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

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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.

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Abbreviations: BC, bladder cancer; DEGs, differentially expressed genes; DCGL, differentially co-expressed genes; KEGG, Kyoto encyclopedia of genes and genomes; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; DCGs, differentially co-expressed genes; DCLs, differentially co-expressed links; DCEA, differential co-expression analysis; DCp, differential co-expression profile; DCe, differential co-expression enrichment

Key words: bladder cancer, hub genes, co-expression network, pathway enrichment analysis, reverse transcription-quantitative polymerase chain reaction

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. Dyrskjøt *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-GEOD-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-GEOD-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, whoch is 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 centricity 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.

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

Gene	Primer sequences (5'-3')	Length (bp)
LGALS4	F: GCCTTCCACTTCAATCCGC R: GGCTGTTCAGCTGTTGATGG	355
PTPRN2	F: GTGGACCATGGAGTAGCTCG R: GTCCGAGAACCTCTCTGTCT	541
TMPRSS11E	F: GTCTCAGGATCGTTGGTGGG R: ACAAGAAAGTTGGCAAGATACCAGT	720
TRIM31	F: GTCTTGTGCAGAAGTGAAGAGTT R: TCACAAAACCAAGCCCGGAT	178
KCND3	F: TTTACACTGGAGGTGGGGGA R: TGCAGTGCGATTTCAGGTCT	506
β-actin	F: AAGTACTCCGTGTGGATCGG R: TCAAGTTGGGGGACAAAAAG	615

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.

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 polymorphic DNA markers, and total RNA was prepared using TRIzol (Beyotime Institute of Biotechnology- Haimen, China). Total RNA was reverse transcribed with an oligo (dT18) primer and was treated with 2 μl RNasin (40 U/μl), 8.0 μl 5X reverse transcriptase buffer, 8.0 μl dNTPs and 2 μl AMV reverse transcriptase (5 U/μl) according to the manufacturer's protocol. The RT reagents were purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). 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-PCR 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 at 37˚C for 2 h, and incubated with rabbit anti-human antibodies (1:10,000) against lectin, galactoside-binding, soluble, 4 (LGALS4) [AP12391a; Abgent Biotech (SuZhou) Co., Ltd., Suzhou, China], protein tyrosine phosphatase, receptor type N2 (PTPRN2) (bs-19591R; BIOSS, Beijing, China), transmembrane protease, serine 11E (TMPRSS11E) [AP16520C; Abgent Biotech (SuZhou) Co., Ltd.], tripartite motif containing 31 (TRIM31) (bs-6220R; BIOSS), potassium voltage-gated channel subfamily D member 3 (KCND3) (bs-20219R; BIOSS) and GAPDH [AP50811; Abgent Biotech (SuZhou) Co., Ltd.] at 37˚C for 2 h. Unbound antibody was washed away by TBST (3 times), and the membrane was incubated with horseradish peroxdiase-labeled sheep anti-rabbit immunoglobulin G secondary antibody (1:5,000; SC-2048; Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China) at 37˚C for 2 h. Following washing with TBST, the substrate was developed on the membrane for 3 min and exposed in the dark. Protein bands were visualized with Amersham ECL Western Blotting Detection kit (GE Healthcare Life Sciences, Chalfont, UK). GAPDH was used as an internal control, and western blots were analyzed using ImageJ 1.36b software (National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/ij/).

Table II. Quantitative polymerase chain reaction amplification conditions for the 5 hub genes.

Gene	Reaction conditions
LGALS4	94˚C 1 min; 35 cycles of 98˚C 10 sec, 54˚C 15 sec, 68˚C 1 min; 72˚C 7 min
PTPRN2	94˚C 2 min; 35 cycles of 98˚C 10 sec, 50˚C 15 sec, 68˚C 1 min; 72˚C 7 min
TMPRSS11E	94˚C 2 min; 33 cycles of 98˚C 10 sec, 55˚C 15 sec, 68˚C 1 min; 72˚C 7 min
TRIM31	94˚C 2 min; 35 cycles of 98˚C 10 sec, 54˚C 15 sec, 68˚C 1 min; 72˚C 7 min
KCND3	94˚C 2 min; 33 cycles of 98˚C 10 sec, 54˚C 15 sec, 68˚C 1 min; 72˚C 7 min
β-actin	94˚C 2 min; 30 cycles of 98˚C 10 sec, 51˚C 15 sec, 68˚C 1 min; 72˚C 7 min

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.

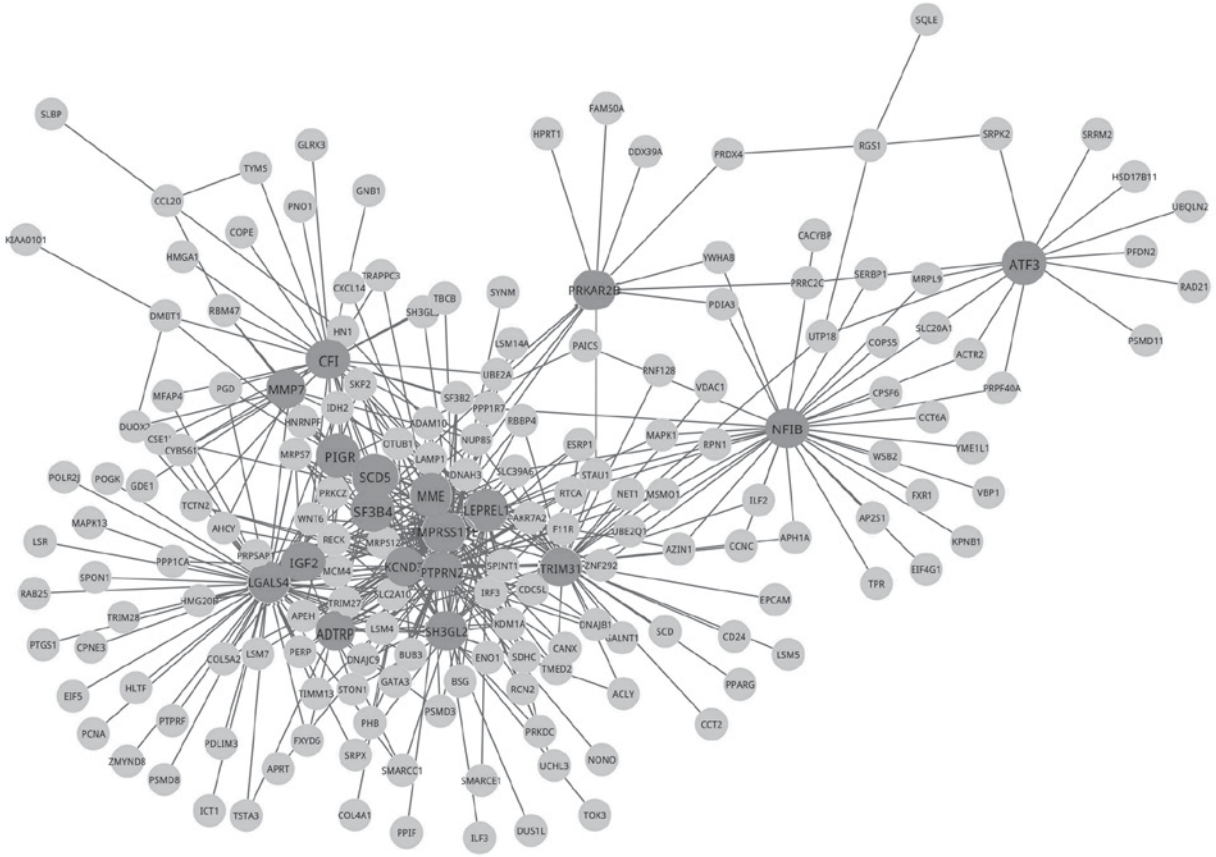

Figure 1. Co-expression network of bladder cancer based on 329 differentially expressed genes. There were 182 nodes and 43 edges in the co-expression network, where nodes referred to genes and edges between nodes indicated interaction of genes in the network; in particular, nodes in dark grey are hub genes.

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 tatistically significanct 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).

Table III. Eighteen hub genes where the degree was ≥9 in the co-expression network.

Genes	Degree
LGALS4	66
PTPRN2	56
TMPRSS11E	45
TRIM31	39
KCND3	35
NFIB	34
LEPREL1	31
SH3GL2	29
CFI	27
ADTRP	21
PIGR	16
MME	15
ATF3	13
MMP7	12
PRKAR2B	12
SCD5	10
IGF2	9
SF3B4	9

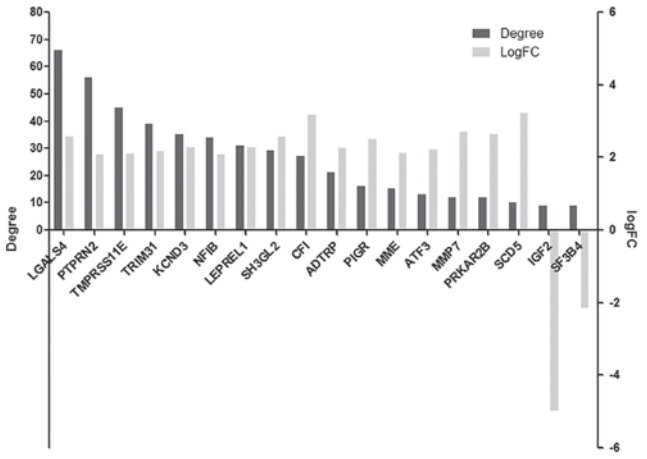

Figure 2. Summary of the degree value and logFC of the hub genes. FC, fold change.

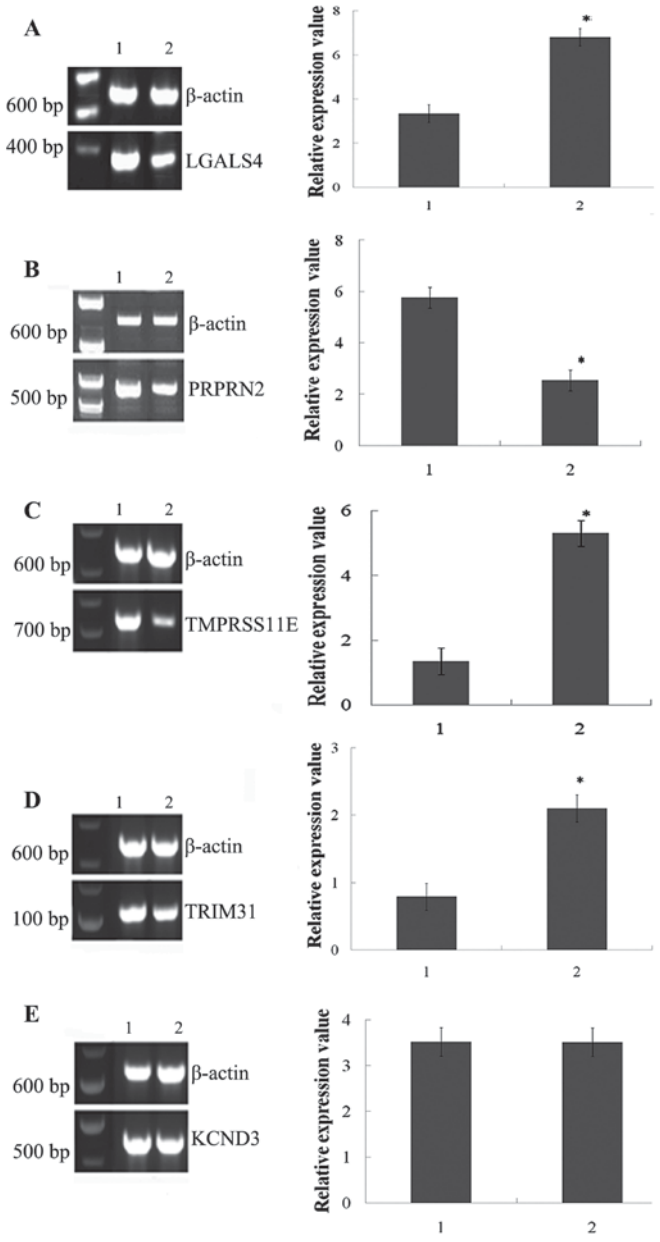

Figure 3. Results of reverse transcription-quantitative polymerase chain reaction analysis for 5 hub genes from the co-expression network. 1 represents the normal control and 2 represents disease. The following genes were investigated: (A) *LGALS4*, (B) *PTPRN2*, (C) *TMPRSS11E*, (D) *TRIM31* and (E) *KCND3*. *P<0.05. *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.

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 ($P=1.95x10^{-3}$), 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, TMPRSS11E* and *TRIM31* were signifi-

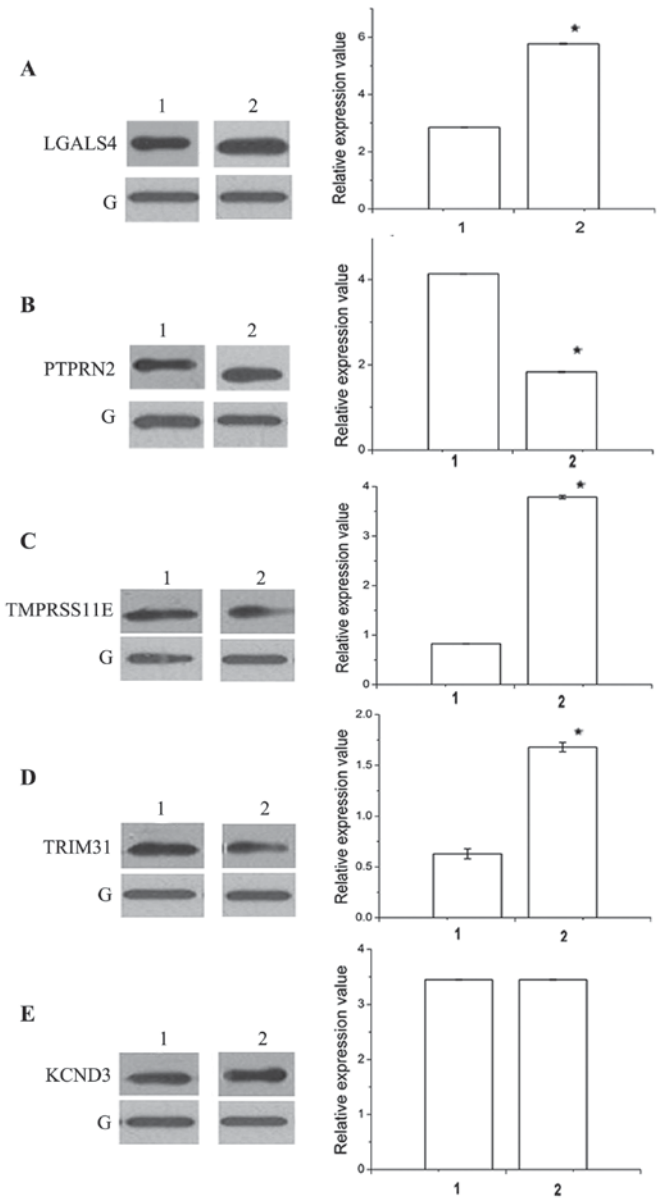

Figure 4. Results of western blot analysis for 5 hub genes. G represents GAPDH, 1 represents normal control and 2 represents disease. The following genes were analyzed: (A) *LGALS4*, (B) *PTPRN2*, (C) *TMPRSS11E*, (D) *TRIM31* and (E) *KCND3*. *P<0.05. *LGALS4*, lectin, galactoside-binding, soluble, 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.

cantly differentially expressed between BC patients and healthy subjects (P<0.05), while *KCND3* was not significantly differentially expressed (P>0.05). Furthermore, it was noted that the relative expression of *LGALS4, TMPRSS11E* and *TRIM31* was increasing, which was consistent with the result of the bioinformatics analysis, while *PTPRN2* demonstrated the opposite trend. *KCND3,* which was downregulated in the bioinformatics investigation, was not significantly differentially expressed.

Discussion

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).

In addition, Li *et al* (45) indicated the effect of the cell cycle on the susceptibility of SAS cells to sonodynamic therapy. p53 is a sequence-specific DNA-binding protein that promotes cell-cycle arrest or apoptosis in response to a variety of cellular stresses (46). The p53 signaling pathway had been suggested as a cellular surveillance mechanism for cancer prevention (47). Furthermore, drug development programs are underway to target the p53 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.

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