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

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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.

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
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.biocoductor.org/packages/2.8/bioc/html/limma.html) package in Bioconductor (8) and Affymetrix annotation files from Brain Array Lab (version 20; http://brainarray.mbi. med.umich.edu/Brainarray/Database/CustomCDF/CFD_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 llog2(fold change)<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. 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.synopspe.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=3.60x10^{-3}), involving DEGs such as CRYAB, TACR2 and MYH3, muscle system process (P=9.48x10^{-6}) associated with DEGs such as CRYAB, TACR2 and MYH3, and actin filament-based process (P=3.19x10^{-4}) involving DEGs such as DCL1, MYH3 and CALDI (Table I). The only two enriched KEGG pathways of downregulated DEGs included focal adhesion (P=3.86x10^{-5}; e.g., PRKCA, LAMA3 and ITGA8) and tight junction (P=8.10x10^{-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=2.46x10^{-5}; Table III).

The top three enriched GO terms in the BP category of upregulated genes included cell cycle (P=1.88x10^{-9}), involving DEGs such as AURKA, CCNA2, CCNE1, CDC20 and CCNB2, M phase (P=1.88x10^{-9}) associated with a number of DEGs, including AURKA, CCNA2, CDC20 and CCNB2, and cell cycle phase (P=2.89x10^{-9}) 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=6.68x10^{-11}; e.g., CCNA2, CCNE1, CDC20 and CCNB2), oocyte meiosis (P=1.76x10^{-7}; e.g., AURKA, CCNE1, CDC20 and CCNB2), DNA replication (P=3.43x10^{-4}; e.g., RFC4 and POLE2) and p53 signaling pathway (P=4.28x10^{-4}; e.g., CCNE1 and CCNB2) (Table V). The five enriched Reactome pathways of upregulated genes included cell cycle, mitotic (P=3.93x10^{-22}; e.g., AURKA, CCNA2, CCNE1, CDC20, CCNB2, RRM2 and KIF20A), cell cycle checkpoints (P=6.55x10^{-7}; e.g., CCNE1, CDC20 and CCNB2), telomere maintenance (P=1.70x10^{-8}; e.g., HIST1H2AC, HIST2H2AA3 and HIST1H2BD), DNA replication (P=3.52x10^{-4}; e.g., RFC4 and POLE2) and metabolism of nucleotides (P=4.04x10^{-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
and CHEK1 were downregulated and 10 tumor suppressor genes (e.g., CCNA2) were downregulated and 12 proto-oncogenes (e.g., BCL3, EZH2 and FOXM1) 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).

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. 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), NUSAPI (degree, 15) and CCNB2 (degree, 14).

**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 | 3.60x10^-3 | CRYAB, TACR2, MYH3, CALD1, VIPR1... |
| GO: 003012 ~ muscle system process | 14 | 9.48x10^-3 | CRYAB, TACR2, MYH3, CALD1, VIPR1... |
| GO: 0030029 ~ actin filament-based process | 16 | 3.19x10^-4 | DLC1, MYH3, CALD1, NF1, FLNA... |
| GO: 0001656 ~ metanephros development | 7 | 3.93x10^-4 | TCF21, BDNF, ITGA8, BCL2, HOXA11... |
| GO: 0040012 ~ regulation of locomotion | 13 | 1.21x10^-3 | RTN4, DCL1, PRKCA, NF1, SMAD3... |
| GO: 0030334 ~ regulation of cell migration | 12 | 1.38x10^-3 | RTN4, DCL1, LAMA3, BCL2, NF1... |
| GO: 0030336 ~ negative regulation of cell migration | 7 | 1.59x10^-3 | DCL1, BCL2, NF1, ILK, TGFB3... |
| GO: 0030036 ~ actin cytoskeleton organization | 14 | 1.62x10^-3 | DLC1, CALD1, NF1, FLNA, CORO2B... |
| GO: 0060284 ~ regulation of cell development | 13 | 2.10x10^-3 | RTN4, NF3, HOXA11, NF1, NLGN1... |
| GO: 0045449 ~ regulation of transcription | 79 | 2.24x10^-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 | 3.86x10^-3 | PRKCA, LAMA3, ITGA8, BCL2, ILK, LAMC1, LAMB1, FLNC, FLNA, PARVA, VCL, MYL9 |
| hsa04530: Tight junction | 9 | 8.10x10^-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 | 2.46x10^-3 | TNNT3, DES, MYH3, DMD, TPM2 |
| Upregulated | | | |
| REACT_152: Cell cycle, mitotic | 49 | 3.93x10^-22 | CDC20, CCNB2, KIF23, EZF2, AURKA... |
| REACT_1538: Cell cycle checkpoints | 17 | 6.55x10^-7 | CDC20, CCNB2, CHEK1, MCM2, UBE2C... |
| REACT_7970: Telomere maintenance | 12 | 1.70x10^-4 | HIST1H2AC, HIST2H2AA3, HIST1H2BD, H2BFS, RFC4... |
| REACT_383: DNA replication | 12 | 3.52x10^-4 | RFC4, POLE2, PSMC4, RFC2, PSMD10... |
| REACT_1698: Metabolism of nucleotides | 9 | 4.04x10^-3 | TYMS, RRM2, DTYMK, DCK, CAD... |

**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, 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), NUSAPI (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 | 1.88x10^-29 | CDC20, CCNB2, KIF23, E2F2, KIFC1, ...            |
| GO:000279 ~ M phase                       | 52 | 1.88x10^-29 | CDC20, CCNB2, KIF23, KIFC1, PRC1, ...            |
| GO:0022403 ~ cell cycle phase             | 56 | 2.89x10^-28 | CDC20, CCNB2, KIF23, KIFC1, PKMY1, ...           |
| GO:0022402 ~ cell cycle process           | 63 | 3.37x10^-27 | CDC20, CCNB2, KIF23, TTK, AURKA, ...             |
| GO:0000278 ~ mitotic cell cycle           | 51 | 4.83x10^-26 | CDC20, CCNB2, KIF23, TTK, ...                    |
| GO:0000280 ~ nuclear division             | 40 | 7.79x10^-25 | CDC20, CCNB2, KIF23, KIFC1, NEK2, ...            |
| GO:0007067 ~ mitosis                      | 40 | 7.79x10^-25 | CDC20, CCNB2, KIF23, KIFC1, PKMY1, ...           |
| GO:0000087 ~ M phase of mitotic cell cycle| 40 | 1.56x10^-24 | CDC20, CCNB2, KIF23, PKMY1, AURKA, ...           |
| GO:0048285 ~ organelle fission            | 40 | 3.64x10^-24 | CDC20, CCNB2, KIF23, ...                        |
| GO:0051301 ~ cell division               | 42 | 6.70x10^-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 | 6.68x10^-11 | CDC20, CCNB2, E2F2, PKMY1, TTK, ...              |
| hsa04114: Oocyte meiosis                  | 16 | 1.76x10^-7  | CDC20, CCNB2, SOLO1, PKMY1, AURKA, ...           |
| hsa03030: DNA replication                 | 7  | 3.43x10^-4  | RFC4, POLE2, RFC2, MCM2, FEN1, ...               |
| hsa04115: P53 signaling pathway           | 9  | 4.28x10^-4  | CCNB2, CCNE2, CCNB1, BID, CCNE1, ...             |
| hsa04914: Progestrone-mediated oocyte maturation | 9  | 2.05x10^-3  | CCNB2, CCNB1, MAD2L1, PLK1, BUB1, ...            |
| hsa05322: Systemic lupus erythematosus    | 9  | 4.93x10^-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, POUSF1, POUSF4, RORB, SMAD3, SP3, TCF21, YAF2, ZNF10, ZNF174 | 21  |
| Upregulated  | BCL3, EZH2, FOXM1, IRF1, RELB, TCEB3, TFDP1, XBP1                      | 8   |
| Oncogenes    | DUSP26, MEIS1                                                          | 2   |
| Downregulated| AURKA, BCL3, CCNA2, CCNE1, CEPI5, DCUN1D1, FGFR1OP, HMMR, MAP3K8, MYB, PTTG1, VEGFA | 12  |
| Upregulated  |                                                                       |     |
| Tumor suppressors | ARHGEF12, BLCAP, CHD5, DLC1, FOXP1, MFHAS1, NDRG4, NF1, PACRG, PEG3, SCARA3, SMAD3, TFGFR2, TFGFR3, 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  | $2.26 \times 10^{-5}$ | CCNB2, PLK1, CDC20, CCNA2, MCM6 |
| hsa04114: Oocyte meiosis                  | 4  | $5.10 \times 10^{-4}$ | CCNB2, PLK1, CDC20, AURKA   |
| hsa04914: Progesterone-mediated oocyte maturation | 3  | $7.41 \times 10^{-3}$ | 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.
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.

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.
To conclude, the present study identified 420 down-regulated and 335 upregulated DEGs. A number of important DEGs, including CDCA20, 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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