune cell infiltration in the training and validation cohorts. The results showed that immune cell infiltration levels were markedly elevated in the high-TRI group (Fig. 6A). Consistently, ESTIMATE analysis confirmed that patients in the high-TRI group exhibited significantly higher immune scores and stromal scores, whereas tumor purity was lower than in the low-TRI group (Fig. 6B). In addition, ssGSEA enrichment scores were used to investigate the correlations between TRI and various immune cell types and immune functional activities. In line with previous findings, patients in the high-TRI group displayed more pronounced immune infiltration, with significantly increased infiltration of myeloid-derived suppressor cells, regulatory T cells, and Th2 cells (Fig. 6C). Moreover, immune function analysis revealed that the high-TRI group not only exhibited higher levels of immune cell infiltration but also showed significant enrichment in CCR, checkpoint pathways, and T-cell co-inhibition processes. This combination of high immune infiltration and strong immunosuppression further indicated an immune-exhausted tumor microenvironment in the high-TRI group (Fig. 6D). To further evaluate the predictive value of TRI in immunotherapy, we analyzed the IMvigor210 urothelial

carcinoma cohort and two additional immunotherapy cohorts, GSE78220 and GSE126044. In the IMvigor210 cohort, patients with partial response (PR) or complete response (CR) exhibited significantly lower TRI scores compared with those with progressive disease (PD) or stable disease (SD) (Fig. 6E). A higher proportion of patients in the low-TRI group achieved disease remission compared with in the high-TRI group (Fig. 6F). Survival analysis showed that patients with higher TRI scores experienced poorer outcomes (Fig. 6G). These findings were further validated in the GSE78220 and GSE126044 cohorts (Fig. 6H–M). Collectively, these results suggest that TRI can serve as a predictor of immunotherapy response, with patients exhibiting low TRI scores more likely to benefit from treatment.

Single-cell characteristics of TRI

To investigate the expression pattern of genes within TRI across different cell types, we analyzed scRNA-seq data from BLCA samples. After stringent quality control, 94,341 cells were retained (Supplementary Fig. S6A–C). Cell annotation identified seven major cell types, including epithelial cells, endothelial cells, T cells, B cells, fibroblasts, myeloid cells, and mast cells (Fig. 7A, B). We further examined the distribution of these cell types in tumor samples (Fig. 7C). The expression levels of the six genes within the TRI model across different cell types are shown in Fig. 7D. Given that bladder cancer originates from epithelial cells, we applied the CopyKat algorithm to infer genomic copy number variations for each epithelial cell and identified 2,226 malignant cells (Supplementary Fig. S6D). Monocle2 was then used to analyze the potential evolutionary trajectories of epithelial cells with different CNV scores. Epithelial cells with low CNV scores gradually transitioned into malignant epithelial cells with high CNV scores (Fig. 7E). In parallel, TRI scores for each cell were calculated using the “AUCcell” function, revealing a positive correlation between TRI and CNV scores, with cells exhibiting higher CNV scores also displaying higher TRI scores (Fig. 7F–G). Furthermore, cell–cell communication analyses to explore pathway information flow between malignant cells with different TRI scores and other cell populations. As shown in Fig. 7H, high-TRI malignant cells displayed stronger communication with fibroblasts and endothelial cells. Signaling pathways such as MK, VEGF, CALCR, GDF, ANGPTL, CD46, and SEMA6 were strongly activated (Fig. 7I), suggesting enhanced cellular activities associated with invasion, angiogenesis, and immune evasion.

Expression and functional validation of the model gene EIF1

To evaluate the biological functions associated with the TRI model, EIF1 was selected as a representative gene for further validation. Western blot analysis showed that EIF1 expression was markedly elevated in recurrent BLCA tissues compared with primary BLCA tissues (Fig. 8A). Similarly, EIF1 expression was significantly higher in BLCA cell lines than in normal bladder epithelial cells (Fig. 8B, C). Kaplan–Meier analysis further demonstrated that patients with higher EIF1 expression had significantly poorer prognostic outcomes (Fig. 8D–E). We next used siRNA to specifically silence EIF1 expression to further investigate its biological functions (Fig. 9A, B). CCK-8 assays showed that EIF1

knockdown suppressed cell proliferation (Fig. 9C). Consistently, colony formation assays confirmed that EIF1 silencing markedly inhibited cell proliferation (Fig. 9D). Transwell assays revealed that EIF1 knockdown significantly reduced cell migration (Fig. 9E, F). Western blot analysis showed that EIF1 silencing substantially decreased the expression of EMT-related proteins, including N-cadherin, Vimentin, Slug, and Snail1 (Fig. 9G). To further validate the role of EIF1 in vivo, a subcutaneous xenograft model was established in nude mice. Tumor volume and growth rate were both lower in the sh-EIF1 group compared with the control group (Fig. 9H–J). Collectively, these findings highlight the critical role of EIF1 in promoting malignant phenotypes of BLCA cells both in vitro and in vivo.

Discussion

Bladder cancer (BLCA) remains a common malignant tumor that seriously threatens human health [19]. Clinical treatment of BLCA is undergoing a historic transformation, particularly with the advent of immuno-targeted therapies such as immune checkpoint inhibitors (ICIs), cellular immunotherapy, and antibody–drug conjugates, which have not only improved overall therapeutic efficacy but also reshaped the treatment landscape of BLCA [20–22]. However, many patients still experience poor outcomes, with approximately 50–70% facing the risk of disease recurrence after treatment [23, 24]. Studies have shown that recurrent BLCA severely impairs bladder function, and subsequent treatments for recurrence may induce multiple severe adverse effects, including urinary tract infections, bladder irritation, dysuria, and even urinary incontinence [8]. At present, reducing postoperative recurrence risk and complications largely depends on the medical conditions of the treatment center and the clinical experience of physicians. Therefore, identifying biomarkers associated with tumor recurrence is crucial for achieving early diagnosis, precise stratification, and effective personalized treatment in BLCA patients. In this study, we systematically elucidated the molecular subtypes and prognostic significance of TRGs in BLCA and integrated multi-omics data to construct a novel prognostic model (TRI) and molecular classification framework. Our findings revealed the critical roles of TRGs in BLCA pathogenesis, prognosis evaluation, and prediction of immunotherapy sensitivity. Moreover, the TRI model demonstrated robust and consistent performance across multiple cohorts, highlighting its potential clinical utility in guiding personalized management of BLCA patients.

We performed an integrated analysis of TRGs identified through differential expression analysis and WGCNA, together with their prognostic value, and defined two molecular subtypes. Among them, cluster R2 exhibited significantly poorer overall survival and was associated with more advanced overall tumor stage and T stage. Analysis of the tumor microenvironment (TME) revealed that cluster R2 displayed a “high-infiltration, high-suppression” paradoxical immune landscape. On one hand, multiple key immune effector cells (such as CD8⁺ T cells and NK cells) showed markedly increased infiltration in cluster R2. Meanwhile, several critical immune checkpoint genes (PDCD1, LAG3, LGALS9, CTLA4) were synchronously upregulated in the R2 subtype, and immunosuppressive factors—particularly Treg cells—also exhibited significantly elevated infiltration. Increased Treg infiltration has been well established to correlate with poor prognosis in previous studies [25, 26]. This phenomenon suggests that although cluster R2 harbors strong immune cell infiltration, these recruited immune cells are profoundly

exhausted and have lost their cytotoxic functionality. In addition, we observed significant activation of multiple oncogenic pathways in the R2 subtype, including the PI3K/AKT, EMT, and hypoxia pathways [27, 28]. These pathways may promote the upregulation of chemokines and remodeling of the extracellular matrix, thereby facilitating Treg accumulation while simultaneously establishing a functionally exclusionary TME barrier. Taken together, TRG-based molecular subtyping effectively distinguishes BLCA patients with distinct pathological and immune characteristics and may provide valuable guidance for individualized therapeutic strategies.

A reliable prognostic model not only enables accurate quantification of patient risk and identification of high-risk individuals but also provides important guidance for clinical decision-making. To this end, we constructed multiple prognostic models using 101 combinations of machine-learning algorithms, among which the Lasso+plsRcox model exhibited the best overall performance and was subsequently incorporated into a multivariate Cox regression analysis to establish the TRI prognostic model. This scoring system demonstrated precise prognostic stratification across all validation cohorts, with patients in the high-TRI group showing markedly poorer overall survival. Higher TRI scores were also associated with more advanced overall tumor stage and T stage. Furthermore, additional analyses indicated that TRI could serve as an independent prognostic indicator for BLCA patients. A nomogram integrating TRI and relevant clinical parameters was also validated, supporting its clinical utility. In addition, we compared the TRI model with several previously published prognostic models, and the results showed that TRI achieved superior predictive accuracy, highlighting its exceptional capability in forecasting survival outcomes in BLCA patients.

The TRI prognostic model consists of six core genes: EIF1, ANAPC5, EGR1, PFDN5, PPP2R2B, and DEXI. Among them, EIF1, ANAPC5, and EGR1 were identified as risk factors associated with poor prognosis, whereas PFDN5, PPP2R2B, and DEXI served as protective factors, collectively contributing to the prediction of patient survival outcomes. ANAPC5 is a member of the anaphase-promoting complex/cyclosome (APC/C) family and primarily participates in cell-cycle regulation by maintaining the structural stability of the E3 ligase complex and targeting cell-cycle-related proteins [29]. EGR1 promotes tumor growth and metastasis through multiple mechanisms [30–32]. PFDN5 plays an important role in protein folding and aggregation, and studies have suggested that it may enhance the efficacy of immunotherapy by modulating the suppressive immune microenvironment [33]. PPP2R2B is an important protein phosphatase subunit involved in signal transduction, cell-cycle regulation, apoptosis, and differentiation, and has been closely linked to the development of bladder cancer [34]. DEXI was initially reported as a gene upregulated by dexamethasone in emphysema, and its methylation-mediated silencing has been shown to be associated with camptothecin (CPT) resistance in colorectal cancer cells [35]. EIF1 is a eukaryotic translation initiation factor involved in mRNA scanning during the initiation stage and assists the ribosome in accurately recognizing the start codon [36, 37]. Takayoshi et al. reported that EIF1 interacts with PDPA1 to help hepatitis A virus counteract ER stress-induced antiviral responses and promote viral protein synthesis [38]. Moreover, EIF1 is aberrantly overexpressed in hepatocellular carcinoma and pancreatic cancer and is closely associated with poor prognosis [39, 40].

To further clarify the role of EIF1 in BLCA, we performed in vitro functional experiments. The results showed that EIF1 was highly expressed in recurrent bladder cancer tissues and significantly correlated with poor patient prognosis. Single-cell sequencing further revealed that EIF1 was highly expressed in epithelial and endothelial cells within BLCA tissues, suggesting that it may influence tumor cell behavior and angiogenesis. In addition, EIF1 silencing markedly inhibited BLCA cell proliferation and migration, and this finding was also validated in vivo.

Despite the preliminary findings obtained in this study, several limitations remain. First, the analyses were primarily based on transcriptomic data from public databases, and the conclusions require further validation in larger and more diverse independent cohorts. Second, although we demonstrated that EIF1 knockdown markedly suppresses the malignant phenotypes of BLCA cells, the underlying molecular mechanisms were not fully explored. Our in vitro and in vivo data strongly support the oncogenic role of EIF1; however, additional studies are needed to systematically investigate its functions and mechanistic pathways in BLCA.

Conclusion

This study systematically analyzed the molecular subtype characteristics associated with TRGs in BLCA and established a novel prognostic model (TRI). The model demonstrated robust prognostic evaluation and therapeutic response prediction across multiple independent cohorts, providing a reliable tool for prognosis assessment and individualized management of BLCA patients. Importantly, we validated the characteristics and functions of the core gene EIF1 within TRI, suggesting that it may serve as a potential therapeutic target to suppress recurrence and malignant progression of BLCA.

Abbreviations

BLCA Bladder cancer

WGCNA Weighted correlation network analysis

OS Overall survival

DSS Disease-specific survival

TME Tumor microenvironment

ROC Receiver operating characteristic

AUC Area under the curve

DCA Decision curve analysis

GSEA Gene set enrichment analysis

GSVA Gene set variation analysis

ssGSEA Single-sample gene set enrichment analysis

ICI Immune checkpoint inhibitor

TIDE Tumor Immune Dysfunction and Exclusion

PCA Principal component analysis

t-SNE t-distributed stochastic neighbor embedding

EMT Epithelial-mesenchymal transition

Declarations

Ethics statement

This study was conducted in strict accordance with the principles of the Declaration of Helsinki. All participants provided informed consent prior to inclusion, and the study protocol was approved by the Ethics Committee of Shenzhen Second People's Hospital (approval no. KS201900604004). All animal experiments were approved by the Animal Ethics Committee of China Technology Industry Holdings (Shenzhen) Co., Ltd (Approval No. 20250002) and were performed in accordance with the Chinese national standard GB/T 35892 – 2018 (Guideline for the Ethical Review of Laboratory Animal Welfare).

Consent for publication

Not applicable

Conflict of interests

The authors declare no conflict of interest.

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Author Contribution

Jia Su and Huarong Luo contributed equally to this work. Jia Su and Huarong Luo designed the study, performed the bioinformatics analyses and experiments, and wrote the original draft. Jiaqi Bo and Zhiming Cai contributed to the data collection and experimental validation. Pingsen Zhao conceptualized, reviewed and edited the manuscript. Xina Xie and Zesong Li acquired the funding, supervised the project and revised the manuscript. All authors have read and approved the final manuscript.

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Data availability statement

All data generated or analyzed during this study are available from the corresponding author on reasonable request.

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Figures

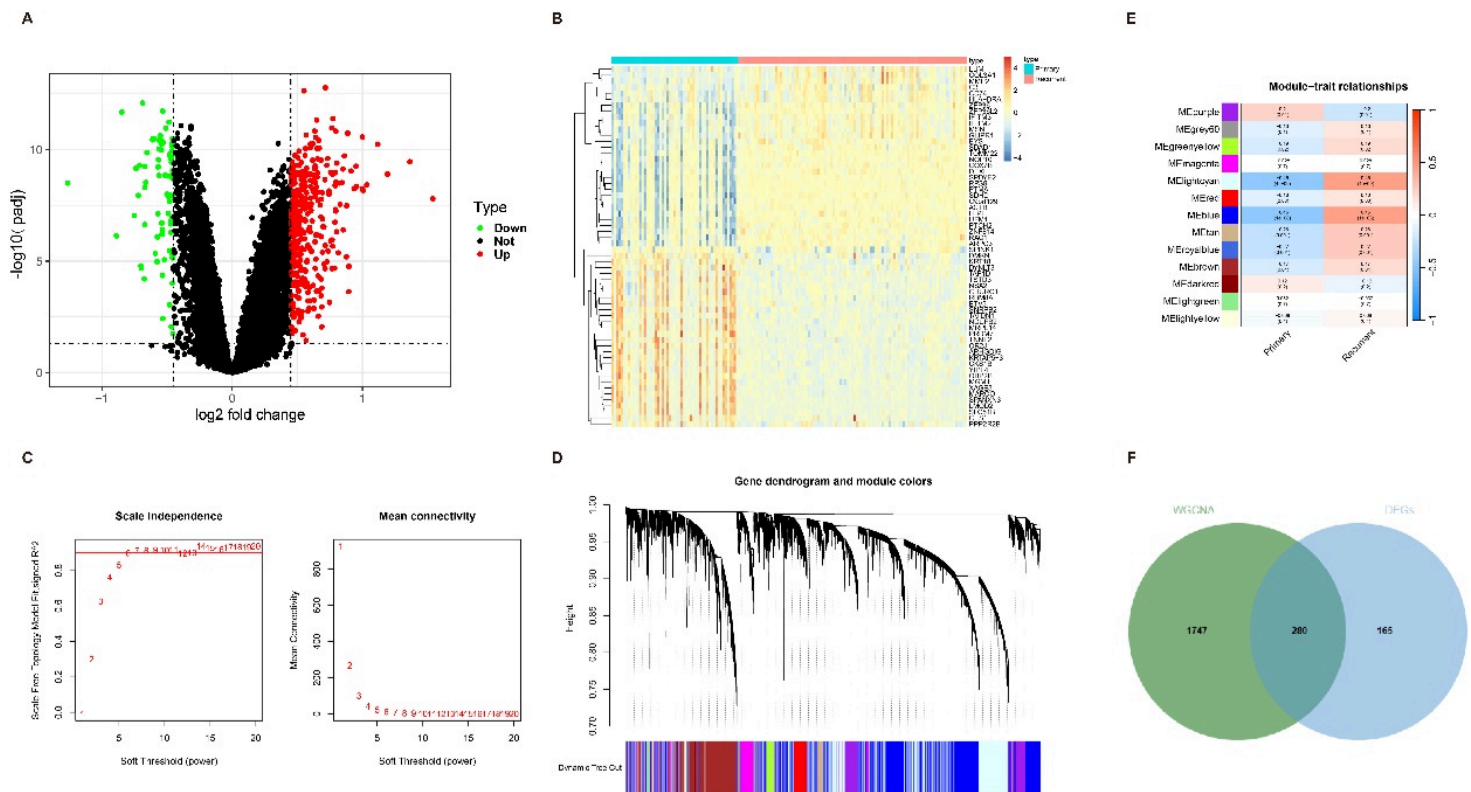
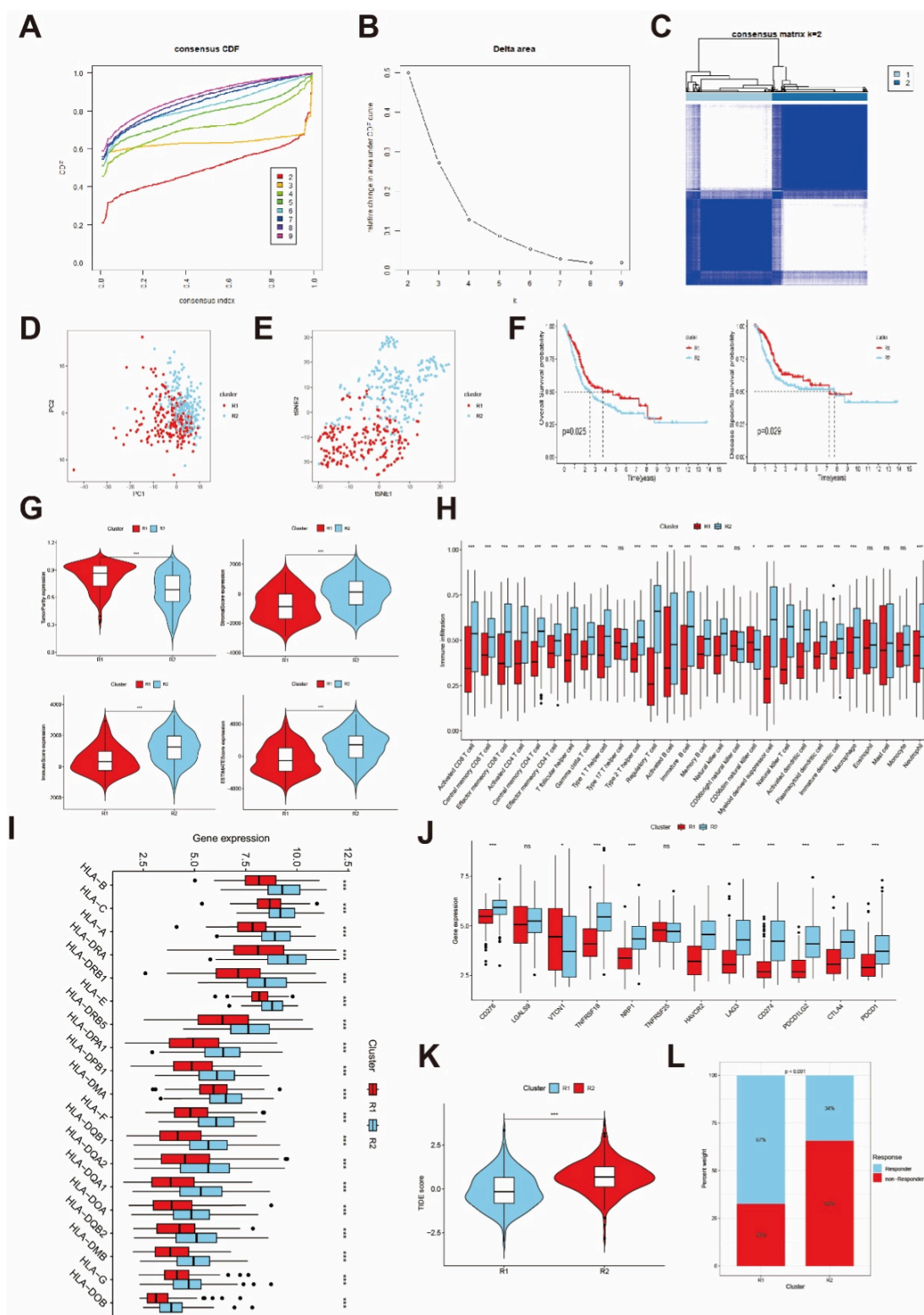


Figure 1

Identification of the tumor relapse related genes in BLCA. (A) Volcano plot of differentially expressed genes. (B) Heatmap of gene expression from primary and recurrent BLCA. (C) Selection of the soft threshold for constructing a scale-free gene co-expression network. (D) Gene clustering dendrogram from WGCNA analysis. (E) Heatmap showing the correlations between clustering modules and clinical traits. (F) Venn diagram illustrating the intersections between differentially expressed genes and module genes.



Identification and immune landscape analysis of BCRGs related molecular subgroups in BLCA. (A) Cumulative distribution function (CDF) curves of consensus scores for different numbers of clusters. (B) Different the area under the CDF curve. (C) Consensus clustering matrix for k = 2. (D, E) PCA and tSNE plots for the two molecular clusters. (F) Kaplan-Meier survival analysis of OS and DSS between cluster R1 and R2. (G) Stromal, ESTIMATE, immune and tumor purity score between the clusters. (H) The

differences of the relative infiltrate abundances of the 28 immune cell types between two clusters. (I) Differences in the expression of HLA-related genes in R1 and R2 clusters. in two clusters(J) The different expression level of ICI-related genes between clusters. (K) Differences in TIDE scores between two cluster. (L) The different proportions of predicted immunotherapy responders and non-responders within each cluster. * P < 0.05; ** P < 0.01; *** P < 0.001

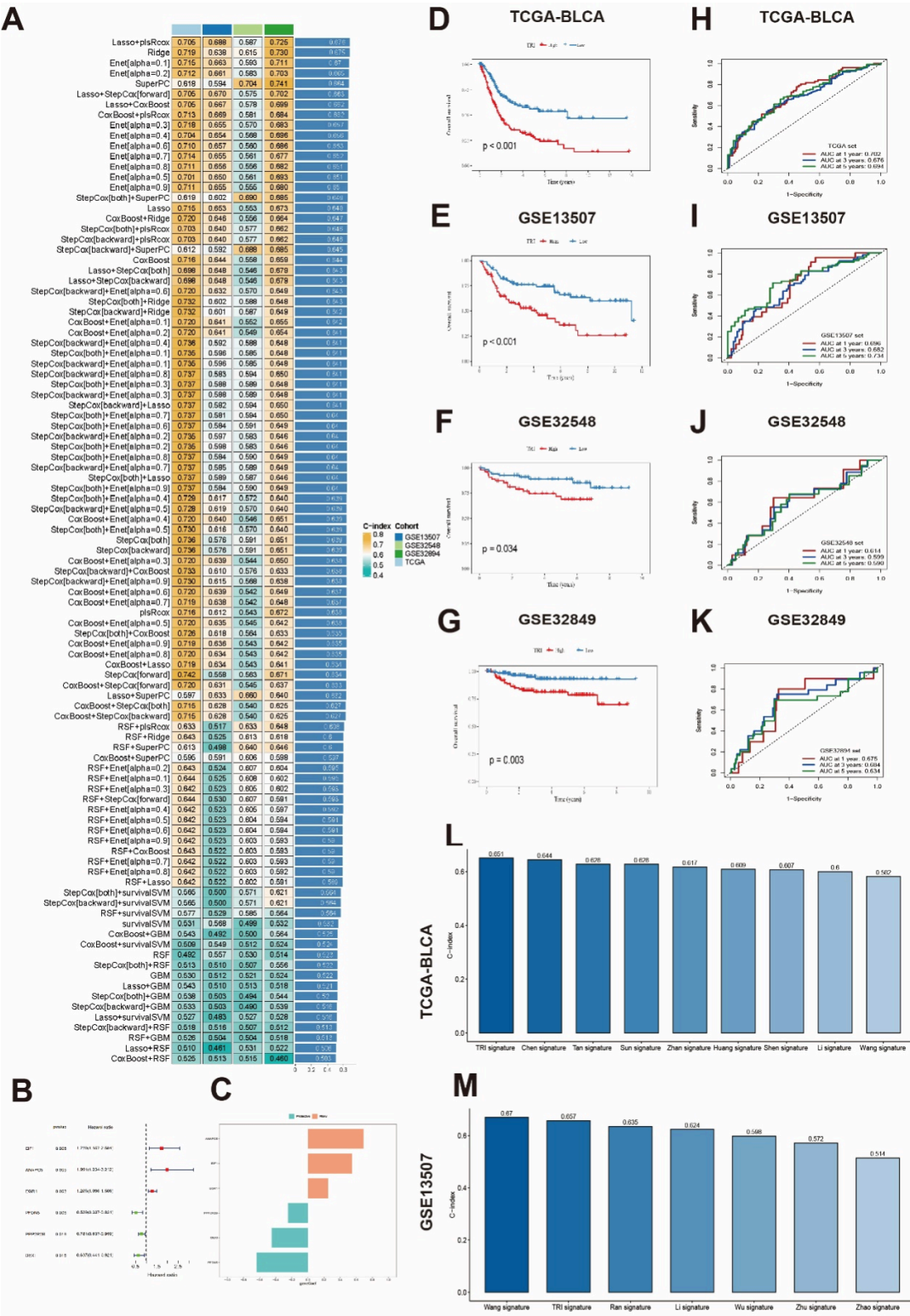


Figure 3

Construction and validation of a prognostic model for bladder cancer. (A) C-index of predictive models constructed by multiple combinations of machine learning algorithms in the training and test cohort. (B) Forest plot showing hazard ratios of the six selected genes from multivariate Cox regression analysis. (C) Coefficients of each gene in the prognostic model. (D-G) Kaplan–Meier survival analyses of patients in the high-TRI and low-TRI groups across different cohorts. (H-K) Time-dependent ROC curves of TRI in different cohorts. (L, M) Comparison of C-index between TRI and previously published prognostic features in different cohorts: TCGA-BLCA cohort (L), GSE13507 cohort (M).

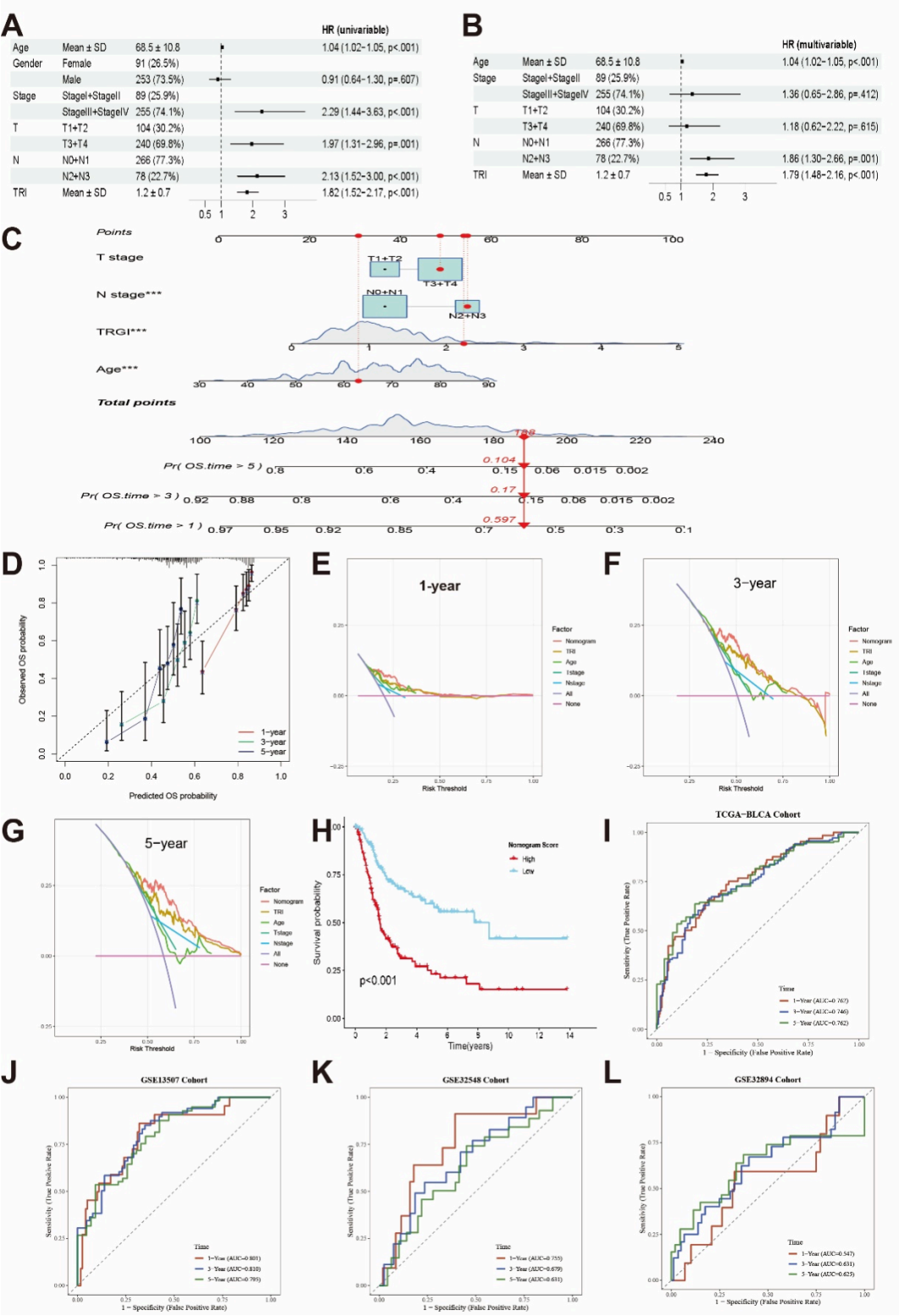


Figure 4

Establishment and validation of the nomogram survival model. (A) Univariate Cox regression analysis of TRI and clinicopathological characteristics in the TCGA-BLCA cohort. (B) Multivariate Cox regression analysis of TRI and clinicopathological characteristics in the TCGA-BLCA cohort. (C) A nomogram constructed for predicting patient prognosis. (D) Calibration curves showing the nomogram's performance in predicting 1-year, 3-year, and 5-year overall survival. (E-G) Decision curve analysis (DCA) evaluating the nomogram's ability to predict 1-year, 3-year, and 5-year overall survival. (H) Survival analysis based on the nomogram score. (I-L) ROC analysis of the nomogram across different cohorts.

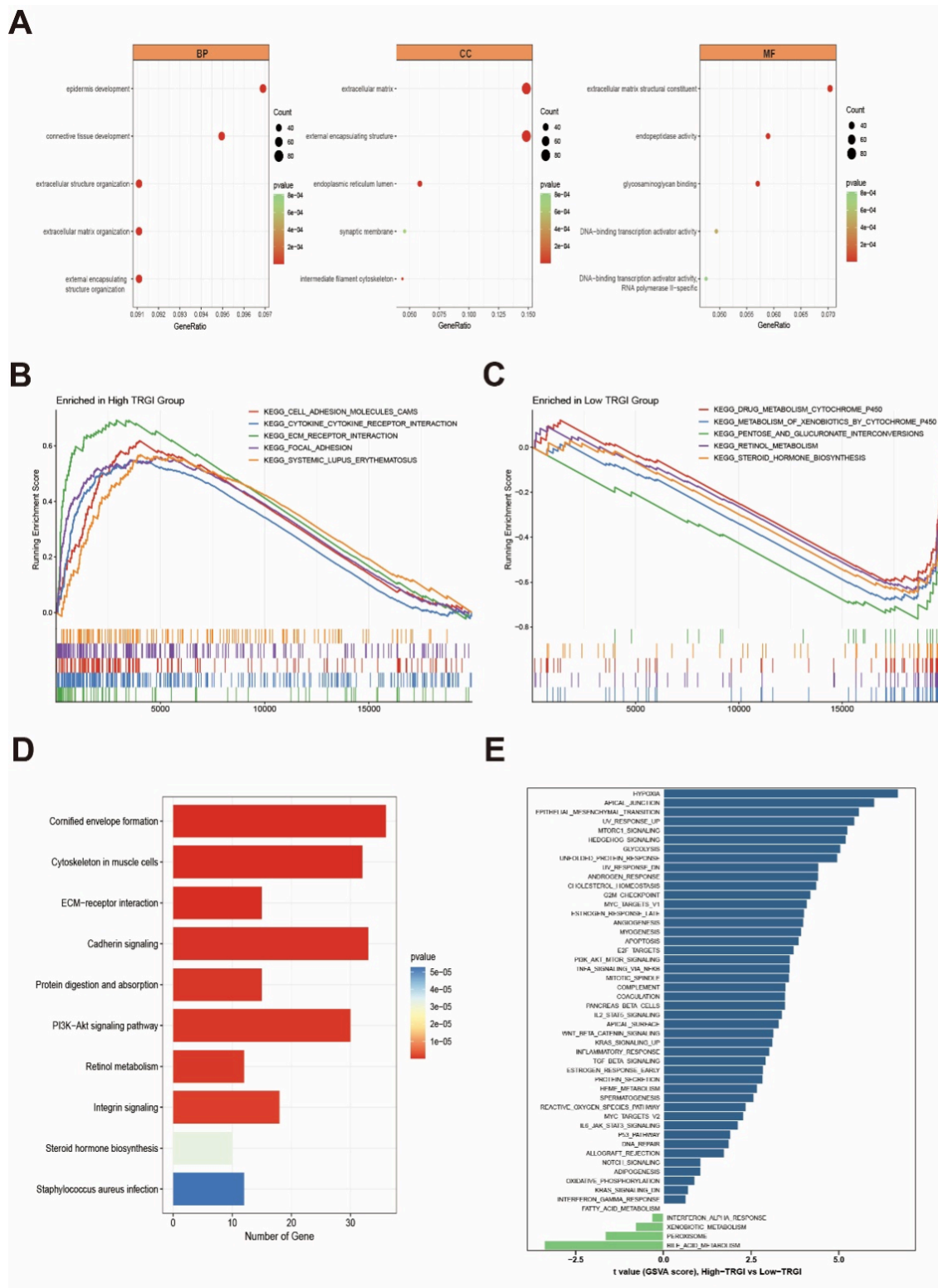
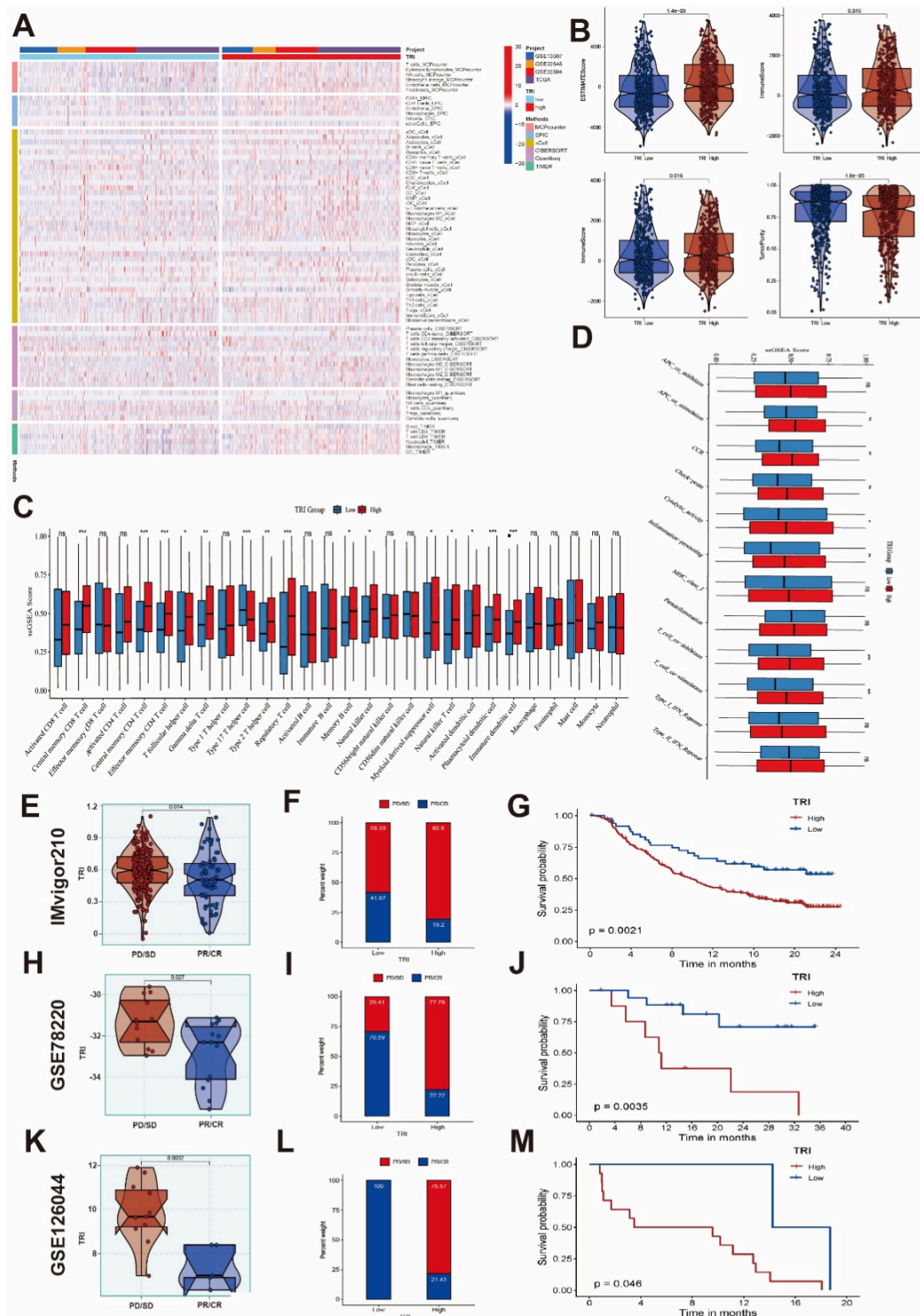


Figure 5

Functional enrichment analysis of TRI groups (A) GO enrichment analysis of TRI groups. (B, C) GSEA analysis of signaling pathways enriched in the high- and low-TRI groups. (D) KEGG enrichment analysis of TRI groups. (E) GSVA analysis showing differences in pathway activities between TRI groups.



TRI-related immune infiltration analysis and prediction of immunotherapy efficacy. (A) Multiple algorithms were used to analyze immune cell infiltration levels between the high- and low-TRI groups. (B) Comparison of Estimate score, Immune score, Stromal score, and tumor purity between the high- and low-TRI groups. (C, D) The ssGSEA analysis of immune infiltration patterns and pathway activation status between the high- and low-TRI groups. (E-G) Analysis of immunotherapy response in the

IMvigor210 cohort. (E) Differences in TRI scores among patients with different clinical response statuses. (F) Distribution of clinical responders and non-responders in the high- and low-TRI groups. (G) Kaplan–Meier survival analysis of the high- and low-TRI groups in the IMvigor210 cohort. (H–J) Validation of the predictive accuracy of TRI for immunotherapy response in the GSE78220 cohort. (K–M) Validation of the predictive accuracy of TRI for immunotherapy response in the GSE126044 cohort. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

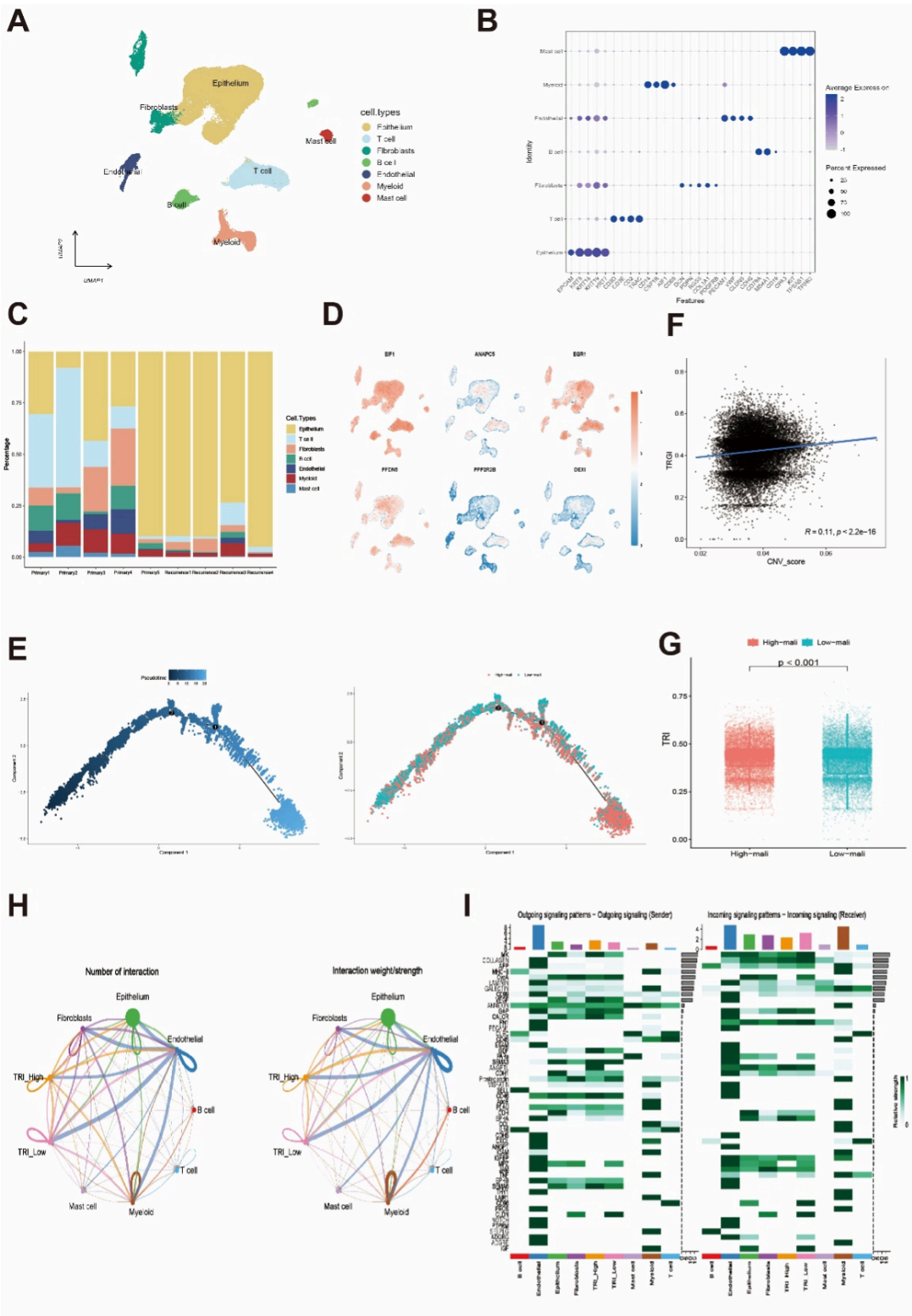


Figure 7

Single-cell analysis based on the TRI signature. (A) UMAP plot showing seven major cell types identified in the GSE277524 dataset. (B) Dot plot displaying the expression of cell type-specific marker genes. (C) Differences in cell type composition between primary and recurrent bladder cancer samples. (D) UMAP plot showing the expression levels of TRI model genes across different cell types. (E) Pseudotime trajectory analysis of malignant cells. (F) Correlation analysis between TRI scores and CNV scores. (G) Comparison of TRI scores between epithelial cells with low and high malignancy. (H) Cell-cell communication analysis among different cell populations. (I) Heatmap showing the activation states of signaling pathways across different cell populations.

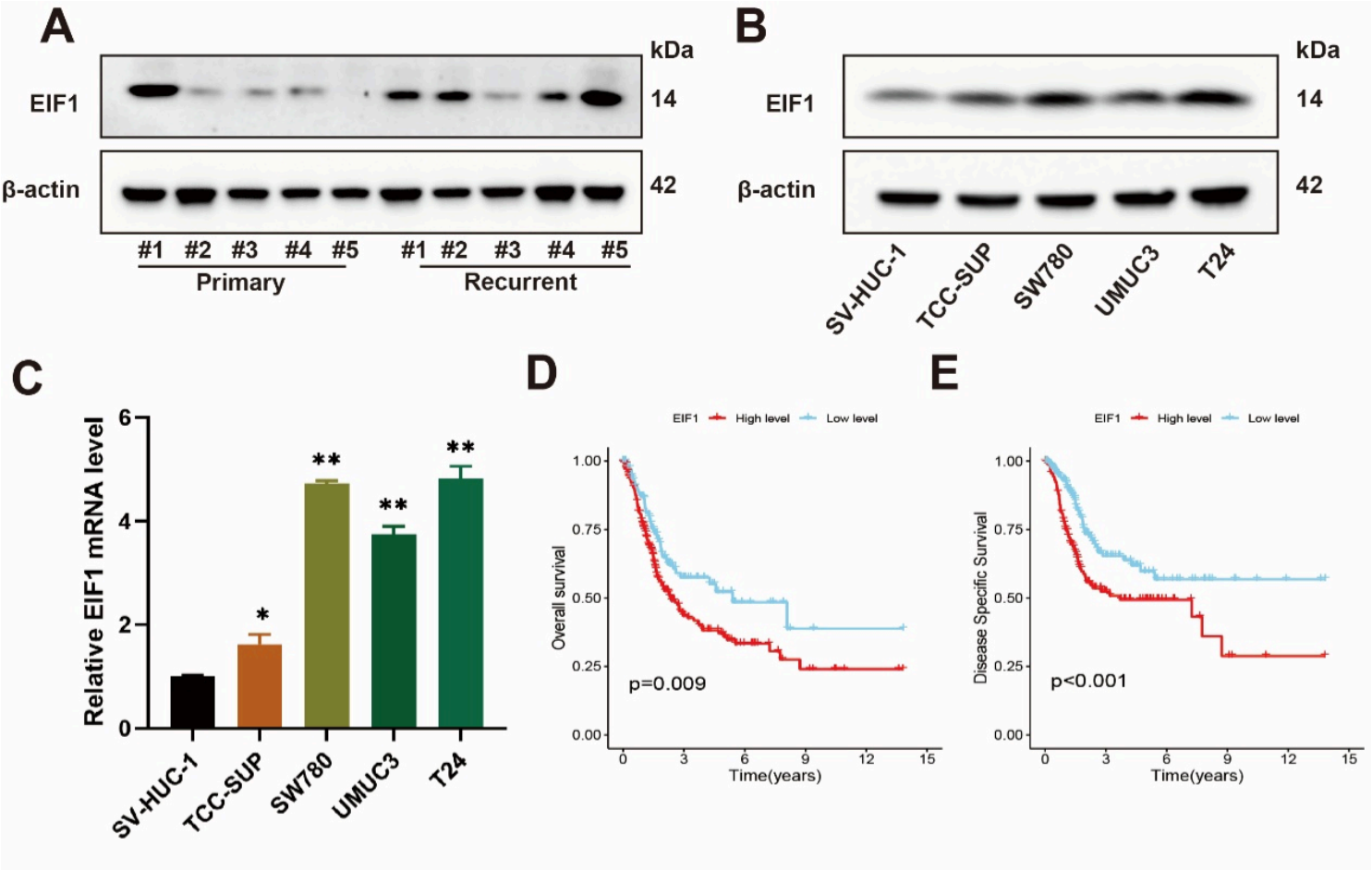


Figure 8

EIF1 is highly expressed in recurrent bladder cancer tissues, and its high expression is associated with poor prognosis. (A) Western blot analysis of EIF1 protein levels in primary and recurrent bladder cancer tissues. (B, C) Western blot (B) and RT-qPCR (C) analysis of EIF1 expression in bladder cancer cell lines and SV-HUC-1. (D, E) The correlation between EIF1 expression and overall survival (OS) as well as disease-specific survival (DSS) in BLCA patients. * $P < 0.05$; ** $P < 0.01$

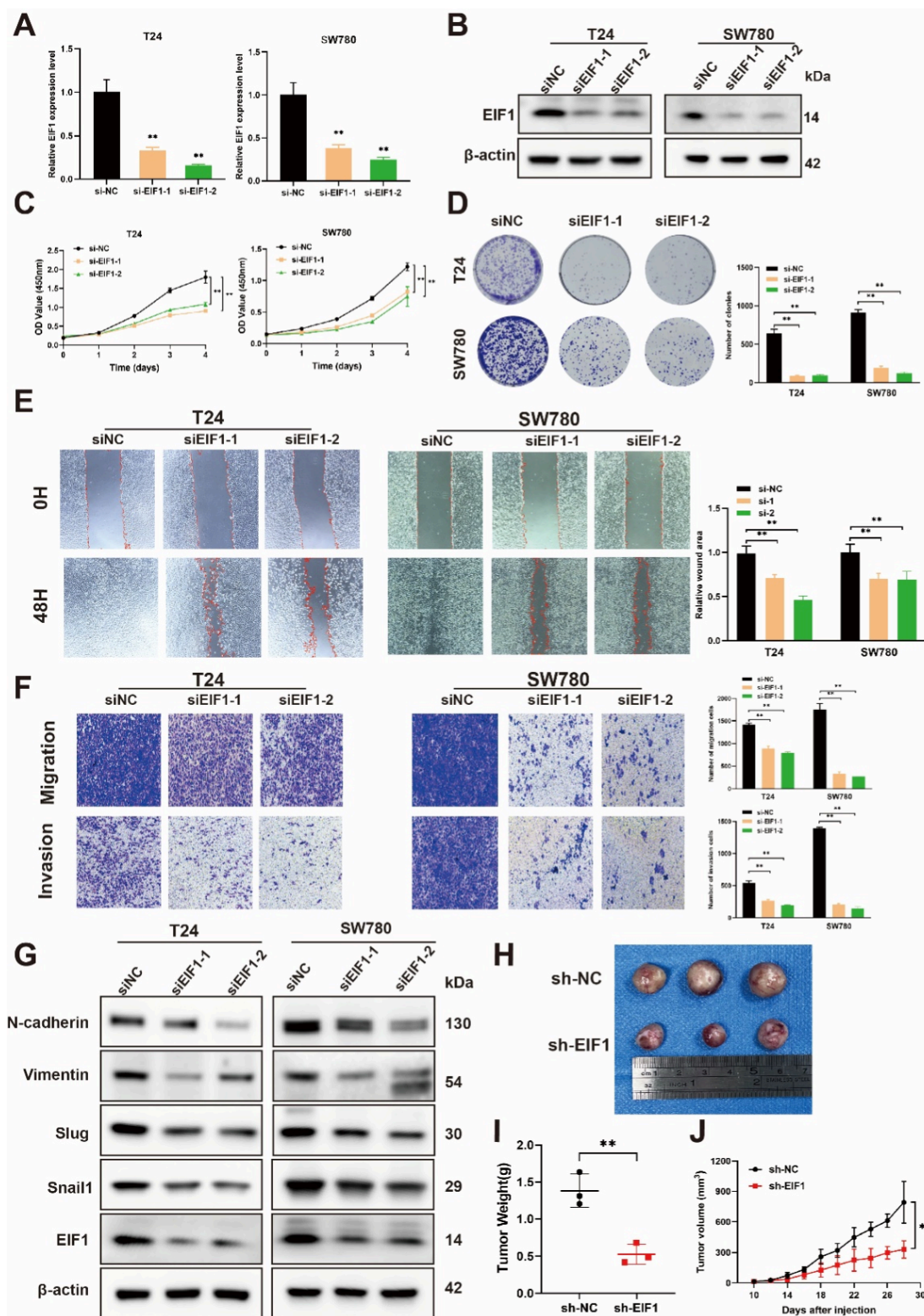


Figure 9

Downregulation of EIF1 impairs the malignant phenotypes of BLCA. (A, B) Relative mRNA expression levels (A) and protein expression levels (B) of EIF1 were detected in T24 and SW780 cells following siRNA-mediated knockdown. (C) CCK-8 assay was performed in T24 and SW780 cells following EIF1 knockdown. (D) Colone formation assays were conducted in T24 and SW780 cells with EIF1 downregulation. (E) Wound-healing assays in T24 and SW780 cells after EIF1 silencing. (F) Cell

migration and invasion experiments were performed in T24 and SW780 cells with EIF1 knockdown. (G) Western blotting assay was to detect the EMT-related proteins in T24 and SW780 cells following EIF1 knockdown. (H–J) Subcutaneous xenograft model using SW780 cells to evaluate the in vivo effects of EIF1 knockdown on tumor growth. Representative images of xenograft tumors (H), tumor weights(I), and tumor growth curves (J). ** $P < 0.01$

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