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Original Article

Comprehensive Analysis of Differential Gene Expression and Correlated Immune Infiltration in Bladder Cancer

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Abstract

Background: Bladder cancer (BCa) is one of the most common urinary tract malignancies. Our study aimed to provide promising biomarkers for BCa screening and prognosis.

Methods: BCa samples were obtained from Gene Expression Omnibus (GEO) datasets. Differentially expressed genes (DEGs) were analysed by GO/KEGG analysis. Univariate Cox hazard analysis and Kaplan Meier Curve clarified the relevance of DEGs and survival. Receiver operating characteristic (ROC) curve showed the discrimination ability of DEGs in BCa patient outcome prediction. RT-PCR was used to validate gene expression.

Results: Overall, 61 common up regulated and 170 common down-regulated genes in BCa were obtained. DEGs were mainly enriched in proliferation and metastasis processes. *CDC20*, *COL14A1*, *SPARCL1*, *TMOD1*, *RHOJ*, *FXYD6* and *MFAP4* had clinical relevance to survival with high accuracy. *CDC20*, *SPARCL1* and *TMOD1* are promising biomarkers of BCa. *CDC20*, *SPARCL1* and *TMOD1* are involved in cancer immune infiltration.

Conclusion: *CDC20*, *SPARCL1* and *TMOD1* are promising biomarkers of bladder cancer. In addition, *CDC20*, *SPARCL1* and *TMOD1* are involved in cancer immune infiltration, which provides new targets in immune therapy in bladder cancer.

Keywords: Bladder cancer; Differentially expressed genes (DEGs); Network analysis; Biomarker; Immune infiltration

Introduction

Bladder cancer (BCa) is one of the most common urinary tract malignancies, associated with high mortality (1). In last decades, several routine therapeutic methods were developed including neoadjuvant chemotherapy, surgical resection, radiotherapy and photodynamic therapy (2). However, it still lacks effective ways to diagnose BCa in early stages without obvious clinical symptoms.

Generally, BCa is categorized into two types including papillary and nonpapillary forms. Different clinical outcomes are observed in two forms due to the various molecular subtypes (3). Recent

researches clarify that different molecular mechanisms are involved in BCa metastasis and progression with differential expression of genes. In line with other cancers, inactivation of p53 leads to BCa progression and invasion (4). Wnt signaling pathway is activated by RAS elevation, which results in bladder tumorigenesis (5). In addition, non-coding RNAs are also involved in BCa progression and regulation. *CASC11*, *SNHG7*, *SPRY4-IT1* are validated to promote BCa metastasis and proliferation (6-8). Hence, exploring differentially expressed genes (DEGs) in BCa is beneficial for finding biomarkers, which predict BCa progression and outcome.

Since recent studies have shown that many types of tumor-infiltrating lymphocytes (TILs) are involved in tumor progression (9), this study could provide new targets in immune therapy in bladder cancer via bioinformatics analysis.

Materials and Methods

In this study, the differentially expressed genes correlated to immune infiltration were analysed through bioinformatics analysis. In addition, the selected genes were validated by q-PCR analysis in bladder cancer cell lines. We compared three independent microarray results downloaded from Gene Expression Omnibus (GEO) and obtained DEGs in bladder cancer. Further analysis was performed through pipelines of gene ontology (GO) enrichment analysis; Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, protein–protein interaction (PPI) network analysis. We clarified the relevance of the selected DEGs and BCa patient survival. Our study provided promising biomarkers for prediction of BCa outcome and survival. Furthermore, we performed analysis on the correlations between TILs and potential biomarkers of bladder cancer.

Patient information

Three independent patient cohorts were obtained from GSE7476, GSE37815 and GSE13507. GSE7476 comprised three groups of normal bladder tissues and nine groups of tumoral bladder tissues from GPL570 (HG-U133_Plus_2) Affymetrix Human Genome U133 plus 2.0 Array platform. GSE37815 comprised 5 groups of normal bladder tissues and 18 groups of bladder cancer tissues from GPL6102 Illumina human-6 v2.0 expression beadchip. GSE13507 comprised 165 primary bladder cancer samples and 23 normal tissues from 14 patients, performed in GPL6102 Illumina human-6 v2.0 expression beadchip.

Because the gene expression profile of patients was from GEO datasets and underwent dual anonymization a specific approval was not deemed necessary by the ethics committee of Chongqing University Fuling Hospital.

Data processing

The datasets were downloaded as MINIL format and DEGs were screened by using Limma package (version: 3.40.2) of R software. "P<0.05 and Log (Fold Change, FC) >1 or Log (FC)< −1" were defined as the thresholds for the screening of differential expression of mRNAs. The volcano plots were implemented by the R software package ggplot2.

GO/KEGG pathway enrichment analysis

To further confirm the underlying function of potential targets, the data were analyzed by Gene Ontology (GO) analysis annotating genes with functions, especially molecular function (MF), biological pathways (BP), and cellular components (CC), and Kyoto Encyclopedia of Genes and Genomes (KEGG) Enrichment Analysis. To better understand the carcinogenesis of mRNA, ClusterProfiler package (version: 3.18.0) in R was employed to analyze the GO function of potential targets and enrich the KEGG pathway. The box plot is implemented by the R software package ggplot2.

PPI network and module analysis

DEGs were uploaded to the online Search Tool for the Retrieval of Interacting Genes (STRING). Protein relations and interactions were obtained and then generated network module with Cytoscape network.

Survival analysis

Univariate Cox hazard analysis and Kaplan Meier Curve were employed to clarify the relevance of selected DEGs and BCa patient survival. The forest plots were generated by the R software package ggplot2. Receiver operating characteristic (ROC) curve was used to show the discrimination ability of selected DEGs in BCa patient outcome prediction. The ROC curve was drawn by R software package pROC (v1.17.0.1) and ggplot2. The area under the curve (AUC) indicates the accuracy. AUC over 0.9 is considered as high accuracy and AUC within 0.7-0.9 is considered as moderate accuracy.

Immune infiltration analysis

Online tool TISIDB (http://cis.hku.hk/TISIDB/index.php) was used to analyse tumor-immune system interactions.

Real time polymerase chain reaction analysis (RT-PCR)

Total RNAs were isolated from bladder cancer cells SW780, HT1197 and HT1376 and a normal urinary bladder epithelial cell (NBEC) following the instruction of High Pure RNA Isolation Kit (Roche). After purification, total 100 ng RNA was used to undergo Reverse transcription reaction to obtain cDNA. Since *CDC20*, *COL14A1*, *SPARCL1*, *TMOD1*, *RHOJ*, *FXYD6* and *MFAP4* may serve as potential biomarkers of bladder cancer via bioinformatics analysis, here we performed q-PCR analysis to confirm the expression of these genes in bladder cancer cells. RT-PCR was performed with SYBR Premix Ex TaqII (Takara) and a LightCycler 480 system (Roche, Indianapolis, IN, USA). The reaction was performed as 95 ℃ 3 min, 45 cycles of 95 ℃ 3 sec and 60 ℃ 30 sec. The relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (cycle threshold (CT)) as described before (10). *GAPDH* was served as an internal control. The primers used were listed in Table 1.

Table 1: Primers used in q-PCR validation

Gene	Forward 5'-3'	Reverse 5'-3'
CDC20	GCACAGTTCGCGTTCGAGA	CTGGATTTGCCAGGAGTTCGG
COL14A1	ATGCCAGACCAGAATTACACAG	ACCATCGACCAGGATTACAATGT
SPARCL1	ACGGTAGCACCTGACAACAC	ATGGTGGGAATCGTCTTCTGT
RHOJ	AGGGGCAACGACGAGAAGA	TTGGCGTAGCTCATCAGCAG
TMOD1	TGCTGGAAAGTGTGACGCTG	CCCAGGATCGCTGCAATGT
FXYD6	ACCCTGAGGATTGGGGGAC	CATTGGCGGTGATGAGGTT
MFAP4	TACCAGTCAGACGGCGTGTA	CCACTCGCAGCTCATACTTCT
GAPDH	CTGGGCTACACTGAGCACC	AAGTGGTCGTTGAGGGCAATG

Statistics

In RT-PCR analysis, results were presented as mean ± SD. Analysis were achieved by two-way analysis of variance and t-test. All analyses were performed using the GraphPad Prism8 software. *P*-values of <0.05 or <0.01 were considered statistically significantly different.

Results

DEGs identification in three independent BCa patient cohorts

To analyse the DEGs of bladder cancer, we obtained gene expression profiles from three independent patient cohorts (GSE7476, GSE37815 and GSE13507) downloaded from GEO datasets. DEGs with $|\log FC| > 1$ and $P<0.05$ were selected and presented in this study, which went through following analysis as shown in Fig.

1. Total 411, 155 and 259 genes were up-regulated and 1148, 359 and 798 genes were down-regulated in GSE7476, GSE37815 and GSE13507 respectively (Fig. 2A). Next, DEGs from three patient cohorts were compared to show the overlapping genes. There were 61 genes overlapped in up-regulated genes while 170 genes were overlapped in down-regulated genes (Fig. 2B).

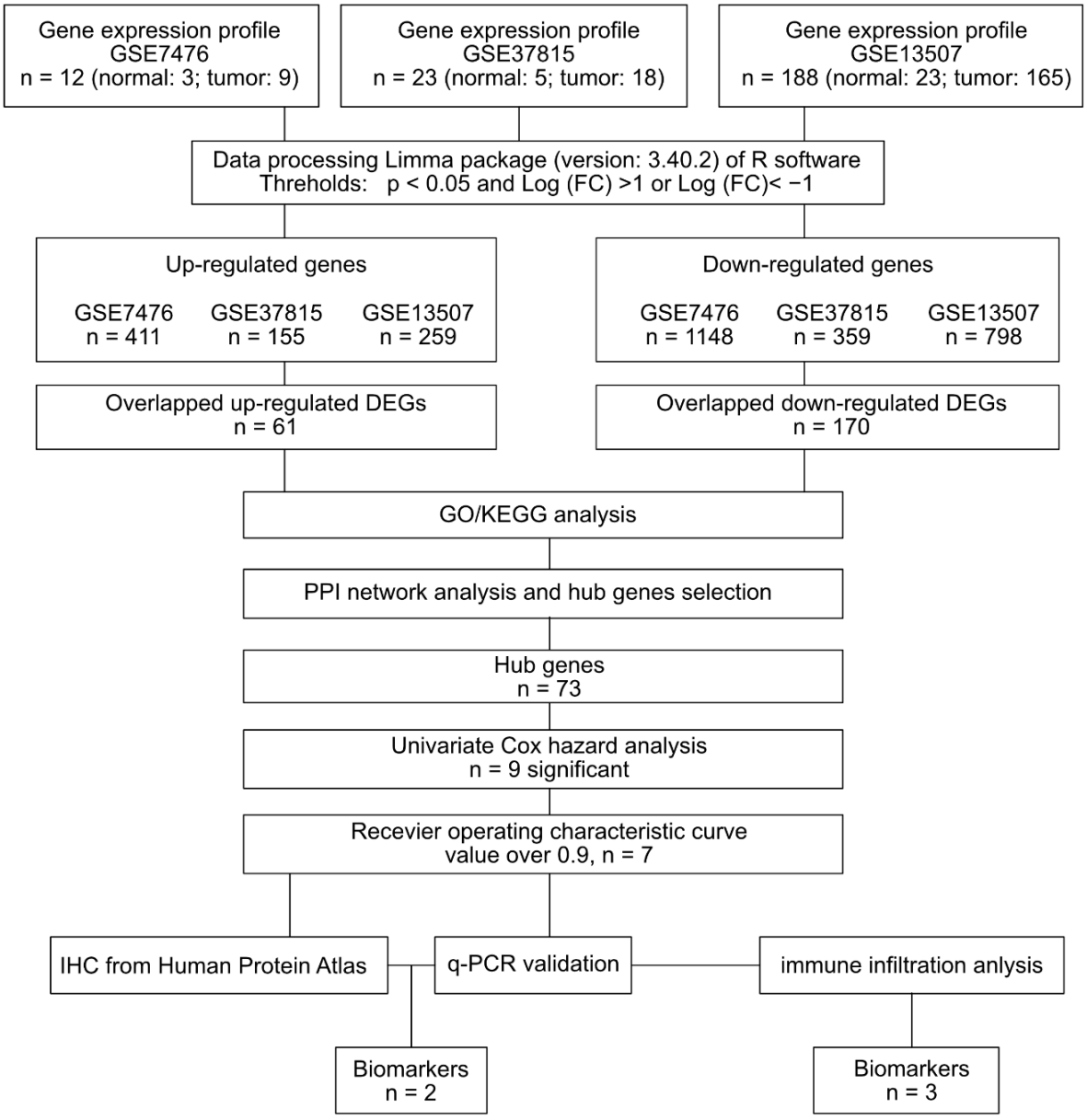

Fig. 1: Flow chart of analysis of patient cohorts and correlated gene expressions

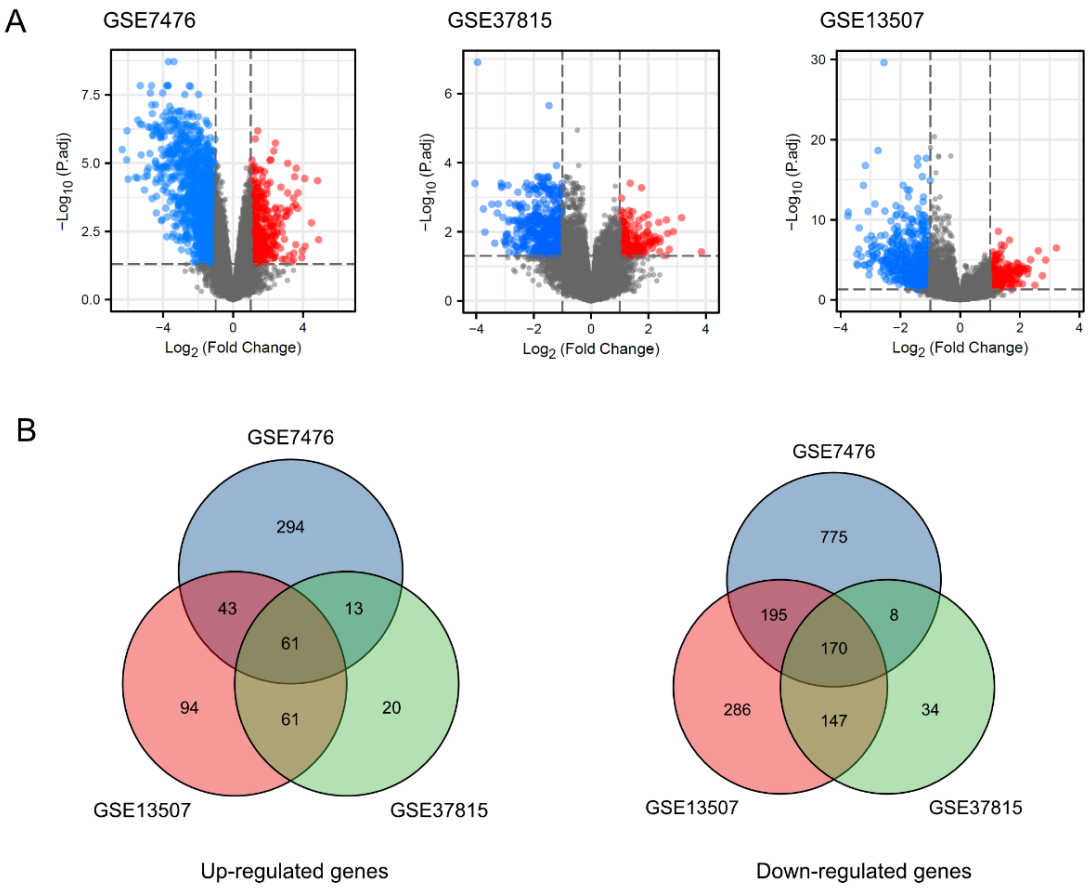

Fig. 2: DEGs identification in three independent BCa patient cohorts

(A) Volcano plots generated based on analysed results of GSE7476, GSE37815 and GSE13507. Up-regulated genes with $|logFC| > 1$ and p value < 0.05 were selected and presented. Up-regulated genes were marked with red dots and down-regulated genes were marked with blue dots. (B) Venn diagrams showed common up-regulated (left) and down-regulated (right) genes from three indicated GEO datasets

DEGs characterization by GO and KEGG pathway analysis

To clarify the function of DEGs obtained, we performed GO/KEGG analysis on all overlapping DEGs. Overall, 61 overlapping up-regulated genes mainly correlated to cell cycle, mitotic nuclear division, nuclear division and organelle fission (Fig. 3A and 3C). Dysregulation of cell cycle and mitosis is possibly involved in bladder cancer progression 170 overlapping down-regulated genes mainly enriched in extracellular matrix (Fig. 3B and 3D), which indicated that potential weaken cell-cell contacting contributes bladder cancer progression.

PPI network and cluster analysis on DEGs in bladder cancer

Total 231 DEGs (61 overlapping up regulated and 170 overlapping down-regulated genes) were imported into the gene PPI network complex (Fig. 4A, interaction score > 0.9, disconnected nodes in the network hided). Next, the interaction network was imported into Cytoscape software for further analysis. A clearer interaction map with three obvious sub-interaction networks was presented (Fig. 4B). Here we selected all presented genes (n=73 in Fig 4B) into further study.

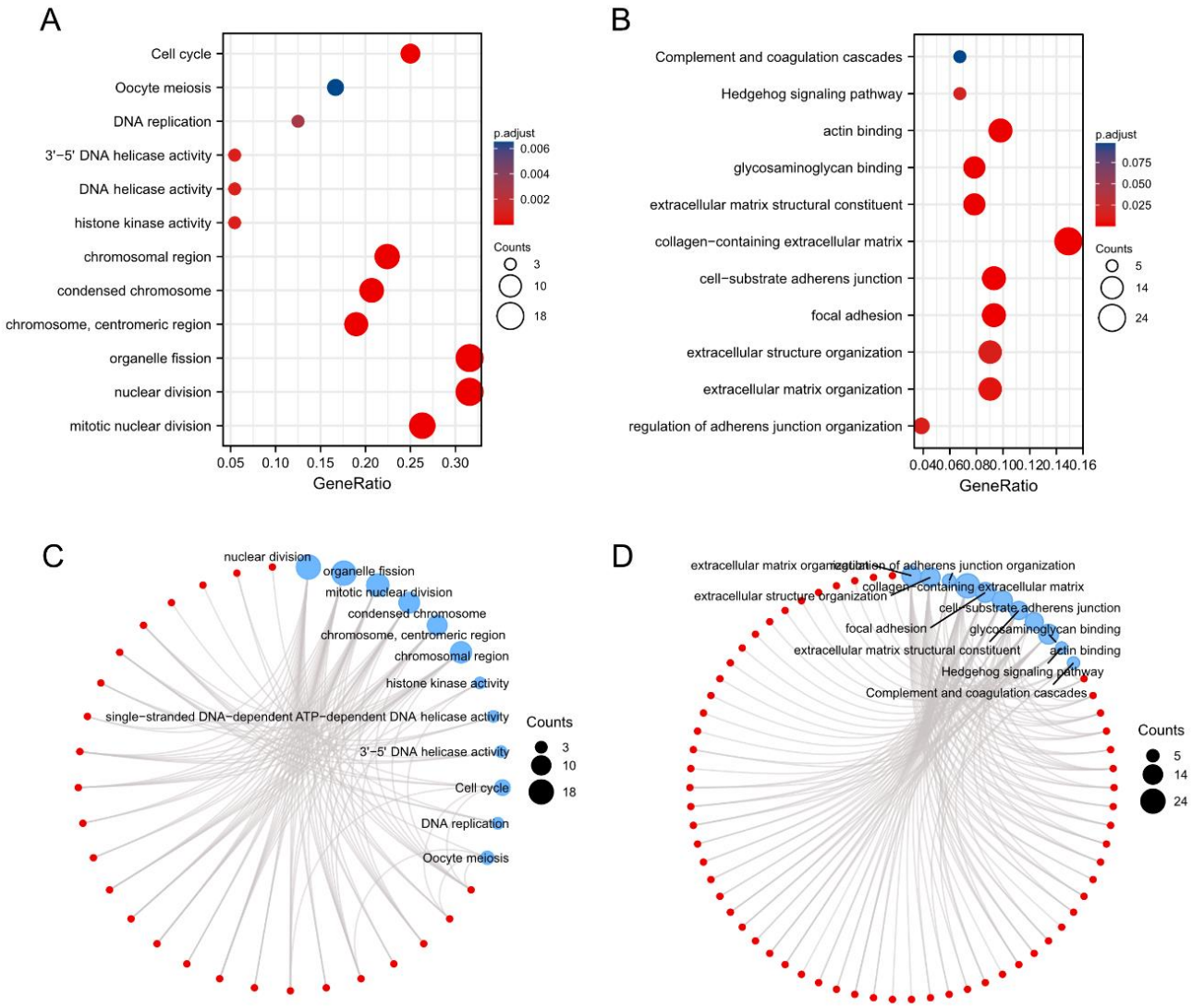

Fig. 3: DEGs characterization by GO and KEGG pathway analysis in BCa

(A) and (C) GO/KEGG analysis of common up-regulated genes. (B) and (D) GO/KEGG analysis of common down-regulated genes. Red indicates higher difference while blue indicates lower difference. The circle area is related to the number of enriched genes

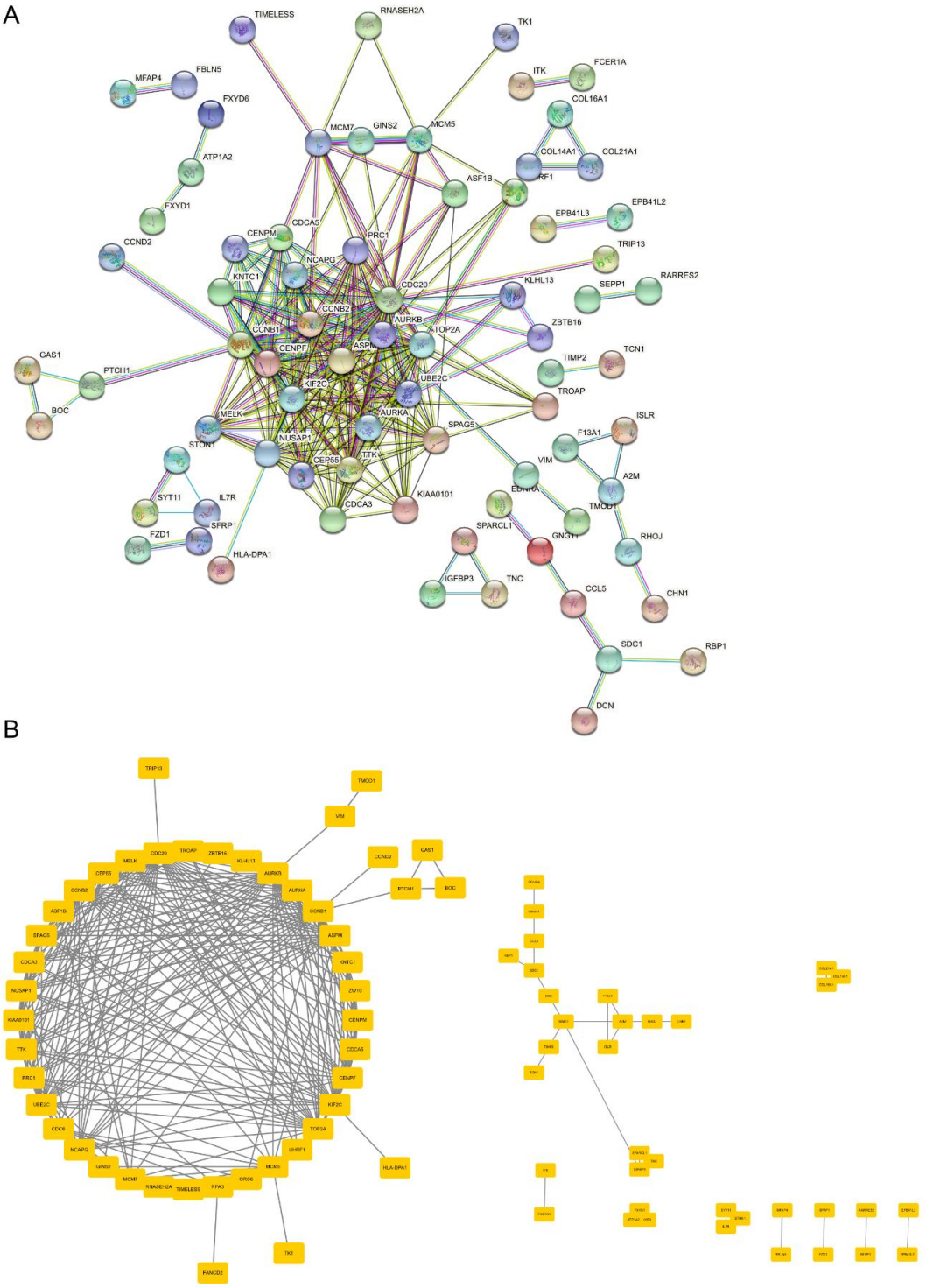

Fig. 4: PPI network and cluster analysis on DEGs in bladder cancer

(A) Total 231 DEGs were uploaded and analysed by using PPI network. The genes with interaction score > 0.9 were presented. (B) Protein interactions obtained were analysed by Cytoscape software and 73 interacted DEGs were presented

Characterization impact of selected DEGs on survival of bladder cancer patients

To clarify the clinical relevance of the selected 73 DEGs in Fig 3B, we performed univariate Cox hazard analysis to show the correlation with survival. Total 11 genes presented significant high hazard ratio (P<0.05, listed in Table 2), indicating high correlation with survival of BCa. Next, the overall survival correlated to 11 significant genes were analysed based on clinical data of TCGA-BLCA patient cohort. In line with the results of univariate Cox hazard analysis, high expression of 11 significant genes indicates a poor survival probability (Fig. 5). To validate the accuracy of 11 DEGs in the ending prediction of bladder cancer, we also used receiver operating characteristic (ROC) curve to show the discrimination ability. In Fig. 6, the area under the curves (AUCs) of *CDC20*, *COL14A1*, *SPARCL1*, *TMOD1*, *RHOJ*, *FXYD6* and *MFAP4* was over 0.9, which suggested a high accuracy of predicting survival in bladder cancer patients.

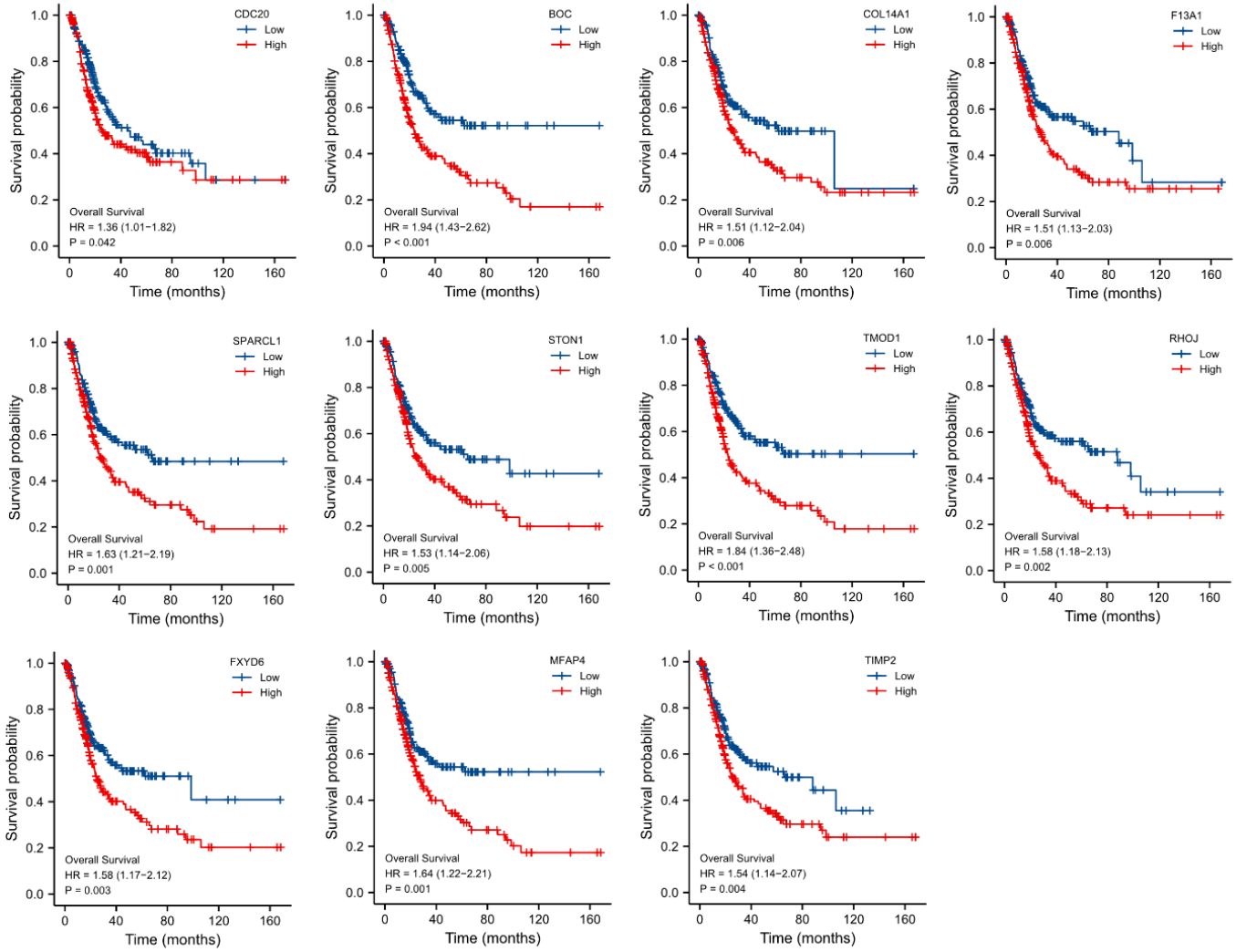

Fig. 5: Effect of CDC20, BOC, COL14A1, F13A1, SPARCL1, STON1, TMOD1, RHOJ, FXYD6, MFAP4 and TIMP2 expression level on the survival of BCa patients from TCGA-BLCA dataset

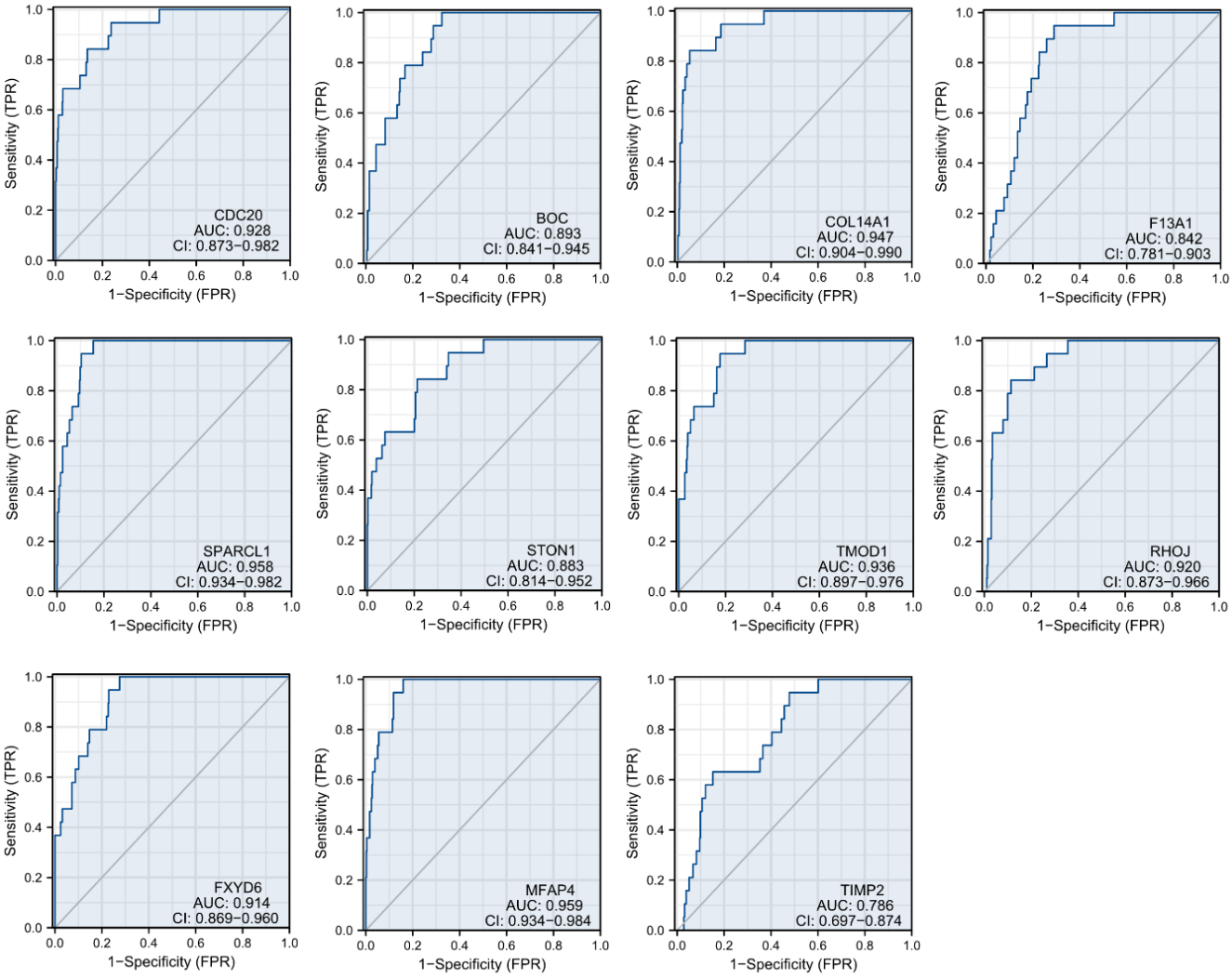

Fig. 6: ROC curve showed the accuracy of 11 indicated DEGs in the ending prediction of bladder cancer from TCGA-BLCA dataset

Table 2: Eleven genes with significant high hazard ratio

Genes	***HR (95 CI)***	***P-value***
CDC20	1.356 (1.012-1.817)	0.042
BOC	1.939 (1.433-2.624)	< 0.001
COL14A1	1.514 (1.124-2.038)	0.006
F13A1	1.511 (1.125-2.029)	0.006
SPARCL1	1.631 (1.212-2.195)	0.001
STON1	1.535 (1.142-2.063)	0.005
TMOD1	1.838 (1.362-2.482)	< 0.001
RHOJ	1.584 (1.179-2.128)	0.002
FXYD6	1.577 (1.172-2.123)	0.003
MFAP4	1.639 (1.217-2.207)	0.001
TIMP2	1.540 (1.144-2.073)	0.004

Validation of selected DEGs in bladder cancer cells by q-PCR analysis

Next, we performed q-PCR analysis to confirm expression of genes with high AUC (>0.9) in three bladder cancer cells including SW780, HT1197 and HT1376. Unexpectedly, not all selected DEGs were up regulated in bladder cancer cells. Only *CDC20*, *SPARCL1* and *TMOD1* were highly expressed in three bladder cancer cell lines compared to NBEC (Fig. 7A). Furthermore, we checked expression of *CDC20*, *SPARCL1* and *TMOD1* in bladder cancer tissues via Human Protein Atlas. However, only *CDC20* and *TMOD1* were highly expressed in tumor tissues, whereas the expression of *SPARCL1* was insignificant between tumor and normal tissues (Fig. 7B). Therefore, *CDC20*, and *TMOD1* are more promising to be developed as biomarkers of bladder cancer in clinical screening.

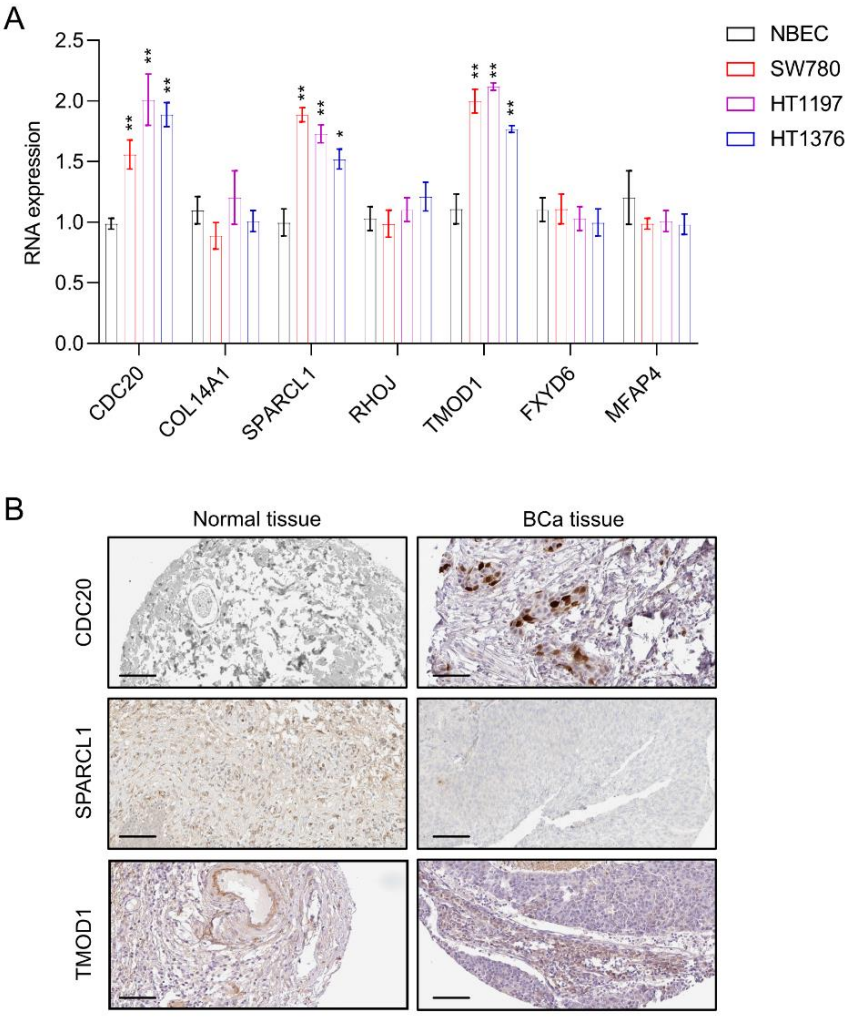

Fig. 7: (A) RT-PCR analysis of selected genes in three BCa cell lines and a normal bladder epithelial cell line (NBEC). (B) Immunohistochemistry of CDC20, SPARCL1 and TMOD1 in bladder cancer tissues compared to normal tissues obtained from Human Protein Atlas. Scale bar: 50 µm

CDC20, SPARCL1 and TMOD1 are involved in immunocyte infiltration

Tumor microenvironment is critical for tumor progression by regulating different immunocyte infiltration. Thereby we checked whether *CDC20*, *SPARCL1* and *TMOD1* are involved in immune infiltration in bladder cancer. Four different immunocytes including activated CD8+ T cell, activated CD4+ T cell, myeloid derived suppressor cell (MDSC) and macrophage were investigated. Indeed, the expression of *CDC20*, *SPARCL1* and *TMOD1* was positively correlated to infiltration of selected immunocytes (Fig. 8A-8C), which indicated that *CDC20*, *SPARCL1* and *TMOD1* induce immune infiltration in bladder cancer. Therefore, high expression of *CDC20*, *SPARCL1* and *TMOD1* also indicates tumor microenvironment in bladder cancer, which may give the hint in immunotherapy.

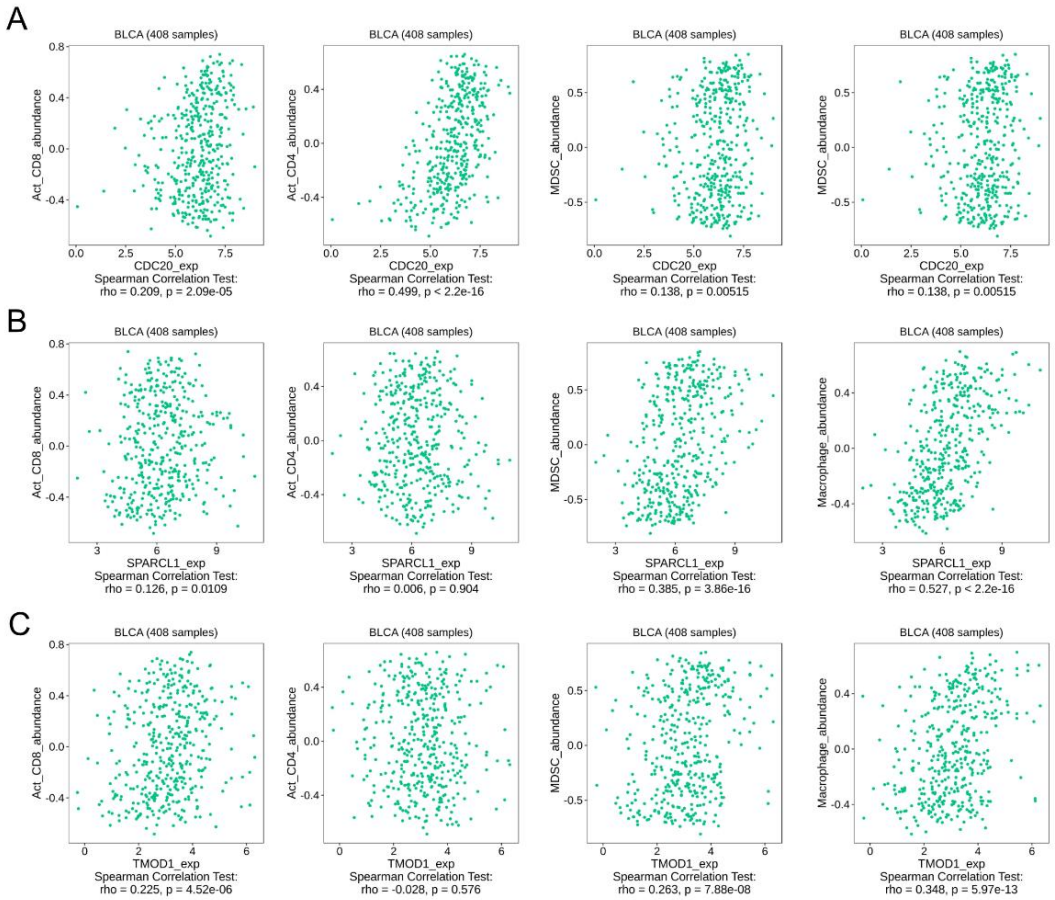

Fig. 8: The correlation between immunocytes and expression of CDC20 (A), SPARCL1 (B) and TMOD1 (C) in bladder cancer. In each panel, four different immunocytes were included: activated CD8+ T cell, activated CD4+ T cell, MDSC and macrophage

Discussion

In this study, we found that *CDC20*, *COL14A1*, *SPARCL1*, *TMOD1*, *RHOJ*, *FXYD6* and *MFAP4* are potential biomarkers of predicting survival in bladder cancer patients with high accuracy. In addition, the high expression of *CDC20*, *SPARCL1* and *TMOD1* indicates tumor microenvironment in bladder cancer; thereby these genes provide opportunities in immunotherapy. Currently, bladder cancer is the most common diagnostic urologic tumor with high

heterogeneity and complexity at the molecular level (1). Here we performed bioinformatics analysis in clinical patient cohorts to uncover potential genes as biomarkers for bladder cancer screening in early stages.

In this study, three independent patient cohorts from GEO datasets were analysed. Sixty-one common up-regulated and 170 common down-regulated genes were obtained. The up-regulated genes were mainly enriched in biological processes including cell cycle, DNA replication, organelle fission, nuclear division and mitotic nuclear division, while down-regulated genes were mainly enriched in regulation of adherent junction organization, extracellular matrix organization and extracellular structure organization. Therefore, our analysis indicates the common DEGs are involved in cell proliferation and migration in bladder cancer. Indeed, inhibition of proliferation and migration is a main direction in bladder cancer prevention researches (11). Thereby, the selected biomarkers also possibly mean high probability of metastasis of BCa.

Through PPI network and Cytoscape screening, 73 DEGs with high interaction score were focused. Based on ROC curve, *CDC20*, *COL14A1*, *SPARCL1*, *TMOD1*, *RHOJ*, *FXYD6* and *MFAP4* were considered having high accuracy as biomarkers for survival prediction. Indeed, previous studies have shown *CDC20* is a promising cancer therapy target in bladder cancer. Furthermore, *CDC20* is overexpressed in different cancer types (12, 13). Depleting *CDC20* causes mitotic arrest and results in cell death (14). Interestingly, *CDC20* is also involved in cancer metastasis as it functions distinctly compared to *CDH1* (15). Therefore, targeting *CDC20* is a promising strategy in bladder cancer therapy. For *COL14A1*, previous researches validated it is correlated to tumor TNM stage (16), suggesting a poor clinical outcome. Our analysis further supports *COL14A1* as a biomarker of outcome prediction in bladder cancer patients. Interestingly, *SPARCL1* is widely repressed in tumour tissues compared to normal tissues in various cancers. However, it is still prognostic biomarkers of poor clinical outcome in prostate cancer (17). Thus, *SPARCL1* is possibly a bi-biomarker for distinguishing both normal/bladder cancer patients and good/poor outcome of bladder cancer.

Previous studies have identified increased immune infiltration in different cancers. Immune infiltration is involved in cancer progression and has been characterized as a promising strategy for cancer therapy (18). Activated CD8+ and CD4+ T cells are considered helpful to prolong the lifespan of bladder cancer patients (19). Interestingly, M2 macrophages, MDSCs and other T regulation cells are commonly found in cancers, associated with poor prognosis (20).

Conclusion

Therefore, *CDC20*, *SPARCL1* and *TMOD1* may have complex functions in bladder cancer immune infiltration. In addition, most chemokines and their receptors are also promoted due to the elevation of *CDC20*, *SPARCL1* and *TMOD1*. Thereby our study provides an alternative explanation that *CDC20*, *SPARCL1* and *TMOD1* promoted immune infiltration is associated with poor prognosis due to the chemokines secreted from MDSCs.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors report there are no competing interests to declare.

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