

Exploring the underlying mechanisms of Hedyotis diffusa and Scutellaria barbata herb pair on the prognosis and treatment efficacy of bladder cancer patients: an integrated approach of network pharmacology and bioinformatics analysis

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Research Article

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Abstract

Background

The development and progression of bladder cancer are closely linked to its complex tumor microenvironment. *Hedyotis diffusa* and *Scutellaria barbata* (HD-SB) are commonly used as a prominent herbal pair for treating bladder cancer. However, the pharmacological targets and molecular mechanisms by which HD-SB impacts bladder cancer require further elucidation. Additionally, it remains uncertain whether this herbal pair affects the prognosis and treatment efficacy of bladder cancer.

Methods

We employed network pharmacology to predict the targets of HD-SB and bladder cancer, identifying overlaps with prognostic genes linked to the overall survival of bladder cancer patients in the TCGA dataset. Subsequently, we utilized least absolute shrinkage and selection operator (LASSO) and Cox regression analyses to pinpoint a prognostic signature and construct a prognostic model. We further explored the correlations between risk scores, immune cells, immune checkpoint genes, and treatment efficacy. Single-cell RNA-sequencing (scRNA-seq) was used to profile the expression of prognostic genes across various cell types, and immunohistochemistry validated the protein levels of these targets. Molecular docking studies were conducted to clarify the interactions between HD-SB components and the identified genes, and in vitro experiments demonstrated the effects of HD-SB on bladder cancer cells.

Results

Venn diagram analysis identified 497 common targets shared between HD-SB and bladder cancer. LASSO and Cox regression identified a 15-gene prognostic signature, including VEGFA, EGFR, MYC, PDGFRA, JUN, FN1, PTPN6, PTGER3, MAP2, CALM1, CTSV, CES1, ADRA1D, PYGL, and PLA2G1B. Kaplan-Meier analysis showed better overall survival in the low-risk group (median 19.8 months) versus the high-risk group (median 15.9 months). Linear regression analysis revealed a significant correlation between risk scores and specific immune cell types, as well as the dysregulated expression of immune checkpoint genes across different groups. The prognostic gene-based risk score was also found to correlate with the efficacy of both immunotherapy and chemotherapy. Six key targets—VEGFA, MYC, JUN, FN1, PTPN6, and CALM1—were validated through scRNA-seq and immunohistochemistry. Molecular docking analysis demonstrated that components of HD-SB bind with high affinity to these signature targets. In vitro experiments showed that HD-SB effectively inhibited bladder cancer cell viability, colony formation, and migration.

Conclusion

This is the first study to explore the potential of HD-SB in enhancing the prognosis and treatment outcomes of bladder cancer through network pharmacology, bioinformatics, and experimental approaches. While the focus is primarily on tumor microenvironment-related factors, the findings provide valuable insights into the molecular mechanisms of HD-SB and identify potential novel targets for bladder cancer therapy.

Introduction

Bladder cancer is one of the most common malignant tumors in the urinary system^[1, 2]. Globally, the incidence of bladder cancer ranks tenth among malignant tumors, with the incidence rate in males being approximately four times that in females^[3]. According to statistics from the International Agency for Research on Cancer, there were approximately 573,000 new cases and 213,000 deaths from bladder cancer worldwide in 2020^[4]. It is projected that by 2040, the number of new cases and deaths from bladder cancer will increase to 991,000 and 397,000, respectively^[5]. Therefore, it is particularly important for the prevention and treatment of bladder cancer.

To improve the prognosis of bladder cancer, platinum-based neoadjuvant or adjuvant chemotherapy is commonly used in clinical practice currently. However, the complete response rate of neoadjuvant chemotherapy for muscle-invasive bladder cancer is only 9–46%, and the 5-year overall survival rate is increased by only about 5%; while the 5-year overall survival rate with adjuvant chemotherapy is increased by about 6%^[6–8]. Additionally, about 50% of bladder cancer patients are not suitable for platinum-based chemotherapy due to comorbidities, poor physical condition, renal insufficiency, and other reasons.

Traditional Chinese Medicine (TCM) has been utilized for centuries and has gradually become an integral component of comprehensive cancer treatment. Recognizing the limitations of contemporary clinical approaches, the integration of TCM may provide additional benefits in enhancing the overall antitumor response and improving patient outcomes in cancer care, thus establishing TCM as a significant focal point in oncological research. *Scutellaria barbata* (SB) is notably rich in flavonoids, whereas *Hedyotis diffusa* (HD) is recognized for its content of both terpenoids and flavonoids. Preclinical studies suggest their potential anticancer effects, and they are often investigated in combination for cancer therapy^[9, 10]. Recent pharmacological studies have confirmed that HD and SB can inhibit tumor cell proliferation, bolster the immune system, induce apoptosis in tumor cells, and counteract drug resistance^[11].

The term “herb-pair” refers to the use of two single herbs that are usually used together to treat a specific disease in order to enhance efficacy or minimize adverse effects^[12]. The clinical drug database analysis showed that HD-SB was the core treatment as the most common herb pair for bladder cancer^[13]. Previous study has shown that HD-SB reduce Akt activation through reducing miR-155 expression, resulting in cell apoptosis of bladder cancer cells^[13]. However, given the complexity of the components in HD-SB, its anticancer effects on bladder cancer have not been thoroughly investigated.

Therefore, utilizing network pharmacology to explore the underlying mechanisms of the HD-SB combination's efficacy in bladder cancer could offer a more profound understanding of its therapeutic potential.

Scientists advocate for extensive bioinformatics analyses of genomic, proteomic, and clinical datasets to identify prognostic biomarkers with aberrant expression and to discover potential drug targets that confer survival benefits. Moreover, identifying appropriate drug targets with prognostic signatures has become a prevalent strategy in drug development. For example, the upregulation of programmed death ligand-1 (PD-L1) has been observed in numerous urological malignancies, including bladder cancer, where it is linked to adverse prognosis and increased tumor immune evasion. The clinical use of PD-L1 inhibitors has demonstrated promising results in the treatment of advanced urological cancers, significantly improving patient outcomes^[14]. Therefore, identifying targets related to bladder cancer prognosis among the various drug targets of the HD-SB combination could be highly valuable for future pharmaceutical development.

In this investigation, we employed a synergistic approach of network pharmacology and bioinformatics to elucidate the impact of the HD-SB combination on bladder cancer. By pinpointing the intersection between HD-SB's targets and genes associated with bladder cancer prognosis, we can determine which targets HD-SB can effectively act upon, thereby improving the prognosis of bladder cancer. Additionally, we determined the relationship between these genes and tumor-infiltrating immune cells and immune checkpoint genes. Moreover, the risk score, derived from prognostic gene expression, correlated with the efficacy of immunotherapy and chemotherapy. Single-cell RNA sequencing (scRNA-seq) revealed that these genes were primarily expressed in urothelial cells but were also detectable in other cell types within the tumor microenvironment (TME). Our study not only identifies the genes through which HD-SB can influence the prognosis of bladder cancer, but also sheds light on the underlying molecular mechanisms of this TCM in the treatment of bladder cancer.

Material and methods

Identification of active ingredients and targets of HD-SB

The overall design of this study is illustrated in Fig. 1. The compounds of HD-SB were retrieved from the TCMSP database (<https://www.tcmsp-e.com/#/database>). Oral bioavailability (OB), defined as the fraction of a drug that reaches the systemic circulation after administration, was considered, with a threshold set at $OB \geq 30\%$. Drug likeness (DL), representing the similarity between a chemical compound and known drugs, was also taken into account, with a cut-off value of $DL \geq 0.18$. Subsequently, the primary components of HD-SB and their corresponding targets were identified. Additionally, the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>) was consulted to obtain compound ID numbers, and the SMILES chemical structure was used for target prediction via the SwissTargetPrediction database (<http://swisstargetprediction.ch/>). Through an online search of targets

for HD-SB components in the TCMSP and SwissTargetPrediction databases, a total of 561 targets were compiled.

Identification of bladder cancer-related targets

The targets associated with bladder cancer were identified through searches in multiple databases, including GeneCards (<https://www.genecards.org/>), MalaCards (<https://www.malacards.org/>), DisGeNET (<https://disgenet.com/>), Therapeutic Target Database (<https://db.idrblab.net/ttd/>), OMIM (<https://www.omim.org/>), and PharmGKB (<https://www.pharmgkb.org/>). The targets retrieved from these databases were consolidated, with duplicates removed, resulting in a final set of 11,352 unique bladder cancer-related targets, which were then used for subsequent analysis.

GO enrichment and KEGG pathway analysis

To identify the overlapping targets between HD-SB and bladder cancer, the intersection was determined using the VennDiagram package (version 1.7.3), and a Venn diagram was constructed for visualization. To explore the biological functions of the potential targets in bladder cancer, we performed functional enrichment analysis of the intersecting targets using the "clusterProfiler" R package (version 4.10.1). Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was conducted to identify key signaling pathways involved in biological processes, while Gene Ontology (GO) analysis was used to examine biological processes (BP), cellular components (CC), and molecular functions (MF) of the intersecting targets.

Construction of the herb-compound-target network

Using Cytoscape (version 3.10.2), a powerful tool for visualizing and analyzing network biology, we constructed a comprehensive network that integrates herbs, their corresponding active compounds, and the associated biological targets. This platform allows for the integration of diverse biological data layers, enabling the exploration of complex interactions and providing valuable insights into the molecular mechanisms underlying the therapeutic effects of the herbs.

Gene expression and clinical data collection

Gene expression profiles and clinical data for bladder cancer patients were obtained from The Cancer Genome Atlas (TCGA) database (<https://www.cancer.gov/tcga>). Genes with low expression (defined as zero expression in more than 50% of samples) were excluded from the analysis. The final cohort included 396 tumor samples, after excluding those with incomplete survival data or survival times shorter than 30 days. Gene expression and clinical data related to responses to PD-L1 inhibitor Atezolizumab in metastatic urothelial carcinoma were retrieved from the Imvigor210 study. To gain a

comprehensive understanding of the expression of key genes within the TME, scRNA-seq data for bladder cancer was obtained from the GEO database (GSE135337).

Characterization and screening of prognostic genes

Kaplan-Meier survival analysis was conducted using the R packages “survival” (version 3.7-0) and “survminer” (version 0.5.0). Univariate Cox regression analysis was performed to identify genes associated with clinical prognosis, which were designated as prognostic genes. These prognostic genes were then intersected with the predicted targets using the VennDiagram package (version 1.7.3). The resulting intersecting genes, which were targets of both HD-SB and bladder cancer, were also associated with the overall survival of bladder cancer patients within the TCGA database. These genes were subsequently selected for further analysis.

Construction of a prognostic signature

Genes with a statistically significant association to prognosis, defined by a P value < 0.01 , were selected from the univariate Cox regression analysis. The “glmnet” package was employed to perform least absolute shrinkage and selection operator (LASSO) regression for further selection of predictors. Genes with non-zero coefficients were then included in a backward stepwise regression analysis. Ultimately, 15 optimal predictive genes and their corresponding coefficients were identified. The risk score for each sample was calculated using the following formula:

$$\text{Risk score} = \sum_i^n \text{Coef}(\text{gene } i) * \text{Expression}(\text{gene } i)$$

The coefficient of each gene in the multivariate Cox regression model is denoted as $\text{Coef}(\text{gene } i)$, while $\text{Expression}(\text{gene } i)$ refers to the expression level of gene i , and n represents the total number of genes. Patients were stratified into high-risk and low-risk groups based on the median risk score. Survival differences between the high- and low-risk groups were compared using Kaplan-Meier survival analysis and log-rank tests. Furthermore, time-dependent receiver operating characteristic (ROC) curves and the area under the curve (AUC) were utilized to evaluate the accuracy and specificity of the risk score in predicting survival outcomes.

Immune microenvironment

The “CIBERSORT” analytical tool was employed to quantify the abundance of 22 immune cell types in the TCGA cohort based on gene expression data. Spearman correlation analysis was conducted to examine the relationships between the risk score and the relative proportions of immune cells. Additionally, the expression levels of immune checkpoint genes were compared between the high-risk and low-risk groups. Furthermore, the Spearman correlation method was used to assess the relationship between model genes and the proportions of immune cells as well as the expression of immune checkpoint genes. The results were subsequently visualized and analyzed using the “corrplot” package.

Analysis of immunotherapy response and chemotherapy sensitivity

Tumor Immune Dysfunction and Exclusion (TIDE) was used to predict patients' responses to immunotherapy based on published data and gene expression profiles. TIDE scores were obtained by uploading gene expression data to the official website (<http://tide.dfci.harvard.edu/>). The difference in TIDE scores between the high- and low-risk groups in the TCGA cohort was analyzed using the Wilcoxon rank sum test. A higher TIDE score was indicative of a poor response to immune checkpoint blockade (ICB) and shorter survival following treatment. Genes associated with overall survival in the Imvigor210 cohort were identified and intersected with the predefined set of 15 prognostic genes. Kaplan-Meier survival analysis was then performed to assess survival differences between patients with distinct expression profiles of the intersecting genes. In addition, we performed drug sensitivity analysis using the Genomics of Drug Sensitivity in Cancer (GDSC) database (<https://www.cancerrxgene.org/>). Based on GDSC online database, the half maximal inhibitory concentration (IC₅₀) value of chemotherapeutic drugs for each patient was calculated, and the differences between high- and low-risk groups were also determined by Wilcoxon rank sum test.

ScRNA-seq data analysis

The analysis was performed using the Seurat package (version 3.1.1). The resulting Seurat objects underwent quality control and normalization, followed by the identification of highly variable genes, dimensionality reduction for cell clustering, and differential expression analysis between clusters. Cell types were then annotated based on the original annotation method provided in the reference literature^[15].

Molecular docking

The primary active components identified in the core target network were selected for molecular docking with the key targets. Firstly, crystal structures of protein receptors were obtained from RCSB Protein Data Bank (PDB) (<https://www.rcsb.org/>) database, Pymol software was applied to remove water molecules and split the ligand and receptor. The center of the original ligand was set as the center of docking box for corresponding receptor. For receptors without original ligands, Proteins.Plus (<https://proteins.plus/>) was used to predict docking boxes. Subsequently, hydrogens were added to the proteins, the charges were calculated and the AD4 atom type was assigned with AutoDockTools V1.5.6 software. The final results were exported in PDBQT format. Secondly, the SDF files for the active ingredients were obtained from the PubChem database. Then we used Chem3D V14.0 software to optimize the structures and saved them in MOL2 format. Subsequently, the MOL2 format files of the bioactive compounds were imported into the AutoDockTools V1.5.6 software to adjust the charges, determine the roots of the ligands, and select the ligands' rotatable bonds, and the results were saved in PDBQT format. Finally, the docking parameters were defined. Molecular docking was performed using AutoDock Vina software, and the docking results were visualized using PyMOL software.

Immunohistochemistry analysis of prognostic-related targets

Immunohistochemical data were obtained from the Human Protein Atlas (HPA) (<http://www.proteinatlas.org/>). The protein expression levels of prognostic-related targets were compared between bladder cancer tissues and normal bladder tissues using data from the HPA.

Cell line and cell culture

Bladder cancer cell lines (T24 and 5637) were obtained from the Cell Bank of the Chinese Academy of Sciences in Shanghai, China. These cell lines were cultured in RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and maintained in a humidified incubator at 37°C with 5% CO₂. Cells were subcultured when they reached approximately 90% confluence, and only those in the logarithmic growth phase were used for experimental analyses.

Preparation of HD-SB

In the present study, HD-SB is an herb pair containing equivalent weights of *Hedyotis diffusa* and *Scutellaria barbata*, both sourced as ready-to-use decoction pieces from the First Affiliated Hospital of Henan University of Chinese Medicine, and conforming to the standards of the Chinese Pharmacopeia. The decoction pieces of *Scutellaria barbata* (batch number: 2404222) and *Hedyotis diffusa* (batch number: 18063002) were processed into decoction formulations, which were then subjected to vacuum freeze-drying. The resulting lyophilized powder was stored in a dark, low-temperature environment to maintain its integrity and stability. A stock solution (100 mg/mL) was prepared using purified water and stored at -20°C for subsequent experiments.

Cell viability assay

The effect of HD-SB on the viability of bladder cancer cells was assessed using the Cell Counting Kit-8 (CCK-8; GK10001; GLPBIO, China). The T24 and 5637 cells were respectively seeded at densities of 2,000 cells and 3,000 cells per well in each of the non-edge wells of 96-well plates and cultured in RPMI-1640 medium containing varying concentrations of HD-SB solution. After 48 hours of incubation, 10 µL of CCK-8 reagent was added to each well, and the cells were further incubated at 37°C with 5% CO₂ for 1 hour. The absorbance (optical density, OD) at 450 nm was measured using a microplate reader (Biotek, Winooski, Vermont, USA). Cell viability was calculated using the following formula: cell viability (%) = (OD value of treatment well/OD value of control well) × 100. The experiment was performed in triplicate.

Colony formation assay

The T24 and 5637 cells were individually seeded at densities of 1,000 cells and 2,000 cells per well in 6-well plates. Following a 48-hour incubation period, the cells were treated with varying concentrations of HD-SB solution. After 7 days, each well was gently washed with chilled phosphate-buffered saline (PBS). The cell colonies were then fixed and stained using a solution of 4% paraformaldehyde and 1% crystal

violet. Subsequently, the wells were washed with PBS until the stained colonies were clearly visible to the naked eye. The entire experiment was conducted in triplicate to ensure reproducibility.

Wound healing assay

The T24 and 5637 cells were seeded into 12-well plates. Once the cells reached near-confluence (approximately 100%), a 200- μ L pipette tip was used to create uniform scratches across each well. The wells were then gently washed with PBS to remove cellular debris. Subsequently, HD-SB solution was diluted in medium containing 1% serum and added to each well. The cells were treated with varying concentrations of the HD-SB solution. Scratch healing was monitored using an inverted microscope at 4 $\times$ magnification, and images were captured to document the progression of wound closure. The experiment was conducted in triplicate to ensure reproducibility and consistency of the results.

Statistical analysis

Statistical analyses were performed using R (version 4.3.3) and GraphPad Prism software (version 10.1.2). The results are presented as values and percentages of the total. The chi-square test is used to determine the difference between two groups. A two-tailed *P*-value of less than 0.05 was considered statistically significant.

Results

Active ingredients and corresponding targets of HD-SB

A network-based pharmacology approach was employed to investigate the mechanisms through which HD-SB influences bladder cancer. The active components of HD-SB were retrieved from the TCMSP database. By applying the selection criteria of oral bioavailability ($OB \geq 30\%$) and drug-likeness ($DL \geq 0.18$), seven active compounds from HD and twenty-nine from SB were identified. After removing three duplicate compounds, a total of 33 unique chemical compounds were retained. An online search for targets associated with these compounds in the TCMSP and SwissTargetPrediction databases identified 516 potential targets.

Enrichment analysis and herb-compound-target-disease network

After eliminating duplicates, a total of 11,352 bladder cancer-related targets were retrieved from various disease databases. Venn diagram analysis revealed 497 common targets shared between HD-SB and bladder cancer (Fig. 2A). These overlapping targets were considered potential critical targets for HD-SB in the treatment of bladder cancer and were selected for subsequent analysis. To explore the functional roles and enriched pathways of HD-SB in bladder cancer, we conducted KEGG pathway and GO enrichment analyses. The PI3K-Akt signaling pathway was found to be strongly associated with both HD-SB and bladder cancer (Fig. 2B). The top five categories from GO BP, CC, and MF were selected for

visualization. As shown in Fig. 2C, the BPs predominantly involved response to xenobiotic stimuli, positive regulation of transferase activity, response to oxidative stress response, peptidyl-serine phosphorylation, and response to reactive oxygen species. MFs primarily included protein serine/threonine kinase activity, protein serine kinase activity, protein tyrosine kinase activity, nuclear receptor activity, and ligand-activated transcription factor activity (Fig. 2C). Furthermore, the data for the 33 active chemical components of HD-SB and their 497 corresponding targets were analyzed using Cytoscape 3.10.2, which facilitated the construction of the herb-compound-target-disease interaction network shown in Fig. 2D.

Identification of key prognostic genes with the best predictive value

Univariate Cox regression was performed to select 1,641 prognostic genes significantly associated with the overall survival of bladder cancer patients in TCGA-BLCA dataset ($P < 0.01$, Table S1). The intersection of these 1,641 prognostic genes and 497 prediction genes was depicted in the Venn diagram (Fig. 3A), with 28 overlapping genes retained for further analysis. To reduce dimensionality and prevent overfitting, LASSO regression was applied (Fig. 3B and 3C), resulting in the selection of 15 prognostic genes. Details of the 15 genes with their gene symbols, Ensembl IDs, coefficients and results of univariate Cox regression analysis were summarized in Table 1. The Sankey diagram illustrates the 15 genes and their associated signaling pathways, including the calcium signaling pathway, focal adhesion, Ras signaling pathway, MAPK signaling pathway, JAK-STAT signaling pathway, and PI3K-Akt signaling pathway (Fig. 3D). The hazard ratios (HRs) and their corresponding confidence intervals for these genes, derived from multivariate Cox regression analysis, are presented in a forest plot (Fig. 3E).

Table 1
Details of the 15 prognostic genes identified through LASSO regression analysis

Gene symbol	Ensembl ID	Coefficient	Univariate Cox regression analysis		
			HR	95% CI	P-value
VEGFA	7422	-0.087437357	0.573	0.424–0.775	< 0.001
EGFR	1956	0.089549156	1.586	1.174–2.142	0.003
MYC	4609	0.035967395	1.561	1.156–2.107	0.004
PDGFRA	5156	0.054562822	1.517	1.123–2.047	0.007
JUN	3725	0.035425798	1.497	1.11–2.02	0.008
FN1	2335	0.012432036	1.605	1.186–2.172	0.002
PTPN6	5777	-0.398539515	0.449	0.329–0.613	< 0.001
PTGER3	5733	0.003567306	1.578	1.164–2.139	0.003
MAP2	4133	0.098577931	1.853	1.367–2.512	< 0.001
CALM1	801	0.201663678	1.653	1.224–2.232	0.001
CTSV	1515	0.021915735	1.489	1.104–2.008	0.009
CES1	1066	0.057060483	1.497	1.109–2.02	0.008
ADRA1D	146	0.06077367	1.542	1.143–2.079	0.005
PYGL	5836	0.057661814	1.588	1.176–2.145	0.003
PLA2G1B	5319	1.521336633	0.618	0.458–0.834	0.002

Development and validation of a prognostic model based on 15 genes

To evaluate the collective prognostic impact of these genes, risk scores were calculated for each patient based on their respective regression coefficients. The 396 patients in the TCGA-BLCA dataset were subsequently classified into high-risk and low-risk groups using the median risk score as the cutoff value. The clinical characteristics of the patients are summarized in Table 2. The high-risk group was associated with more advanced disease features, including higher stage, grade, and pathological TN stages, as well as lower survival rates and shorter overall survival.

We further evaluated the distribution of risk scores in bladder cancer patients stratified by survival status. By ranking patients based on increasing risk scores, we observed poorer survival outcomes and a higher incidence of death in the high-risk group (Fig. 4A and 4B). A heatmap of the 15 genes revealed the altered expression patterns associated with varying risk scores (Fig. 4C). Kaplan-Meier analysis demonstrated significantly better overall survival in the low-risk group compared to the high-risk group

($P < 0.0001$; Fig. 4D), with median survival times of 19.8 months and 15.9 months, respectively. The gene-based risk score model demonstrated satisfactory predictive accuracy, with AUC values of 0.66, 0.72, and 0.71 for 1-, 3-, and 5-year survival predictions, respectively (Fig. 4E).

Correlation between risk score, tumor immune infiltrating cells and immune checkpoint genes.

CIBERSORT analysis was performed to assess the infiltration of immune cells across different risk groups. Linear regression analysis further revealed a negative correlation between risk scores and the levels of immune infiltration in CD8 T cells, naive CD4 T cells, follicular helper T cells, regulatory T cells, gamma delta T cells, memory B cells, activated NK cells, monocytes, and activated dendritic cells. In contrast, the levels of immune infiltration in memory resting CD4 T cells, M0 macrophages, M1 macrophages, and M2 macrophages were positively correlated with risk scores (Fig. 5 and Figure S1).

Additionally, we compared the expression levels of immune checkpoint genes between the high-risk and low-risk groups. As shown in Fig. 6, the expression of immune checkpoint genes was significantly dysregulated between the two groups. Notably, most of these genes, including CD274, CD44, CD86, and TNFRSF4, were upregulated in the high-risk group.

Table 2
Clinical characteristics of bladder cancer patients in the TCGA cohort

Clinical features	Bladder cancer patients			
	Total	Low-risk group	High-risk group	<i>P</i> -value
Patients, no (%)	396 (100)	198 (50)	198 (50)	
Median age (years)	68	67.0	69.5	0.050
Gender, no (%)				0.016
Female	104 (26.3)	41 (20.7)	63 (31.8)	
Male	292 (73.7)	157 (79.3)	135 (68.2)	
Stage, no (%)				< 0.001
I	2 (0.5)	2 (1.0)	0 (0)	
II	124 (31.3)	83 (42.3)	41 (20.7)	
III	138 (34.8)	57 (29.1)	81 (40.9)	
IV	130 (32.8)	54 (27.6)	76 (38.4)	
NA	2 (0.5)	2 (1.0)	0	
Grade, no (%)				< 0.001
High grade	375 (94.7)	178 (89.9)	197 (99.5)	
Low grade	18 (4.5)	17 (8.6)	1 (0.5)	
NA	3 (0.8)	3 (1.5)	0 (0.0)	
Pathologic T, no (%)				< 0.001
T0 + T1	4 (1.0)	4 (2.0)	0 (0.0)	
T2	113 (28.5)	72 (36.4)	41 (20.7)	
T3	190 (48.0)	76 (38.4)	114 (57.6)	
T4	57 (14.4)	24 (12.1)	33 (16.7)	
NA	32 (8.1)	22 (11.1)	10 (5.1)	
Pathologic N, no (%)				0.061
N0	229 (57.8)	122 (61.6)	107 (54.0)	
N1	44 (11.1)	16 (8.1)	28 (14.1)	
N2	75 (18.9)	31 (15.7)	44 (22.2)	

Clinical features	Bladder cancer patients			
	Total	Low-risk group	High-risk group	<i>P</i> -value
N3	7 (1.8)	5 (2.5)	2 (1.0)	
NA	41 (10.4)	24 (12.1)	17 (8.6)	
Pathologic M, no (%)				< 0.001
M0	189 (47.7)	115 (58.1)	74 (37.4)	
M1	10 (2.5)	5 (2.5)	5 (2.5)	
Unknown	197 (49.7)	78 (39.4)	119 (60.1)	
Status, no (%)				< 0.001
Alive	220 (55.6)	140 (70.7)	80 (40.4)	
Dead	176 (44.4)	58 (29.3)	118 (59.6)	
Median survival (months)	18.0	19.8	15.9	< 0.001

Correlation analysis of model genes, tumor immune infiltrating cells, and immune checkpoint genes

The expression of all model genes was found to be correlated with tumor immune infiltrating cells (Fig. 7A). Furthermore, these model genes were also associated with the expression of immune checkpoint genes (Fig. 7B). For instance, CD274 exhibited a positive correlation with the expression of EGFR, MYC, PDGFRA, JUN, FN1, PTGER3, MAP2, CALM1, CTSV, ADRA1D, and PYGL in bladder cancer. In contrast, CD274 showed a negative correlation with VEGFA and PLA2G1B in bladder cancer (Figure S2).

The prognostic genes-based risk score was associated with immunotherapy and chemotherapeutic efficacy

TIDE analysis was conducted to evaluate the potential clinical benefit of immunotherapy across different risk groups. Our results showed that patients in the low-risk group had lower TIDE scores (Fig. 8A), suggesting a better predicted response to immunotherapy. In the Imvigor210 cohort, patients with high expression of EGFR and MYC exhibited a poorer response to immune checkpoint inhibitor treatment compared to those with low expression of EGFR and MYC (Fig. 8B and 8C).

We conducted a sensitivity analysis on 198 drugs from the GDSC database and found that the IC50 values for many chemotherapeutic agents were significantly lower in the low-risk group (Fig. 9), indicating that patients in this group were more sensitive to chemotherapy. We then focused on cisplatin, a widely used first-line chemotherapy drug for bladder cancer. The results showed that patients in the low-risk group had lower IC50 values for cisplatin (Fig. 9), suggesting a better response to cisplatin treatment. These findings collectively indicate that our risk signature is associated with therapeutic

efficacy in bladder cancer patients, and that targeting these prognostic genes with HD-SB may enhance the efficacy of both immunotherapy and chemotherapy.

Single-cell RNA-sequencing data

In the present study, we profiled the transcriptome of single cells from the scRNA-seq data of GSE135337, creating a comprehensive atlas of the TME. After quality control and correction for batch effects, the core cells were classified into 15 distinct clusters using the UMAP algorithm (Fig. 10A). These clusters were annotated by identifying marker genes, resulting in four primary cell types: urothelial cells, myeloid/macrophage cells, T cells, and fibroblasts (Fig. 10B). By intersecting prognostic genes with marker genes obtained from the single-cell analysis, we identified six dysregulated genes across multiple cell clusters. These genes were primarily expressed in urothelial cells but were also detectable in other cell types within the TME (Figs. 10C and 10D).

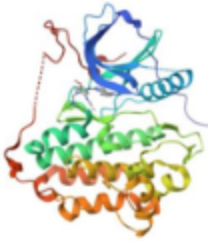
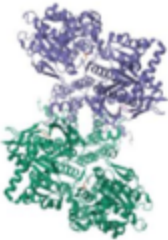
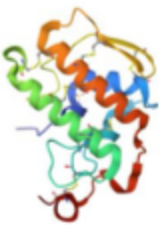
Validation based on immunohistochemistry

To further validate the prognostic signature, we compared the expression levels of six prognostic targets —VEGFA, MYC, JUN, FN1, PTPN6, and CALM1—between normal bladder and bladder cancer tissues using data from the HPA. Immunohistochemical analysis revealed that the expression of these proteins was upregulated in bladder cancer tissues. These findings confirm the differential expression of these targets between normal bladder and bladder cancer tissues, as illustrated in Fig. 11.

Molecular docking identification of pharmaceutical constituents in HD-SB

Based on the results of network pharmacology, we identified the top three compounds by degree centrality: quercetin, luteolin, and wogonin. These compounds were subjected to molecular docking analysis with four specific targets among the fifteen prognostic targets, namely EGFR (PDB ID: 6LUD), JUN (PDB ID: 4IZY), PLA2G1B (PDB ID: 3ELO), and PYGL (PDB ID: 2QLL). The binding affinities of the compounds with these proteins are presented in Table 3, and the optimal docking configurations of the receptor-ligand complexes are visualized in Fig. 12 and Figure S3. Our results demonstrate that all three compounds can interact with EGFR, JUN, PYGL, and PLA2G1B. It is generally accepted that a binding energy of less than -4 kcal/mol between a ligand and a receptor indicates potential binding activity, while a binding energy of less than -5 kcal/mol suggests strong binding affinity. The molecular docking results show that the binding energies of all interactions are less than -5 kcal/mol, indicating high-affinity binding between the compounds and the target proteins.

Table 3. The binding energy of primary active components and specific targets (kcal/mol)

Target	PDB ID	Target Structure	Components	Affinity (kcal/mol)
EGFR	6LUD		Luteolin	-7.6
			Quercetin	-7.9
			wogonin	-7.5
JUN	4IZY		Luteolin	-7.6
			Quercetin	-7.4
			wogonin	-7.2
PYGL	2QLL		Luteolin	-8.3
			Quercetin	-8.4
			wogonin	-8.0
PLA2G1B	3ELO		Luteolin	-6.5
			Quercetin	-6.8
			wogonin	-6.4

The impact of HD-SB on the viability and colony formation of bladder cancer cells

As shown in Fig. 13, HD-SB effectively inhibited bladder cancer cell viability in a dose-dependent manner (Fig. 13A-B). This inhibitory effect was further confirmed by the colony formation assay, which revealed a significant decrease in the number of cell colonies as drug concentrations increased (Fig. 13C-D). These results collectively underscore the cytotoxic effects of HD-SB on bladder cancer cells.

The impact of HD-SB on the migration of bladder cancer cells

To explore the impact of HD-SB on the migratory capacity of bladder cancer cells, a wound healing assay was conducted using different concentrations of HD-SB. The results demonstrated that HD-SB

significantly inhibited the lateral migration capacity of bladder cancer cells in a concentration-dependent manner (Fig. 14).

Discussion

According to the ancient texts of Shennong's Herbal Classics, a multitude of TCM are recognized for their anti-tumor capabilities, with a history of use in China spanning millennia. We employed an integrative approach of network pharmacology and bioinformatics to investigate the mechanisms of the TCM prescription HD-SB within the bladder cancer microenvironment. Network pharmacology was instrumental in delineating the material basis of the HD-SB formula, while bioinformatics analysis deepened our understanding of prognostic targets and the molecular underpinnings of its clinical benefits. TCM has been recognized for its dual therapeutic roles in oncology: bolstering the body's immune defenses (Fu Zheng) and targeting malignant elements (Qu Xie)^[16, 17]. This research not only sheds new light on the pharmacological effects of TCM in the context of "Fu Zheng and Qu Xie" but also paves the way for precision medicine strategies in bladder cancer immunotherapy.

In this study, we utilized network pharmacology to identify 497 HD-SB targets associated with bladder cancer. To elucidate the intricate interplay between drug targets and the material foundation of the herbal formula HD-SB, we constructed a unique herb-compound-target network. Subsequently, we performed Cox regression analysis on data from TCGA database, identifying 1,641 genes correlated with bladder cancer patient prognosis. By conducting intersection analysis, we identified 28 genes that were both potential targets of the HD-SB and prognostic markers for bladder cancer. Using LASSO regression, we further refined this list to 15 prognostic genes, as presented in Table 1, which exhibit substantial correlation with bladder cancer patient outcomes. To validate our computational analysis, we corroborated the expression patterns of these 15 prognostic genes with the existing scientific literature. Abnormal expression of these genes in bladder cancer has been widely documented. For instance, PTGER3, a gene involved in alternative splicing, has been linked to survival, clinicopathological features, the TME, and responses to immunotherapy in bladder cancer^[18]. ADRA1D, associated with lymph node status, has been crucial for the development of a genomic-clinicopathologic nomogram aimed at preoperative lymph node metastasis prediction^[19]. Notably, PYGL and PLA2G1B are being reported in this study for the first time.

In addition, we developed a novel gene-signaling pathway network map (Fig. 3D) to elucidate the interactions among prognostic genes and the enrichment of signaling pathways. Our findings suggest that HD-SB may target specific genes, thereby influencing these pathways and ultimately affecting the prognosis of bladder cancer patients. To further validate the prognostic significance of these genes in bladder cancer patients, we constructed a prognostic model incorporating 15 genes. By applying the coefficients of each gene, we calculated a risk score for individual patients. This prognostic signature enabled us to categorize bladder cancer patients into high- and low-risk groups based on the median risk score. Our analysis showed that patients in the high-risk group had significantly poorer overall survival compared to those in the low-risk group. Additionally, the ROC curve confirmed the accuracy and

reliability of this signature in predicting patient prognosis. These findings further suggest that HD-SB may influence prognosis by targeting these genes.

Recent research indicates that the synergy between TCM and immunotherapy can significantly enhance anti-tumor efficacy^[20]. This aligns with the immunotherapeutic strategies that aim to reconfigure the TME to exert anti-neoplastic effects. The TME is populated by a multitude of immune cells, which may influence tumor initiation and progression^[21]. Consequently, we explored the correlation between risk scores and the distribution of immune cells within the TME. Notably, there was a strong association between risk scores and the presence of immune cells in the TME. Furthermore, immune checkpoints are pivotal in tumor evolution and resistance to therapy^[22–24]. It is noteworthy that the expression levels of immune checkpoint genes are also deregulated in patients with varying risk scores. Considering that immune scores are derived from 15 prognostic genes, we also conducted analyses to elucidate the relationships among these genes, immune cell populations, and immune checkpoint expression. Our findings revealed significant correlations, suggesting that HD-SB may exert an influence on both immune cell populations and immune checkpoint expression through its interactions with these model genes.

Prior research has established that molecular subtypes of bladder cancer can predict clinical outcomes following ICB therapy^[25, 26]. Building on the aforementioned relationship, we posit that risk scores may also correlate with responses to immunotherapy. To test the hypothesis, we examined this association using the TIDE score, a metric that assesses the probability of immunotherapy success, with higher scores suggesting a higher risk of immune evasion and a diminished potential for ICB therapy benefits. Our findings supported our hypothesis, as bladder cancer patients classified in the high-risk group exhibited elevated TIDE scores, which are indicative of a poorer response to immunotherapy. In the Imvigor210 cohort^[27–29], a comprehensive analysis was conducted on 298 pre-treatment tumor samples through transcriptome RNA sequencing to assess integrated biomarkers. Upon investigating the prognostic roles of model genes within this cohort, we identified that elevated expression levels of two genes, EGFR and MYC, were associated with reduced survival in patients with metastatic urothelial carcinoma undergoing PD-L1 blockade with atezolizumab. EGFR is a receptor on the surface of cells that, when activated, triggers a cascade of signaling pathways involved in cell growth, survival, and proliferation. In EGFR-mutant cancers, especially in non-small cell lung cancer, the presence of EGFR mutations is generally associated with lower response rates to immune checkpoint inhibitors^[30]. Previous study has shown that MYC inhibition induced tumor cell-intrinsic immune responses, and promoted CD8⁺ T cell infiltration. Mechanistically, MYC inhibition increased CD8⁺ T cell-recruiting chemokines by inducing the DNA damage related cGAS-STING pathway^[31]. Moreover, the combination of c-Myc inhibitor and anti-PD-L1 antibody exerted synergistic inhibition of human pancreatic ductal adenocarcinoma growth^[32]. Noteworthy, resistance to PD-L1 inhibitors, whether intrinsic or acquired, continues to pose a significant challenge, and the majority of bladder cancer patients do not respond to immune checkpoint inhibitors^[33, 34]. Interestingly, our results suggest that HD-SB may modulate the response to immunotherapy by influencing the expression of key model genes, particularly EGFR and MYC. This investigation may offer novel insights into the nexus between the TME immune theory and the

TCM principles of “Fu Zheng” (strengthening the body’s defenses) and “Qu Xie” (expelling pathogenic factors).

We also aimed to assess whether prognostic genes were associated with chemotherapy response. The TCGA has significantly advanced our understanding of cancer genomics by providing extensive datasets linking genomic alterations to various cancer types, subtypes, and clinical outcomes. The GDSC database further complements this by offering drug sensitivity data correlated with genetic profiles, thereby aiding in the identification of chemotherapy drugs that may be most effective for cancers with specific genetic alterations. The integration of TCGA risk scores and chemosensitivity data holds substantial promise for refining treatment strategies in oncology. In the present study, we calculated the IC50 values for various chemotherapy drugs in different patient groups using the GDSC database. Notably, the IC50 values were elevated for many drugs in the high-risk group. As a platinum-based chemotherapy agent, cisplatin remains one of the most widely used and effective chemotherapy agents for muscle invasive and metastatic bladder cancer. Our analysis also reveals that patients in the high-risk group exhibited higher IC50 values, suggesting a greater likelihood of cisplatin resistance. However, HD-SB may enhance chemosensitivity by modulating model genes and influencing the associated risk score. These findings offer insights into the potential for improving chemotherapy response through TCM.

Although the molecular characterization of bladder cancer has been extensively studied, the TME remains heterogeneous due to its diverse components. Consequently, exploring the various components of the TME is crucial for elucidating the mechanisms of bladder cancer and developing innovative therapeutic strategies. In light of this, an increasing number of studies have employed scRNA-seq to mitigate tissue heterogeneity by clustering individual cells^[35, 36]. Furthermore, scRNA-seq enables the precise evaluation of gene functions within specific cell types, thereby facilitating the investigation of molecular mechanisms involved in the initiation, progression, and metastasis of malignancies^[35, 36]. To identify cell clusters potentially affected by HD-SB, we conducted an scRNA-seq analysis using publicly available data from the GEO database. By distinguishing cell clusters and identifying highly variable marker genes, we observed that six prognostic genes were differentially expressed across different cell clusters. Notably, these genes were primarily expressed in urothelial cells but were also detectable in other cell types within the TME. This finding suggests that HD-SB may influence the prognosis of bladder cancer through its effects on various cells within the TME.

Molecular docking is a computational method employed in bioinformatics, computational chemistry, and structural molecular biology to predict the physical interactions between small molecules and proteins or other biomacromolecules. The process is often referred to as “docking”, which is analogous to how two molecules “dock” together like ships in a harbor. In the context of network pharmacology, HD-SB comprises over 33 components that can target 561 potential targets. Notably, a single component may interact with multiple targets, and a single target may be influenced by several components. Based on the aforementioned analysis, we conducted molecular docking to confirm the interactions between these components and their targets. As a powerful tool in the drug discovery pipeline, molecular docking

facilitates a detailed understanding and prediction of molecular interactions, which is crucial for gaining insights into biological processes and developing new therapeutics for bladder cancer.

In our comprehensive analysis of HD-SB, we acknowledge several limitations in our study. First, we did not perform animal experiments. Future research could employ a murine orthotopic bladder cancer model to assess whether HD-SB enhances the efficacy of chemotherapy and immunotherapy. Second, although HD-SB is widely used in clinical practice for bladder cancer patients, its impact on survival remains unclear. A randomized controlled trial would be crucial to address this question. Third, the specific mechanisms of HD-SB require further validation through multimodal analyses, including RNA sequencing, metabolomics, and proteomics.

Conclusion

In summary, we employed network pharmacology, bioinformatics analyses, and experimental validation to explore the potential active compounds, targets, and pathways of HD-SB in the treatment of bladder cancer. Our findings suggest that HD-SB may improve the prognosis of bladder cancer patients by interacting with multiple genes, modulating immune cell populations, and influencing immune checkpoint expression within the TME. Consequently, HD-SB could potentially enhance the response to chemotherapy and immunotherapy in bladder cancer patients. This study is the first to investigate the potential of HD-SB in improving prognosis and treatment outcomes for bladder cancer. These insights help clarify the molecular mechanisms of HD-SB and identify novel targets for bladder cancer therapy.

Declarations

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Not applicable.

Author Contributions

Y.L.: Methodology, Formal analysis, Investigation, Validation, Visualization, Writing-original draft, Writing - review & editing.; L.D.: Methodology, Data curation, Formal analysis, Investigation, Validation, Visualization, Writing - review & editing; M.Z.: Methodology, Resources, Software; Z.Z. (Zhenzhen Zhou): Methodology, Formal analysis. P.Z.: Methodology, Data curation; Y.Z.: Conceptualization, Project administration, Resources, Supervision, Writing - review & editing; X.Z.: Conceptualization, Resources, Supervision, Writing - review & editing; Z.Z. (Zhaowei Zhu): Conceptualization, Resources, Software, Supervision, Project administration, Writing - review & editing. All authors have read and agreed to the published version of the manuscript.

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Data Availability

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Declaration

Ethics approval and consent to participate

Not applicable.

Competing interest

The authors declare no competing interests.

Conflicts of Interest

The authors declare no conflicts of interest.

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Figures

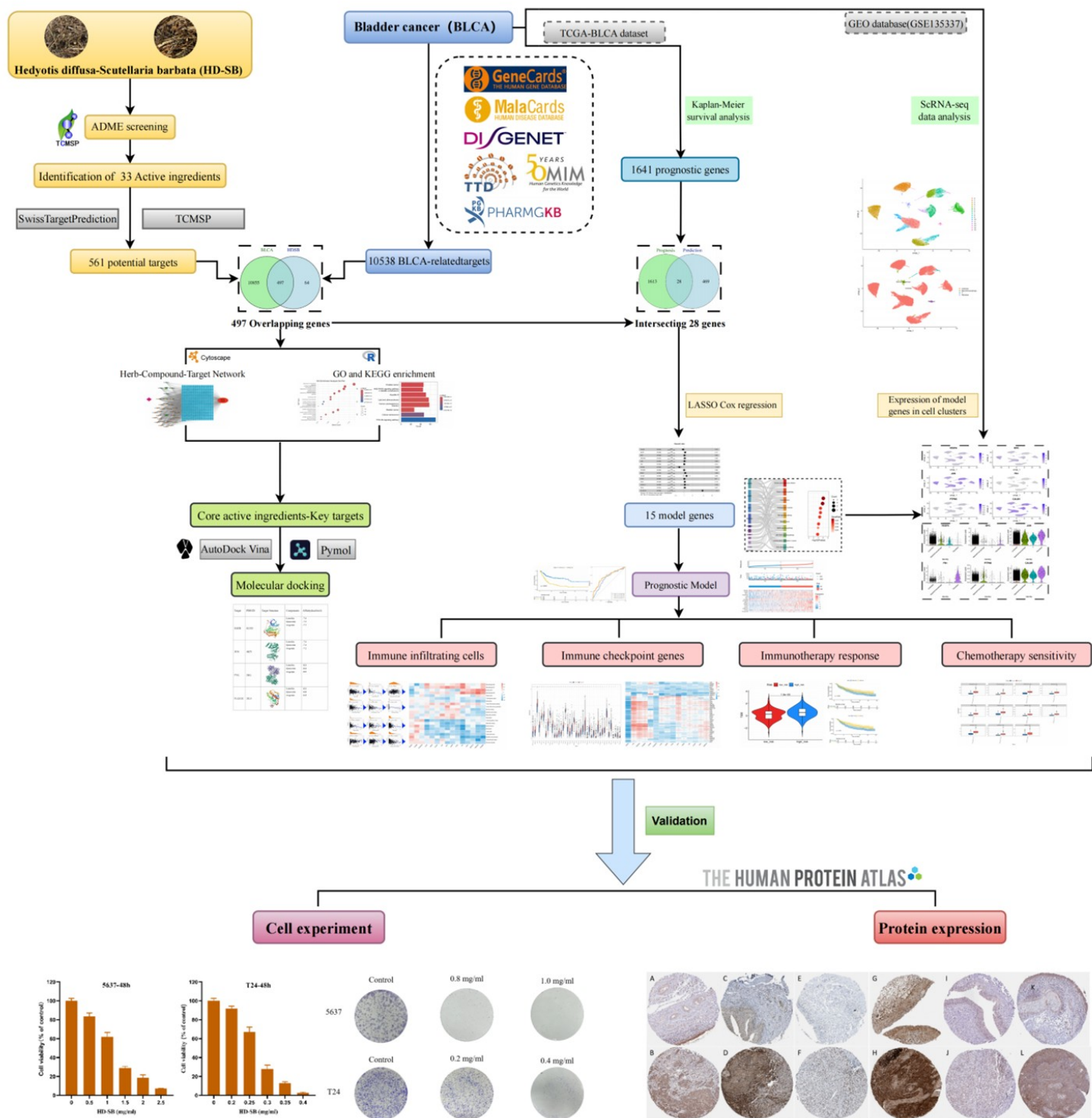


Figure 1

The graphical abstract illustrates the research process.

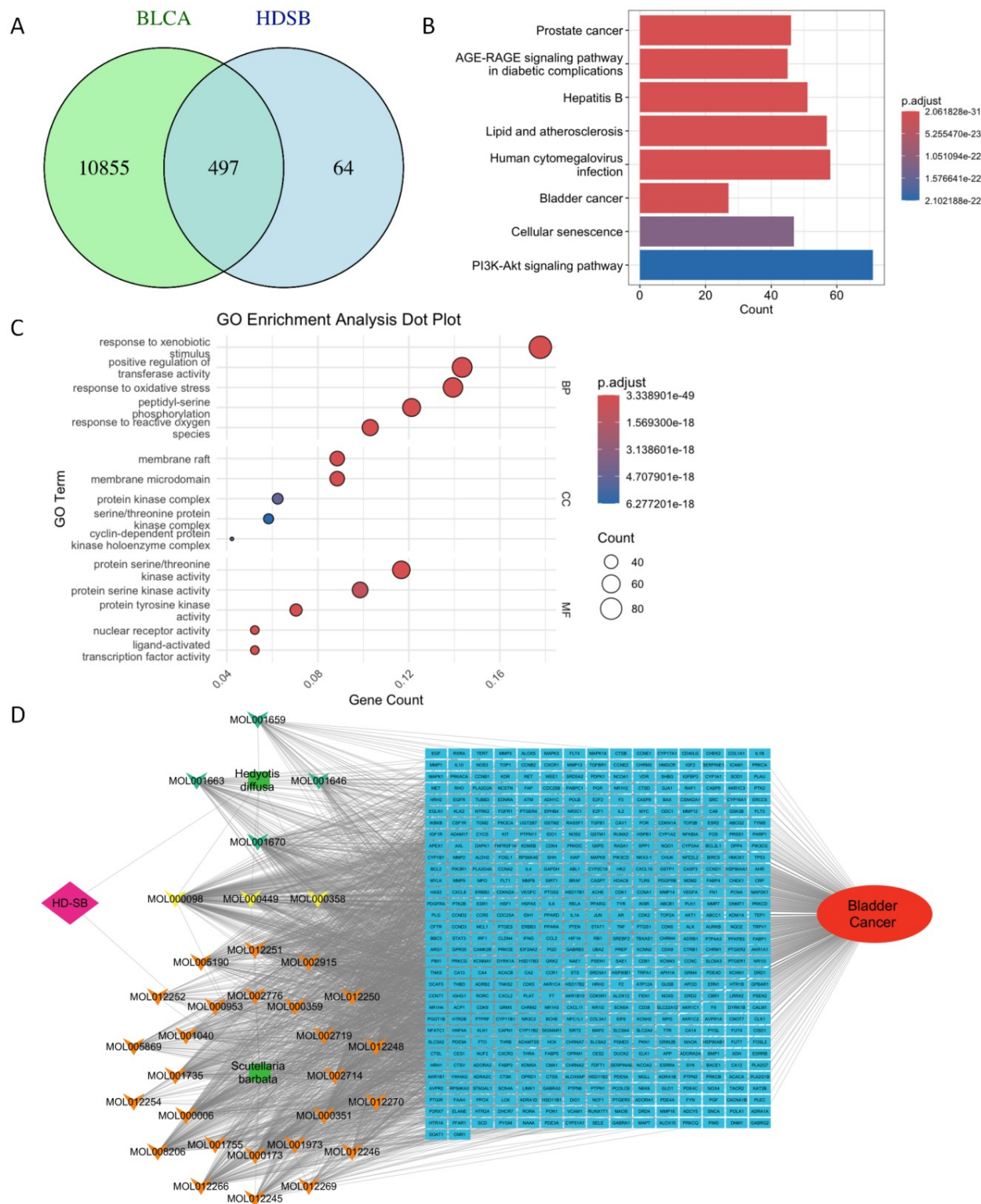


Figure 2

Network pharmacology and enrichment analysis. (A) Venn diagram displays the overlapping targets between bladder cancer (BLCA) and the herbal combination of Hedyotis diffusa and Scutellaria barbata (HD-SB); (B) KEGG enrichment analysis highlights the top eight pathways associated with these overlapping targets; (C) GO enrichment analysis outlines the biological processes (BP), cellular

components (CC), and molecular functions (MF) of the overlapping targets; (D) The herb-compound-target-disease network is presented.

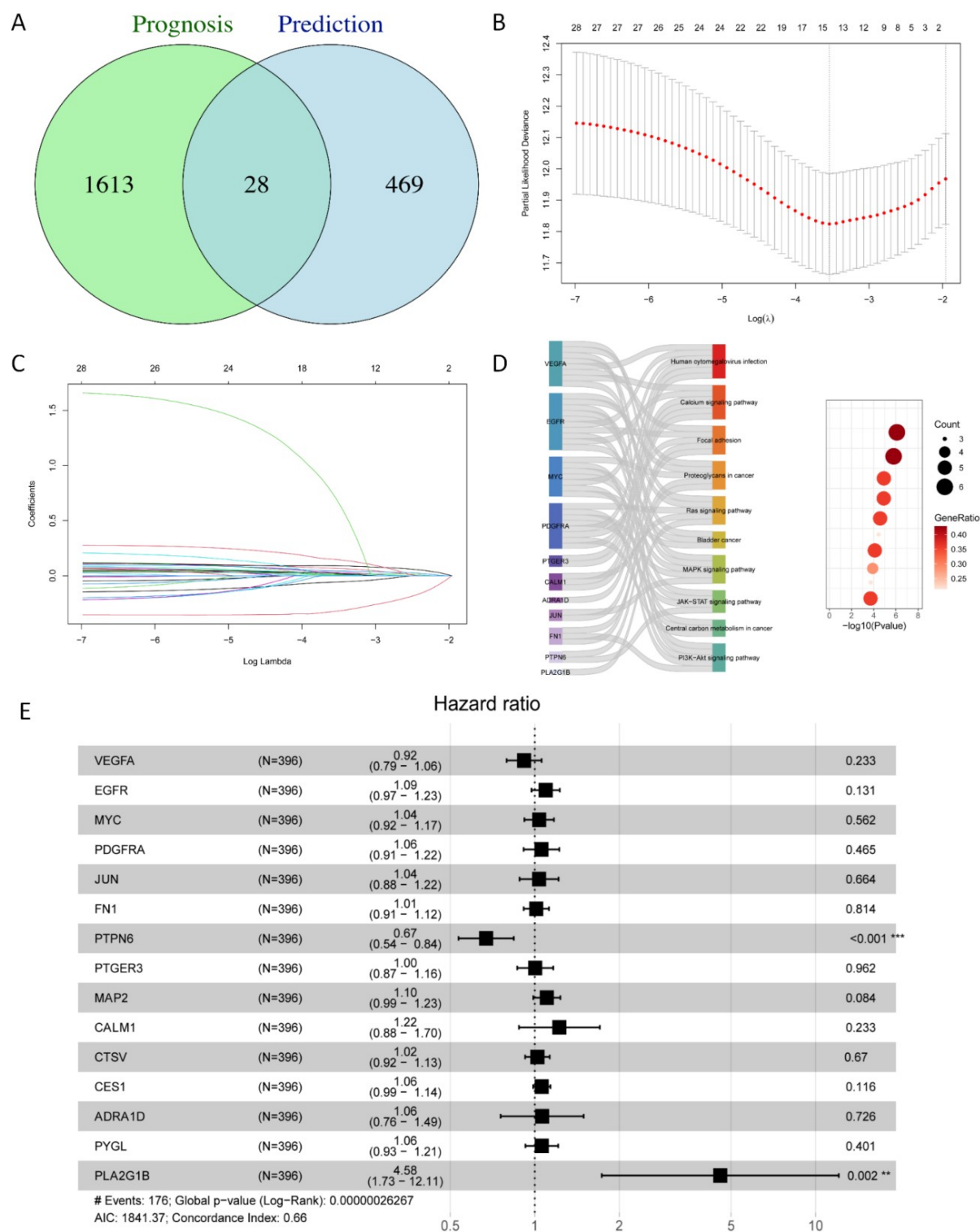


Figure 3

Identification of prognostic genes and their associations with pathways and overall survival. (A) A Venn diagram illustrates the overlapping genes between prognostic genes (derived from the TCGA-BLCA database) and predicted genes (as shown in Figure 2); (B) The profile of the lasso coefficient for the 15

prognostic genes in the TCGA-BLCA database; (C) The profile of partial likelihood deviation for the 15 prognostic genes in the TCGA-BLCA database; (D) A Sankey diagram depicts the relationships between the 15 prognostic genes and their associated pathways; (E) A forest plot presents the results of multivariate Cox regression analysis, assessing the predictive ability of the 15 prognostic genes for overall survival in the TCGA-BLCA cohort.

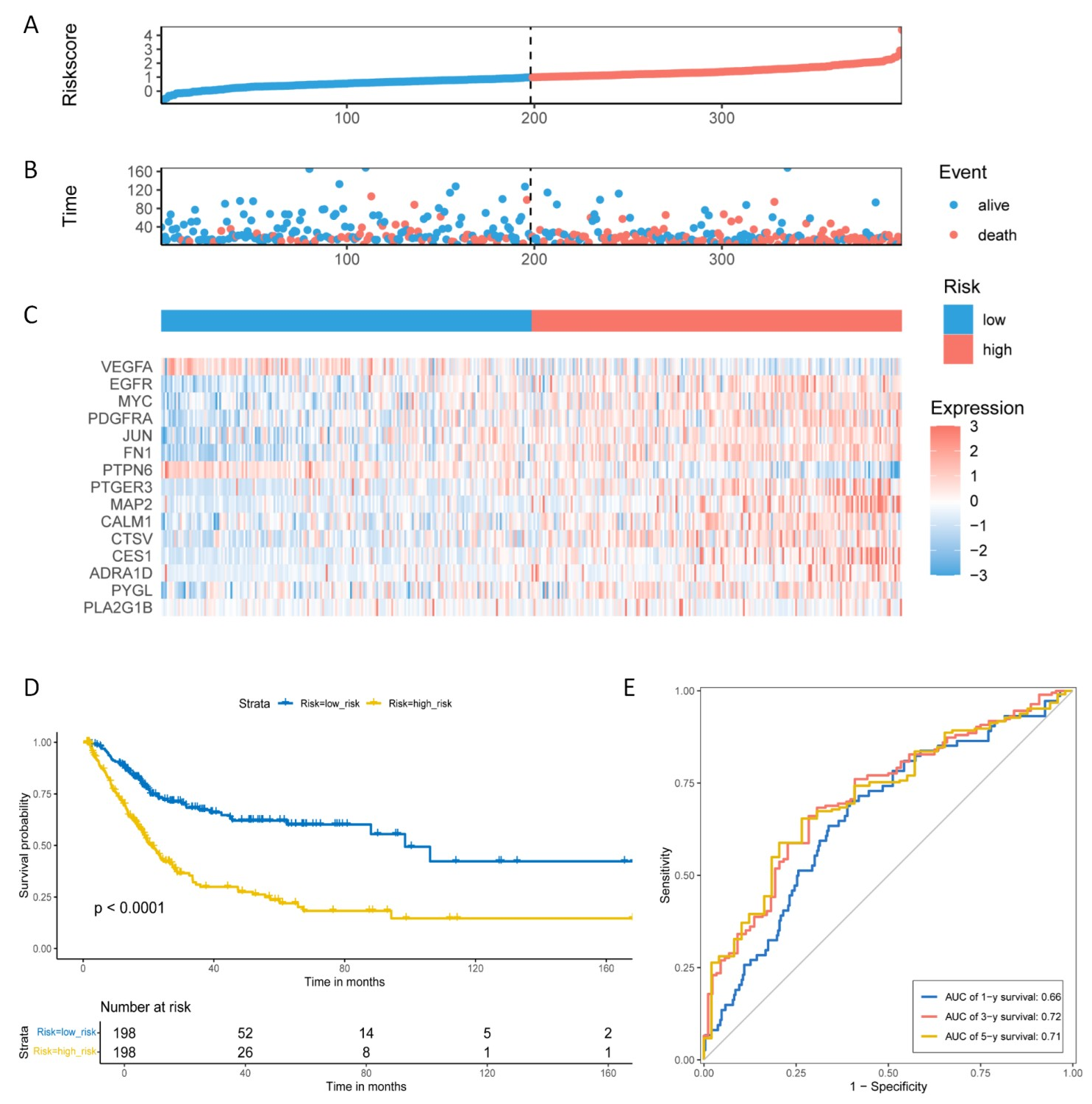


Figure 4

Development and validation of a prognostic model based on 15 genes in the TCGA-BLCA cohort. (A) The survival status of bladder cancer patients is ranked according to their risk scores; (B) The survival time of bladder cancer patients is ordered by their risk scores; (C) A heatmap was generated to illustrate the expression levels of 15 genes in patients categorized into high-risk and low-risk groups; (D) Kaplan-Meier analysis illustrates the difference in overall survival between the high-risk and low-risk groups; (E) A time-dependent ROC curve assesses the predictive accuracy of the risk score for 1-, 3-, and 5-year overall survival.

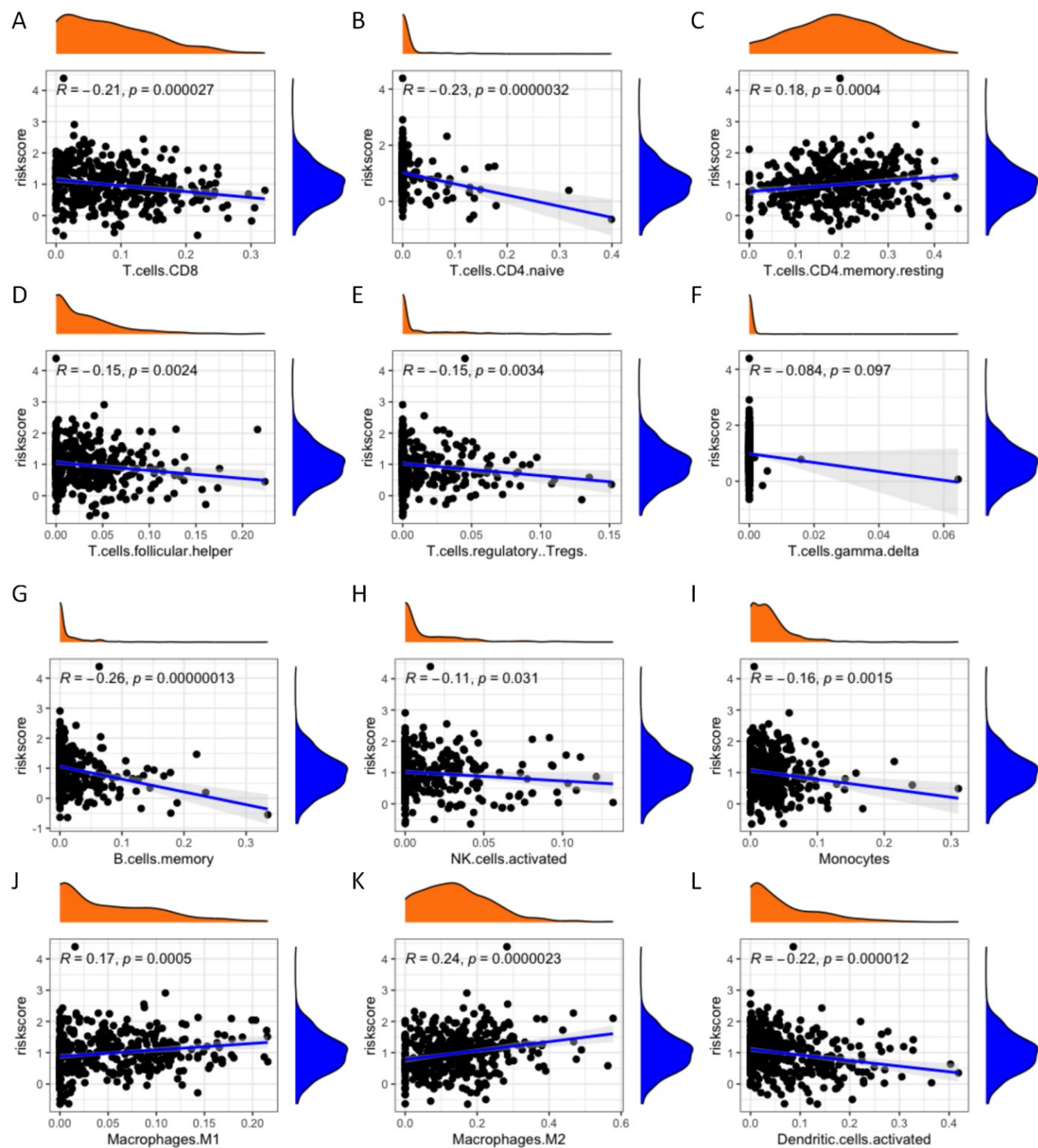


Figure 5

Association of risk score with tumor immune cell infiltration.

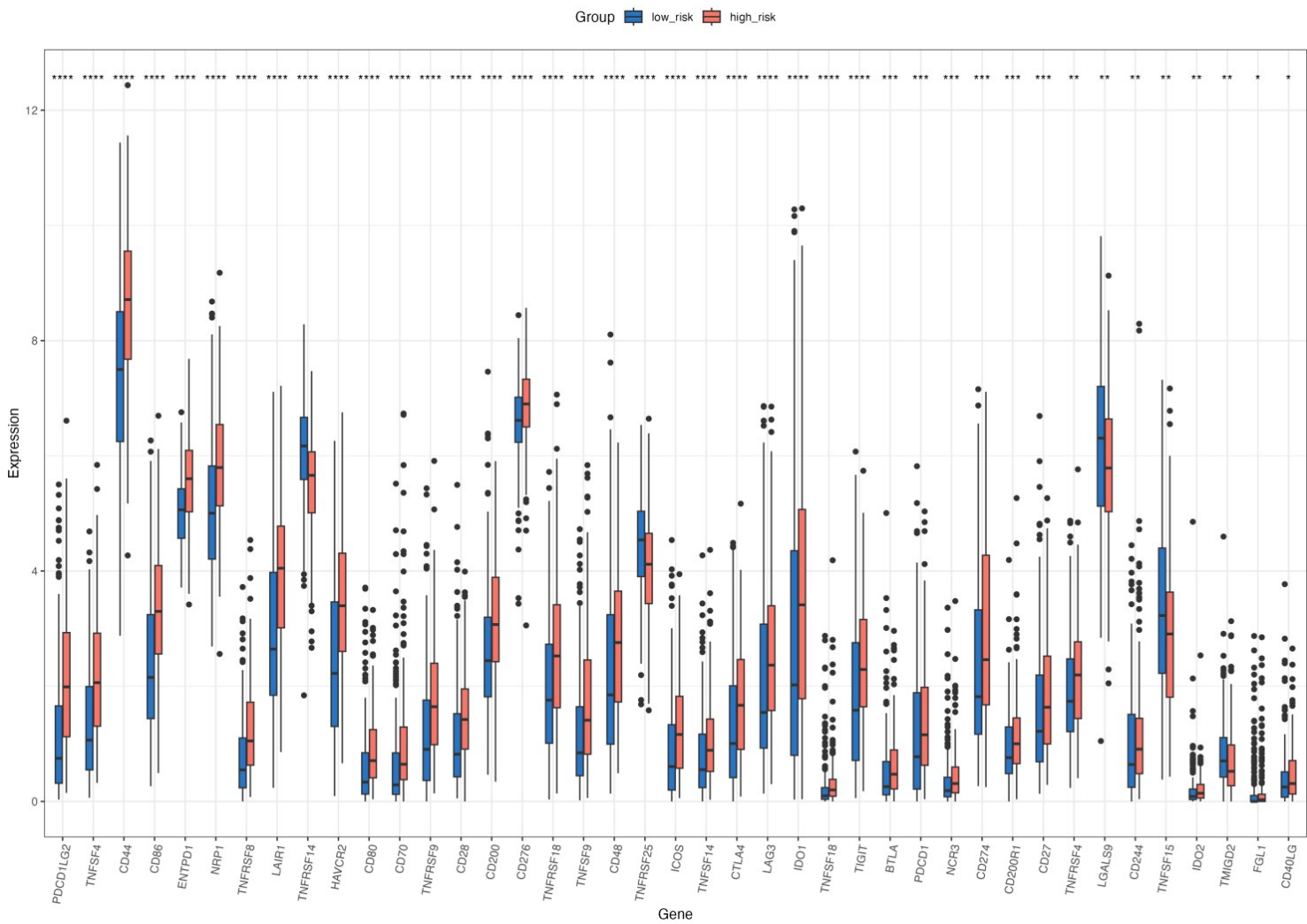


Figure 6

Relationship between risk score and immune checkpoint genes.

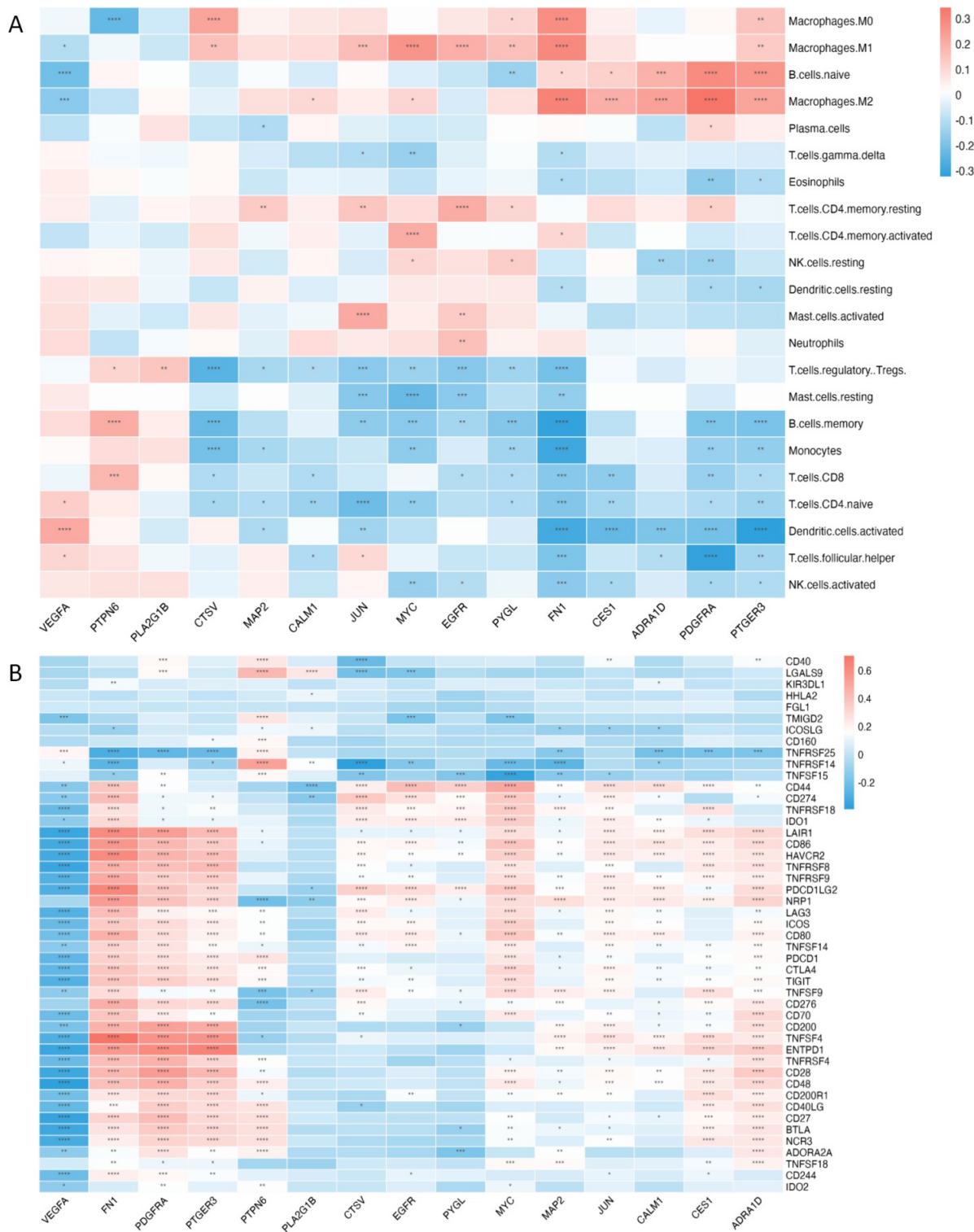


Figure 7

Correlation analysis of model genes, tumor immune infiltrating cells, and immune checkpoint genes. (A) The relationship between model genes and tumor immune infiltrating cells; (B) The relationship between model genes and immune checkpoint genes.

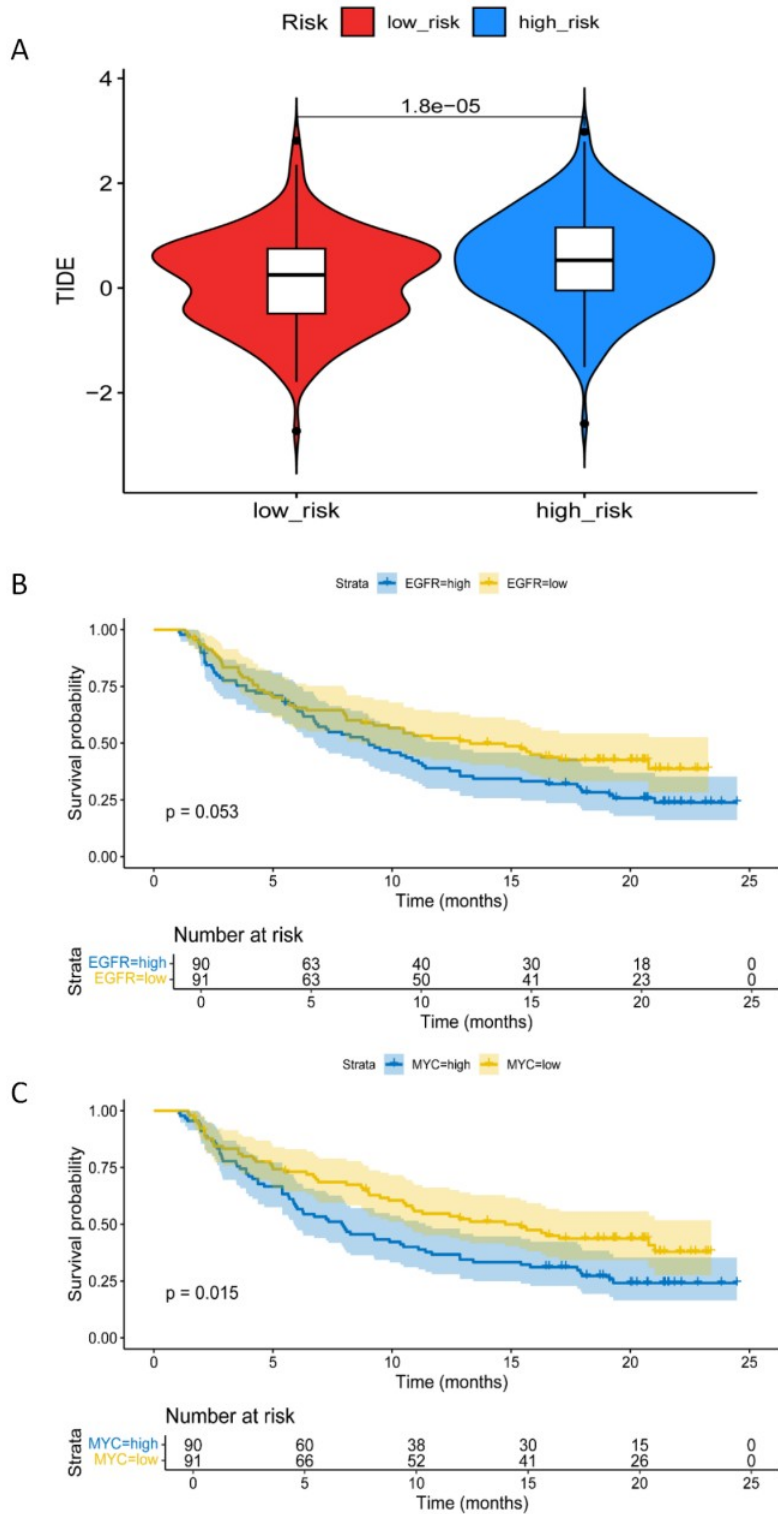


Figure 8

Association between risk score based on prognostic genes and immunotherapy efficacy. (A) The TIDE algorithm was utilized to predict the response to immune checkpoint therapy in bladder cancer patients based on the risk score. (B) Survival analysis comparing overall survival between patients with high and low EGFR expression in bladder cancer patients of the IMvigor210 cohort. (C) Survival analysis

comparing overall survival between patients with high and low MYC expression in bladder cancer patients of the IMvigor210 cohort.

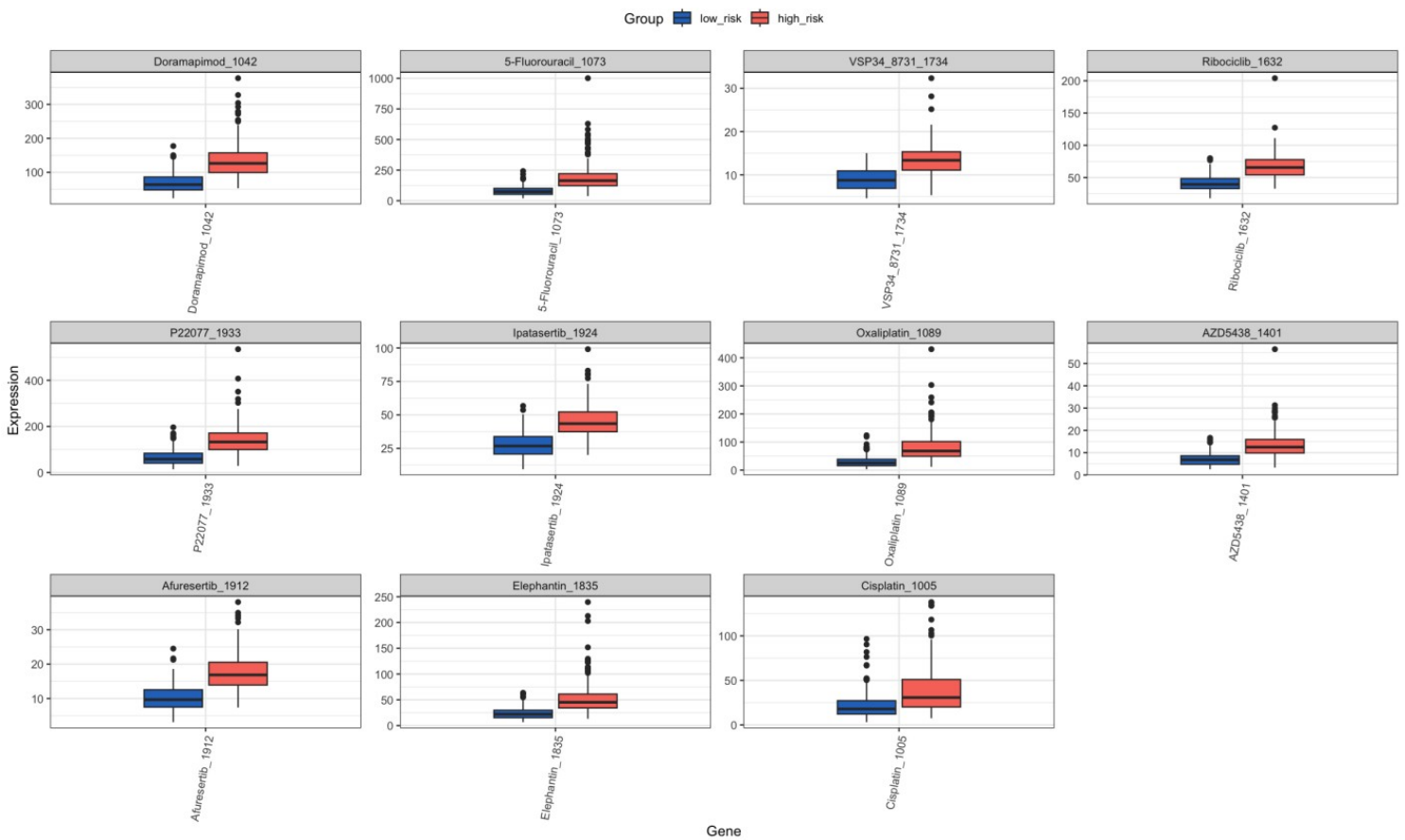


Figure 9

Association between risk score derived from prognostic genes and chemotherapy sensitivity.

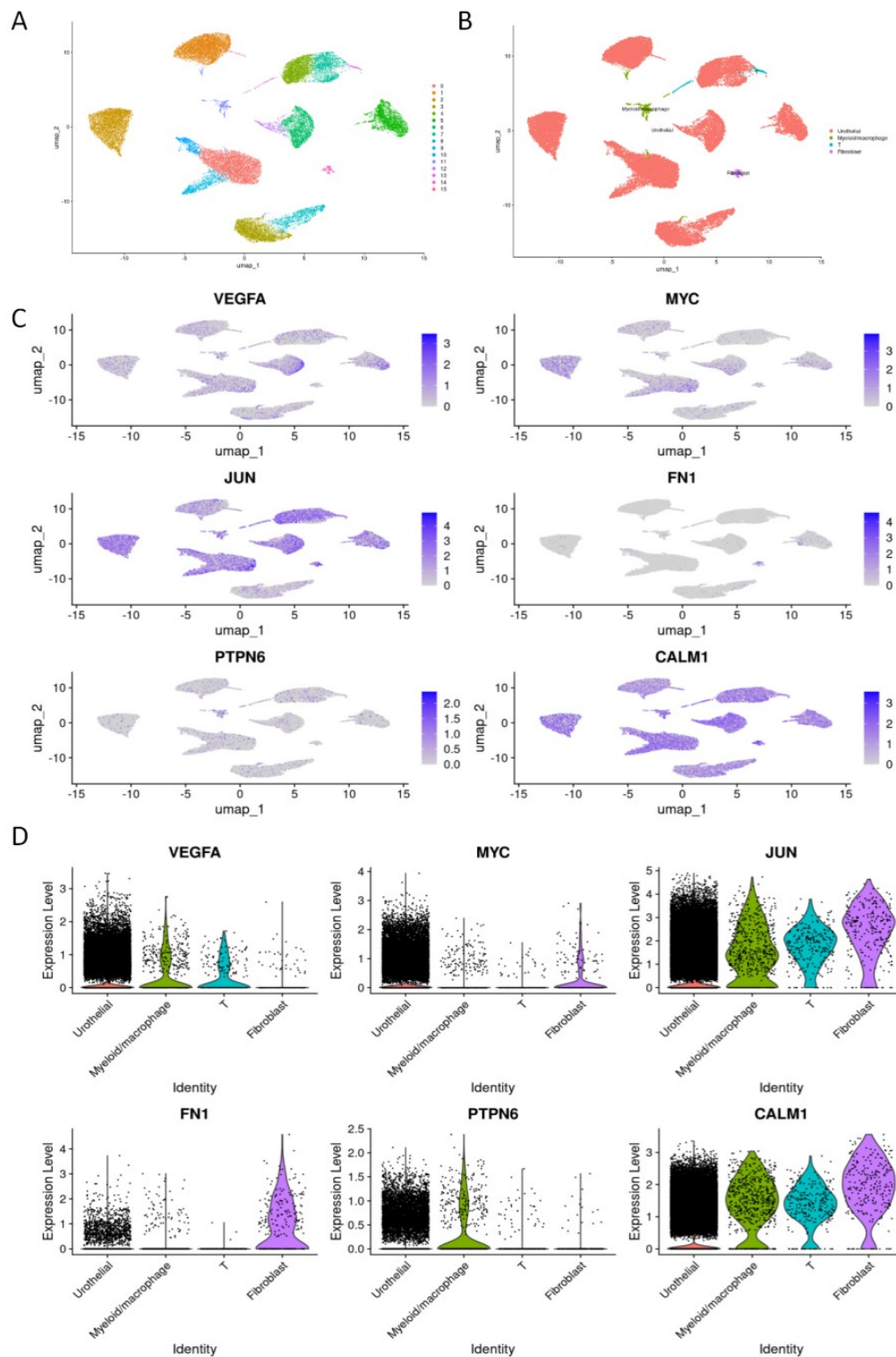


Figure 10

Identifying infiltrated cell types in bladder cancer tissues and expression of prognostic genes in each cell type. (A) The UMAP algorithm was applied to the top 15 PCs for dimensionality reduction, and 16 cell clusters were successfully classified; (B) All four cell clusters in bladder cancer were annotated according to the composition of marker genes; (C) Expression of six model genes in different cell populations; (D) Violin plot for expression of six model genes in different cell populations.

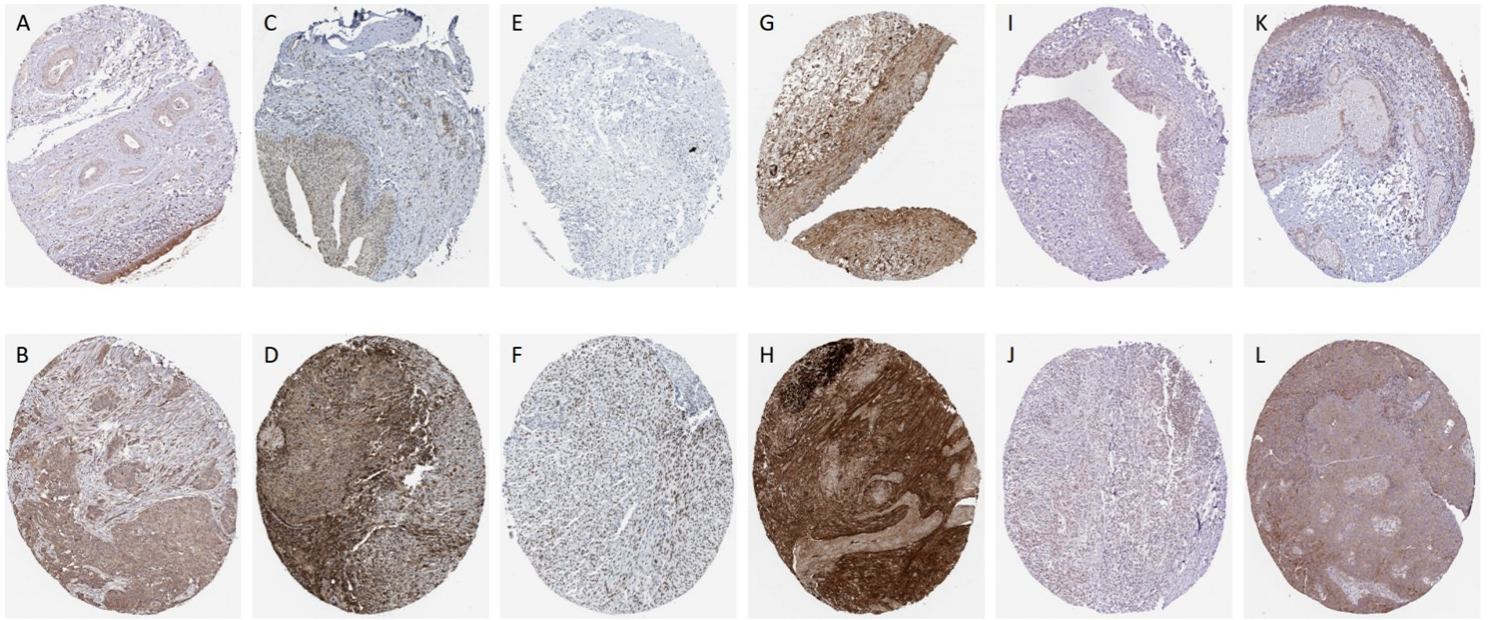


Figure 11

Validation of protein expression of six prognostic targets in normal bladder tissues (upper panel) and bladder cancer tissues (lower panel) from the HPA database. (A) VEGFA; (B) MYC; (C) JUN; (D) FN1; (E) PTPN6; (F) CALM1.

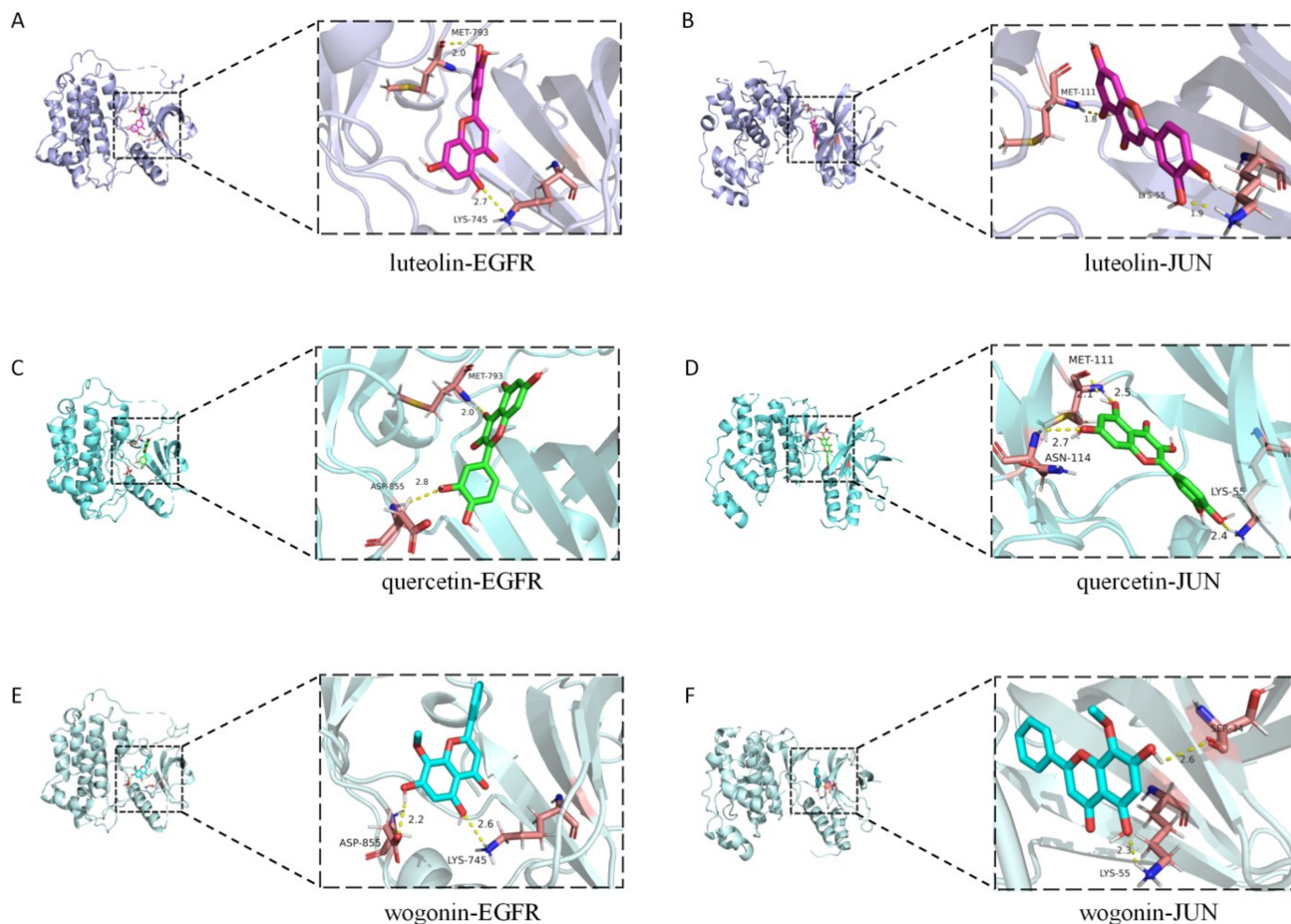


Figure 12

Molecular docking identification of pharmaceutical constituents in HD-SB. (A) Luteolin forms one hydrogen bonds with MET-793 and LYS-745 in EGFR; (B) Luteolin forms one hydrogen bond with MET-111 and LYS-55 in JUN; (C) Quercetin forms one hydrogen bond with MET-793 and Asp-855 in EGFR; (D) Quercetin forms one hydrogen bond with ASN-114 and LYS-55 in JUN, and also interacts with MET-111 through two hydrogen bonds; (E) Wogonin is linked to Asp-855 and LYS-745 in EGFR through one hydrogen bond; (F) Wogonin can interact with SER-34 and LYS-55 in JUN through one hydrogen bond.

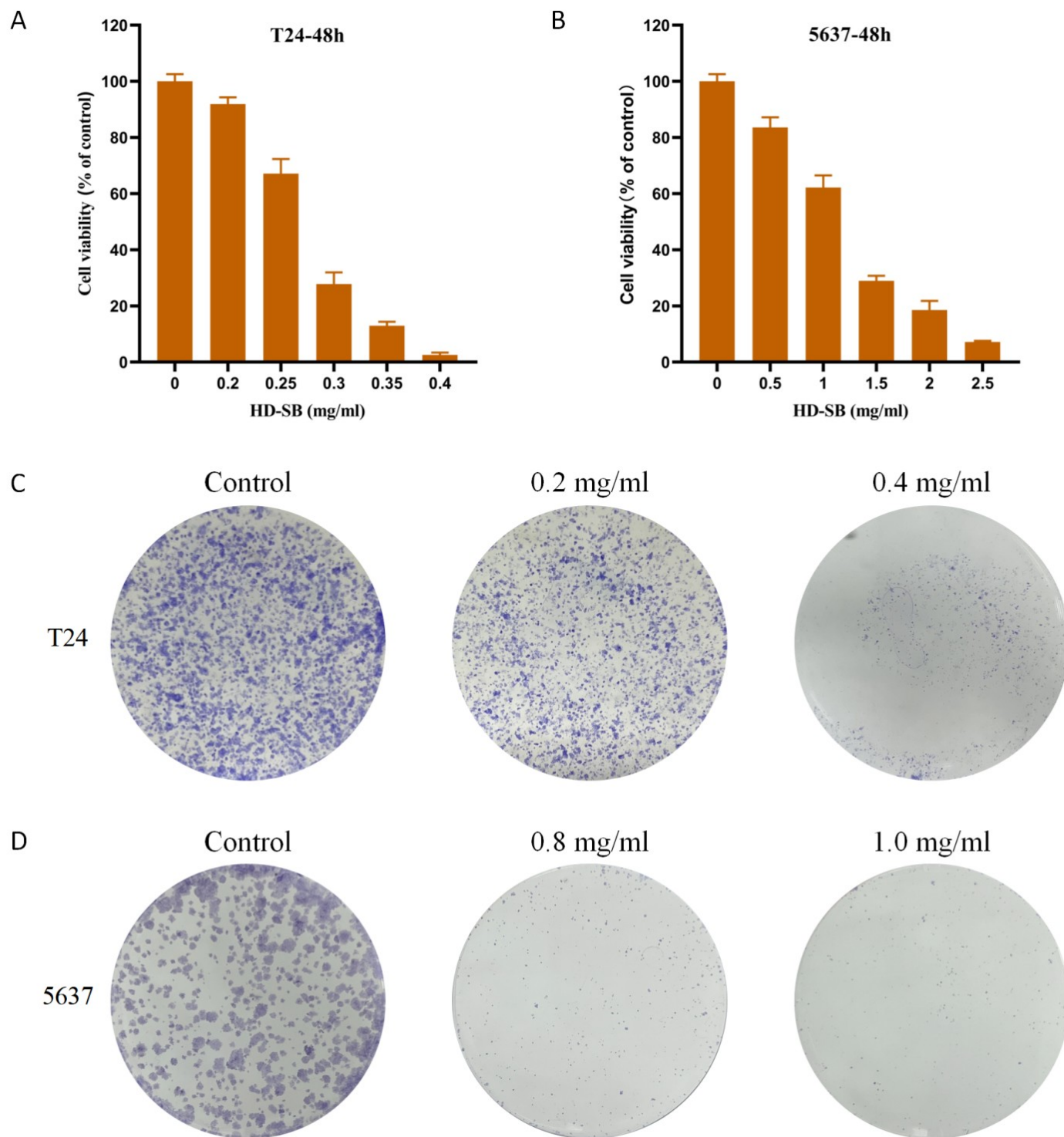


Figure 13

The impact of HD-SB on the cell viability and colony formation of bladder cancer cells. HD-SB inhibited the cell viability of both T24 (A) and 5637 (B) cells, as well as the colony formation of T24 (C) and 5637 (D) cells.

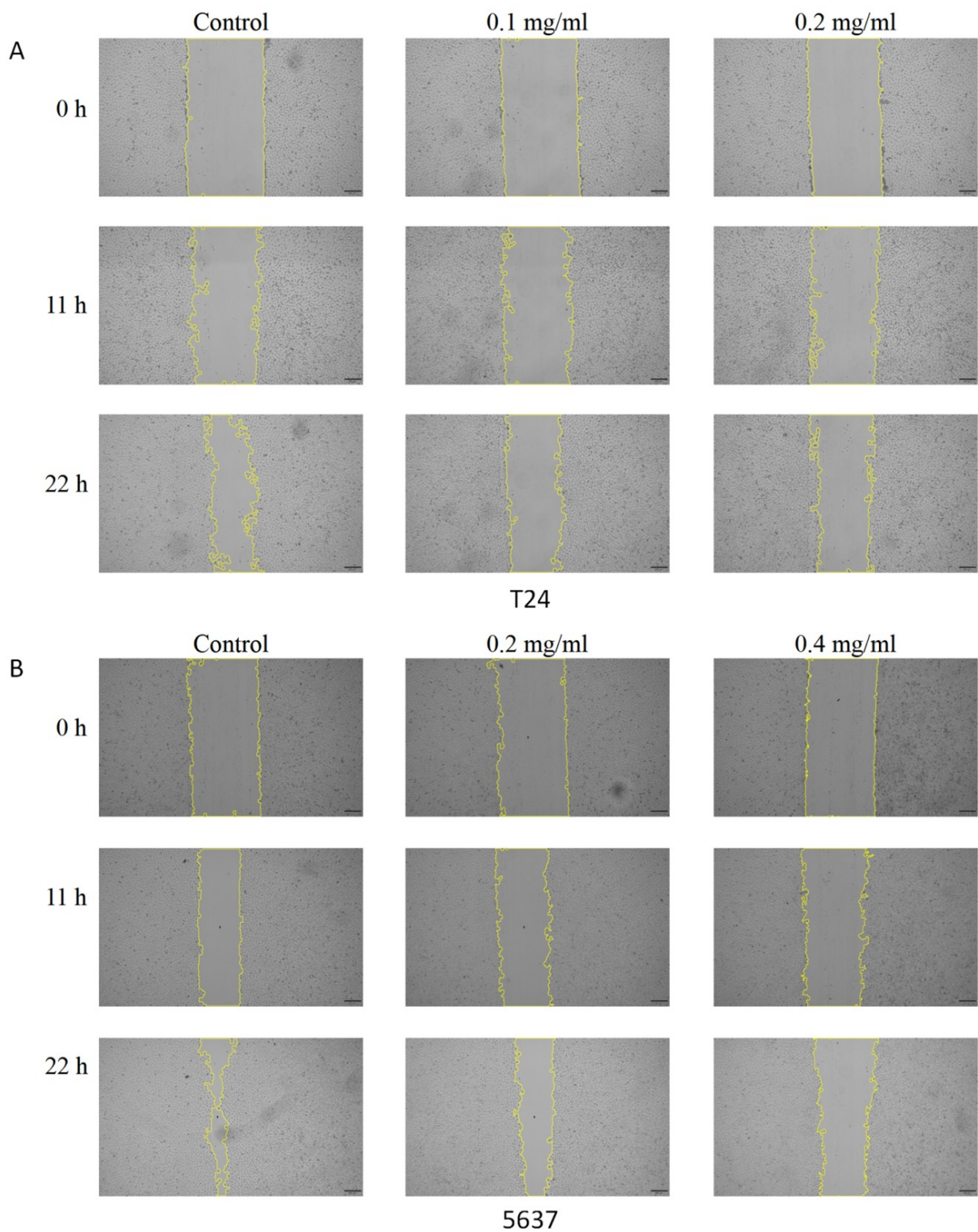


Figure 14

The inhibitory effect of HD-SB on the migration of bladder cancer cells. HD-SB significantly reduced the migratory capacity of both T24 (A) and 5637 (B) cells.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryMaterial1.xlsx](#)
- [SupplementaryMaterials.docx](#)