Identification and Validation of A Hypoxia-Related Prognostic and Immune Microenvironment Signature in Bladder Cancer

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Abstract

Bladder cancer is the main leading causes of cancer-related deaths and seriously affects population health. Hypoxia plays a key role in tumor development and immune escape, which contributes to malignant behaviors. In this study, we analyzed the RNA-seq and clinical information of bladder cancer patients from The Cancer Genome Atlas (TCGA) database. In order to investigate the hypoxia-related prognostic and immune microenvironment in bladder cancer, we constructed a hypoxia-related risk model for overall survival (OS). Moreover, the RNA-seq and clinical information of bladder cancer patients from Gene Expression Omnibus (GEO) database were used to verified. Our research revealed that the hypoxia risk signature significantly correlated with clinical outcome and independently predict the OS. Furthermore, the hypoxia risk signature could effectively reflect the level of immune cell type fraction and the expression of critical immune checkpoint genes were higher in the high-risk group compare to low-risk group. We also confirmed the expression level of the prognostic genes in bladder cancer and paracancerous tissue samples using qRT-PCR analysis. In summary, the present study identified 7 hypoxia-related genes (HRGs) signature that may be served as an independent clinical predictor and provide a potential mechanism in bladder cancer immunotherapy.

Introduction

Hypoxia is a highly crucial process for cells acquiring specific futures to adapt low oxygen level situation (1). Previous studies have been reported that hypoxia promotes cell proliferation, invasion and regulates immune response (2-3). Bladder cancer is the common urinary cancer worldwide, causing more than 199,922 deaths in 2018 (4). Despite the current treatment including surgery, chemotherapy, radiotherapy and some novel immunotherapy applied, the clinical outcome are still not satisfactory (5). High recurrence rate in bladder cancer is the main obstruction to conquer the disease. Therefore, we are eager to develop valued precision prediction to promote clinical diagnosis and treatment. Recent studies have demonstrated that hypoxia is associated with tumor progression and recurrence in bladder cancer (6-7). Hypoxic cancer cells regulate tumor microenvironment to facilitate tumor progression and development through releasing exosomes in bladder cancer (8). Circular RNA involved in the adaptive response to hypoxia and contributes to bladder cancer progression and drug resistance (9). Moreover, the recent researches have illustrated the mechanisms between tumor hypoxia and immune escape, which indicated that hypoxia can be utilized to predict immunotherapy outcomes (10-12).

It has been revealed that tumor microenvironment function as key roles in bladder cancer. The immune checkpoint molecules are significantly associated with the regulation of tumor microenvironment and the inhibitors have been approved for various cancers such as programmed cell death protein 1 (PD-1) and programmed cell death-ligand 1 (PD-L1). Previous evidences support that hypoxia induced factors-1 (HIF-1) promotes the expression of PD-L1 via binding to the hypoxia response element in the specific proximal promoter. Blockade of PD-L1 under hypoxia enhanced myeloid-derived suppressor cells (MDSCs) regulated T cell activation and down-regulated the expression level of IL-6 and IL-10 (13). Intermittent

hypoxia can reduce autologous T-cell proliferation and the cytotoxic activity of $CD8^+$ T-cells (14). Therefore, tumor hypoxia may be regarded as a potential therapeutic target for immunotherapy.

In this study, we developed a hypoxia-related risk signature as a prognostic symbol to reflect the immune landscape in bladder cancer. We screened 7 HRGs that were significantly connected with the OS of bladder cancer from The Cancer Genome Atlas bladder cancer cohort (TCGA-BLCA). We divided the samples into high-risk and low-risk groups according to the median risk score. Furthermore, survival and Cox analysis estimated the prognostic value of the hypoxia risk model. Further tools were used to explore the different mechanisms in signaling pathways and the fractions of immune cell types between the high-risk and low-risk groups. The purpose of the present study was to analyzed the expression of HRGs in bladder cancer and explored the potential prognostic value. Importantly, we constructed and verified a hypoxia-related signature that could improve the precise prognosis prediction in bladder cancer.

Materials And Methods

Data source

The RNA-seq transcriptome information and clinical characteristics of the BLCA cohort were downloaded from TCGA (https://portal.gdc.cancer.gov/) database. Gene expression profiles and clinical data in GSE32894 were obtained from GEO (https://www.ncbi.nlm.nih.gov/geo/) database. The detail patients information were showed in supplemented Table S1 and Table S2.

Construction a protein–protein interaction (PPI) network

The PPI network was analyzed by STRING database (http://string-db.org). Cytoscape software (https://cytoscape.org/) platform was used to visualize and integrate these associated protein networks. The Network Analyzer plug-in calculated the node degree between these networks and defined the most key genes through the network.

Development and validation the hypoxia-related prognostic signature

The prognostic HRGs in bladder cancer obtained from univariable and multivariable Cox regression were screened to further calculate the each patient's risk score. The risk score was calculated as following:

$$RiskScore=Expression_{gene1}\times Coefficient_{gene1}+Expression_{gene2}\times Coefficient_{gene2}+\ldots Expression_{genen}\times Coefficient_{genen}$$

The formula was utilized to figure the risk score of each bladder cancer patient in our model. Next, we investigated whether the risk score was correlated to OS.

Evaluation of the immune cell type fractions

The CIBERSORT (https://cibersort.stanford.edu/) was utilized to evaluate the proportions of tumor-infiltrating lymphocyte in a mixed cell population. 22 immune cell types in this tool were used to estimate relative abundance of immune cell infiltration between low- and high-risk groups.

Gene Set Enrichment Analysis (GSEA)

Based on the median value of the risk scores, the patients were divided into low-risk and high-risk groups. GSEA was performed to analyze the difference pathway signaling genes between the groups. The analysis was carried out by GSEA3.0 (http://www.broad.mit.edu/gsea/) and the nominal $P < 0.05$ was considered statistically significant.

RNA extraction and quantitative real-time (qRT-PCR)

Total RNA was extracted with Trizol reagent (Invitrogen, CA, USA). Next, qRT-PCR was performed using SYBR-Green Mix (Vazyme, Nanjing, China) with the different primers (Sangon Biotech, China). We used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal standard. Fold-changes were calculated by the $2^{-\Delta\Delta Ct}$ method. Primer information is listed in supplemented Table S3.

Clinical patients and bladder specimens

45 paired badder cancer and paracancerous tissues samples were collected from patients at Shanghai Tenth People's Hospital (Shanghai, China). All patients provided informed consent, and this study was approved by the Ethics Committee of Shanghai Tenth People's Hospital. The patients were diagnosed according to WHO classification without any treatment preoperatively. The samples were frozen and stored in liquid nitrogen. We isolated total RNA from the samples and qRT-PCR analysis were used to validate the expression of 7 HRGs in human samples.

Statistical analysis

Data analyses were performed using the R programming language (https://www.r-project.org/). Univariate and multivariate Cox proportional hazard regression analyses were used to screen genes and evaluate the correlation between the risk score and OS. The receiver operating characteristic (ROC) analysis was used to assess the sensitivity and specificity of the risk model to predict survival with the "survivalROC" package. The area under the ROC curve (AUC) was used to show prognostic accuracy. Student's t test was used to compare the difference between two groups. P values less than 0.05 were considered statistically significant.

Results

Identification of the hypoxia-related risk signature to predict bladder cancer prognosis.

Figure 1 showed the flow chart of this study process. We downloaded the RNA-seq and clinical prognostic information in the TCGA-BLCA cohort. The hypoxia-related gene set was obtained from GSEA (hallmark-

hypoxia). Next, we developed PPI network analysis by the STRING online database and Cytoscape software to further find out the interactions between these genes (Figure 2A). A total of 50 genes with the most significant interaction degrees were screened (Figure 2B).

To construct the hypoxia-related risk model, univariate and multivariate Cox analysis were analyzed with the top 50 genes in the TCGA-BLCA cohort. In the univariate Cox analysis, 14 HRGs were significantly associated with patients' OS (Figure 2C). After multivariate Cox regression analysis, 7 HRGs were identified and chosen to construct the signature for OS (Figure 2D). The risk score signature was developed as follows: risk score = (0.119 × SLC2A3 expression level) + (-0.387 × ALDOB expression level) + (0.320 × FOXO3 expression level) + (-0.216 × SDC4 expression level) + (-0.227 × VEGFA expression level) + (0.165 × EGFR expression level) + (0.232 × GPC1 expression level).

Prognostic significance of the hypoxia-related risk signature in bladder cancer patients.

For investigating the prognostic significance of the hypoxia-related risk signature. As shown in the Figure 3A, the expressions of the 7 HRGs were upregulated in high risk score in both TCGA and GSE32894 set. The risk score of patients in the low- and high- risk groups were visualized (Figure 3B). As the hypoxia risk score increased, an increasing rate of mortality in the patients (Figure 3C,D). Moreover, KaplanMeier analysis was used to evaluate the prognostic significance of the hypoxia risk signature. We found that high hypoxia risk score was correlated to poor OS in the TCGA and GSE32894 set compare to low risk score (Figure 3E,F).

The hypoxia-related risk signature for OS is an independent prognostic factor of bladder cancer patients.

To determined the predictive accuracy of the hypoxia risk signature, we used the ROC curve to assess the model. The AUC of the signature for prediction of 1-, 3-, and 5-year OS were 0.661, 0.676 and 0.710, respectively, in the TCGA set and 0.600, 0.594, 0.636 respectively in the GSE32894 set (Figure 4A,B). Then, the univariate and multivariate Cox analysis were used to evaluate whether the independent prognostic value of hypoxia risk signature for OS. The univariate Cox analyses showed that the risk score was associated with OS like other variables including age, gender and WHO grade (Figure 4C). Next, multivariate Cox analysis indicated that the risk score was independently correlated with the OS (Figure 4E). The results were validated in GSE32894 set (Figure 4D,F).

Relationships between the prognostic signature with clinicopathological variables

To investigate whether the prognostic signature correlated with clinicopathological variables in TCGA-BLCA. Bladder cancer patients were stratified according to age, gender, satge, T stage and N stage. The result showed that high-risk patients in those clinical parameters had significantly shorter OS time than low-risk patients (Figure 5), which suggest that the hypoxia-related signature could be applicable to clinical factors.

Correlation between the hypoxia risk signature and immune cell infiltration

For investigating the utility of the risk signature in reflecting the immune cell environment. We used the CIBERSORT analysis to estimate expression level of the 22 immune cell types infiltration between different risk level bladder cancer patients. The composition of the immune cell population in the patients was summarized (Figure 6A). The low hypoxia risk patients performs notably higher proportions of follicular helper T cells (p=0.011), CD8 T cells (p=0.0032) and plasma cells (p=0.0077) compared to the high-risk group (Figure 6E-G). However, a higher proportion of mast resting cells (p=0.023), neutrophils cells (p=0.0061) and CD4 memory resting T cells (p=0.0023) were enriched in high-risk group (Figure 6B-D).

Functional analysis of the prognostic signature

We further verify the underlying mechanism involved in the low- and high-risk groups. GSEA analysis showed that the signaling pathways such as hypoxia, epithelial-mesenchymal transition, inflammatory response and complement were the most significantly enriched pathways in the high risk groups (Figure 7).

Potential of the hypoxia risk signature associated with immunosuppressive microenvironment

Immunotherapy has been a promising treatment for advanced urothelial carcinoma. It was confirmed that Cancer-Immunity Cycle regulating cancer cells and immune response, which affects the utility to immune therapies. The process of Cancer-Immunity Cycle is initiated by the release of cancer-associated antigen. Then the related antigens are identified with dendritic cells and transfer to lymph nodes, accompanied by activating T cells. Those effector cells next migrate and infiltrate the tumor stroma, specifically recognize and eliminate cancer cells. Every step of the cycle needs the coordination of stimulatory and inhibitory factors. Here, we focused on the genes negatively mediating the process in low- and high- risk groups. The related genes signatures were obtained from Tracking Tumor Immunophenotype website (http://biocc.hrbmu.edu.cn/TIP/index.jsp). Genes enriched in the negative regulation of the cycle were significantly increased in the high risk score group (Figure 8A), which indicates that high hypoxia risk patients may associated with poor immunotherapy efficacy.

Moreover, the association between the hypoxia-related signature and the expression levels of important immune checkpoint genes (i.e., PD-L1, PD-1, CTLA-4 and LAG-3) were investigated. As shown in Figure 8B-E, the four immune checkpoints were correlated with hypoxia risk score and upregulated in the high hypoxia risk group. Furthermore, we evaluated the expression of some immunosuppressive cytokines in the low- and high- risk groups. High hypoxia risk group significantly showed a high expression level of immunosuppressive cytokines (Figure 8F).

These results demonstrated that high hypoxia risk patients may develop an immunosuppressive microenvironment and insensitive to immunotherapy.

Validation of 7 HRGs expression results using qRT-PCR analysis

We analyzed the expression of 7 HRGs mRNAs by qRT-PCR in 45 paired paracancerous and cancer tissues. As show in Figure 9 A-G, expression level of SLC2A3, FOXO3, EGFR and GPC1 were higher in tumor samples. No difference was found in ALDOB, SDC4 and VEGFA expression. Moreover, Gene Expression Profling Interactive Analysis (GEPIA) database was used to analyze the HRGs with patients' OS in TCGA-BLCA. The results showed that high expression of FOXO3 and EGFR and low expression of VEGFA were closely correlated with poorer survival of bladder cancer (Figure 9 H-K). Immunohistochemistry data obtained from the Human Protein Atlas were used to verify the expression of HRGs in normal and tumor tissues was showed in supplemented Figure S1.

Discussion

Bladder cancer is the tenth most common cancer over the world with high morbidity and mortality rates. Due to tumor recurrence and drug resistance, the clinical outcome has not improved obviously over the past years (15). Precise diagnostic and predictive methods are urgently need to promote the treatment and prognosis of bladder cancer. Recently, immunotherapy is a novel treatment method to bladder cancer by blocking immune checkpoints, while the the therapeutic efficacy were not very high (16).

A large number of reports indicate that tumor hypoxia is associated with offering a growth condition for tumor cells and contributing to the convert of malignant phenotype. In addition, tumor hypoxia has been regarded as a prognostic factor for cancer. Lin et al constructed a prognostic model of HRGs in glioma (17). Zhang et al defined a hypoxia-related signature in pan-cancer using multi-omics data (18). Here, we identified a hypoxia risk signature and investigated its prognostic value to predict OS and evaluated the immune microenvironment among the low and high risk score groups. We employed univariate Cox regression to analyze HRGs correlated with the prognosis of bladder cancer. 14 HRGs were screened to be significantly correlated with the prognosis. Next, multivariate Cox regression analysis identified 7 HRGs (SLC2A3, ALDOB, FOXO3, SDC4, VEGF, EGFR, GPC1) to construct the risk score model. High expression of solute carrier family 2 facilitated glucose transporter member 3 (SLC2A3) is closely related with poor prognosis in papillary thyroid cancer (19) and colorectal cancer (20). ALDOB is upregulated in liver metastases and enhances fructose metabolism (21). FOXO3 promotes tumor angiogenesis in neuroblastoma (22). SDC4 gene silencing could obviously attenuate the epithelial-mesenchymal transition and promote apoptosis in the papillary thyroid cancer cells (23). VEGF is primarily responsible for angiogenesis and promote cell proliferation (24). EGFR can be used as a potential therapeutic target for muscle-invasive bladder cancer presenting a basal-like phenotype (25). GPC1 is an independent prognostic factor in oesophageal squamous cell carcinoma (26).

GSEA showed that the regulation of hypoxia, epithelial-mesenchymal transition and inflammatory response were more enriched in the high-risk group compare to low-risk group. However, the mechanisms of these genes negatively expressed in the low-risk group requires further investigation. In addition, we demonstrated that the hypoxia risk signature for OS could independently predict the prognosis of bladder cancer patients. High risk score patients probably associated with worse prognosis and the result also adapted to different clinicopathological variables. Moreover, we validated the risk model in GSE32894 set

to test the applicability of the hypoxia risk signature. Furthermore, our qRT-PCR analysis demonstrated that SLC2A3, FOXO3, EGFR and GPC1 were upregulated in tumor samples.

Hypoxia can affect the function of immune cell types, thereby inducing tumour development directly or indirectly. Under the hypoxic environment, macrophages synthesise chemokines and cancer cells can attract regulatory T-cells from the circulation and suppress the anti-tumour response from other T-cells (27). Hypoxic microenvironment contribute to repress anti-tumor immune effector cells and facilitate immune escape for the growth of tumor cells (28). Hypoxia promotes the FOXP3 transcription factor expression levels, which is a potent regulator of Treg cells (29). Hypoxia also increases the expression of CCL28 and TGF-β, which play a key role in attracting Treg cells, regulating the inhibition efficacy of Teff cell responses, as well as contributing to angiogenesis and tumour tolerance (30). Tumour-associated macrophages can promote malignant progression by inducing angiogenesis in the tumor hypoxic environment. Hypoxia significantly promoted the positive percentage of PD-L1 related myeloid-derived suppressor cells (MDSCs) in the tumor-bearing mice (13). In this study, CIBERSORT showed that patients with high risk score had a higher proportions of neutrophils and mast resting cells phenotype. However, immunosuppressive cells, like follicular helper T cells and CD8 T cells, were increased in the low-risk group, indicating that an immune disability status between the groups.

Immune checkpoints have become the potential targets of cancer therapy and inhibitors that block the key molecules have showed impressive efficacy against cancer. In the present study, high-risk group also correlated with the associated immune checkpoints like PD-L1, PD-1, CTLA-4, and LAG3. Moreover, we analyzed some immunosuppressive cytokines were also increased in high-risk group, which further affected the immune response.

In summary, our study was the first time to construct and validate a hypoxia risk signature based on 7 HRGs in bladder cancer and reflected the degree of immune microenvironment. This research provide a novel understanding of hypoxia-related gene signature to estimate prognosis for patient survival and may benefit for making individualized treatment strategies for the cancer, but more specific experiments are still required to verify the findings in the future.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

XS, ML, XY and LY designed the experiments. ZZ, LJ, TZ, XL, JZ, WM and BZ performed the statistical analysis. YZ, JW and XZ participated in coordination of the study. XS and LY wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The authors declare that the data supporting the findings of this study are available within the article.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Shanghai Tenth People's Hospital.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

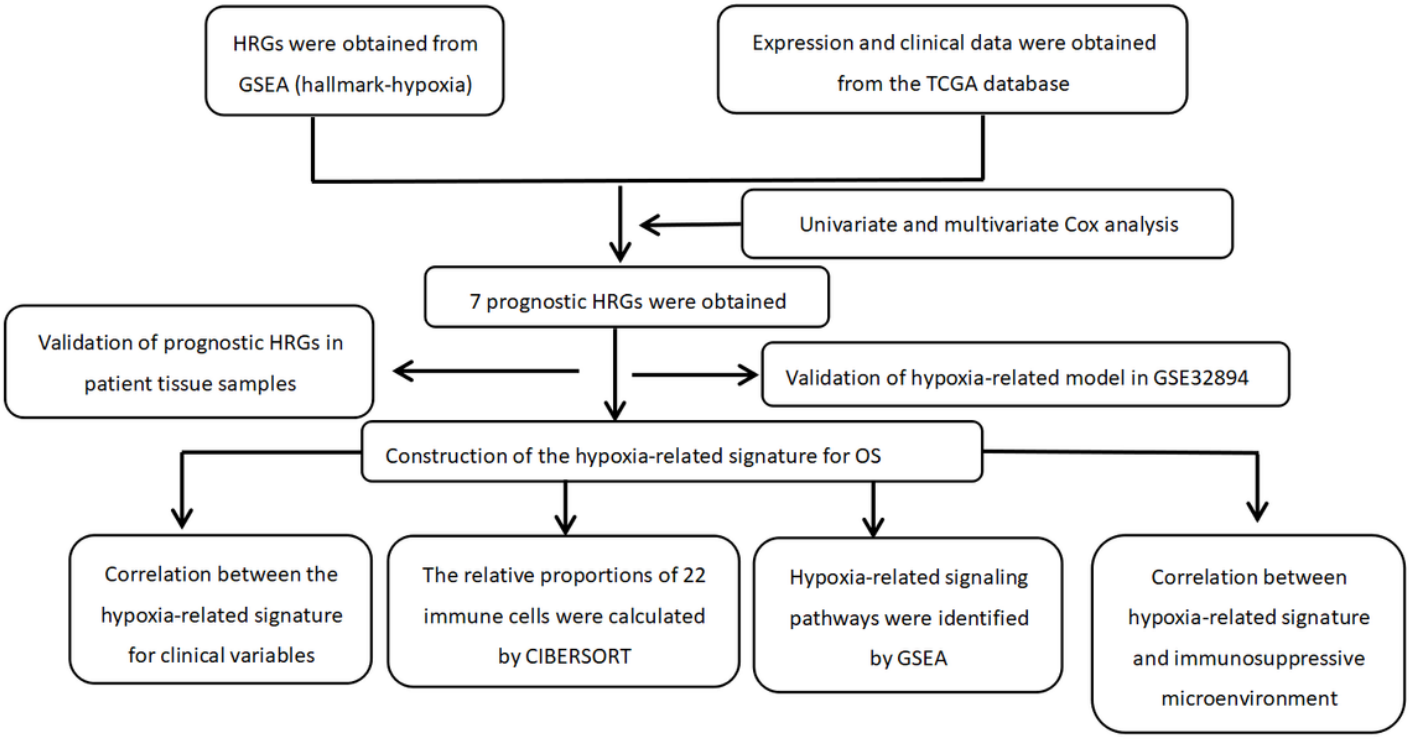

Figure 1

The flowchart of this study process.

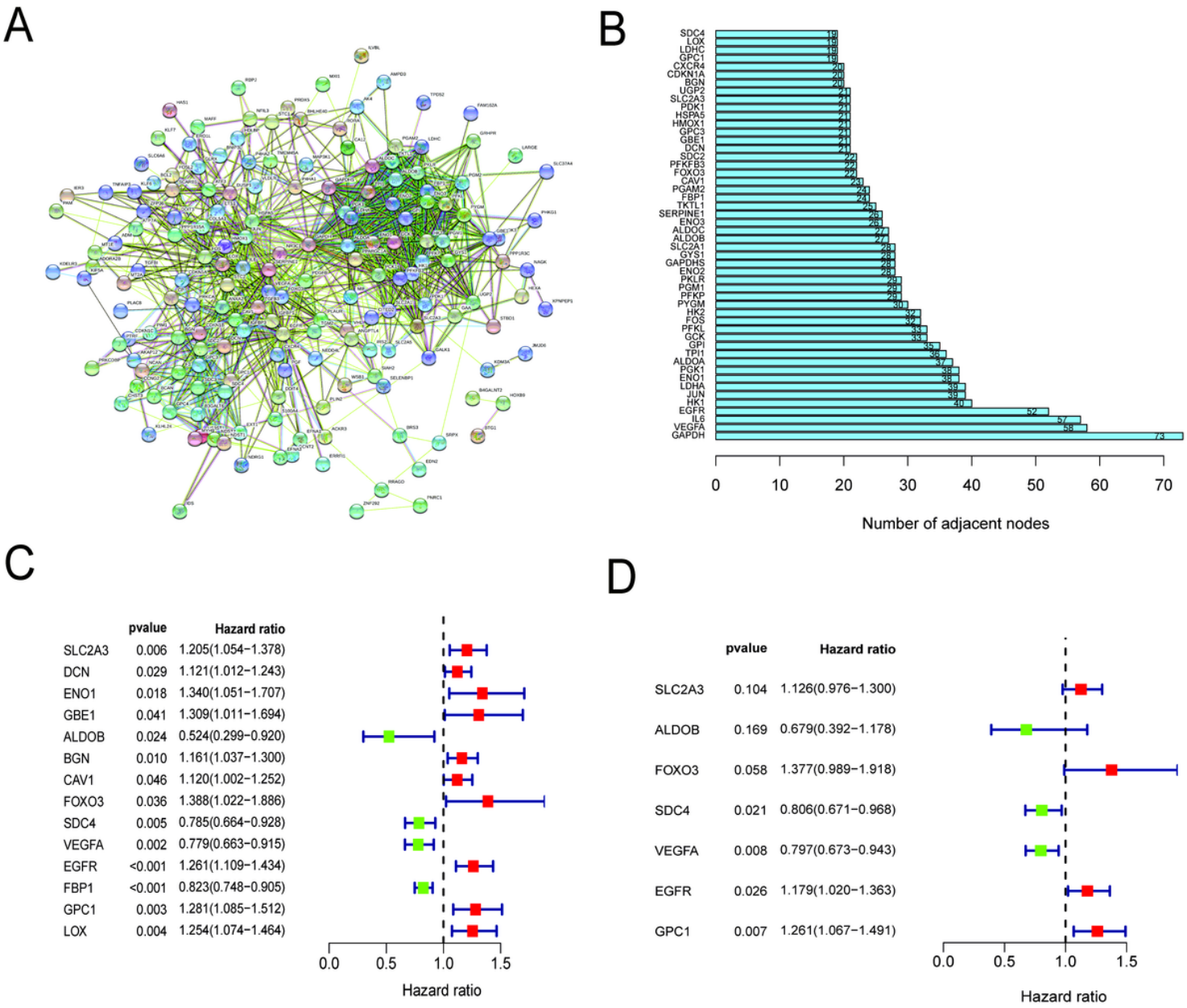

Figure 2

Identification of hypoxia-related genes in bladder cancer. (A) Protein-Protein network interactions including 200 hypoxia-associated genes. (B) The 50 genes with the most associated interaction degrees were selected. (C) Univariate Cox regression analysis of hypoxia-related genes. (D) Multivariate Cox regression analysis of hypoxia-related genes.

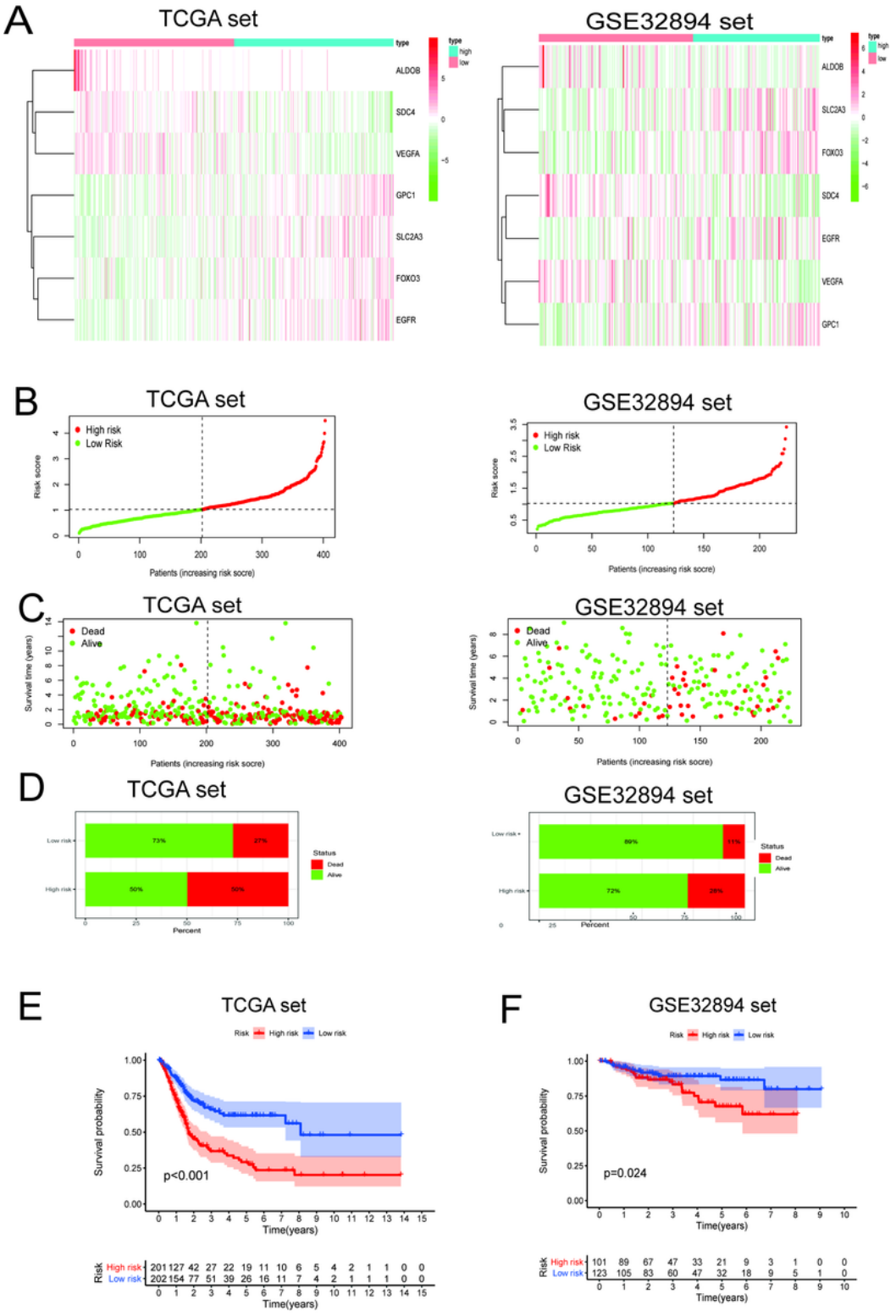

Figure 3

Prognostic significance of the hypoxia-related risk signature in bladder cancer. (A) The heatmap shows 7 hypoxia gene expression level in low- and high- risk groups from the TCGA and GSE32894 set. (B) Distribution of the risk scores of bladder cancer patients. (C) Patient status distribution in the low- and high- risk groups. The dot presents patient status ranked by the increasing risk score. The X axis is patient

number and Y axis is survival time. (D) Mortality rates of the low- and high- risk groups. (E,F) The prognosis significance of hypoxia signature in TCGA and GSE32894 data.

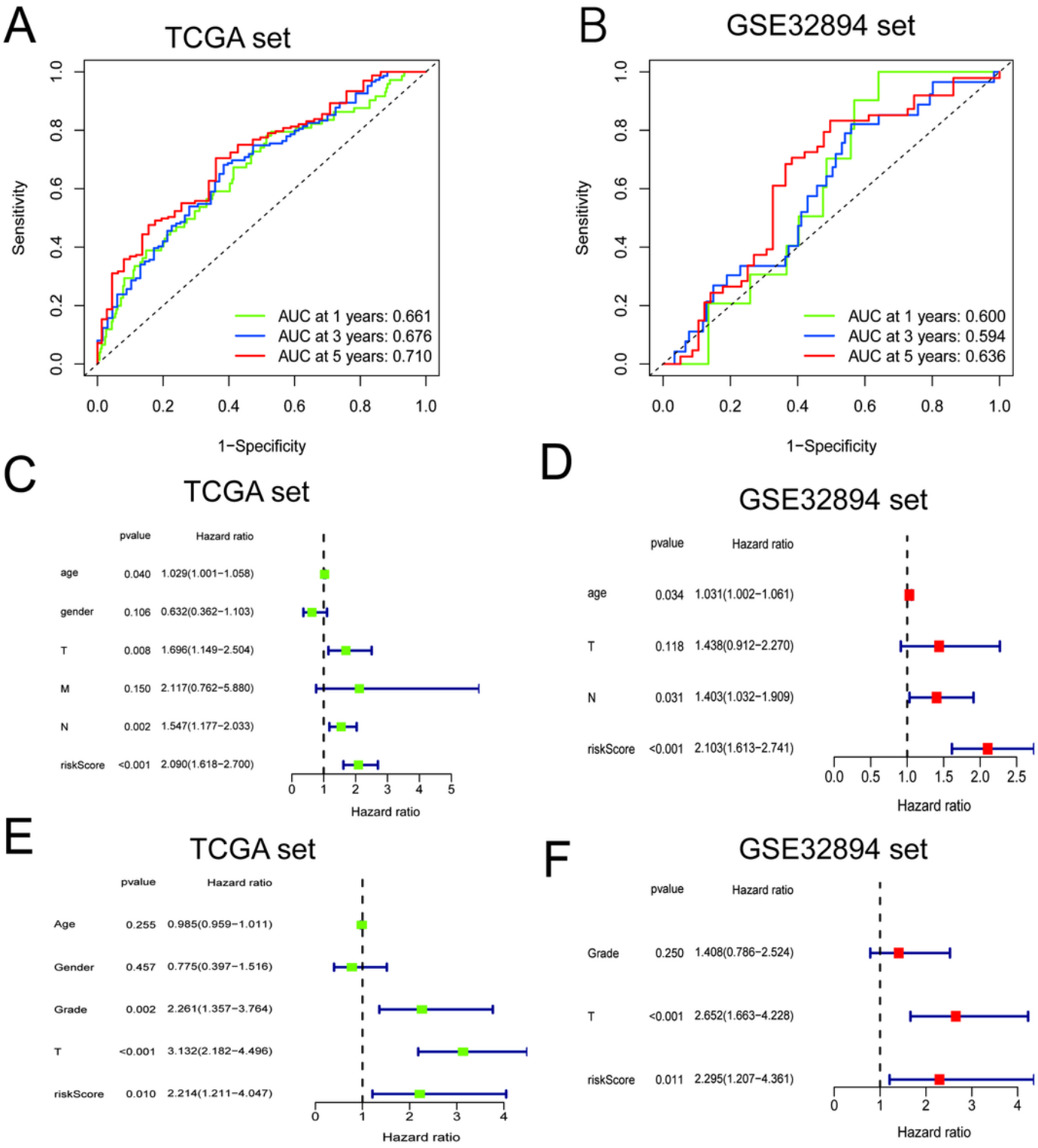

Figure 4

The hypoxia-related signature for OS is an independent prognostic factor for bladder cancer. (A,B) ROC curves showing the predictive efficiency of the hypoxia-related risk signature on the 1-, 3-, and 5-years

survival rate. (C-F) Univariate and multivariate Cox analysis of correlations between the risk score for OS and clinical variables.

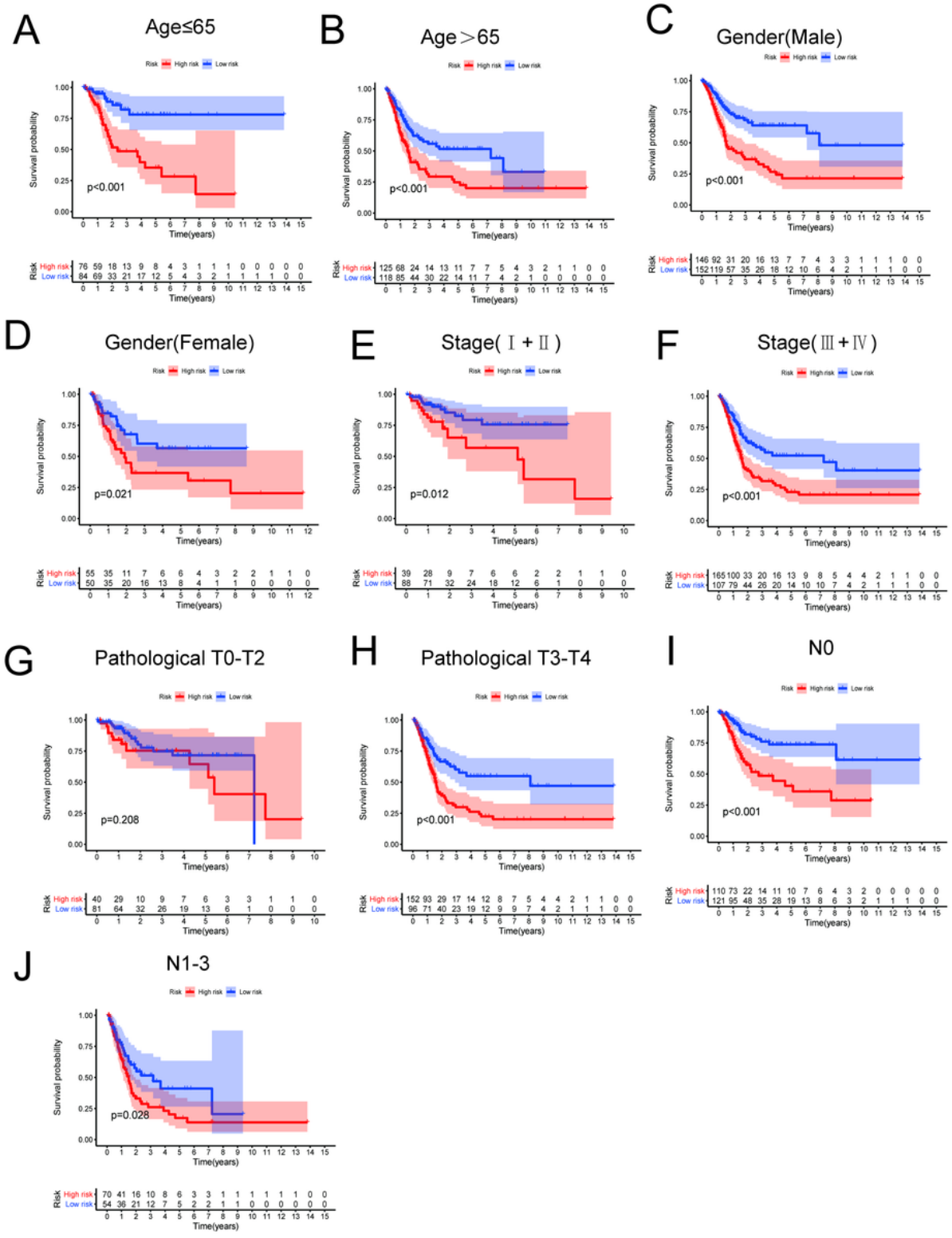

Figure 5

Kaplan-Meier survival curves for the low- and high-risk groups stratified by clinicopathological variables. (A,B) Age. (C,D) Gender. (E,F) Stage. (G,H) Pathological T stage. (I,J) N Stage.

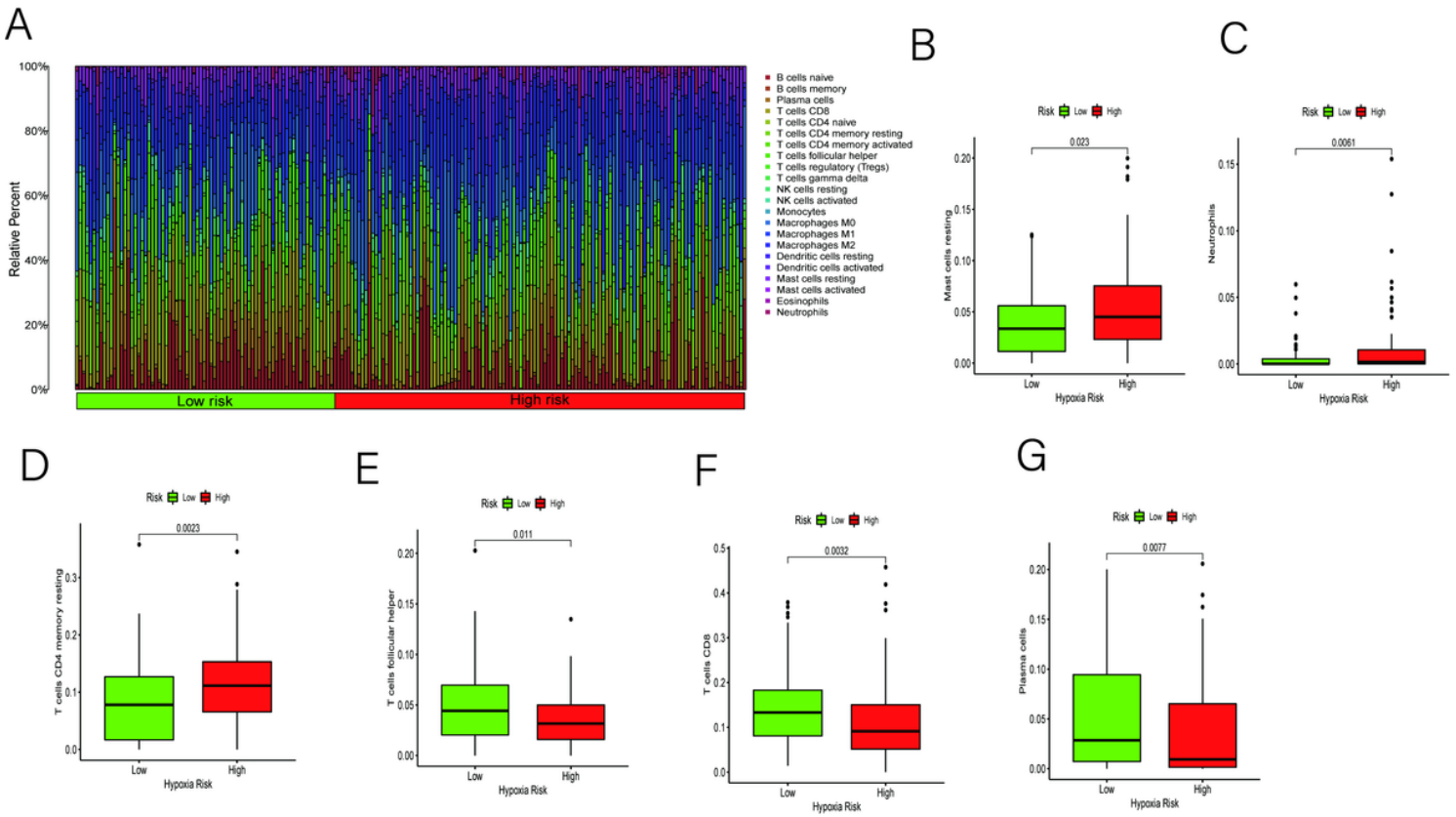

Figure 6

Immune landscape between low and high hypoxia risk bladder cancer patients. (A) The proportion of immune infiltration in low and high hypoxia risk patients. (B-G) Box plots visualizing significantly different immune cells between low and high hypoxia risk patients.

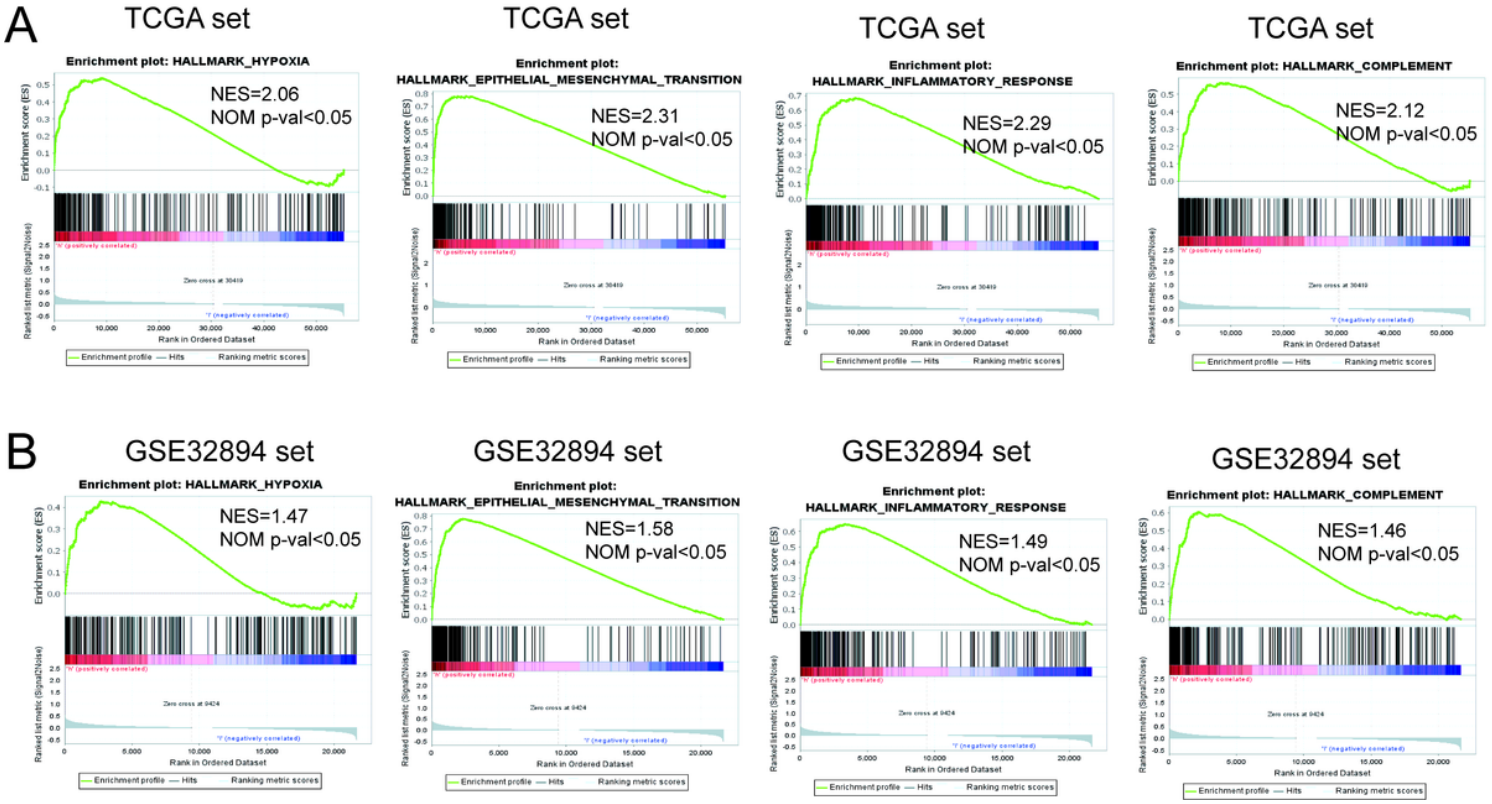

Figure 7

GSEA comparing the low and high hypoxia risk groups. The result listed the common functional gene sets enriched in high risk group compare to low risk group in TCGA (A) and GSE32894 set (B).

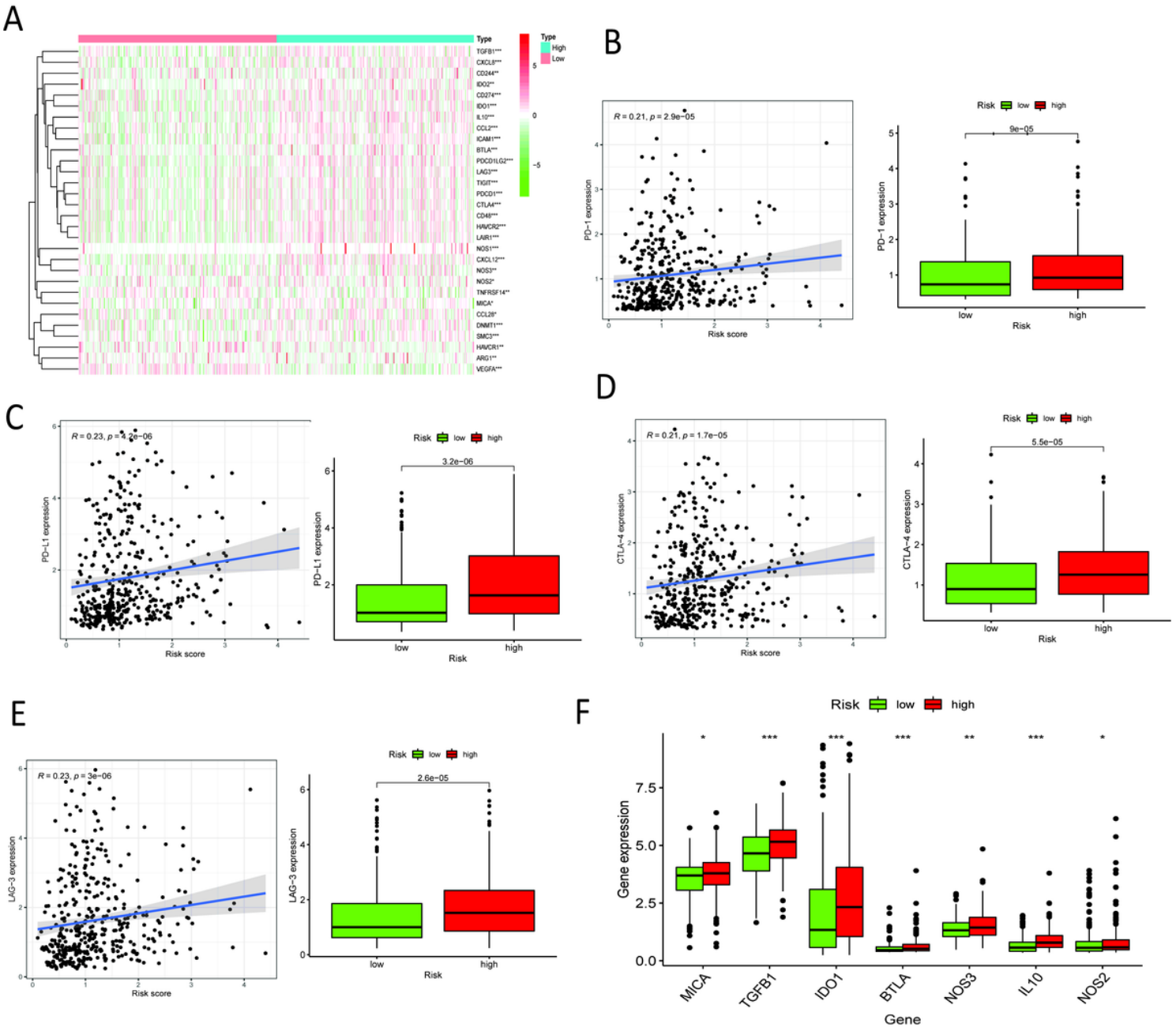

Figure 8

The hypoxia risk score was associated with immune microenvironment. (A) The heatmap of related negative genes in regulation of the Cancer-Immunity Cycle in low and high risk groups in TCGA-BLCA. (B) Correlation between PD-1 expression and hypoxia risk score. (C) Correlation between PD-L1 expression and hypoxia risk score. (D) Correlation between CTLA-4 expression and hypoxia risk score. (E) Correlation between LAG-3 expression and hypoxia risk score. (F) Tumor immunosuppressive cytokine expression in low and high hypoxia risk groups. *P < 0.05, **P < 0.01, and ***P < 0.001.

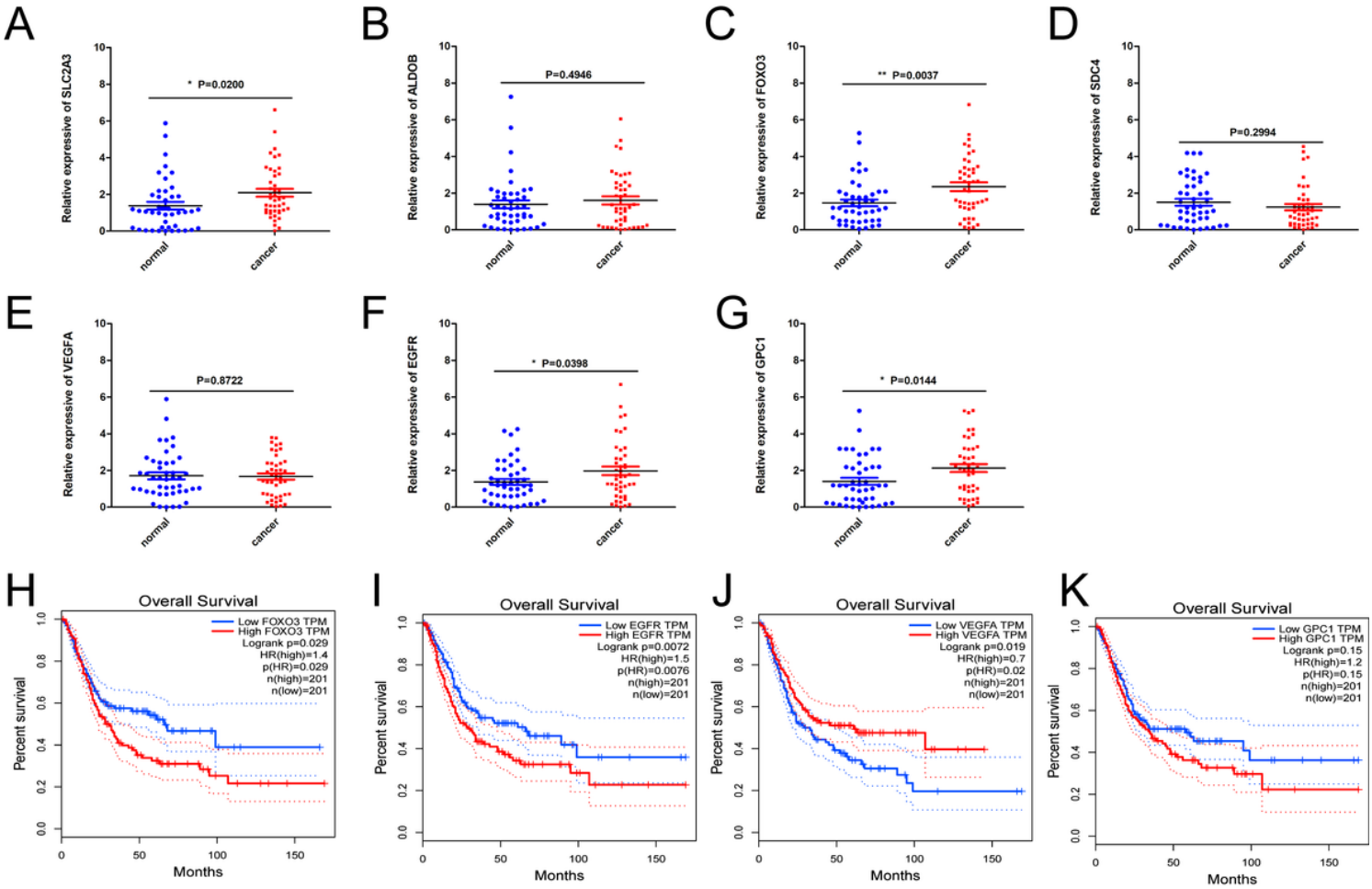

Figure 9

Validation of genes expression using qRT-PCR. A-G qRT-PCR analysis validation of the expression of SLC2A3, ALDOB, FOXO3, SDC4, VEGF, EGFR and GPC1 in paracancerous and cancer tissues. H-K Survival analysis of FOXO3, EGFR, VEGFA and GPC1. *P < 0.05, **P < 0.01.

Supplementary Files

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

- FigureS1.tif
- TableS1.docx
- TableS2.docx
- TableS3.docx