Screening of potential therapy targets for prostate cancer using integrated analysis of two gene expression profiles

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Received December 31, 2015; Accepted May 23, 2017

DOI: 10.3892/ol.2017.6879

Abstract. The aim of the present study was to analyze potential therapy targets for prostate cancer using integrated analysis of two gene expression profiles. First, gene expression profiles GSE38241 and GSE3933 were downloaded from the Gene Expression Omnibus database. Differentially expressed genes (DEGs) between prostate cancer and normal control samples were identified using the Linear Models for Microarray Data package. Pathway enrichment analysis of DEGs was performed using Gene Ontology and the Kyoto Encyclopedia of Genes and Genomes. Furthermore, protein-protein interaction (PPI) networks of DEGs were constructed, on the basis of the Search Tool for the Retrieval of Interacting Genes/Proteins database. The Molecular Complex Detection was utilized to perform module analysis of the PPI networks. In addition, transcriptional regulatory networks were constructed on the basis of the associations between transcription factors (TFs) and target genes. A total of 529 DEGs were identified, including 129 upregulated genes that were primarily associated with to the cell cycle. Additionally, 400 downregulated genes were identified, which were principally enriched in the pathways associated with vascular smooth muscle contraction and focal adhesion. Cell Division Cycle Associated 8, Cell Division Cycle 45, Ubiquitin Conjugating Enzyme E2 C and Thymidine Kinase 1 were identified as hub genes in the upregulated sub-network. Furthermore, the upregulated TF *E2F,* and the downregulated TF Early Growth Response 1, were identified to be critical in the transcriptional regulatory networks. The identified DEGs and TFs may have critical roles in the progression of prostate cancer, and may be used as target molecules for treating prostate cancer.

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Key words: prostate cancer, differentially expressed gene, enrichment analysis, transcription factor

Introduction

Prostate cancer is a malignancy that occurs in the prostate epithelial cells and it is the most common type of reproductive system cancer in males worldwide (1,2). Cancer statistics in 2016 revealed that prostate cancer accounts for ~20% novel cancer cases in males in the USA (3). Radical prostatectomy is an effective treatment to improve patient survival time (4), but it is only suitable for ~10% of all cases (5). A number of other therapies, including radiotherapy, hormonal therapy, chemotherapy and immunotherapy, have been developed for prostate cancer treatment (6); however, there is limited information regarding the long-term survival rate, and the mortality rate of patients with prostate cancer remains high (7). Therefore, investigations into novel treatment strategies for patients with prostate cancer are required.

Gene therapy and small molecule drugs are novel strategies for cancer treatment, and have received increasing attention over the past few decades (8). Recently, a number of studies have been conducted to reveal the underlying molecular mechanisms and identify treatment targets for prostate cancer (9-20). Specific genes involved in the DNA damage response, including breast cancer 1, breast cancer 2 and tumor protein 53 genes, are mutated during the progression of prostate cancer (9-11). A number of activated carcinogenic signaling pathways, including c-Myc, protein kinase B and Ras, induce the replication and genomic instability of prostate cancer cells (12-14). The histone-lysine N-methyltransferase gene is overexpressed in prostate cancer and may act as a therapeutic target (15). Previous studies have primarily focused on a certain gene or pathway; therefore, it is necessary to explore the underlying molecular mechanisms and therapy targets for prostate cancer using other methods.

Identification and analysis of differentially expressed genes (DEGs) is an effective method to acquire multiple novel targets for the treatment of diseases (16,17). An expression profiling analysis for prostate cancer has been studied previously (18). In addition, DNA methylation alterations in prostate cancer have been analyzed using the gene expression profile GSE38241 (19), and clinically relevant subtypes, including subgroups I (the clinically least aggressive subclass) and subgroups II/III (clinically aggressive tumor subclasses), of prostate cancer have been studied using the gene expression profile GSE3933 (20). However, DEGs and their regulatory

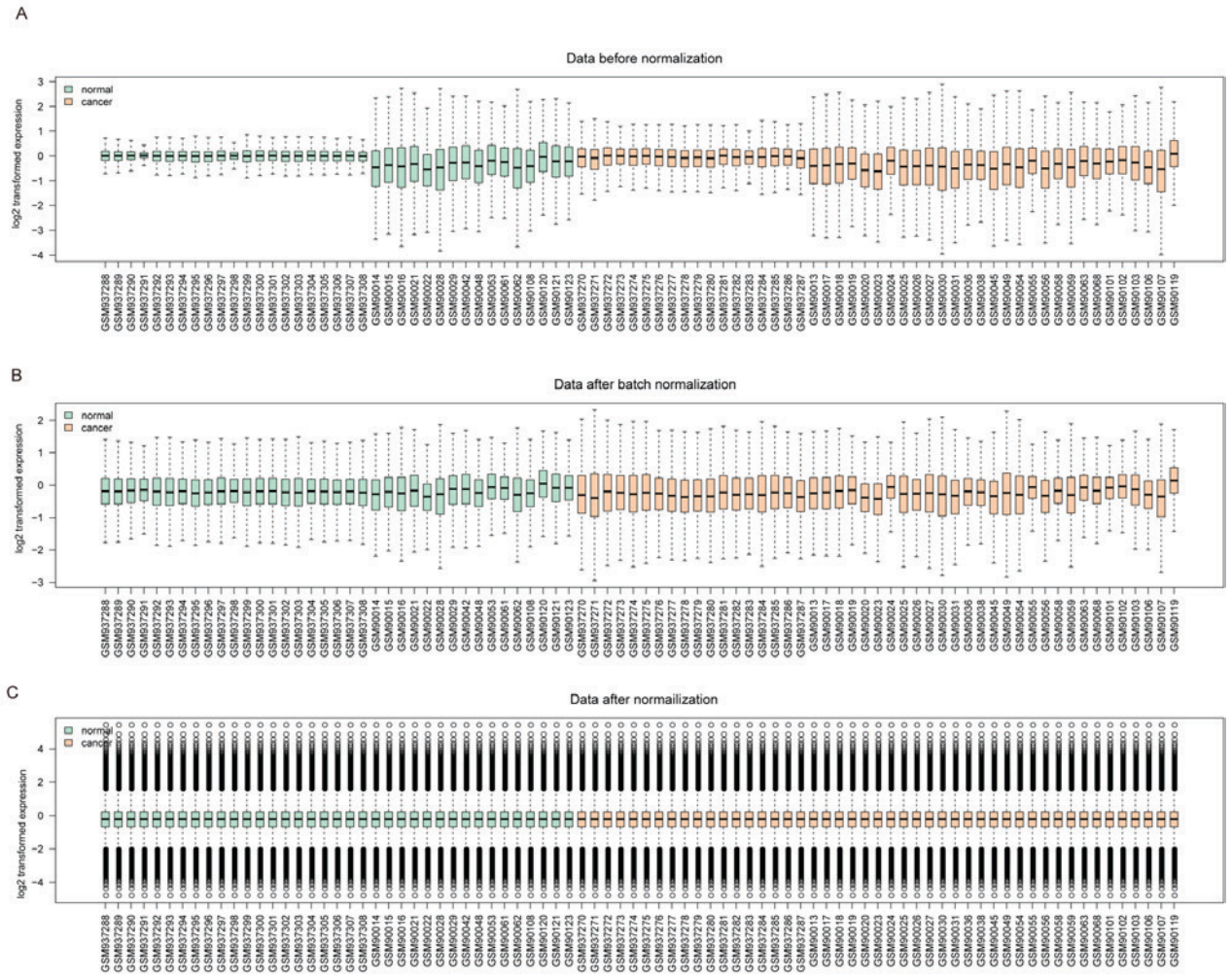

Figure 1. Box plots of the expression profiles. Green box represents the 37 normal samples; yellow box represents the 47 prostate cancer samples. The black line in each box represents the median of data, the distribution of which determines the standardization degree. An excellent degree of standardization is indicated when there is little variation in the median values. (A) Box plots of the expression profiles prior to standardization. (B) Box plots of the expression profiles following the removal of the batch error. (C) Box plots of the expression profiles following standardization.

factors between prostate cancer and normal samples were not analyzed as part of the present study.

In the present study, the expression-profiling data GSE38241 and GSE3933 were integrated to identify DEGs between prostate cancer samples and normal samples. The functions of DEGs were analyzed using Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis. Furthermore, protein-protein interactions (PPIs) of DEGs were investigated and hub genes, genes identified to be key genes, in the PPI network were identified. In addition, transcriptional regulatory networks were constructed on the basis of the associations between transcription factors (TFs) and DEGs. The results of the present study may identify the underlying molecular mechanisms of prostate cancer and provide targets for the treatment of prostate cancer.

Materials and methods

mRNA expression profiles of prostate cancer. The datasets of prostate cancer gene expression profiling by array with large sample size and high data quality were searched in the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo). Prostate cancer and normal control samples were included in the eligible dataset, and samples were not treated by additional treatments such as drugs and radiation. As a result, two prostate cancer expression profiling datasets were chosen for analysis, GSE38241 (18) and GSE3933 (19). Data of 39 samples (18 prostate cancer samples and 21 normal samples) in GSE38241 were produced using platform GPL4133 Agilent-014850 Whole Human Genome Microarray 4x44K G4112F. Data of 112 samples in GSE3933 were produced using three platforms, consisting of GPL2695 SHBB (26 samples), GPL3044 SHCQ (45 samples) and GPL3289 SHBW (41 samples). In the dataset GSE3933, only the data of 45 samples (29 prostate cancer samples and 16 normal samples) obtained from platform GPL3044 were selected for additional analysis, since gene probes detected by platform GPL3044 overlapped more with those from platform GPL4133. Data and probe annotation files were downloaded for analysis.

Data preprocessing. Subsequent to obtaining the raw data, probe IDs of the matrix data were first translated into corresponding gene symbols. If one gene symbol was matched by a number

Table I. Top 5 most significant upregulated and downregulated DEGs from GO analysis across 3 categories including BP, CC and MM.

A, Upregulated DEGs

ID	Term	Count	P-value
GOTERM_BP_FAT	GO:0000279-M phase	27	$1.68x10^{-20}$
GOTERM_BP_FAT	GO:0022403-cell cycle phase	29	$2.93x10^{-20}$
GOTERM_BP_FAT	GO:0000280-nuclear division	22	$2.64x10^{-18}$
GOTERM_BP_FAT	GO:0007067-mitosis	22	$2.64x10^{-18}$
GOTERM_BP_FAT	GO:0000087-M phase of mitotic cell cycle	22	$3.84x10^{-18}$
GOTERM_CC_FAT	GO:0005819-spindle	10	$4.39x10^{-7}$
GOTERM_CC_FAT	GO:0000775-chromosome, centromeric region	9	$1.34x10^{-6}$
GOTERM_CC_FAT	GO:0000793-condensed chromosome	9	$1.81x10^{-6}$
GOTERM_CC_FAT	GO:0000777-condensed chromosome kinetochore	7	$1.93x10^{-6}$
GOTERM_CC_FAT	GO:0000779-condensed chromosome, centromeric region	7	$4.16x10^{-6}$
GOTERM_MF_FAT	GO:0005524-ATP binding	27	$1.39x10^{-5}$
GOTERM_MF_FAT	GO:0030554-adenyl nucleotide binding	28	$1.49x10^{-5}$
GOTERM_MF_FAT	GO:0032559-adenyl ribonucleotide binding	27	$1.76x10^{-5}$
GOTERM_MF_FAT	GO:0001883-purine nucleoside binding	28	$1.97x10^{-5}$
GOTERM_MF_FAT	GO:0001882-nucleoside binding	28	$2.23x10^{-5}$

B, Downregulated DEGs

ID	Term	Count	P-value
GOTERM_BP_FAT	GO:0007517-muscle organ development	23	$2.94x10^{-9}$
GOTERM_BP_FAT	GO:0008285-negative regulation of cell proliferation	27	$2.35x10^{-7}$
GOTERM_BP_FAT	GO:0007155-cell adhesion	39	$6.17x10^{-7}$
GOTERM_BP_FAT	GO:0022610-biological adhesion	39	$6.27x10^{-7}$
GOTERM_BP_FAT	GO:0003012-muscle system process	16	$7.10x10^{-6}$
GOTERM_CC_FAT	GO:0044421-extracellular region part	59	$2.25x10^{-12}$
GOTERM_CC_FAT	GO:0005576-extracellular region	90	$4.38x10^{-11}$
GOTERM_CC_FAT	GO:0031012-extracellular matrix	29	$3.94x10^{-9}$
GOTERM_CC_FAT	GO:0005578-proteinaceous extracellular matrix	26	$5.96x10^{-8}$
GOTERM_CC_FAT	GO:0043292-contractile fiber	16	$8.23x10^{-8}$
GOTERM_MF_FAT	GO:0046870-cadmium ion binding	7	$3.11x10^{-8}$
GOTERM_MF_FAT	GO:0003779-actin binding	27	$5.32x10^{-8}$
GOTERM_MF_FAT	GO:0008092-cytoskeletal protein binding	34	$9.74x10^{-8}$
GOTERM_MF_FAT	GO:0005507-copper ion binding	12	$5.45x10^{-7}$
GOTERM_MF_FAT	GO:0005198-structural molecule activity	33	$3.76x10^{-5}$

DEGs, differentially expressed genes; count, number of DEGs; BP, biological process; CC, cell component; MF, molecule function; ATP, adenosine 5'-phosphate; GO, Gene Ontology.

of probe IDs, the mean expression value was selected as the expression level of this gene. In order to obtain reliable results, only the common genes in the two datasets were selected for the following analysis. During the process of merging the two different datasets, batch errors (21) were removed using the ComBat command of sva package in R language (http://www.bioconductor.org/packages/release/bioc/html/sva.html version 3.5) (22). Subsequently, quantile normalization of genes was performed by preprocessCore package in R (http://www.bioconductor.org/packages/release/bioc/html/preprocessCore.html; version 1.38.1) and an expression profile matrix was generated consisting of 12,621 genes.

Identification of DEGs. The linear models for microarray data package (http://www.bioconductor.org/packages/release/bioc/html/limma.html; version 3.22.7) (23), a widely-used tool for the identification of DEGs, was applied to identify DEGs between prostate cancer samples and normal samples. The raw P-value for each gene was calculated and subsequently adjusted into the false discovery rate (FDR) using

Table II. KEGG pathway analysis of the upregulated and downregulated differentially expressed genes.

A, Upregulated genes

Pathway term	Count	P-value
hsa04110: Cell cycle	7	3.79×10^{-4}
hsa04114: Oocyte meiosis	5	1.04×10^{-2}
hsa00983: Drug metabolism	3	4.45×10^{-2}

B, Downregulated genes

Pathway term	Count	P-value
hsa04270: Vascular smooth muscle contraction	15	2.66×10^{-7}
hsa04510: Focal adhesion	16	6.41×10^{-5}
hsa00982: Drug metabolism	9	8.87×10^{-5}
hsa05414: Dilated cardiomyopathy	9	1.35×10^{-3}
hsa00980: Metabolism of xenobiotics by cytochrome P450	7	2.70×10^{-3}

Count, number of differentially expressed genes; KEGG, Kyoto Encyclopedia of Genes and Genomes; hsa, human.

the Benjamini-Hochberg method (24). Only the genes that met the threshold criteria of $|\log_2$ fold change$| > 1$ and FDR<0.05 were identified as DEGs.

Functional enrichment analysis of DEGs. In order to investigate the signaling pathways and biological processes which may be involved in the progression of prostate cancer, GO (25) and KEGG (26) enrichment analysis were performed using the Database for Annotation, Visualization and Integrated Discovery (27,28). This provided a number of functional annotation tools to reveal the biological function of genes. Functional terms with P<0.05 were considered to indicate a statistically significant difference.

PPI networks and module analysis. PPIs of DEGs were searched in the Search Tool for the Retrieval of Interacting Genes/Proteins database (29), which integrates a number of known and predicted associations between proteins. The PPI network was visualized using Cytoscape (30), an open source software for integrating biomolecular networks. In the network, 'node' represents a gene or protein, and 'line' represents an interaction between two nodes. The degree of each node is equal to the number of nodes that the node interacted with. The node degree represents its topological importance; the higher the degree, the more important the node is (31). Hub genes were identified on the basis of the degree of genes in the PPI network. Molecular Complex Detection (MCODE) (32) is a tool used to determine the dense connections in large PPI networks, which may represent molecular complexes. In the present study, MCODE was utilized to screen the modules from the PPI network with a network aggregation score >10.

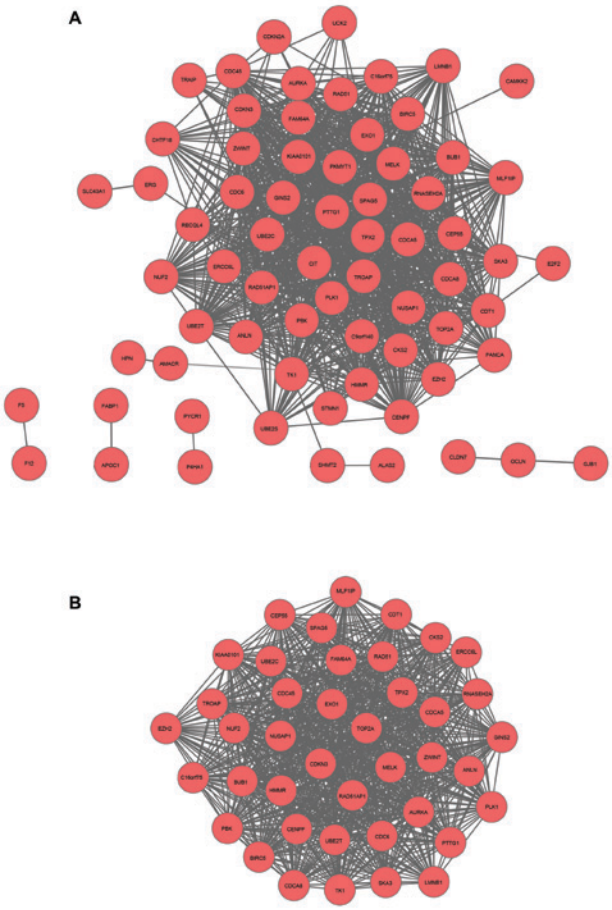

Figure 2. Networks of upregulated genes. (A) Protein-protein interaction network of upregulated genes. (B) Sub-network extracted from the protein-protein interaction network of upregulated genes.

Constructing the transcriptional regulatory networks. TFs targeting DEGs were identified from DEGs on the basis of information in the TRANSFAC database (http://gene-regulation.com/pub/databases.html; version 7.0) (33), which provided data on eukaryotic transcription factors, consensus binding sequences (positional weight matrices), experimentally proven binding sites and regulated genes. Transcription regulatory networks were visualized using Cytoscape, as aforementioned, in order to observe the interactions between TFs and target DEGs.

Results

Identified DEGs. Prior to normalization, the medians of gene expression in each sample were markedly distinct (Fig. 1A). However, the medians became consistent and were at a similar level following normalization (Fig. 1B and C), suggesting that the normalization process was successful and the normalized data may be used for additional analysis.

On the basis of the threshold criteria, a total of 529 DEGs were obtained, including 129 upregulated and 400 downregulated genes in prostate cancer samples, compared with normal samples.

Enrichment analysis of DEGs. To reveal the biological functions of DEGs, GO and KEGG pathway enrichment

Table III. Top 5 most significant genes within the sub-network of upregulated genes from GO analysis of 3 categories including BP, CC and MM.

Category	Term	Count	P-value
GOTERM_BP_FAT	GO:0000279-M phase	23	$4.23x10^{-27}$
GOTERM_BP_FAT	GO:0022403-cell cycle phase	24	$1.28x10^{-26}$
GOTERM_BP_FAT	GO:0007067-mitosis	20	$3.12x10^{-25}$
GOTERM_BP_FAT	GO:0000280-nuclear division	20	$3.12x10^{-25}$
GOTERM_BP_FAT	GO:0000087-M phase of mitotic cell cycle	20	$4.44x10^{-25}$
GOTERM_CC_FAT	GO:0005819-spindle	11	$4.94x10^{-13}$
GOTERM_CC_FAT	GO:0000775-chromosome, centromeric region	10	$4.83x10^{-12}$
GOTERM_CC_FAT	GO:0000777-condensed chromosome kinetochore	8	$3.94x10^{-11}$
GOTERM_CC_FAT	GO:0015630-microtubule cytoskeleton	14	$5.31x10^{-11}$
GOTERM_CC_FAT	GO:0000779-condensed chromosome, centromeric region	8	$1.01x10^{-10}$
GOTERM_MF_FAT	GO:0005524-ATP binding	13	$3.65x10^{-5}$
GOTERM_MF_FAT	GO:0032559-adenyl ribonucleotide binding	13	$4.17x10^{-5}$
GOTERM_MF_FAT	GO:0030554-adenyl nucleotide binding	13	$7.02x10^{-5}$
GOTERM_MF_FAT	GO:0001883-purine nucleoside binding	13	$8.15x10^{-5}$
GOTERM_MF_FAT	GO:0001882-nucleoside binding	13	$8.72x10^{-5}$

Count, number of differentially expressed genes; GO, Gene Ontology; BP, biological process; CC, the cell component; MF, molecule function; count, number of differentially expressed genes; ATP, adenosine 5'-phosphate.

Table IV. KEGG pathway analysis of the sub-network of upregulated genes.

Category	Term	Count	P-value
KEGG_ PATHWAY	hsa04110: Cell cycle	5	$1.48x10^{-4}$
KEGG_ PATHWAY	hsa04114: Oocyte meiosis	4	$1.88x10^{-3}$

Count, number of differentially expressed genes; KEGG, Kyoto Encyclopedia of Genes and Genomes; hsa, human.

analyses were performed for the up- and downregulated genes. Upregulated genes were primarily enriched in cell cycle-associated GO terms, including cell cycle phase, spindle and adenosine 5'-phosphate binding (Table I). Downregulated genes were identified to be significantly involved in a set of GO terms including muscle organ development, negative regulation of cell proliferation and cell adhesion (Table I).

According to KEGG pathway enrichment analysis, upregulated genes were significantly associated with cell cycle and oocyte meiosis signaling pathways (Table II). Downregulated DEGs were principally implicated in vascular smooth muscle and focal adhesion signaling pathways (Table II).

PPI networks construction and MCODE analysis. To investigate interactions between the DEGs, PPI networks for the DEGs were constructed. There were 69 nodes and 180 edges in the PPI network of the upregulated genes (Fig. 2A). According to the degrees of nodes, four genes were selected as the hub nodes of the PPI network, cell division cycle associated 8 (*CDCA8*), cell division cycle associated 5 (*CDCA5*, ubiquitin-conjugating enzyme E2C (*UBE2C*) and thymidine kinase 1 (*TK1*). These four DEGs interacted with >45 nodes in the PPI network, suggesting the four DEGs served crucial roles in the PPI network. One sub-network was selected from the upregulated PPI network (network aggregation score, 19.366), containing 41 nodes and 794 edges (Fig. 2B). Enrichment analysis of genes in the sub-network revealed that genes in the sub-network were primarily associated with cell cycle and cell division (Tables III and IV). Furthermore, 257 nodes and 594 edges were included in the PPI network of the downregulated genes (Fig. 3). However, no significant module was screened with the threshold of network aggregation score >10.

Construction of the transcriptional regulatory networks. As an important regulatory element, TFs regulate the expression of certain genes (34). In the present study, 14 upregulated genes were regulated by three upregulated TFs, and 10 genes [e.g. cell division cycle 6 (*CDC6*) and RAD51 recombinase (*RAD51*)] were regulated by E2F transcription factor 2 (E2F2) (Fig. 4A). Furthermore, six TFs were predicted to target the downregulated DEGs. Notably, early growth response 1 (EGR1) regulated a number of downregulated genes and one TF, NK3 homeobox 1, in the downregulated transcriptional regulatory network (Fig. 4B).

Discussion

Prostate cancer is the most common type of reproductive system cancer in males (1,2), particularly in men over 65 years of age. In the present study, analysis of GSE38241 and GSE3933 gene expression profiles identified a total of

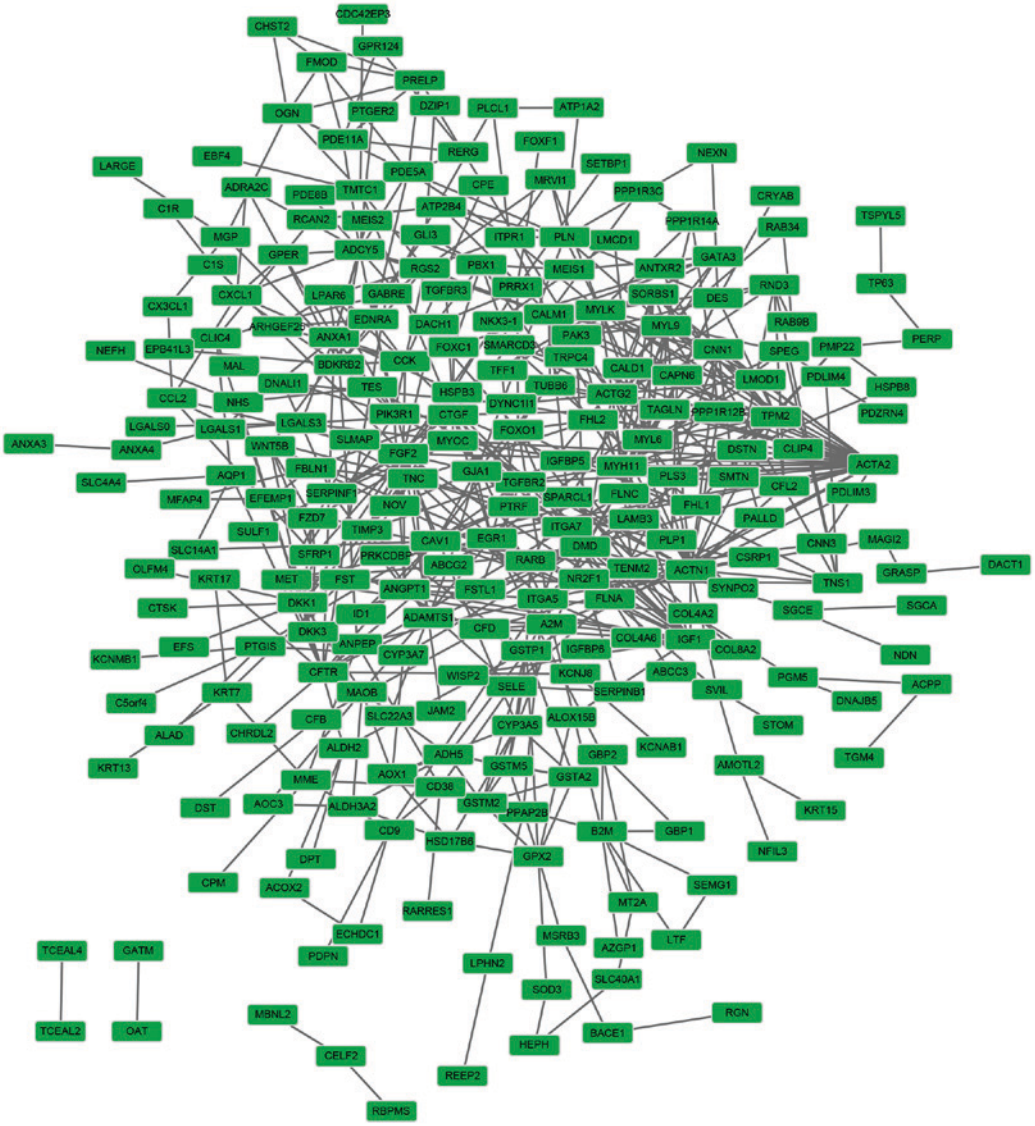

Figure 3. Protein-protein interaction network of downregulated genes.

529 DEGs (129 up- and 400 downregulated DEGs) between the prostate cancer and normal samples. Integrative analysis of two microarray data enhanced the reliability of the present study. Enrichment analysis of the upregulated genes predicted the cell cycle to be the primary biological process in the GO function and the KEGG pathway analyses. In addition, focal adhesion pathway was identified as a significant pathway of downregulated genes. The results of the present study were consistent with those of previous studies, which demonstrated that the cell cycle and focal adhesion are required for the progression of cancer (35,36).

A total of four genes, consisting of *CDCA8*, *CDCA5*, *UBE2C* and *TK1*, exhibited a high degree of interaction in the upregulated PPI network. All four genes were involved in cell cycle-associated biological processes and signaling pathways. In the sub-network, *CDCA8* interacted with pituitary tumor-transforming gene-1 (*PTTG1*), which was upregulated in prostate cancer. There is evidence that knockdown of *PTTG1* suppresses the proliferation and invasive potential of prostate cancer cells (37). Therefore, it was hypothesized that *CDCA8* may be used as a target for prostate cancer treatment, and that the interaction between *CDCA8* and *PTTG1* may have a role in the progression of prostate cancer. In addition, *UBE2C* belongs to the ubiquitin-conjugating enzyme family and participates in the process of cell mitosis (38). A previous study identified that *UBE2C*, as an androgen receptor target gene, was involved in the progression of prostate cancer (39). Furthermore, serological *TK1* protein concentration was used as a reliable marker for the risk assessment of pre/early cancerous progression (40). However, to the best of our knowledge, there is no evidence that *TK1* is a target for cancer treatment. It was hypothesized that *CDCA8*, *CDCA5*, *UBE2C* and *TK1* may be associated with the progression of prostate cancer, and these genes were expected to be used as potential treatment targets for prostate cancer. Limited information is known about the roles and underlying molecular mechanisms of these four genes in prostate cancer; therefore, the present study may provide novel insights into the study of treatment targets for prostate cancer.

In addition to the hub genes in the PPI network, TFs targeting DEGs were identified on the basis of the transcriptional regulatory network analysis. TFs are well known to regulate the

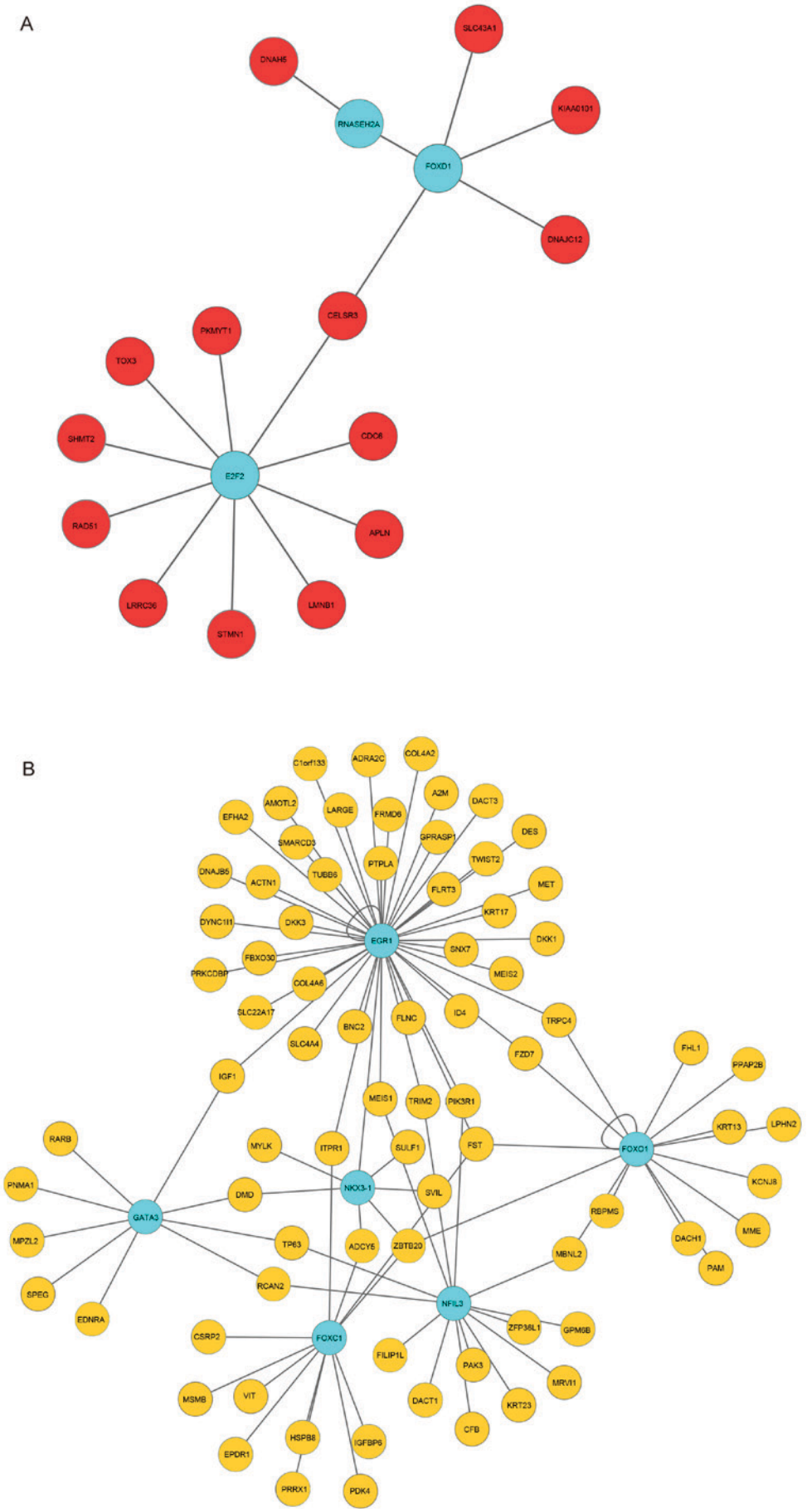

Figure 4. Transcriptional regulatory networks. (A) Transcriptional regulatory network of the upregulated genes. Blue nodes represent transcription factors of the upregulated genes and red nodes represent upregulated genes. (B) Transcriptional regulatory network of the down-regulated genes. Blue nodes represent transcription factors of the downregulated genes and yellow nodes represent downregulated genes.

transcription of a number of genes involved in distinct signaling pathways and biological processes (34). In the present study, the upregulated TF, E2F2, and the downregulated TF EGR1 regulated a number of DEGs. E2F2 regulates genes by binding the target sequence 5'-TTTSSCGC-3' (S=C/G) (41). In the transcriptional regulatory network, DEGs, including *CDC6* and *RAD51*, which contain the aforementioned sequence, may be bound by E2F2 (42). CDC6 is a protein that is required for the initiation of DNA replication and has been previously identified to be deregulated in prostate cancer (43). RAD51, a protein that catalyzes DNA repair via homologous recombination, is highly expressed in cancer cells (44). Additionally, overexpression of E2F2 leads to uncontrolled proliferation of ovarian cancer cells (45) and EGR1 regulates gene expression by binding the target sequence 5'-GCGC(G/T)GGGCG-3' (46). The downregulated gene Dickkopf WNT Signaling Pathway Inhibitor 3 (*DKK3*) contained this sequence and was predicted to be regulated by EGR1. *DKK3* promotes the proliferation and differentiation of fibroblasts and has a function in the pathogenic stromal remodeling of prostate cancer (47). Therefore, the TFs, E2F2 and EGR1, may have marked roles in the progression of prostate cancer.

The cell cycle signaling pathway may be closely associated with prostate cancer. A total of four genes (*CDCA8*, *CDCA5*, *UBE2C* and *TK1*) and two TFs (E2F2 and EGR1) were selected, and may have important roles in the progression of prostate cancer. The selected DEGs and TFs may be used as target genes for the treatment of prostate cancer and, although they were identified using bioinformatics, the specific roles and underlying molecular mechanisms in prostate cancer require further confirmation.

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