

SMC4, CCNB1 and CKS1B as potential targets and new critical biomarkers for the prognosis of human bladder cancer

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Research

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Abstract

Background

As a common malignant cancer of the urinary system, the precise molecular mechanisms of bladder cancer remain to be illuminated. The purpose of this study was to identify core genes with prognostic value as potential oncogenes for the diagnosis, prognosis or novel therapeutic targets of bladder cancer.

Methods

The gene expression profiles GSE3167 and GSE7476 were available from the Gene Expression Omnibus (GEO) database. Next, PPI network was built to filter the hub gene through the STRING database and Cytoscape software and GEPIA and Kaplan-Meier plotter were implemented. Frequency and type of hub genes and sub groups analysis were performed in cBioportal and ULCAN database. Finally, We used RT-qPCR to confirm our results.

Results

Totally, 251 DEGs were excavated from two datasets in our study. We only founded high expression of SMC4, TYMS, CCNB1, CKS1B, NUSAP1 and KPNA2 was associated with worse outcomes in bladder cancer patients and no matter from the type of mutation or at the transcriptional level of hub genes, the tumor showed a high form of expression. However, only the expression of SMC4,CCNB1and CKS1B remained changed between the cancer and the normal samples in our results of RT-qPCR.

Conclusion

In conclusion,These findings indicate that the SMC4,CCNB1 and CKS1B may serve as critical biomarkers in the development and poor prognosis.

Introduction

Bladder cancer (BLCA) is one of the most prevalent cancers among all human malignant cancers all over the world(1). It is figured out that there were approximately 549,000 new bladder cancer patients and 200,000 patients with bladder cancer-related deaths in 2018, making bladder cancer become the tenth most frequent cause of cancer death in both genders worldwide(1). Despite surgery, intravesical treatment, and various adjuvant treatments for BLCA(2, 3), it has been reported that it is likely to develop into muscle-invasive diseases and has high recurrence(4, 5), due to these, the five-year survival rate of this cancer type is still unsatisfied. Therefore, precisely targeted therapy is particularly important. Although there have been a lot of reports on the exact targets and mechanisms of the occurrence, development and recurrence of bladder cancer for diagnosis, prognosis and treatment. However,

chemotherapeutic resistance and low five-year survival rate still remains(6–8). The mechanism of tumorigenesis is relatively complex. Genomic and its transcriptional abnormalities and genomic methylation are the causes of tumorigenesis(9–11). Therefore, it is urgent to explore more reliable biomarkers for diagnosis, prognosis or precise therapy and better understanding the potential mechanism.

Due to the development of gene chip detection technology, more and more studies use these techniques to analyze and mine differential genes.(12). Gene expression omnibus (GEO), a comprehensive database of gene expression, is an open data set of gene expression profiles, which stores numerous kinds of tumor gene expression data(13). Therefore, on the basis of these data, we can explore numerous of available things for new research. Furthermore, many bioinformatics studies on bladder cancer have found a large number of diagnostic and prognostic markers of bladder cancer(14–16), which indicated that bioinformatical methods could help us to identify critical biomarkers.

In the present study, we downloaded two microarray datasets from the GEO database, namely GSE3167 and GSE7476, which included 50 tumor and 12 normal samples. Secondly, the common DEGs of the above two datasets is obtained by using GEO2R online tool in the GEO databases and Venn diagram tool. The function of DEGs was analyzed by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis in DAVID database, and the important pathways related to DEGs were studied. The protein-protein interaction network was constructed by using the STRING database, and then the most important modules containing core genes were screened from the PPI network by using the MCODE plug-in in Cytoscape software. Moreover, each core gene was furtherly verified the expression patterns through GEPIA online tool. Finally, Kaplan-Meier Plotter was utilized to assess the prognosis of the core genes. Frequency and type of hub genes and Subgroups analysis were performed in cBioportal and ULCAN database, and then used the RT-qPCR to confirm the expression of these genes.

Methods

Sample collection

Nine bladder Cancer samples and paired adjacent normal tissues were obtained from the Third Affiliated Hospital of Sun Yat-Sen University. The information of the patients was list in Supplemental_Table_S1. All fresh tissues were immediately stored at – 80°C after radical resection. Our studies were approved by the ethics committee of the Third Affiliated Hospital of Sun Yat-Sen University.

Data source

In our study, gene expression profile datas of GSE3167 and GSE7476 were downloaded from the NCBI-GEO database (<http://www.ncbi.nlm.nih.gov/geo>)(17). Microarray data of GSE3167, including 41 bladder cancer samples and 9 normal samples, was based on GPL96 ([HG-U133A] Affymetrix Human Genome U133A Array). Based on the GPL 570 platform ([HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array), the GSE7476 dataset contained 9 bladder cancer specimens and 3 normal specimens in this

profile. The dataset information is shown in Table 1, and the clinical information of the samples in each dataset is shown in Supplemental_Table_S2.

Data processing

DEGs between cancer and normal bladder samples were screened by using GEO2R, an online tool of GEO. Adjust P value < 0.05 and $|\log FC| > 1$ were chosen as a threshold criteria. Next, the raw data were processed by venn diagram software, and the common DEGs in two data sets is selected. The DEGs with $\log FC > 1$ and $\log FC < -1$ were considered as an up-regulated gene and down-regulated genes respectively.

GO and KEGG pathway analysis

The Database for Annotation, Visualization, and Integrated Discovery (DAVID: <https://david.ncifcrf.gov/>) tools was used to analyse Gene ontology(GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment(18). The cut-off standard of functional and related pathway enrichment analysis is P -value < 0.05 . Besides, during the GO enrichment analysis among all the common DEGs, we also showed the top 7 gene counts.

Protein-protein interaction (*PPI*) network and module analysis. The Search Tool for the Retrieval of Interacting Genes (STRING) online tool (<http://string-db.org>) was applied to exhibit the PPI information of the common DEGs(19). The data of PPI information in TSV format were downloaded from STRING to view in Cytoscape software as a cutoff criterion that a maximum number of interactors = 0 and confidence score ≥ 0.4 . The most significant module of PPI network was screened by the MCODE plug-in in Cytoscape(degree cut-off ≥ 2 , node score cut-off ≥ 0.2 , K-core ≥ 2 , and max depth = 100).

Core gene validation by GEPIA

GEPIA (Gene Expression Profiling Interactive Analysis) dataset (<http://gepia.cancer-pku.cn/>), an online tool based on The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx) data(20), was used to verify the expression of core genes identified in the module.($P < 0.05$ and $|\log FC| > 1$).

Survival analysis

In this study, the prognostic value of the core genes was evaluated on the Kaplan-Meier plotter online tool (<http://www.kmplot.com>). $P < 0.05$ indicates that there is a statistically significant difference in survival between the high expression group and the low expression group, and the figure also shows the 95% confidence interval hazard ratio (HR).

cBioportal analysis

CBioportal (<http://cbioportal.org>) is an open access web-based resource for exploring and visualizing multidimensional cancer genome data sets(21). The bladder urothelial carcinoma (TCGA, Firehose

Legacy) dataset, mutations, putative copy number alterations from GISTIC, RNASeq V2 RSEM and protein expression Z-scores was selected(RPPA) were selected for further analyses.

UALCAN dabase analysis

UALCAN is an online network database based on TCGA database and contained RNA-seq and clinical data of 31 cancer types(22). We can use the transcriptional and clinical information in the database to do some correlation analysis of clinical features. In this study, we used this database to analyze the difference of expression between tumor group and its subgroups and normal group.

Quantitative real-time PCR

Total RNA of all samples was extracted by RaPure Total RNA Kits(Magen,China). Then, RNA was reverse transcribed to cDNA using the HiFiScript cDNA Synthesis Kit(CoWin,China). Quantitative PCR was performed using SYBR Green qPCR Master Mix in the BIO-RAD CFX96 Real-Time PCR System. $2^{-\Delta\Delta Ct}$ method was used to analyse the relative fold change of genes. qPCR primers used were as follows: SMC4 (forward primer ACAATGACTGGTGGTGAAGC and reverse primer CATTTCTCGTTCACTATGCCGT), CCNB1 (forward TGGTGACTTTGCTTTTGTGACTG and reverse CTCGACATCAACCTCTCCAATCT), CKS1B (forward primer CGGACAAATACGACGACGAGG and reverse primer TTGGTTTCTTGGGTAGTGGGC), TYMS (forward GTTCTGGAAGGGTGTTTTGGAG and reverse ATTGGCATCCCAGATTTTCACT), KPNA2 (forward TGATGATGCTACTTCTCCGCTG and reverse TGTCTATGGGGGCTGTTTTTC), NUSAP1 (forward TTGGTGATCCATCTTCCGAGTA and reverse CTTTTAACAACTTGGTTGCCCTC), GAPDH (forward GGAAGCTTGTCATCAATGGAAATC and reverse TGATGACCCTTTTGGCTCCC).

Statistical analysis

The qPCR data was analyzed by Student's t-test in GraphPad 6.0software. The p -value < 0.05 was considered to have statistical differences.

Results

Identification of DEGs in bladder cancers

In the present study, we downloaded two gene expression profiles (GSE3167 and GSE7476) from the GEO database. GSE3167 contained 41 tumor cases and 13 normal cases, and GSE7476 included 9 tumor cases and 3 normal cases respectively (Table 1). Based on GEO2R online tools, $P < 0.05$ and $|\log FC| > 1$, 1520 and 2098 DEGs from GSE3167 and GSE7476 respectively were screened(Fig. 1A-B). Then, venn diagram online tool was performed to select the commonly DEGs in all two datasets (Fig. 1C-D). Finally, a total of 251 genes were identified, including 173 genes were significantly upregulated and 78 genes were significantly downregulated (Supplemental_Table_S3).

GO and KEGG Pathway Analysis in bladder cancers

DAVID software was utilized to analyze the gene ontology and KEGG pathway analysis among all 251 common changed DEGs. The results of GO analysis were divided into three groups: biological process (BP), molecular function (MF) and cellular component (CC). In the BP group, up-regulated DEGs were mainly found in response to drug, positive regulation of transcription, DNA-templated, cell division, cell proliferation, and down-regulated DEGs in muscle contraction, positive regulation of GTPase activity, extracellular matrix organization. Additionally, in the CC group, up-regulated DEGs were mainly associated with cytoplasm, nucleus extracellular exosome, down-regulated DEGs mainly with cytoplasm, extracellular exosome, extracellular space. As for the MF group, the results show that the up-regulated DEGs were mainly enriched in protein binding, ATP binding, poly(A) RNA binding, and down-regulated DEGs in actin-binding, heparin binding, oxidoreductase activity (Fig. 2A-B, Supplemental_Table_S4).

KEGG pathway analysis was also through DAVID online. The results show that the up-regulated differentially expressed genes were significantly gathered in Pathways in cancer, Cell cycle, Biosynthesis of antibiotics, p53 signaling pathway, Oocyte meiosis, while down-regulated DEGs in Vascular smooth muscle contraction, Proteoglycans in cancer, Regulation of actin cytoskeleton, Dilated cardiomyopathy, cGMP-PKG signaling pathway and Melanoma (Fig. 2C-D, Supplemental_Table_S5-6).

PPI network construction and significant module selection

To further understand the relationship among identified DEGs, STRING online tool and Cytoscape software were used. We filtered 210 genes of the 251 commonly altered DEGs into the DEG PPI network (as presented in Fig. 3A), which possessed 210 genes and 1075 edges. Among the 210 genes, there were 144 upregulated genes and 66 downregulated genes.

Then Cytotype MCODE (Molecular Complex Detection) plug-in was used to detect significant modules in the PPI network. The results showed that 19 nodes were identified from the PPI network which were all up-regulated genes (Fig. 3B).

Core genes expression between cancer and normal bladder tissues

GEPIA, the website-based GTEx and TCGA database, was utilized to verify the expression level of the 19 core genes between cancerous and normal people. We found that the expression trends were consistent with the two GEO datasets, and 18 of 19 genes were statistically significantly upregulated in bladder cancer tissue compared with normal bladder tissue through analysing RNA-Seq profiles of 28 normal and 404 cancer samples from the GTEx and TCGA database (Fig. 4).

Prognostic value analysis of core genes

Survival datas of 18 core genes were analyzed on the Kaplan Meier plotter prognostic analysis platform (<http://kmplot.com/analysis>). According to the results in prognostic analysis platform, we found that 11 of 18 genes were associated with the prognosis. However, the high expression group for SMC4, TYMS,

CCNB1, CKS1B, NUSAP1 and KPNA2 had significantly unfavorable OS than those in the low expression group while the other five high expression groups contribute to favorable outcomes, which is conflicted with their high expression level in the tumor (Fig. 5).

Frequency and type of hub genes alterations in BLCA

Next, we use the cBioportal database to verify the mutation types and frequencies of six genes in the above steps. As shown in Fig. 6 and Table 2, the mutation frequencies of SMC4, CCNB1, CKS1B, NUSAP1, KPNA2 are 18.45% 7.28% 5.58% 4.61% 9.47% and 14.32% respectively. There is no doubt that gene amplification is the largest type of mutation.

Subgroups analysis of patients with bladder carcinoma

We further used the UALCAN database based on TCGA database to evaluate the transcriptional level of SMC4, and showed that the expression of SMC4 mRNA in bladder cancer tissues was significantly higher than that in normal tissues(Fig. 7). Further subgroup analysis of multiple clinicopathological features of 408 bladder cancer samples in TCGA, in the subgroup analysis of the two clinical characteristics of disease staging and lymph node metastasis, the transcriptional levels of six hub genes in bladder cancer patients were significantly higher than those in healthy people (Fig. 8).

Quantitative real-time PCR validation

After comparing the expression level of six genes between the tumor and normal tissue, we found that the expression levels of SMC4, CCNB1, and CKS1B were upregulated in tumor tissues in 9 pairs of patients(Fig. 9G-I). Different from the results in the database, our results showed that there were no significant change of TYMS, NUSAP1 and KPNA2 in BLCA tissues compared to the normal samples (Fig. 9J-L).

Discussion

Bladder cancer is one of the most common cancers worldwide(1). In recent years, great progress, including surgery, intravesical treatment, and various adjuvant immunotherapy has made to treat the patients(2, 3), unfortunately, these methods have limitations, including tumor recurrence, drug resistance, resulting in poor 5-year survival(4, 5). Therefore, it is quite important to exploit new therapeutic strategies and biomarkers for diagnosis, prognosis or precise therapy.

In this study, a series of online database and tools were performed to screen useful diagnostic and prognostic biomarkers. Two expression profile datasets (GSE3167 and GSE7476) were downloaded from the GEO. There were 50 cancer tissues and 12 normal tissues in our present study. Totally, 251 common DEGs (173 upregulated and 78 downregulated) were identified. Then, GO and KEGG pathway enrichment analysis of the 251 common DEGs were performed. In GO enrichment analysis, the DEGs genes were mainly enriched in positive regulation of transcription, cell division, cell proliferation, muscle contraction, extracellular exosome, ATP binding and actin binding. These results suggest that these DEGs are involved

in the transcriptional regulation and cell proliferation of bladder cancer cells. In KEGG pathway analysis, results revealed that the DEGs were mainly gathered in Pathways in cancer, cell cycle, biosynthesis of antibiotics, p53 signaling pathway, vascular smooth muscle contraction, proteoglycans in cancer, regulation of actin cytoskeleton. As we all know, the shortening of cell division cycle is accompanied by cell proliferation, which is a remarkable feature of tumor cells. At the same time, the increase of material metabolism is necessary for tumor cell proliferation. The p53 signaling pathway has been shown to play an important role in BLCA, Madka(23) and Wang(24) reported that targeting p53 signaling may inhibit bladder cancer cells growth and metastasis in vivo. Most of the Chemotherapeutic drugs in inducing apoptosis of bladder cancer cells through inhibiting p53 pathway(25, 26). Cytoskeleton is involved in the deformation of cancer cells, which may be an important basis for cell migration, invasion and metastasis(27). Many studies have been discovered that dysfunction or inhibition of actin cytoskeleton can inhibit migration and invasion in many cancers(28, 29) including bladder cancer(30, 31). Therefore, the study of these pathways will help to clarify the potential mechanism of proliferation, invasion and metastasis of bladder cancer, and help to predict tumor progression.

The protein-protein interaction network complex was established to understand the interrelationship of the DEGs via the STRING online database, and there are 210 genes and 1075 edges in the protein-protein interaction network. Then, 19 up-regulated core genes were screened out by Cytotype MCODE plug-in. We then performed a validation of these 19 hub genes using the GEPIA websites. Compared with normal tissues, the result in the GEPIA box plots showed that only 18 hub genes expression levels were statistically significantly overexpressed in bladder cancer samples. Furthermore, we found that 6 of 18 genes were significantly associated with the prognosis($P < 0.05$). In the 6 qualified genes, high expression group for SMC4, TYMS, CCNB1, CKS1B, NUSAP1, and KPNA2 had significantly unfavorable OS than those in the low expression group, suggesting that the six genes may play a crucial role in bladder cancer for diagnosis, prognosis or precise therapy. In the next mutation type analysis, we found that gene amplification is the main type of mutation. Further UALCAN analysis found that in the TCGA database, the expression of tumor group, clinical stage and lymph node metastasis were higher than that of the normal group. However, the expression of TYMS, NUSAP1 and KPNA2 gene remained unchanged between the BLCA patients and the normal cases in our qPCR validation. The possible reason is that our sample size is so small, a larger samples was needed to confirm this conclusion.

As a member of the SMC family, structural maintenance of chromosomes 4 (SMC4), is a chromosomal ATPase which plays an important role in maintaining the chromosome structure during chromosome assembly and segregation. Previous studies have demonstrated that SMC4 was overexpression in multiple cancers and promoted proliferation, migration/invasion and tumorigenicity, including primary hepatocellular carcinoma, colorectal cancer, glioma, breast cancer and lung adenocarcinoma(32–36). In addition, inhibition of SMC4 could suppress cell proliferation(37). Further vitro studies have been reported that overexpression of SMC4 promotes aggressiveness of cancer cells via activating JAK2/Stat3 and TGF β /Smad pathway(32, 34). Besides, higher mRNA expression of SMC4 was also significantly associated with unfavorable OS of some cancers patients, indicating SMC4 took part in the tumorigenesis(33, 35). However, to date, the expression pattern or prognostic value of SMC4 in BLCA

remains unknown. Thus, we are the first to report significant overexpression of SMC4 in BLCA tissues associated with poor survival in BLCA patients.

Cyclin B1(CCNB1), a monitoring protein initiates the process from the G2 phase to mitosis. Many studies reported that CCNB1 is overexpressed in many tumors and promotes tumor cell proliferation, including hepatocellular carcinomas(38), colorectal cancer(39) and bladder cancer(40). Furthermore, it was also pointed out that inhibition of CCNB1 could reduce cell proliferation(41). Similarly, over-expression of CCNB1 was possessed clinical significance in the diagnosis and prognosis of various cancers, such as breast cancer(42), pancreatic cancer(43). Our study also indicated the expression of CCNB1 was upregulated in BLCA tissues and reported their poor prognostic value for the patients.

Cyclin-dependent kinase regulatory subunit 1B (CKS1B), as a member of the Cks/Suc1 family, regulate their function of cyclin-dependent protein kinases through binding the catalytic subunit. Frequent up-regulation of CKS1B had been found in multiple cancer tissues and cell lines and was associated with the poor prognosis of these patients(44–46). Recently, targeting of CKS1B by a variety of microRNAs, including miR-204 and miR-1258 has been found to inhibit the proliferation, invasion and migration of cancer cells(47, 48). Moreover, CKS1B has been found to play a critical role in chemoresistance via counteraction of Hsp90 and MEK1/2 or JAK2/Stat3 pathways(49, 50). Increased CKS1B gene expression significantly correlated with tumor stage and grade, indicating that overexpression of CKS1B contributes to the progression of human bladder carcinoma(51). In the present study, higher CKS1B expression was significantly related to poor survival of BLCA patients, suggesting that CKS1B may be exploited as a novel and promising therapeutic target for BLCA treatment.

Conclusion

In conclusion, through an integrated bioinformatics analysis of two gene profiles, we identified 251 common DEGs (173 upregulated and 78 downregulated), which contain core genes in bladder cancer pathogenesis. Three of the 19 hub genes including SMC4, CCNB1 and CKS1B were filtered out through our analysis and may be potential biomarkers for diagnosis, prognosis or precise therapy in bladder cancer.

Tables

Tables 1&2 are provided in the supplemental file section.

Abbreviations

BLCA
Bladder cancer; GEO:Gene Expression Omnibus ;TCGA:The Cancer Genome Atlas; DAVID:Database for Annotation, Visualization, and Integrated Discovery; GO:Gene ontology; KEGG:Kyoto Encyclopedia of Genes and Genomes; DEGs:differentially expressed genes; STRING:Search Tool for the Retrieval of

Interacting Genes; GEPIA:Gene Expression Profiling Interactive Analysis; SMC4:Structural maintenance of chromosomes 4; CCNB1:Cyclin B1; CKS1B:Cyclin-dependent kinase regulatory subunit 1B;

Declarations

Availability of data and materials

All analyzed data related to this paper are included in this paper.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All the authors have consented for the publication.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author Contributions

Hongpeng Fang, and Jiaming Wan conceived, designed and validation the study. Hongpeng Fang and Xianzi Zeng analyzed the microarray datasets and prepared the figures and/or tables. Hongpeng Fang and Zhansen Huang wrote the article. Jieying Wu and Jinming Di revised the manuscript critically for important content, Obtained funding, Supervision. All authors read and approved the final manuscript.

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Figures

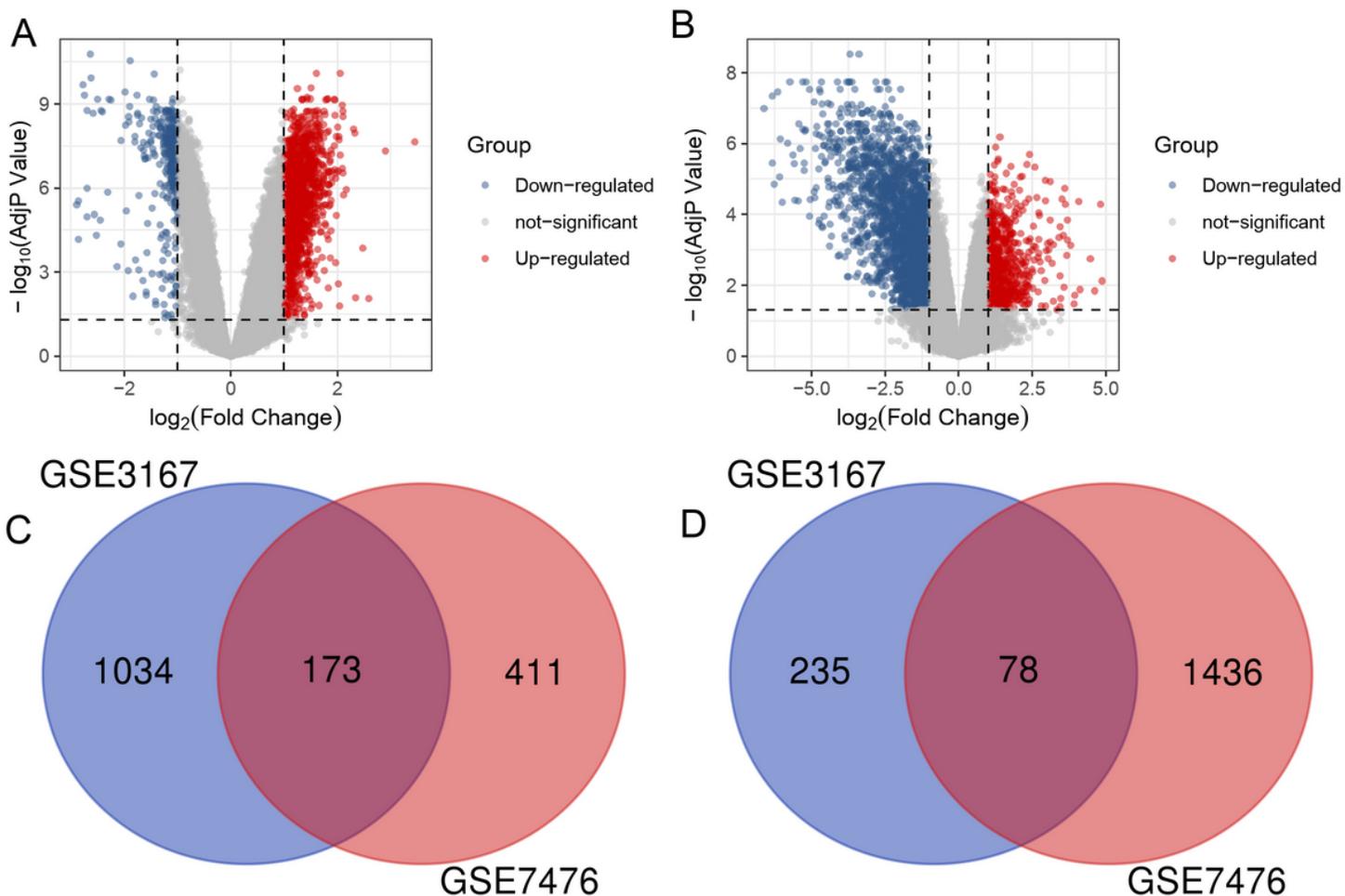


Figure 1

A-B. Results of differentially expressed genes in GSE3167(A) and GSE7476(B). C-D.Venn diagram of commonly changed DEGs in two GEO datasets. (C) Upregulated genes. (D) Downregulated genes.

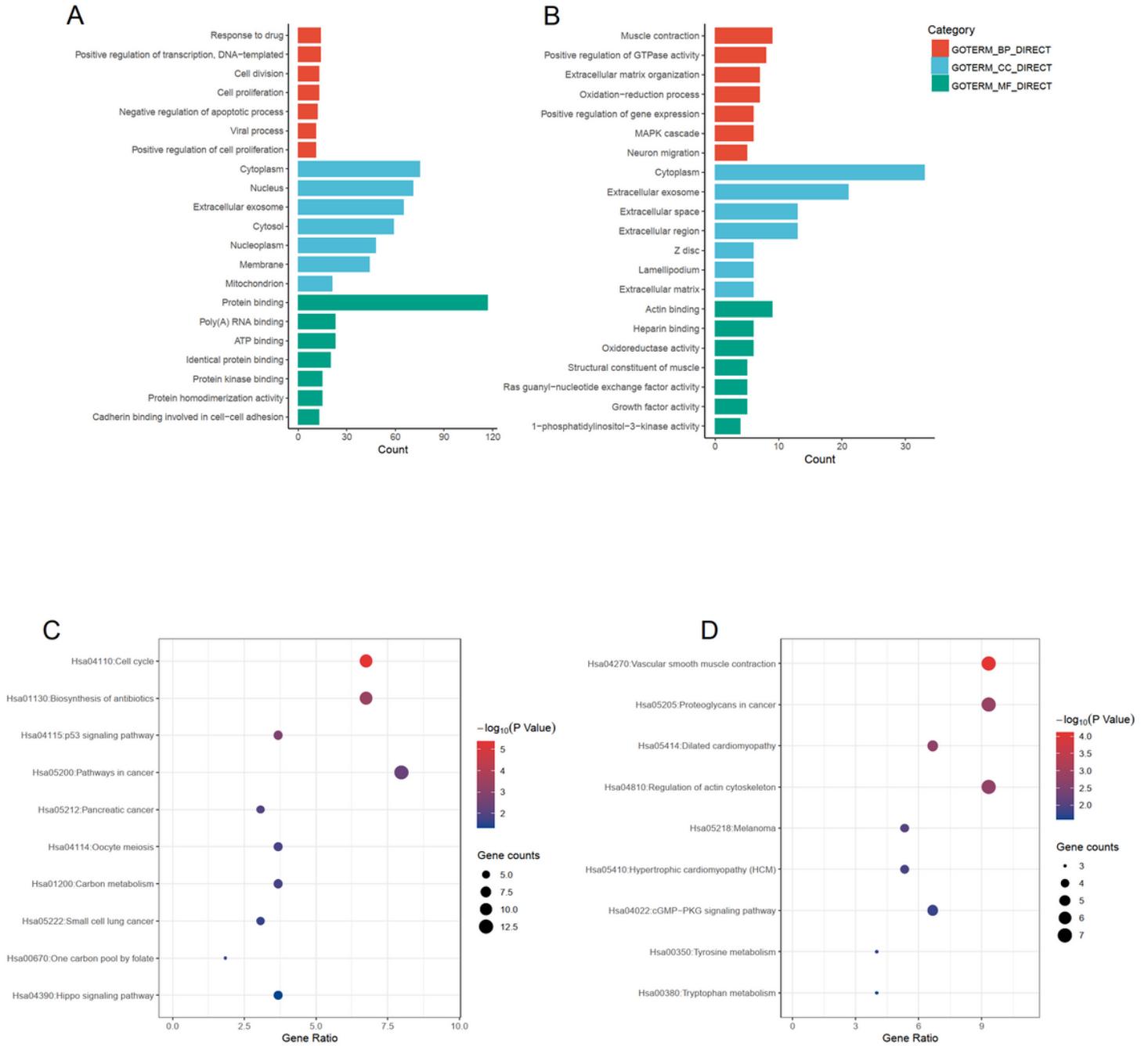


Figure 2

Differential genes GO and KEGG pathway enrichment analysis.

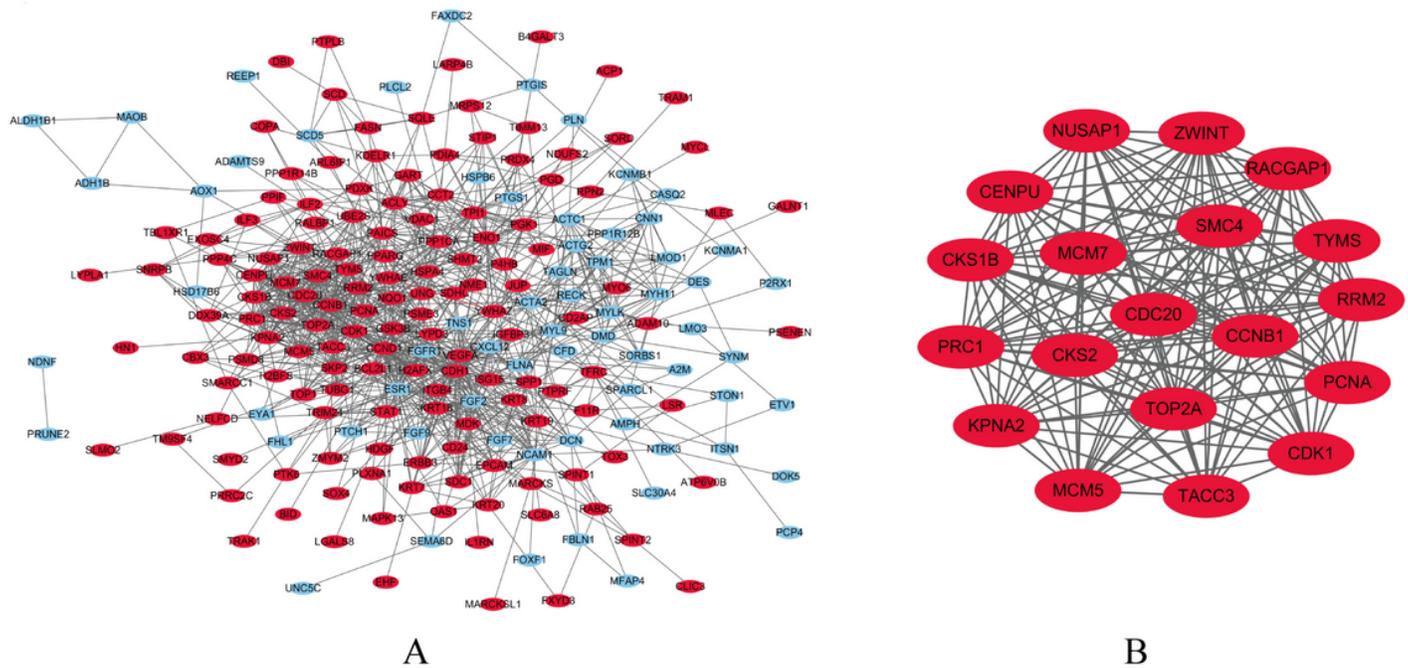


Figure 3

PPI network and module analysis of Common DEGs. A . PPI network of DEGs.B. One significant module screened out from PPI network. Red nodes stand for upregulated genes; blue nodes stand for downregulated genes.

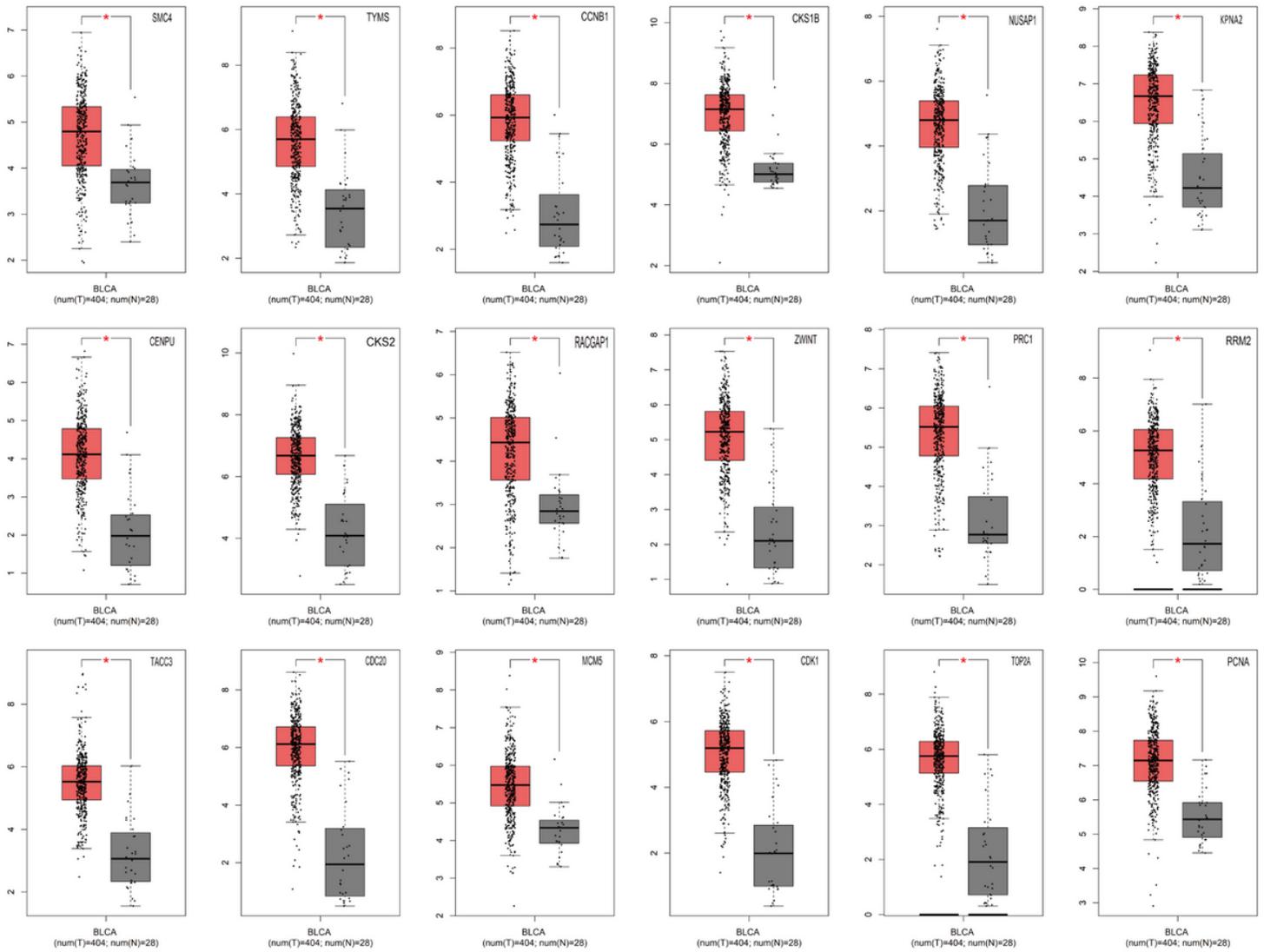


Figure 4

Boxplots showing significantly expressed 18 genes in bladder cancer patients compared to normal people (* P < 0.05). Red color means cancer tissues and grey means normal tissues.

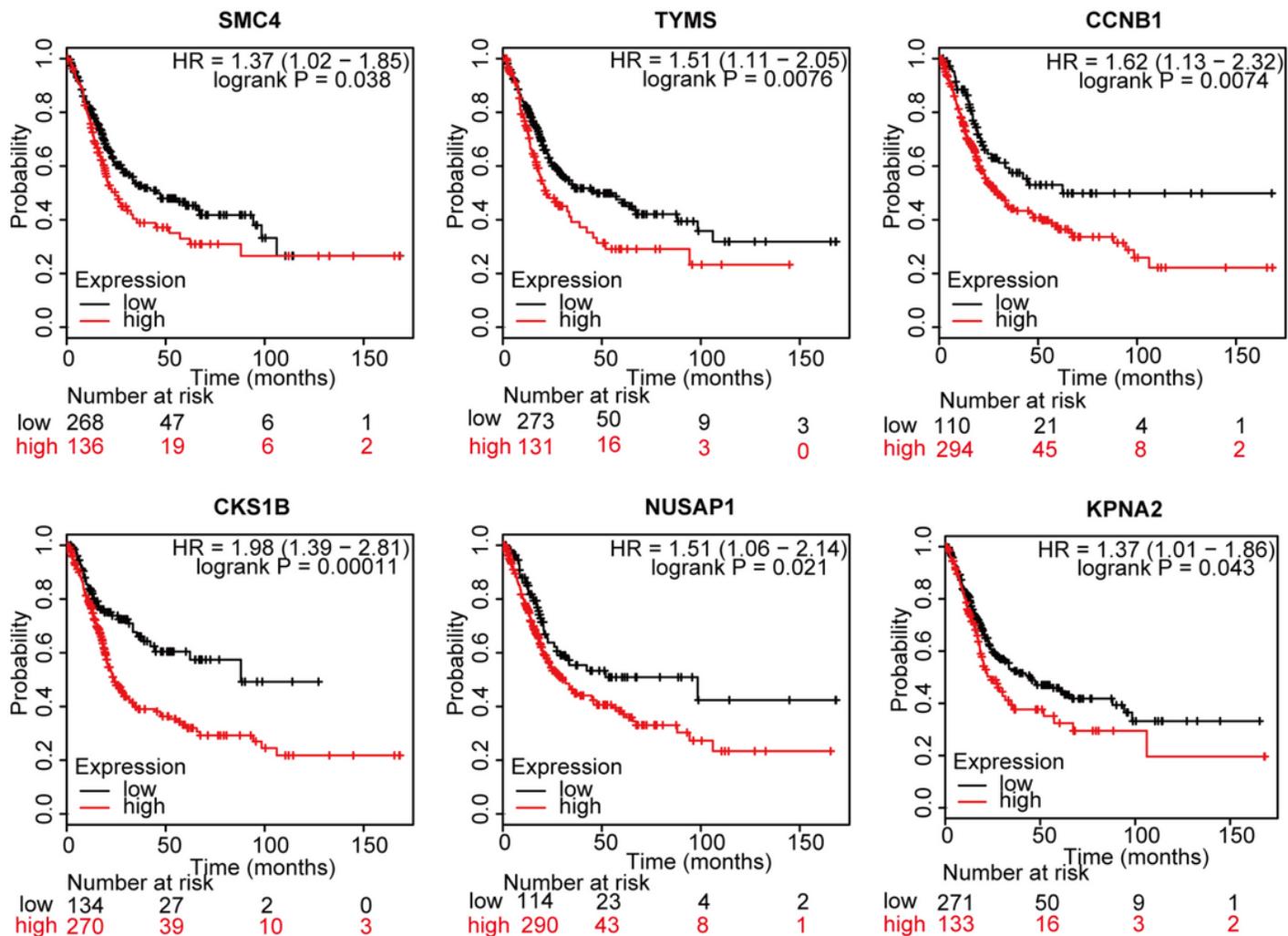


Figure 5

Kaplan-Meier overall survival curve for 18 core genes in bladder cancer and 6 of 18 genes had a significantly associated with the poor prognosis($P < 0.05$).

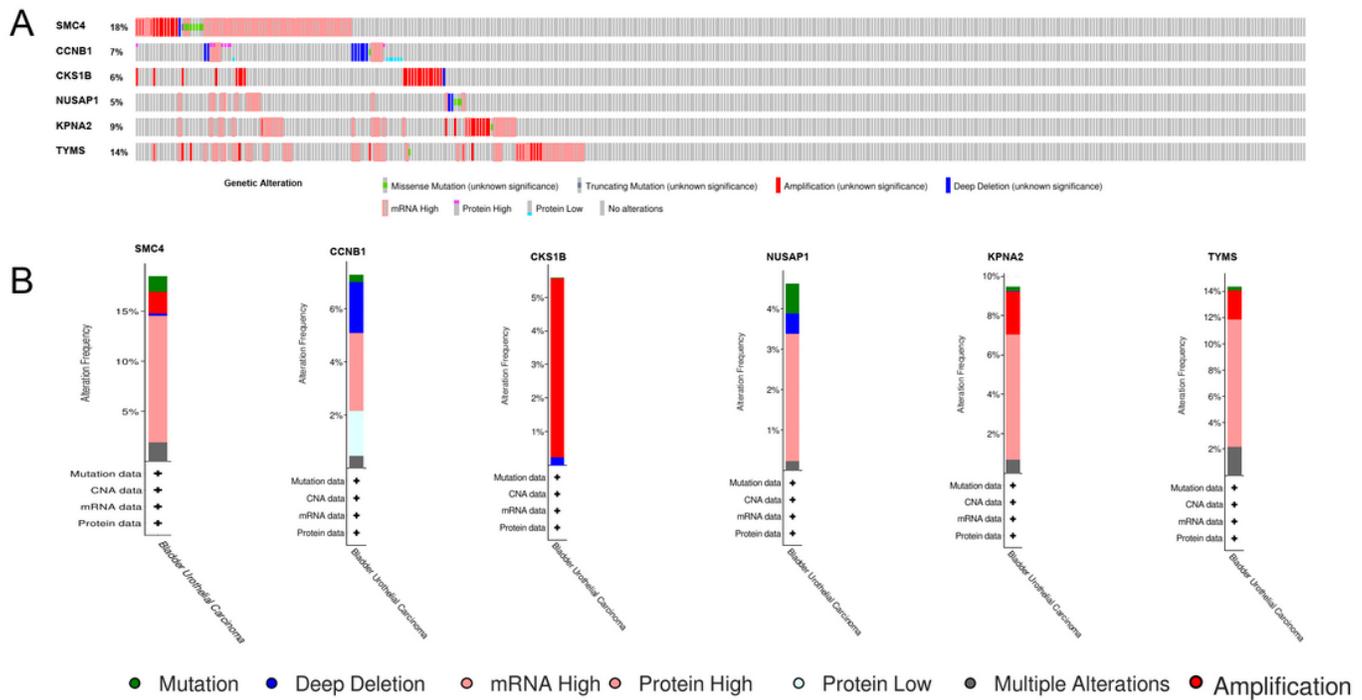


Figure 6

Frequency and type of hub genes alterations in bladder cancer.

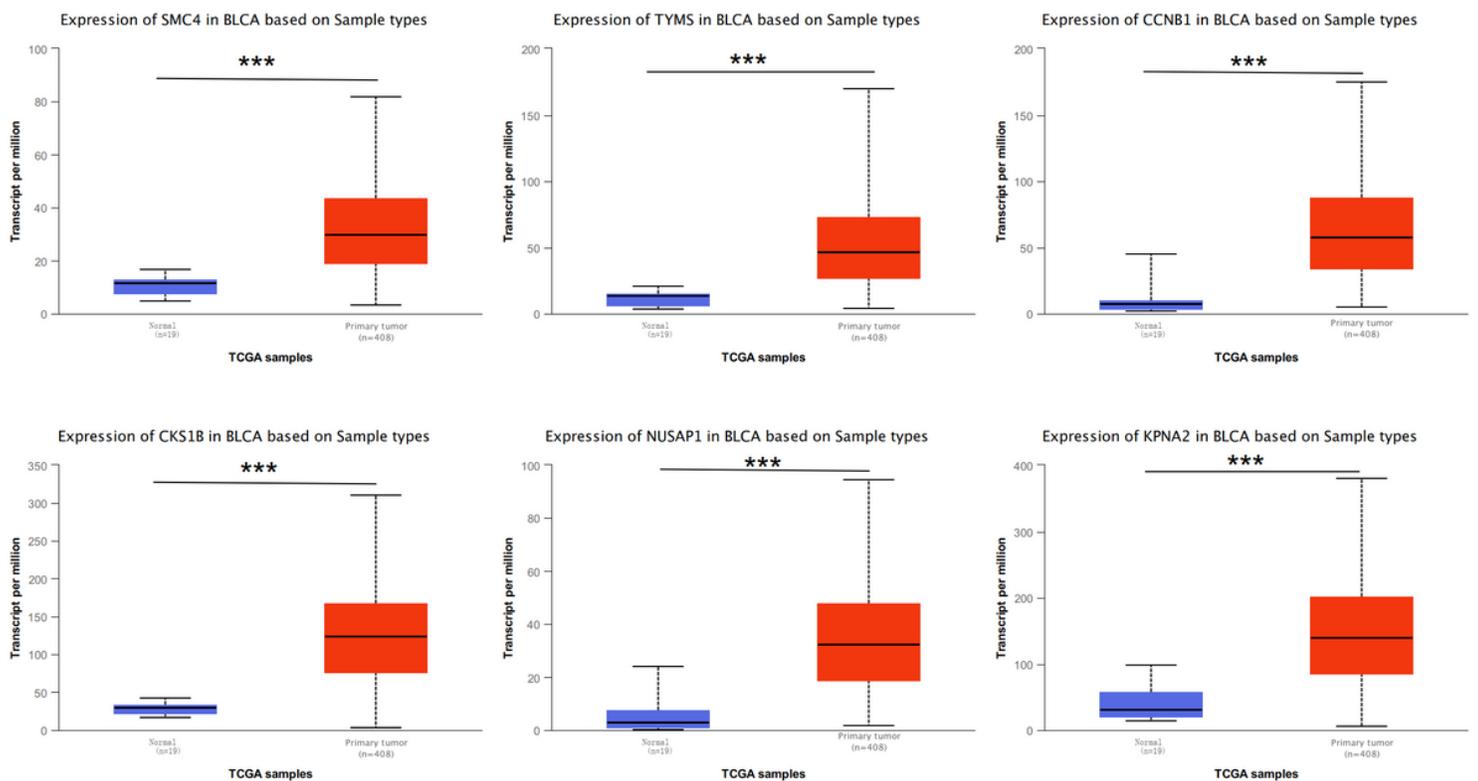


Figure 7

Hub genes transcription in bladder carcinoma (ULCAN). (***) $p < 0.001$.

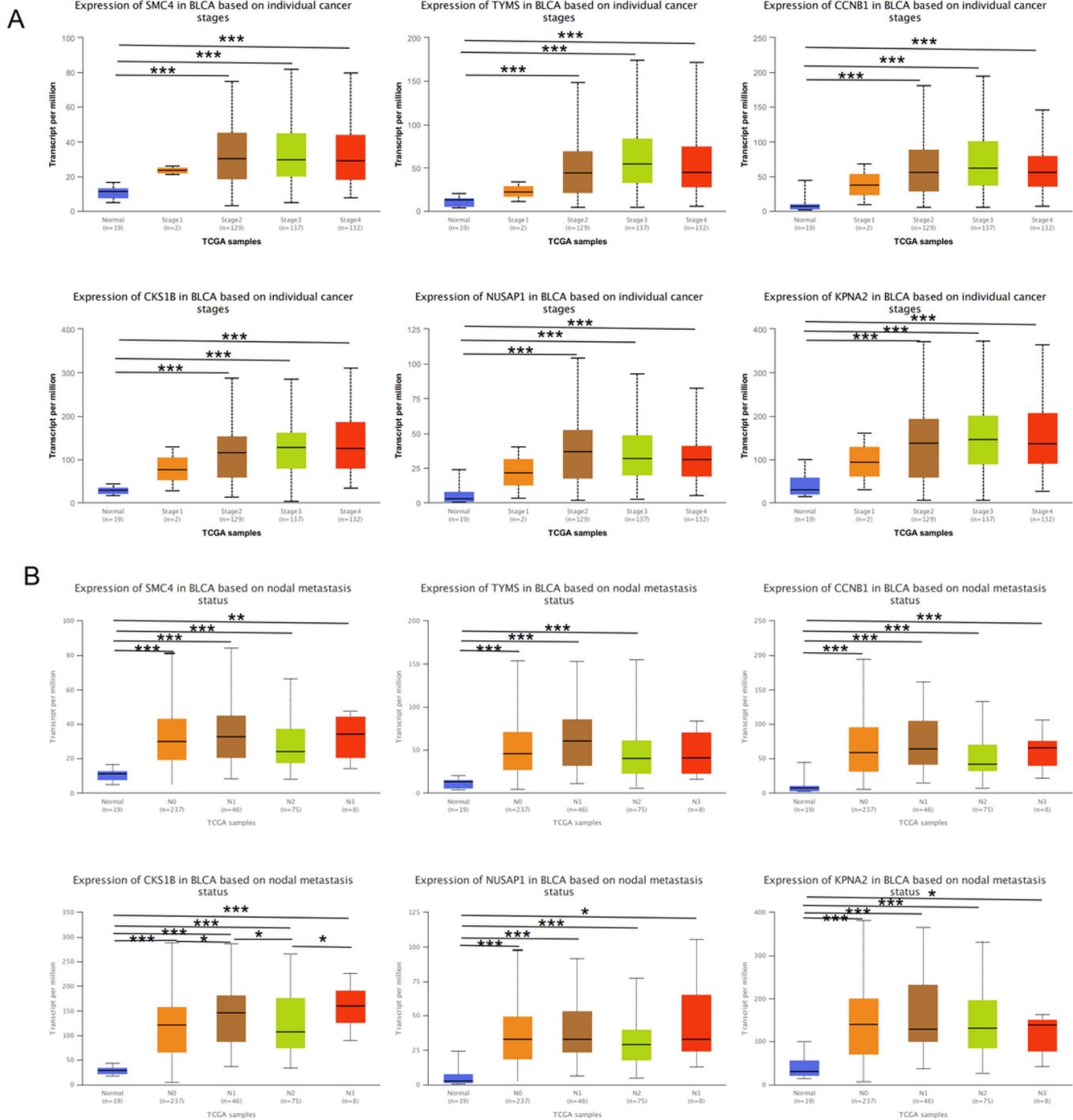


Figure 8

The subgroup analysis of cancer stages and lymph node metastasis in bladder carcinoma (* $P < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

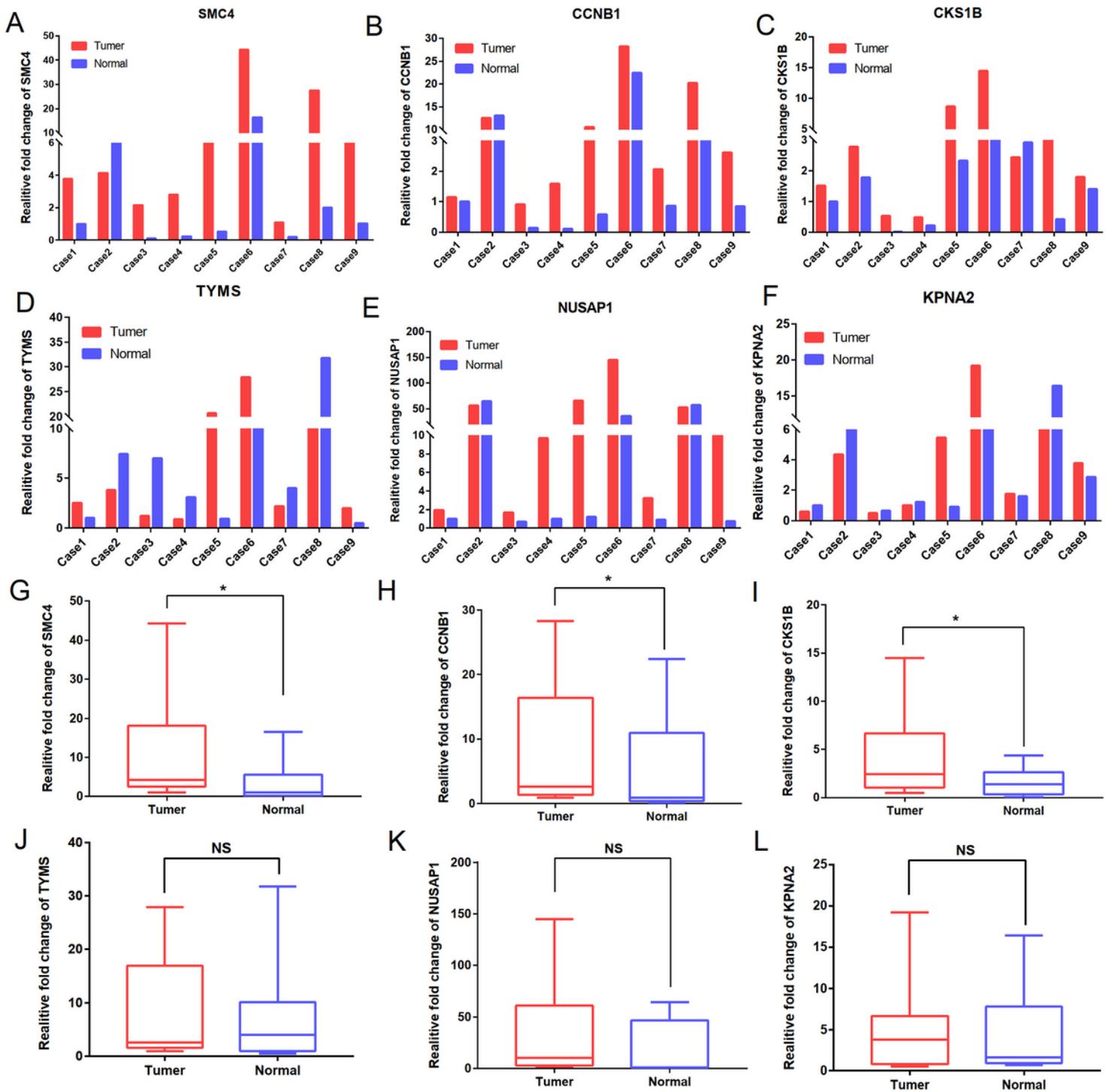


Figure 9

RT-qPCR results showed the expression levels of the SMC4, CCNB1, CKS1B, TYMS, NUSAP1 and KPNA2 in 9 pairs of bladder cancer (A-F), and statistical analysis also identified the expression changes between the cancer and the normal samples (G-L) * $p < 0.05$, NS $p > 0.05$.

Supplementary Files

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