Identification of hub genes and pathways associated with bladder cancer based on co-expression network analysis

DONG-QING ZHANG, CHANG-KUO ZHOU, SHOU-ZHEN CHEN, YUE YANG and BEN-KANG SHI

Department of Urology, Qilu Hospital of Shandong University, Jinan, Shandong 250012, P.R. China

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Abstract. The aim of the present study was to identify hub genes and signaling pathways associated with bladder cancer (BC) utilizing centrality analysis and pathway enrichment analysis. The differentially expressed genes (DEGs) were screened from the ArrayExpress database between normal subjects and BC patients. Co-expression networks of BC were constructed using differentially co-expressed genes and links, and hub genes were investigated by degree centrality analysis of co-expression networks in BC. The enriched signaling pathways were investigated by Kyoto Encyclopedia of Genes and Genomes database analysis based on the DEGs. The hub gene expression in BC tissues was validated using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting. A total of 329 DEGs were screened, including 147 upregulated and 182 downregulated genes. The co-expression network constructed between BC and normal controls consisted of 182 nodes and 434 edges, and the two genes in each gene pair were differentially co-expressed genes. Centrality analysis of co-expression networks suggested that the top 5 hub genes with high degree included lectin, galactoside-binding, soluble, 4 (LGALS4), protein tyrosine phosphatase, receptor type N2 (PTPRN2), transmembrane protease, serine 11E (TMPRSS11E), tripartite motif containing 31 (TRIM31) and potassium voltage-gated channel subfamily D member 3 (KCND3). Pathway analysis revealed that the 329 DEGs were significantly enriched in 5 terms (cell cycle, DNA replication, oocyte meiosis, p53 signaling pathway and peroxisome proliferator-activated receptor signaling pathway). According to RT-qPCR and western blot analysis, 4/5 hub genes were significantly expressed, including LGALS4, PTPRN2, TMPRSS11E, TRIM31; however, KCND3 was not significantly expressed. In the present study, 5 hub genes were successfully identified (LGALS4, PTPRN2, TMPRSS11E, TRIM31 and KCND3) and 5 biological pathways that may be underlying biomarkers for early diagnosis and treatment associated with bladder cancer were revealed.

Introduction

Bladder cancer (BC) is the fourth most common cancer and the eighth leading cause of cancer-associated mortality worldwide. BC is an important public health issue as it is biologically very aggressive and highly prevalent in Western countries (1). In 2017, an estimated 79,030 new cases of BC and 16,870 mortalities will occur in the USA (2). In China, the incidence and mortality rates of BC rank the highest among tumors of the male urogenital system (3). Painless hematuria is the main symptom of BC, and its diagnosis is established based on urinary cytology and transurethral tumor resection (4). There is an increasing trend in incidence and mortality rates of BC. Numerous studies have identified various risk factors that may induce BC, including geography, race, gender, schistosomiasis infection, environmental or occupational exposure, smoking and genetic susceptibility (5-7).

Tumor progression is a complicated procedure of cancer cell development from normal epithelial cells, which involves changes in various genes, including oncogenes, cell cycle-associated genes, tumor suppressor genes and DNA damage repair genes. These are potential tumor markers in clinical practice; however, additional clinical studies are required to confirm their clinical utility (8-10). Development of molecular biology has increased the understanding of the mechanism underlying BC. Dyrekjot et al (11) detected carcinoma in situ gene expression that is reflected in carcinoma in situ biopsies and superficial transitional cell carcinoma. Biton et al (12) demonstrated that a molecular urothelial differentiation program was maintained by applying independent component analysis to bladder cancer transcriptome data and exploiting additional molecular and clinic pathological data.
Network-based approaches, particularly co-expression networks, offer a more effective means to identify potential malignancy diagnostic molecules based on connecting them together. Co-expression networks are generally used to study disease mechanisms (13) and provide a systems level view of dysregulated signaling pathways (14). The basic premise of co-expression analysis is that strongly correlated genes are likely to be functionally associated. Furthermore, it is possible to gain a clear insight into the important tumorigenic genes and signaling pathways of a variety of diseases, many of which are applicable to early detection and treatment (15).

In order to investigate the molecular and genetic mechanisms of BC, the present study identified the differentially expressed genes (DEGs) between BC and normal controls and constructed co-expression networks of BC via differentially co-expressed genes and links (DCGL); subsequently, the hub genes and pathways were further investigated by centrality analysis and using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, respectively. In addition, the hub genes that were potential tumor markers for BC progression were validated in BC tissues using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting.

Materials and methods

Data collection and preprocessing. A total of two gene expression datasets from healthy people and BC patients, including E-MTAB-1940 and E-GEO-D-3167, were obtained from the ArrayExpress database (www.ebi.ac.uk/arrayexpress/). A total of 146 samples (128 cases and 18 controls) were collected in the present study. The dataset E-MTAB-1940 (12) included 4 controls (samples from normal bladders) and 82 cases (samples from BC tissue); the dataset E-GEO-D-3167 (11) included 14 controls and 46 cases.

Data preprocessing for all original expression information was performed prior to the analysis. In order to reduce the influence of nonspecific factors about, the background correction and normalization were performed using the robust multichip average method (16) and the quantile based algorithm (17), respectively. Perfect match and mismatch values were revised and selected using the Micro Array Suite 5.0 (MAS 5.0) algorithm (18) and the median method, respectively. Subsequently, the data were screened by the feature filter method of the genefilter package (http://bioconductor.org/packages/release/bioc/html/genefilter.html). Each probe was mapped to one gene using getSYMBOL, which is a function in package annotate of the genefilter package (http://www.bioconductor.org/packages/release/bioc/html/annotate.html), and the probe was discarded if it did not match any genes. The two expression datasets were merged and synthetically analyzed using Batch Mean-centering, a merged data method (19), following adaptation according to Support Vector Machines, through the inSilicoMerging package (20).

Detection of DEGs. In the present study, the DEGs between BC patients and normal controls were screened by the linear models for microarray data (LIMMA) package (21). t-tests and F-tests were performed on the matrix, and the P-values were transformed to -log10. Empirical Bayes (22) statistics and false discovery rate (23) calibration of P-values for the data were conducted using the lmFit function (lmFit:http://lmfit.github.io/lmfit-py/). The DEGs were selected from following inspection for the following cut-off criteria: values of log fold change (FC) ≥2 and P<0.05.

Construction of differential co-expression networks by DCGL. It is critical to construct a co-expression network for identifying modules and the intra-modular connectivity. DCGL 2.0 (24) is an R package for identifying differentially co-expressed genes (DCGs) and differentially co-expressed links (DCLs) from gene expression microarray data. It examines the expression correlation based on the exact co-expression changes of gene pairs between two conditions, and thus is able to differentiate significant co-expression changes from relatively trivial ones (25). It has four functional modules: Gene filtration, link filtration, differential co-expression analysis (DCEA) and differential regulation analysis. Differential co-expression profile (DCp) and differential co-expression enrichment (DCE) are involved in the DCEA module for extracting DCGs and DCLs. DCp worked on the filtered set of gene co-expression value pairs, where each pair was composed of two co-expression values worked out under two different conditions separately (24,26). The present study used a length-normalized Euclidean distance to measure differential co-expression (dC) of the co-expression value pairs associated with a particular gene. A permutation test was performed to assess the significance of dC. The sample permutation was repeated N times, and a large number of permutation dC statistics formed an empirical null distribution. The P-value for each gene could then be estimated.

DCe was also used to identify DCGs and DCLs, which were based on the ‘Limit Fold Change’ (LFC) model. Initially, correlation pairs were divided into 3 parts according to the pairing of signs of co-expression values and the multitude of co-expression values: Pairs with the same signs, pairs with different signs and pairs with differently-signed high co-expression values. The first two groups were processed with the ‘LFC’ model separately to produce two subsets of DCLs, while the third group was added to the set of DCLs directly. Therefore, a total of DCLs was determined from all gene links.

Centrality analysis. To additionally assess the key genes of BC, a centrality analysis was performed based on the nodes degree in the networks (27). Centrality measures mainly contain degree centrality, closeness centrality and shortest path between centrality, in which degree, the equivalent of the number of nodes directly adjacent to a given node (indicating the degree the vertex), is the simplest topological index (27). In the present study, centrality analysis, which was particularly useful to identify key players in biological processes, was implemented to study the differential co-expression networks. Calculation of the degree allows determining the ‘degree distribution’, which gives the probability that a selected node has exact links. Nodes with high degree (highly connected) are called ‘hubs’, which interact with several other genes, suggesting a central role in the interaction network (28). An obvious order of the vertices of a graph may be established by sorting them according to their degree (29). Genes with degree ≥9 were defined as hub genes in the present study.
Pathway enrichment analysis. To investigate the enriched signaling pathways of the DEGs, enrichment analysis was performed based on the KEGG database (www.genome.jp/kegg/). The DEGs were applied to this database for investigating the association between the biochemical pathways and the occurrence of bladder cancer. The analysis was conducted by DAVID (30) (david.abcc.ncifcrf.gov/tools.jsp). The enrichment pathway analysis of the DEGs contributed to additional observation of these genes at the macroscopic level. Categories were obtained according to the Expression Analysis Systematic Explorer (31) score for 0.01.

Patients. Tumor tissues from 10 BC patients were obtained during biopsy in Qilu Hospital of Shandong University (Jinan, China) between January 2015 and March 2015. Normal samples were collected from 2 cm away from the tumor. The present study was approved by the Ethical Committee of Qilu Hospital of Shandong University. Written informed consent was obtained from patients who agreed to participate in the study.

Validation of hub genes by RT-qPCR and western blotting. The tumor tissues of 10 BC patients and normal tissue samples obtained from 2 cm away from the tumor were analyzed with RT-qPCR and western blotting. For RT-qPCR, primer sequences of the 5 hub genes are listed in Table I. The qPCR mix composition was as follows: 10 µl of 10X qPCR buffer, 1 µl of Taq DNA polymerase, 3 µl of each forward and reverse primer and 8 µl of dNTPs. The qPCR reagents were purchased from Invitrogen (Thermo Fisher Scientific, Inc.). The qPCR conditions are shown in Table II. Complementary DNA was used as a template, and β-actin was used as an internal reference. RT-qPCR products were separated using 1.5% agarose gel electrophoresis and gels were visualized using Quantity One Software v4.62 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Every sample was run 3 times along with the internal control.

Proteins were extracted from tumor tissues and adjacent normal tissues according to the method reported by Yoon et al (32). Protein concentration was measured with a BCA Protein Assay kit (CW Biotech, Beijing, China). SDS-PAGE (12%) was performed for 10 µg protein, and the protein was electrotransferred (4˚C, 300 mA, 2 h) to a nitrocellulose membrane. The membrane was blocked in TBST containing 5% skimmed milk powder for 30 min and exposed in the dark. Protein bands were visualized using ImageJ 1.36b software (National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/ij/).

### Table I. Primer sequences and product length of the 5 hub genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences (5‘-3’)</th>
<th>Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LGALS4</td>
<td>F: GCCTTCCACTCAATCCGC R: GGCCTGTCAGCTGTTGATGG</td>
<td>355</td>
</tr>
<tr>
<td>PTPRN2</td>
<td>F: GTGGACCAGTGAGTAGCTCG R: GTCGGAGAACCTCTCTGTCT</td>
<td>541</td>
</tr>
<tr>
<td>TMPRSS11E</td>
<td>F: GTCTCAGGATCTGGTTGGG R: ACAAGAAAGTGGGCAAGATCAGT</td>
<td>720</td>
</tr>
<tr>
<td>TRIM31</td>
<td>F: GTCTTGTGCAAGAATGAGATT R: TCACAAAACCAAGCAGGAT</td>
<td>178</td>
</tr>
<tr>
<td>KCND3</td>
<td>F: TTTACACTGGAGGTGGGGA R: TGCAGTGGGATTTCTGCT</td>
<td>506</td>
</tr>
<tr>
<td>β-actin</td>
<td>F: AAGTACTCCGTGTGGATCGG R: TCAAGTTGGGGGCAAAAAAG</td>
<td>615</td>
</tr>
</tbody>
</table>

LGALS4, lectin, galactoside-binding, soluble; 4; PTPRN2, protein tyrosine phosphatase, receptor type N2; TMPRSS11E, transmembrane protease, serine 11E; TRIM31, tripartite motif containing 31; KCND3, potassium voltage-gated channel subfamily D member 3; F, forward; R, reverse; bp, base pairs.
Statistical analysis. Statistical analysis was conducted using SPSS 20.0 software (IBM SPSS, Armonk, NY, USA). All data were presented as mean ± standard deviation. Statistical differences among groups were assessed using one way analysis of variance. P<0.05 was considered to indicate a statistically significant difference. Graphs were designed using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Detection of DEGs. In the present study, a total of 146 samples of two datasets associated with BC were preprocessed to identify DEGs using the LIMMA package. A total of 329 DEGs, including 147 upregulated and 182 downregulated genes between BC patients and normal subjects were detected under the criteria of |logFC|≥2 and P<0.05.

Construction of differential co-expression networks by DCGL. The present study applied the DCGL 2.0 package in R to identify DCGs and DCLs, in which DCp and DCe methods involved in DCEA module were employed. A total of 434 DCLs were included in the co-expression network, and the two genes in each link were DCGs. A gene co-expression network containing 182 nodes and 434 edges was constructed in the analysis (Fig. 1).
Centrality analysis. By accessing degree centrality analysis of the co-expression network, 18 hub genes were obtained with degree ≥9 (Table III), of which 16 genes were upregulated and 2 were downregulated (Fig. 2). The genes LGALS4, PTPRN2, TMPRSS11E, TRIM31 and KCND3 were the top 5 hub genes with high degree, and all of them were upregulated.

Pathway enrichment analysis. Pathway analysis based on the KEGG database revealed that these DEGs were significantly enriched in 5 terms, which were cell cycle (P=4.37x10^{-7}), DNA replication (P=1.95x10^{-5}), oocyte meiosis (P=6.47x10^{-3}), p53 signaling pathway (P=7.17x10^{-3}) and the peroxisome proliferator-activated receptor (PPAR) signaling pathway (P=7.70E-03). Among the 5 terms, the cell cycle pathway was the most significant term, which also enriched more genes compared with the other terms.

Validation by RT-qPCR and western blotting. In the present study, to confirm the messenger RNA (mRNA) and protein expression levels of 5 hub genes (LGALS4, PTPRN2, TMPRSS11E, TRIM31 and KCND3) from the co-expression network, RT-qPCR and western blotting was performed on BC patient samples. The results of the relative expression of all the hub genes are shown in Figs. 3 and 4. It was observed that 4/5 hub genes and the corresponding proteins including LGALS4, PTPRN2, TRIM31, and KCND3 were signifi-
BC is a heterogeneous disease, with 30% of cases presenting as muscle-invasive disease associated with a high risk of death from distant metastases, which may be managed with transurethral resection (33). However, BC has a notable tendency to recur (30-85%), therefore the present study investigated an adequate method for investigating biomarkers in BC, that may contribute to our understanding of the pathogenesis and diagnoses of the disease, substantially reducing the mortality associated with this disease.

In the present study, hub genes and pathways of BC were identified based on degree centrality analysis of the co-expression network and pathway enrichment analysis. A total of 18 hub genes were obtained, the top 5 of which were LGALS4, PTPRN2, TMPRSS11E, TRIM31 and KCND3, by conducting centrality analysis on the co-expression network. Furthermore, cell cycle, DNA replication, oocyte meiosis, the p53 signaling pathway and the PPAR signaling pathway were observed to be significant pathways of BC. In addition, RT-qPCR and western blotting were performed to verify network-based results and investigate significant genes of BC.

RT-qPCR analysis revealed that 3 hub genes (LGALS4, TMPRSS11E and TRIM31) were significantly upregulated in BC patients when compared with normal subjects, which was consistent with the bioinformatics analysis; however, KCND3 was not significantly differentially expressed between the conditions, and in contrast to the bioinformatics result, the relative content of the upregulated PTPRN2 was significantly reduced. This result was not entirely consistent with the network analysis. The probable reason for this was variations of samples; the microarray data was downloaded from the ArrayExpress database and RT-qPCR and western blotting were performed on patient samples. Therefore, it can be speculated that the 3 consistent hub genes (LGALS4, TMPRSS11E and TRIM31) may be potential markers of BC. LGALS4 has been identified as one of the genes involved in numerous types of human tumor, including sinonasal adenocarcinoma tumors (34), colorectal cancer (35) and breast cancer (36). There has also been a previous report that compared expression changes at mRNA and protein levels in the rat model and identified the gene exhibiting concordant changes with LGALS4 levels in bladder tumors (37). TRIM family proteins are involved in various cellular processes, including tumor development and antiviral response (38). One of the family proteins, TRIM31, was originally identified as a gene induced by growth-suppressive retinoid (39). A previous study showed that TRIM31 had the ability to regulate cell proliferation negatively in gastric adenocarcinoma (40) and its expression was reduced in lung cancer cell lines (41). However, the expression level of TRIM31 was increased in BC patients in the present study. Additional research is required to verify the biological properties of TRIM31.

Pathway analysis revealed that several significantly enriched signaling pathways included cell cycle, DNA replication, oocyte meiosis, the p53 signaling pathway and the PPAR signaling pathway. The cell cycle is the universal process through which cells reproduce and grow in all living organisms, and is concerned with the copying of the hereditary material, including replication of the chromosomal DNA during mitosis (42). Previous studies have indicated that several tumor suppressor candidates exert growth inhibitory effects by inducing cell cycle arrest at G2/M phase in leukemia cells (43), and exerted different tumor suppressive effects (44).
Furthermore, drug development programs are underway to target the p35 signaling pathway (48).

In conclusion, the present study identified 5 hub genes associated with BC, and 3 of these were verified via molecular experiments, RT-qPCR and western blotting. The signaling pathways associated with these genes were presented systematically. These genes and pathways may be potential biomarkers for early detection and therapy for BC.

References