Identification of key genes associated with bladder cancer using gene expression profiles

YUPING HAN[1], XUEFEI JIN[1], HUI ZHOU[2] and BIN LIU[1]

Departments of [1]Urology and [2]Gastrointestinal Colorectal and Anal Surgery,
China-Japan Union Hospital of Jilin University, Changchun, Jilin 130033, P.R. China

Abstract. The aim of the present study was to further investigate the molecular mechanisms of bladder cancer. The microarray data GSE52519 were downloaded from Gene Expression Omnibus, comprising 9 bladder cancer and 3 normal bladder tissue samples. Differentially expressed genes (DEGs) were identified using Limma package analysis. Subsequently, Gene Ontology, Kyoto Encyclopedia of Genes and Genomes and Reactome pathway enrichment analyses were performed for down- and upregulated DEGs. Transcription factors and genes associated with cancer from DEGs were identified. Protein-protein interaction (PPI) networks were constructed using STRING, and pathway enrichment analysis was also conducted for genes in the core sub-network that was identified using BioNet. In total, 420 downregulated and 335 upregulated DEGs were identified. Functional and pathway enrichment analyses identified that a number of DEGs, including *AURKA*, *CCNA2*, *CCNE1*, *CDC20* and *CCNB2*, were enriched in the cell cycle. Furthermore, a total of 12 upregulated proto-oncogenes were identified, including *AURKA* and *CCNA2*. In the PPI sub-network, a number of DEGs (e.g., *CCNB2*, *CDC20*, *CCNA2* and *MCM6*) with higher degrees were enriched in the KEGG pathway of the cell cycle. In conclusion, the DEGs associated with the cell cycle (e.g., *CDC20*, *CCNA2*, *CCNB2* and *AURKA*) may serve pivotal roles in the pathogenesis of bladder cancer.

Correspondence to: Dr Xuefei Jin, Department of Urology, China-Japan Union Hospital of Jilin University, 126 Xiantai Street, Changchun, Jilin 130033, P.R. China
E-mail: xuefeijinxfj@sina.com

Abbreviations: CIN, chromosomal instability; GO-BP, Gene Ontology-Biological Process; KEGG, Kyoto Encyclopedia of Genes and Genomes; DEGs, differentially expressed genes; DAVID, Database for Annotation, Visualization and Integrated Discovery

Introduction

Bladder cancer is one of the most common types of cancer worldwide and was the most common urological tumor in China in 2012 (1). In 2012, of the individuals with bladder cancer, 90% were diagnosed at >65 years-of-age worldwide (2). This disease is a common malignancy characterized by a poor clinical outcome (3); therefore, investigations into the underlying molecular mechanisms are urgently required in order to facilitate improvements in early diagnosis and treatments.

Bladder cancer is considered a genetic disease and is driven by the multistep accumulation of genetic and epigenetic factors that usually result in uncontrolled cellular proliferation, cell cycle deregulation or a decrease in cell death (3). The two different types of genetic alterations that are observed in bladder cancer are tumor protein p53 mutations and a number of single-nucleotide and structural variants, as well as chromosome shattering (4). Di Pierro *et al* (1) revealed that mutations in *FGFR3* and *TP53* are usually predictive of bladder malignancy. Overexpression of PIN2/TRF1-interacting telomerase inhibitor 1 in urothelial carcinoma of the bladder inhibited cell proliferation by inhibiting telomerase activity and the p16/cyclin D1 signaling pathway (5). Furthermore, chromosomal instability (CIN) characterized by loss or gain of chromosomal fragments or entire chromosomes is most prevalent in invasive urothelial cancer, compared with other less malignant papillary subtypes (6). Checkpoint dysfunction serves an important role in the development of CIN and is caused by defects in cell cycle regulation, p53 function and checkpoint signaling (7). Although certain studies have reported that gene mutations, telomerase activity and chromosomal instability are connected with bladder cancer (4-7), the exact molecular mechanism of bladder cancer remains unclear. A profound understanding of the molecular mechanism of action may be useful to provide an improved, more efficient handling of bladder cancer.

In the present study, the raw microarray data GSE52519 were downloaded to investigate the underlying molecular mechanisms of bladder cancer. Gene Ontology-Biological Process (GO-BP) functional analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis and Reactome pathway enrichment analysis were performed for down- and upregulated differentially expressed genes (DEGs). Subsequently, transcription factors and genes associated with cancer for DEGs were identified. In addition, a protein-protein interaction (PPI)

network and its core sub-network were constructed, and KEGG pathway enrichment analysis of the genes in the identified core PPI sub-network was also performed.

Materials and methods

Microarray data and data preprocessing. The raw microarray data GSE52519 were downloaded from Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE52519). GSE52519 comprises 9 bladder cancer tissue samples obtained during cystectomy and 3 normal tissue samples derived from post mortem donors without bladder cancer. The microarray platform of GSE52519 was GPL13497 Illumina HumanWG-6 v3.0 Expression BeadChip (Illumina, Inc., San Diego, CA, USA).

The gene expression profile was preprocessed using Limma (version 3.83; linear models for microarray data; www.bioconductor.org/packages/2.8/bioc/html/limma.html) package in Bioconductor (8) and Affymetrix annotation files from Brain Array Lab (version 20; http://brainarray.mbni.med.umich.edu/Brainarray/Database/CustomCDF/CDF_download.asp) (9). The background correction, quantile normalization and probe summarization of the microarray data were performed using the Robust Multi-Array Average algorithm (10) to obtain the gene expression matrix.

Identification of DEGs. The normalized data were calculated with the Limma package (8), and genes with $P<0.01$ and $|\log_2 \text{fold change}| \geq 2$ were considered to indicate a statistically significant difference between the bladder cancer group and the normal group.

Enrichment analysis of DEGs. The Database for Annotation, Visualization and Integrated Discovery (DAVID; david.abcc.ncifcrf.gov) is a comprehensive functional annotation tool (10,11). Based on DAVID online analysis, GO-BP functional enrichment analysis was performed for DEGs (12); $P<0.01$ was selected as the threshold criterion.

KEGG is a knowledge base for systematic analysis of gene functions, and the PATHWAR database (www.kegg.jp/kegg/pathway.html) is supplemented by the information of conserved sub-pathways (13). The Reactome database (www.reactome.org) is an open-source open-data resource of human pathways and reactions (14). Pathway enrichment analysis was performed for the DEGs using these two databases, with the threshold of $P<0.01$.

Identification of transcription factors and genes associated with cancer from DEGs. Transcription factor analysis using the TRANSFAC database (www.biobase-international.com/product/transcription-factor-binding-sites#resources) (15) was performed on DEGs to determine whether the genes were transcription factors. The DEGs were also submitted to the Tumor Suppressor Gene database (bioinfo.mc.vanderbilt.edu/TSGene) (16) and the Tumor-Associated Gene database (blog.synapse.info/tag/Database) (17) to obtain all known proto-oncogenes and tumor suppressor genes.

Construction of PPI network and PPI sub-network analysis. DEGs were submitted to STRING version 9.1 (Search Tool for the Retrieval of Interacting Genes; string.embl.de) (18) to search interaction associations of the proteins; the confidence score >0.9 was used as the threshold criterion. Then, visualization of the PPI network was performed using Cytoscape software (cytoscape.org) (19). The HUB nodes with the top 5 degrees in the PPI network were also obtained.

The PPI sub-network of DEGs was identified by BioNet (20), and a false discovery rate of <0.01 was selected as the threshold criterion. The pathway enrichment analysis of genes in the core PPI sub-network was performed using the KEGG database, and $P<0.01$ was selected as the threshold criterion.

Results

DEG analysis. A total of 779 transcripts and 755 DEGs were identified in the bladder cancer and the normal group combined, including 431 downregulated transcripts that corresponded to 420 downregulated DEGs, and 348 upregulated transcripts that corresponded to 335 upregulated DEGs.

Enrichment analysis of DEGs. The top three enriched GO terms in the BP category of downregulated DEGs included muscle contraction ($P=3.60 \times 10^{-5}$), involving DEGs such as *CRYAB*, *TACR2* and *MYH3*, muscle system process ($P=9.48 \times 10^{-5}$) associated with DEGs such as *CRYAB*, *TACR2* and *MYH3*, and actin filament-based process ($P=3.19 \times 10^{-4}$) involving DEGs such as *DLC1*, *MYH3* and *CALD1* (Table I). The only two enriched KEGG pathways of downregulated DEGs included focal adhesion ($P=3.86 \times 10^{-3}$; e.g., *PRKCA*, *LAMA3* and *ITGA8*) and tight junction ($P=8.10 \times 10^{-3}$; e.g., *PRKCA*, *GNAI1* and *MYH3*) (Table II). Additionally, five downregulated DEGs (*TNNT3*, *DES*, *MYH3*, *DMD* and *TPM2*) were significantly enriched in the Reactome pathway of muscle contraction ($P=2.46 \times 10^{-3}$; Table III).

The top three enriched GO terms in the BP category of upregulated genes included cell cycle ($P=1.88 \times 10^{-29}$), involving DEGs such as *AURKA*, *CCNA2*, *CCNE1*, *CDC20* and *CCNB2*, M phase ($P=1.88 \times 10^{-29}$) associated with a number of DEGs, including *AURKA*, *CCNA2*, *CDC20* and *CCNB2*, and cell cycle phase ($P=2.89 \times 10^{-28}$) associated with DEGs such as *AURKA*, *CCNA2*, *CCNE1*, *CDC20* and *CCNB2* (Table IV). A total of six pathways were significantly enriched for upregulated genes, including cell cycle ($P=6.68 \times 10^{-11}$; e.g., *CCNA2*, *CCNE1*, *CDC20* and *CCNB2*), oocyte meiosis ($P=1.76 \times 10^{-7}$; e.g., *AURKA*, *CCNE1*, *CDC20* and *CCNB2*), DNA replication ($P=3.43 \times 10^{-4}$; e.g., *RFC4* and *POLE2*) and p53 signaling pathway ($P=4.28 \times 10^{-4}$; e.g., *CCNE1* and *CCNB2*) (Table V). The five enriched Reactome pathways of upregulated genes included cell cycle, mitotic ($P=3.93 \times 10^{-22}$; e.g., *AURKA*, *CCNA2*, *CCNE1*, *CDC20*, *CCNB2*, *RRM2* and *KIF20A*), cell cycle checkpoints ($P=6.55 \times 10^{-7}$; e.g., *CCNE1*, *CDC20* and *CCNB2*), telomere maintenance ($P=1.70 \times 10^{-6}$; e.g., *HIST1H2AC*, *HIST2H2AA3* and *HIST1H2BD*), DNA replication ($P=3.52 \times 10^{-4}$; e.g., *RFC4* and *POLE2*) and metabolism of nucleotides ($P=4.04 \times 10^{-3}$; e.g., *TYMS* and *RRM2*) (Table III).

Analysis of transcription factors and genes associated with cancer. Transcription factor analysis of DEGs revealed that 21 transcription factors (e.g., *ARNT*, *FOXP1* and *HEY1*) were

Table I. Top 10 enriched GO terms in the Biological Process category for downregulated differentially expressed genes in bladder cancer.

Term	n	P-value	Example genes
GO: 0006936 ~ muscle contraction	14	3.60×10^{-5}	*CRYAB, TACR2, MYH3, CALD1, VIPR1…*
GO: 0003012 ~ muscle system process	14	9.48×10^{-5}	*CRYAB, TACR2, MYH3, CALD1, VIPR1…*
GO: 0030029 ~ actin filament-based process	16	3.19×10^{-4}	*DLC1, MYH3, CALD1, NF1, FLNA…*
GO: 0001656 ~ metanephros development	7	3.93×10^{-4}	*TCF21, BDNF, ITGA8, BCL2, HOXA11…*
GO: 0040012 ~ regulation of locomotion	13	1.21×10^{-3}	*RTN4, DLC1, PRKCA, NF1, SMAD3…*
GO: 0030334 ~ regulation of cell migration	12	1.38×10^{-3}	*RTN4, DLC1, LAMA3, BCL2, NF1…*
GO: 0030336 ~ negative regulation of cell migration	7	1.59×10^{-3}	*DLC1, BCL2, NF1, ILK, TGFBR3…*
GO: 0030036 ~ actin cytoskeleton organization	14	1.62×10^{-3}	*DLC1, CALD1, NF1, FLNA, CORO2B…*
GO: 0060284 ~ regulation of cell development	13	2.10×10^{-3}	*RTN4, NTF3, HOXA11, NF1, NLGN1…*
GO: 0045449 ~ regulation of transcription	79	2.24×10^{-3}	*ZNF383, LCOR, MAP3K13, RNF20, KCNH4…*

GO, gene ontology.

Table II. The two enriched Kyoto Encyclopedia of Genes and Genomes pathways for downregulated differentially expressed genes in bladder cancer.

Term	n	P-value	Genes
hsa04510: Focal adhesion	12	3.86×10^{-3}	*PRKCA, LAMA3, ITGA8, BCL2, ILK, LAMC1, LAMB1, FLNC, FLNA, PARVA, VCL, MYL9*
hsa04530: Tight junction	9	8.10×10^{-3}	*PRKCA, GNAI1, MYH3, CNKSR3, MYH11, MYH14, CLDN11, TJAP1, MYL9*

hsa, *homo sapiens*.

Table III. Enriched Reactome pathways for downregulated and upregulated differentially expressed genes in bladder cancer.

Term	n	P-value	Example genes
Downregulated			
REACT_17044: Muscle contraction	5	2.46×10^{-3}	*TNNT3, DES, MYH3, DMD, TPM2*
Upregulated			
REACT_152: Cell cycle, mitotic	49	3.93×10^{-22}	*CDC20, CCNB2, KIF23, E2F2, AURKA…*
REACT_1538: Cell cycle checkpoints	17	6.55×10^{-7}	*CDC20, CCNB2, CHEK1, MCM2, UBE2C…*
REACT_7970: Telomere maintenance	12	1.70×10^{-6}	*HIST1H2AC, HIST2H2AA3, HIST1H2BD, H2BFS, RFC4…*
REACT_383: DNA replication	12	3.52×10^{-4}	*RFC4, POLE2, PSMC4, RFC2, PSMD10…*
REACT_1698: Metabolism of nucleotides	9	4.04×10^{-3}	*TYMS, RRM2, DTYMK, DCK, CAD…*

significantly downregulated in bladder cancer tissues and 8 transcription factors (e.g., *BCL3, EZH2* and *FOXM1*) were upregulated (Table VI).

Analysis of screening for genes associated with bladder cancer identified that 2 proto-oncogenes, *DUSP26* and *MEIS1*, were downregulated and 12 proto-oncogenes (e.g., *AURKA, CCNA2* and *CCNE1*) were upregulated. Furthermore, 17 tumor suppressor genes (e.g., *ARHGEF12, BLCAP* and *CHD5*) were downregulated and 10 tumor suppressor genes (e.g., *BLM, CHEK1* and *CST6*) were upregulated (Table VI).

PPI network and PPI sub-network analysis. A PPI network of DEGs was constructed (Fig. 1). The top five genes/proteins with the highest degree in the PPI network were *CCNA2, BUB1, CDC20, CCNB1* and *MAD2L1*, with degrees of 57, 53, 52, 50 and 44, respectively.

The obtained core sub-network from the PPI network included 24 DEGs (Fig. 2). The *CDC20* has the highest degree (degree, 18). A number of DEGs exhibited degrees >10, including *CCNA2* (degree, 17), *KIF11* (degree, 16), *AURKA* (degree, 15), *NUSAP1* (degree, 15) and *CCNB2* (degree, 14).

Table IV. Top 10 enriched GO terms in Biological Process category for upregulated differentially expressed genes in bladder cancer.

Term	n	P-value	Example genes
GO: 0007049 ~ cell cycle	76	1.88×10^{-29}	*CDC20, CCNB2, KIF23, E2F2, KIFC1…*
GO: 0000279 ~ M phase	52	1.88×10^{-29}	*CDC20, CCNB2, KIF23, KIFC1, PRC1…*
GO: 0022403 ~ cell cycle phase	56	2.89×10^{-28}	*CDC20, CCNB2, KIF23, KIFC1, PKMYT1…*
GO: 0022402 ~ cell cycle process	63	3.37×10^{-27}	*CDC20, CCNB2, KIF23, TTK, AURKA…*
GO: 0000278 ~ mitotic cell cycle	51	4.83×10^{-26}	*CDC20, CCNB2, KIF23, KIFC1, TTK…*
GO: 0000280 ~ nuclear division	40	7.79×10^{-25}	*CDC20, CCNB2, KIF23, KIFC1, NEK2…*
GO: 0007067 ~ mitosis	40	7.79×10^{-25}	*CDC20, CCNB2, KIF23, KIFC1, PKMYT1…*
GO: 0000087 ~ M phase of mitotic cell cycle	40	1.56×10^{-24}	*CDC20, CCNB2,* KIF23, *PKMYT1, AURKA…*
GO: 0048285 ~ organelle fission	40	3.64×10^{-24}	*CDC20, CCNB2, KIF23, PTTG1, CEP55…*
GO: 0051301 ~ cell division	42	6.70×10^{-22}	*CDC20, CCNB2, KIF23, CKS1B, PRC1…*

GO, gene ontology.

Table V. The six enriched Kyoto Encyclopedia of Genes and Genomes pathways for upregulated differentially expressed genes in bladder cancer.

Term	n	P-value	Example genes
hsa04110: Cell cycle	21	6.68×10^{-11}	*CDC20, CCNB2, E2F2, PKMYT1, TTK…*
hsa04114: Oocyte meiosis	16	1.76×10^{-7}	*CDC20, CCNB2, SGOL1, PKMYT1, AURKA…*
hsa03030: DNA replication	7	3.43×10^{-4}	*RFC4, POLE2, RFC2, MCM2, FEN1…*
hsa04115: P53 signaling pathway	9	4.28×10^{-4}	*CCNB2, CCNE2, CCNB1, BID, CCNE1…*
hsa04914: Progesterone-mediated oocyte maturation	9	2.05×10^{-3}	*CCNB2, CCNB1, MAD2L1, PLK1, BUB1…*
hsa05322: Systemic lupus erythematosus	9	4.93×10^{-3}	*HIST1H2AC,HIST2H2AA3,CD86,HIST1H2BD,HIST1H2BK…*

hsa, *Homo sapiens*.

Table VI. Transcription factors and genes associated with cancer in the differentially expressed genes in bladder cancer.

Terms	Genes	n
TF genes		
Downregulated	*ARNT, FOXP1, HEY1, HOXA11, HOXA3, HOXA9, ISL1, LMO3, MEIS1, NR1H3, NR1H4, NR3C2, POU3F1, POU3F4, RORB, SMAD3, SP3, TCF21, YAF2, ZNF10, ZNF174*	21
Upregulated	*BCL3, EZH2, FOXM1, IRF1, RELB, TCEB3, TFDP1, XBP1*	8
Oncogenes		
Downregulated	*DUSP26, MEIS1*	2
Upregulated	*AURKA, BCL3, CCNA2, CCNE1, CEP55, DCUN1D1, FGFR1OP, HMMR, MAP3K8, MYB, PTTG1, VEGFA*	12
Tumor suppressors		
Downregulated	*ARHGEF12, BLCAP, CHD5, DLC1, FOXP1, MFHAS1, NDRG4, NF1, PACRG, PEG3, SCARA3, SMAD3, TGFBR2, TGFBR3, VCL, YAP1, ZDHHC2*	17
Upregulated	*BLM, CHEK1, CST6, ERRFI1, IRF1, MT1G, PLEKHG2, RASSF1, SLC9A3R1, TNFAIP3*	10

TF, transcription factor.

Furthermore, *CDC20, CCNA2, CCNB2* and *AURKA* interacted with each other.

KEGG pathway enrichment analysis of the genes in the core sub-network revealed that DEGs were significantly involved in

Table VII. Enriched Kyoto Encyclopedia of Genes and Genomes pathways for differentially expressed genes of protein-protein interaction sub-network.

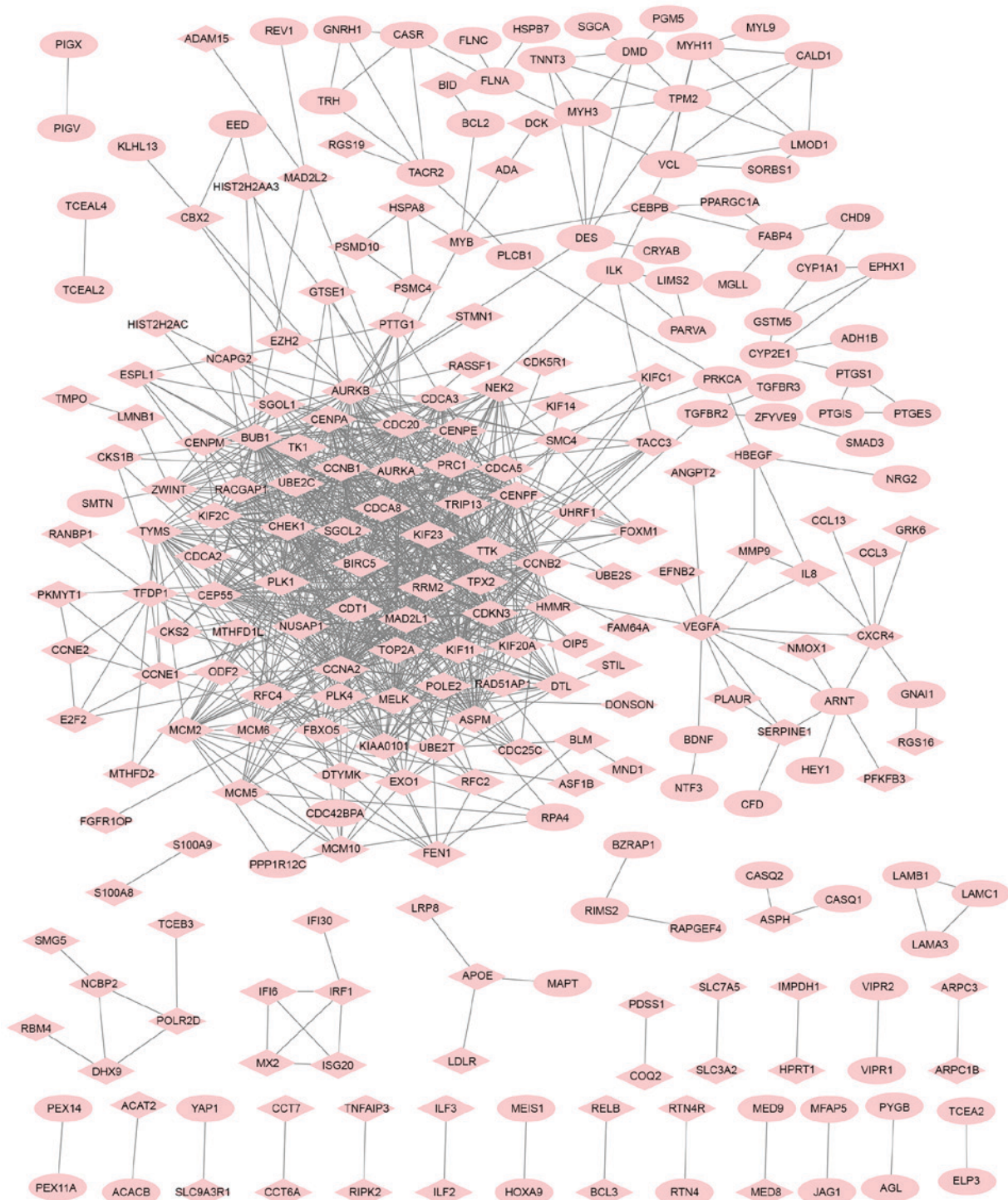| Term	n	P-value	Genes
hsa04110: Cell cycle	5	2.26×10^{-5}	*CCNB2, PLK1, CDC20, CCNA2, MCM6*
hsa04114: Oocyte meiosis	4	5.10×10^{-4}	*CCNB2, PLK1, CDC20, AURKA*
hsa04914: Progesterone-mediated oocyte maturation	3	7.41×10^{-3}	*CCNB2, PLK1, CCNA2*

hsa, *homo sapiens*.

Figure 1. Protein-protein interaction network of differentially expressed genes. The diamond nodes represent upregulated differentially expressed genes and the round nodes represent downregulated differentially expressed genes.
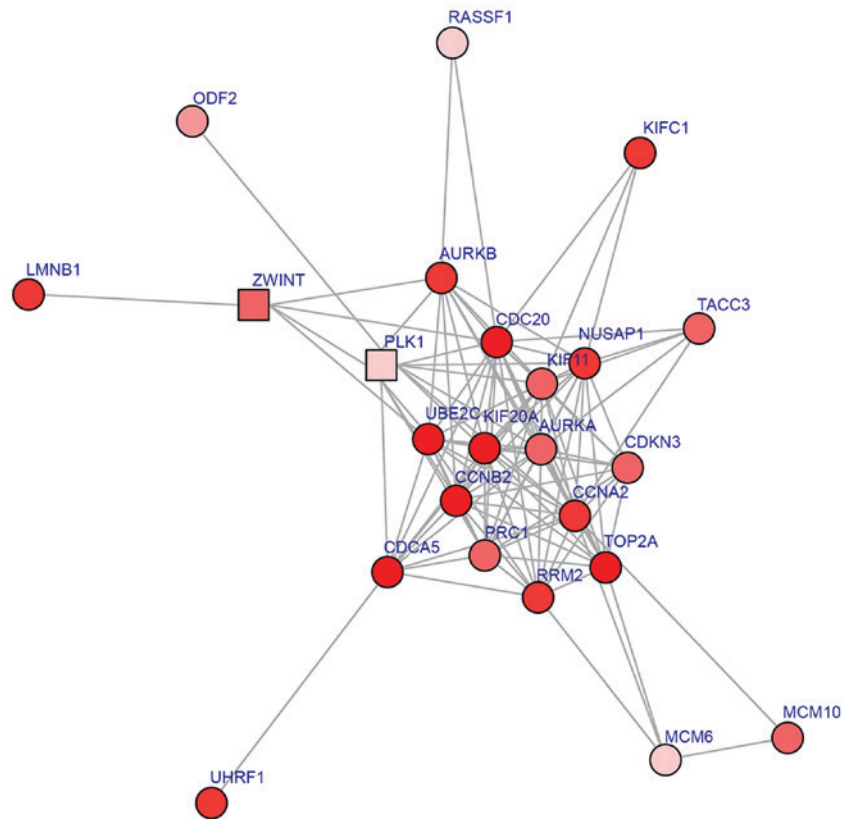
Figure 2. Protein-protein interaction sub-network of differentially expressed genes. The circles represent upregulated differentially expressed genes. The fold change of gene expression is presented through color (deeper color indicates higher fold change of gene expression). The square nodes represent the genes with lower importance in the sub-network.

cell cycle (P=2.26x10^{-5}; *CCNB2*, *PLK1*, *CDC20*, *CCNA2* and *MCM6*), oocyte meiosis (P=5.10x10^{-4}; *CCNB2*, *PLK1*, *CDC20* and *AURKA*) and progesterone-mediated oocyte maturation (P=7.41x10^{-3}; *CCNB2*, *PLK1* and *CCNA2*; Table VII).

Discussion

In the present study, gene expression profiling was used to investigate the molecular mechanisms underlying bladder cancer. A set of 335 upregulated and 420 downregulated DEGs were identified between bladder cancer samples and normal controls. Analysis of the PPI sub-network demonstrated that 24 DEGs were obtained, and *CDC20*, *CCNA2*, *CCNB2* and *AURKA* had >10 degrees and interacted with each other. The pathway enrichment analysis revealed that these four genes were enriched in the cell cycle signaling pathway.

   CDC20 serves a key role in the spindle assembly checkpoint and is necessary for anaphase onset and cell cycle progression (21). The abnormal expression of spindle assembly checkpoint proteins during mitosis, including *CDC20*, is associated with chromosome aneuploidy, and results in poor differentiation, tumor aneuploidy and poor prognosis (22). Kidokoro *et al* (23) demonstrated that p53 inhibits tumor cell growth by indirectly regulating the expression levels of *CDC20*.

   CCNA2 and *CCNB2* encode cyclin and function as regulators of CDKs. In the present study, *CCNA2* was identified as an upregulated proto-oncogene. Lu *et al* (24) and Lee *et al* (25) have demonstrated that *CCNA2* is upregulated in bladder cancer. Increased expression of *CCNA2* has been associated

with poor prognosis for individuals with bladder cancer (26). Furthermore, in the present study, the KEGG pathway enrichment analysis identified that *CCNB2* was enriched in the p53 signaling pathway. It has been demonstrated that the expression level of *CCNB2* is upregulated in bladder tumors during interphase and proteolysis (24), which is consistent with the results of the present study. Additionally, deletion of p53 in bladder epithelium has been demonstrated to lead to invasive cancer in a novel mouse model (27), indicating a key role for p53 in bladder cancer. Therefore, *CDC20*, *CCNA2* and *CCNB2* may contribute to the development of bladder cancer.

   AURKA, another upregulated proto-oncogene identified in the present study, encodes a cell cycle-regulated kinase (28). A previous study identified *AURKA* to be a biomarker for the detection of bladder cancer, due to *AURKA* aneuploidy resulting in chromosomal loss or gain (29). Genomic instability, which may be caused by checkpoint loss and perturbation of cell cycle control, results in the development of bladder cancer (30). A recent study demonstrated that *AURKA* is associated with the presence and grade of urothelial bladder cancer, suggesting a potential role as a diagnostic and prognostic biomarker (31). Hence, *AURKA* may serve a key role during the progression of bladder cancer.

   However, the present study has a number of limitations. For example, the results of the present study were only predicted by bioinformatical analysis and must be further confirmed by experimental test. The studies should also be conducted using a larger sample sizes. These limitations are to be addressed in a further study.

To conclude, the present study identified 420 downregulated and 335 upregulated DEGs. A number of important DEGs, including *CDC20*, *CCNA2*, *CCNB2* and *AURKA*, may serve pivotal roles in the development of bladder cancer by regulating the cell cycle, as well as mutual interactions. These results provide a theoretical basis for a subsequent experimental study, and may contribute to an improved understanding of the molecular mechanisms that underlie bladder cancer.

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