GRB2-associated binding protein 2 regulates multiple pathways associated with the development of prostate cancer

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Abstract. The development of prostate cancer is complicated and involves a number of tumor-associated gene expression level abnormalities. Gene chip technology is a high-throughput method that can detect gene expression levels in different tissues and cells on a large scale. In the present study, gene chip technology was used to screen differentially expressed genes in PC-3 human prostate cancer cells following GRB-associated binding protein 2 (GAB2) gene knockdown, and the corresponding biological information was analyzed to investigate the role of *GAB2* in prostate cancer. The PC-3 human prostate cancer cell *GAB2* gene was knocked out and gene chip hybridization and bioinformatics methods were used to analyze the classical pathway and predict upstream regulatory molecules, disease and function associations and genetic interaction networks. According to the screening conditions |fold change|>1 and P<0.05, 1,242 differential genes were screened; 665 genes were upregulated, and 577 genes were downregulated. Ingenuity Pathway Analysis software demonstrated that *GAB2* regulates pathways, such as the superpathway of cholesterol biosynthesis and p53 signaling in cells, and serves a role in diseases and functions such as 'non-melanoma solid tumors', 'viral infections' and 'morbidity or mortality'. In the occurrence and development of prostate cancer, factors such as the activation of genes involved in the proliferative cycle, abnormalities in metabolism-associated enzyme gene activities and viral infection play key roles. The present study provides novel research directions and therapeutic targets for prostate cancer.

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Key words: prostate cancer, GRB-associated binding protein 2, gene chip technology, signaling pathway

Introduction

Prostate cancer (PCa) is a common malignant tumor in the male genitourinary system, and its incidence rate is the second highest among all types of tumor in men, following only lung cancer. There are approximately 1,276,106 new cases and 358,989 deaths worldwide annually (1). With the aging population, changes in dietary structure and widespread implementation of prostatic-specific antigen screening, the incidence rate of PCa in China has increased from 35.2/100,000 in 1998 to 5,300/100,000 in 2012 (2,3). For early or localized PCa, close observation, radiotherapy and radical prostatectomy (open prostatectomy, laparoscopic or robotic-assisted laparoscopy) are effective treatment options. For advanced PCa, androgen deprivation therapy (ADT) is one of the most commonly used treatments. However, the average effective duration of ADT treatment is 12-18 months. The majority of patients develop resistance, progress to castration-resistant PCa (CRPC), and succumb due to PCa (4,5). Furthermore, the median survival time of patients with metastatic CRPC (mCRPC) is <2 years (6). Consequently, there is an urgent need for more effective alternative or supplemental treatment options. The development of biotechnology provides more opportunities for investigating novel potential targets for gene-targeted PCa treatment.

The GRB-associated binding protein 2 (GAB2) scaffold protein is a member of the Gab family and is encoded by the *GAB2* (11q14.1) gene (7). *GAB2* serves a role in a number of physiological processes, such as cell proliferation, differentiation, apoptosis and migration, by binding to multiple receptors and participating in numerous signal transduction pathways, such as the PI3K/AKT, SHP2/ERK and JAK/STAT pathways (8,9). Previous studies have reported that the GAB2 protein is abnormally expressed in numerous types of malignant tumor, including glioma, leukemia and melanoma, as well as ovarian, breast and lung cancer (10-13). Abnormal GAB2 signaling is associated with tumor progression, indicating that *GAB2* may be a novel oncogene (14). In the present study, the Oncomine and Gene Expression Omnibus (GEO) databases were searched for PCa gene expression level analyses; the expression level of the *GAB2* gene was higher in PCa tissues than in benign prostatic hyperplasia tissues. Thus, it was

hypothesized that the *GAB2* gene may possess a regulatory effect on the progress of PCa, but its specific function and mechanism have not yet been elucidated.

In the present study, the *GAB2* gene was knocked down in PCa cells using small interfering (si)RNA, and the differentially expressed genes in the GAB2 knockdown cells were screened via gene chip technology. The aim of the present study was to investigate the downstream gene expression levels and signaling associated with GAB2 and to provide theoretical and experimental evidence for novel therapeutic targets for PCa.

Materials and methods

Data download and analysis. The GSE55945 chip data set was downloaded from the GEO database (ncbi.nlm.nih.gov/geo/). The chip data set is based on the GPL570 platform. In total, 21 samples were obtained including 13 cases of PCa and 8 cases of benign prostatic hyperplasia. The GEO2R online analysis website (ncbi.nlm.nih.gov/geo/geo2r/) was used to process GSE55945 gene chips in the GEO database to compare differences in the expression levels of *GAB2*. The Oncomine database (oncomine.org/) was used to analyze *GAB2* expression levels in prostate adenocarcinoma and acinar prostate adenocarcinoma.

Gene chip. The gene chip used in the present study was the GeneChip™ PrimeView™ Human Gene Expression Array (cat. no. 901838; Affymetrix; Thermo Fisher Scientific, Inc.) and was provided by Shanghai Genechem Co., Ltd. This single GeneChip array can yield data for >530,000 probes and 36,000 transcripts and variants, which represent more than 20,000 genes mapped via UniGene or RefSeq annotation. Sequences for the array design were selected from the UniGene database 219 https://www.ncbi.nlm.nih.gov/unigene (constructed 30 March 2009), RefSeq 36th edition https://www.ncbi.nlm.nih.gov/refseq/ (13 July 2009) and full-length human mRNA from GenBank™ http://www.ncbi.nlm.nih.gov/genbank (downloaded 12 May 2009).

Cell culture and small interfering (si)RNA knockdown experiments. PC-3 human PCa cells from the Cell Bank of Xi'an Jiaotong University were cultured in medium containing 10% fetal bovine serum (Hyclone Laboratories; GE Healthcare Life Sciences), 1% streptomycin mixture and ~90% RPMI-1640 complete medium (Gibco; Thermo Fisher Scientific Inc.). The cells were cultured at a constant temperature of 37˚C with 95% O_2, and 5% CO_2 in an incubator (relative humidity, ~95%). Adherent cells were transfected with *GAB2* siRNA as follows: The commercial LV3-*GAB2* lentivirus vector (containing green fluorescent protein and the puromycin sequence) and *GAB2* siRNA sequences were constructed by Shanghai GenePharma Co., Ltd. The sequence of *GAB2* siRNA was 5'-GGGACCTCCTGGTAGACAATA-3', and the sequence of scrambled fluorescein-labeled negative control siRNA was: 5'-TTCTCCGAACGTGTCACGT-3'. When cells reached 40-50% confluence in the presence of 8 μg/ml polypropylene, lentiviral vectors were used to infect PC-3 cells at 50 multiplicity of infection for 72 h. The stable *GAB2* low expression level subclones were maintained via 2-3 μg/ml puromycin-resistant culturing (puromycin, Sigma-Aldrich; Merck KGaA). PC-3 human PCa cells transfected with negative control (NC) siRNA were used as the NC group, and cells transfected with *GAB2* siRNA were used as the knockdown (KD) group. Three samples in each group were subjected to gene chip analysis.

Total RNA isolation and RT-qPCR. Total RNA was isolated via TRIzol extraction (cat. no. 3101-100; Shanghai Pufei Biotechnology Co., Ltd.) according to the manufacturer's instructions. First strand cDNA was synthesized using M-MLV reverse transcriptase (cat. no. M1701; Promega Corporation) and analyzed via qPCR using SYBR Ex Taq™ II (Takara Bio, Inc.). Gene-specific primers were designed and synthesized by Shanghai Genechem Co., Ltd. and their sequences were as follows: *GAB2* (forward primer: 5'-AGACCGCCAATCAGT GAAAAT-3', reverse primer: 5'-GGTGAAGTCGGCTGT TGTC-3', 91bp) and GAPDH (forward primer: 5'-TGACTT CAACAGCGACACCCA-3', reverse primer: 5'-CACCCTGTT GCTGTAGCCAAA-3', 121bp). Real-time chain reactions were performed using an iQ5TM Multicolor Real-Time PCR and Detection System (Bio-Rad Laboratories, Inc.). Amplification conditions used were 95˚C for 5 min followed by 30 cycles of 94˚C for 30 sec, 55˚C for 30 sec and 72˚C for 35 sec, and finally 72˚C for 3 min. Relative quantitative analysis was performed according to the following equation: $F=2^{-\Delta\Delta Cq}$, where $2^{-\Delta\Delta Cq}$ is the expression level of the target gene for each KD group sample (PC-3 human PCa cells with *GAB2* gene knockdown) relative to that of each NC group sample (PC-3 human PCa cells) (15).

Chip hybridization. Chip hybridization was performed using an Affymetrix (Thermo Fisher Scientific, Inc.) expression profile chip and GeneChip Hybridization Wash and Stain kit (cat. no. 900720; Affymetrix; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Following hybridization at 45˚C for 16 h at 20 x g in a GeneChip Hybridization Oven 645 (Affymetrix; Thermo Fisher Scientific, Inc.), the hybridized chips were eluted and stained using a GeneChip Fluidics Station 450 (Affymetrix; Thermo Fisher Scientific, Inc.).

Chip scanning and detection analysis. Chip hybridization results were scanned using a GeneChip Scanner 3000 (cat. no. 00-0210; Affymetrix; Thermo Fisher Scientific, Inc.), and the raw data were read using Command Console software (version 4.0; Affymetrix; Thermo Fisher Scientific, Inc.). The quality control data were analyzed using R software (version 3.6.0) (16). The package was normalized, and the algorithm used was MAS 5.0 (Affymetrix; Thermo Fisher Scientific, Inc.).

Chip data bioinformatics analysis. The association between *GAB2* gene expression levels and PCa occurrence was found by mining the gene literature (17,18). The IPA online integration analysis software was used to analyze differentially expressed genes in PCa cells before and after *GAB2* gene knockdown. IPA is an all-in-one online integrated analysis software (www.ingenuity.com) which helps to understand data from gene expression data, microRNA data and small-scale experimental data. IPA establishes a visual experimental system

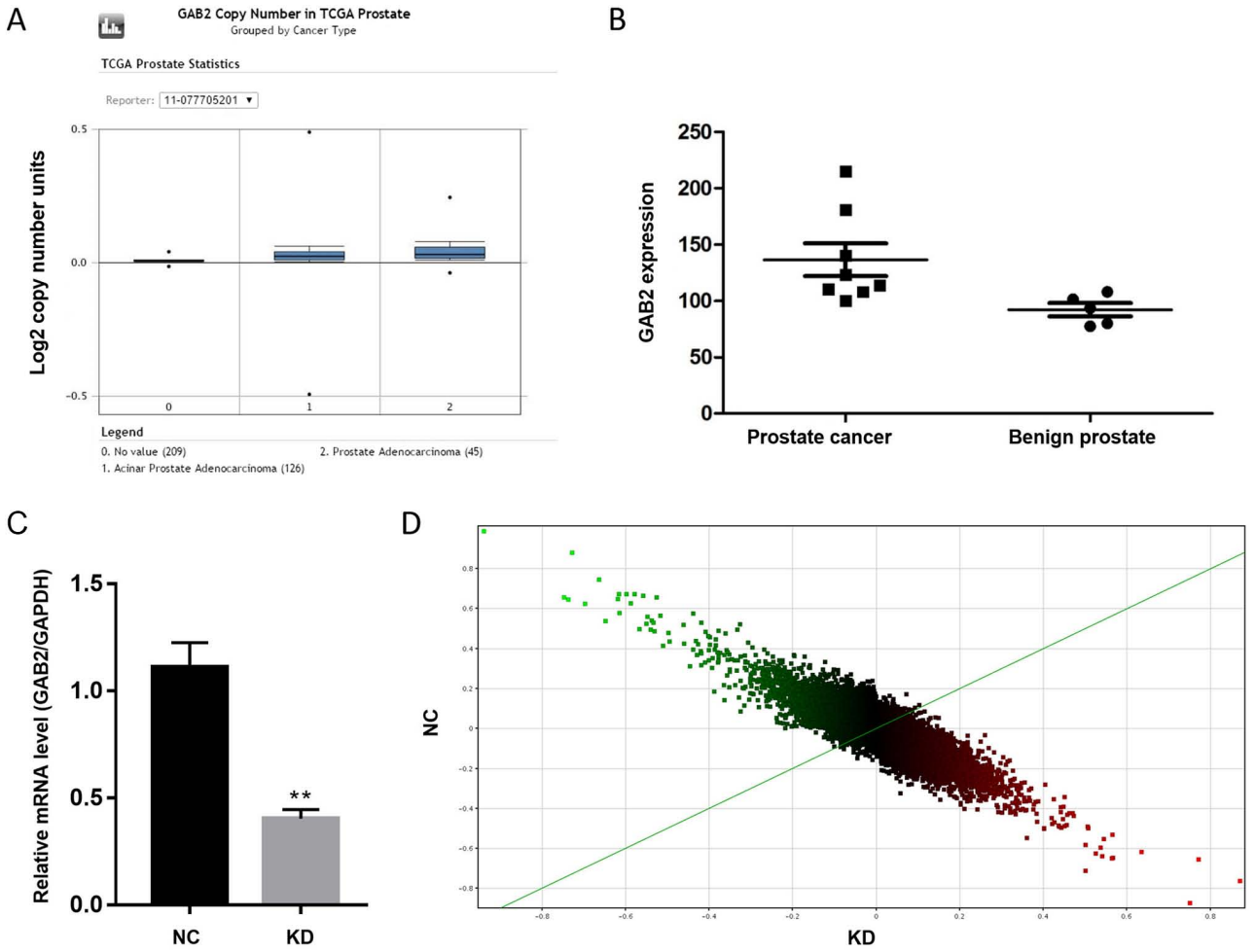

Figure 1. GAB2 expression is associated with PCa. Expression levels of the *GAB2* gene in PCa tissues and benign prostatic hyperplasia tissues from the (A) Oncomine and (B) Gene Expression Omnibus databases. (C) Relative mRNA expression level of GAB2 (GAB2/GAPDH) in the NC and KD group. (D) Scatter plot. The ordinate and abscissa value of each point in the figure represent the expression level value of a probe in the experimental and control groups. **P<0.01 vs. control. GAB2, GRB-associated binding protein 2; NC, negative control; KD, knockdown; PCa, prostrate cancer.

to understand the properties of various molecules in genes, proteins, chemicals, drugs and their interaction networks.

Statistical analysis. Data are expressed as the mean ± SD of three replicate samples. Except for the IPA, all statistical analyses were performed using SPSS software (version 22.0; IBM Corp.). Between-group differences were compared using an unpaired t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Significant differences in gene expression levels in PCa cells following GAB2 knockdown. The Oncomine and GEO databases were searched for PCa gene expression level data, which demonstrated that the expression level of the *GAB2* gene was higher in PCa tissues than in benign prostatic hyperplasia tissues (Fig. 1A and B). These results indicate that *GAB2* may be an oncogene of PCa and possess regulatory effects on tumor development. The *GAB2* gene was knocked down in PC-3 human PCa cells and gene chip technology was used to compare gene expression levels. *GAB2* gene knockdown efficiency in the KD group was 60.7%, compared with *GAB2* gene expression levels in the NC group (Fig. 1C). The scatter plot demonstrates the distribution of signal values between the experimental and control groups on the plane of the rectangular coordinate system. The ordinate and abscissa values of each point represent the expression level value of a probe in the experimental and control groups, respectively. The upper part of the green line indicates KD is downregulated relative to NC; the lower part of the green line indicates KD is upregulated relative to NC (Fig. 1D). The red dots on the volcano map indicate genes with fold difference >1 and significance level <0.05 (Fig. 2A). The points to the left of X=-1 and to the right of X=1 are genes with differences >2-fold. The majority of gene expression level changes are notably different and differ >2-fold (Fig. 2A). The heat map shows the expression levels of genes in each sample (Fig. 2B). Green indicates a relatively decreased signal value; black indicates a moderate signal value; gray indicates that the signal value was not detected. In the tree structure, two adjacent samples or genes have higher similarity. The expression profiles of the KD and NC groups are notably different (Fig. 2B). The results demonstrated significant differences in 1,242 genes; 665 genes in the KD group were upregulated, and 577 were downregulated compared with the NC group.

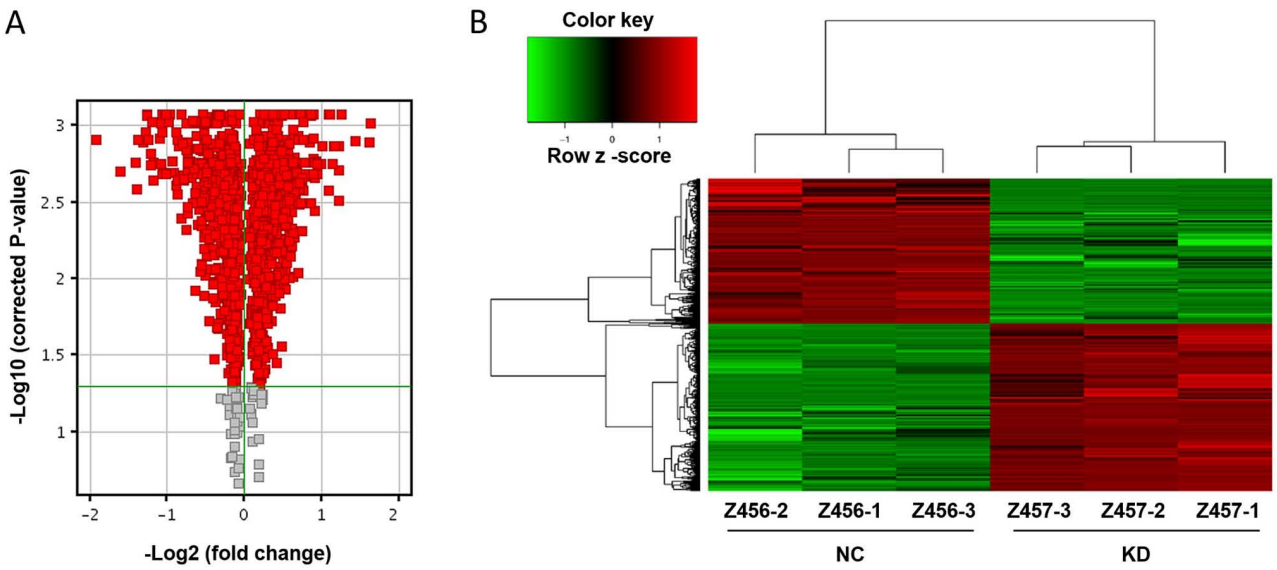

Figure 2. Changes in gene expression of prostate cancer cells after knocking down GAB2. (A) Volcano map shows the difference in gene distribution between the experimental group and the control group. (B) Heatmap shows the distribution of the signal values between the experimental group and the control group on the rectangular coordinate system plane. Data are presented as the mean ± SD of 3 independent experiments. NC, negative control; KD, knockdown; GAB2, GRB-associated binding protein 2.

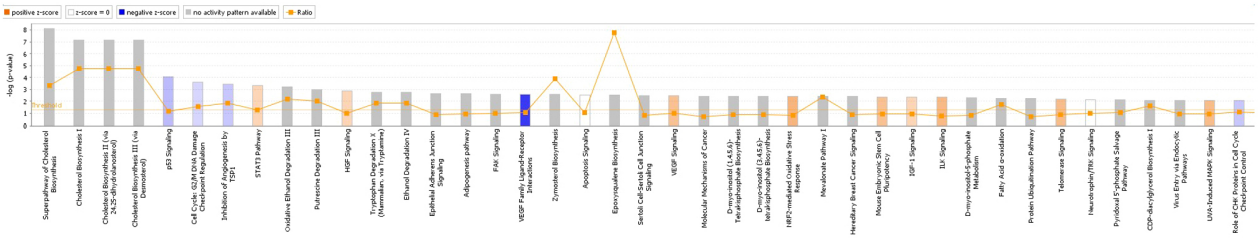

Figure 3. Signaling pathway histograms. Orange indicates Z-score>0; blue indicates Z-score<0. The ratio is the number of differential genes compared with the total number of genes in the signaling pathway.

Classical pathway and upstream regulation analysis. IPA online integration analysis software was used to analyze the differential gene list and the enrichment of the gene sets contained in each pathway. p53 signaling was inhibited after knocking down *GAB2* (Z-score=-0.535), and *GAB2* was involved in regulating the superpathway of cholesterol biosynthesis (Fig. 3). The behavior of each molecule in the p53 signaling pathway in the present study is shown in Fig. 4. Green represents a decrease; red represents an increase; color intensity is associated with the change magnification. Magenta indicates molecules on the differential gene list. Fig. 5 shows the performance of each gene when the pathway is activated, according to the analysis of IPA commercial software (www.ingenuity.com). In the present study, DNA-dependent protein kinase (DNA-PK), checkpoint kinase 1 (Chk1), AKT, thrombospondin 1 (TSP1) and death receptor (DR) 4/5 were abnormally expressed compared with previously reported results, indicating that these molecules may participate in tumorigenesis via novel regulatory pathways (Figs. 4 and 5). The shape and color of the molecule, and the interaction relationship between the molecules are shown in Figures S1-4. Next, activation or inhibition of upstream regulators, including transcription factors, cytokines, small RNAs, receptors, kinases, chemical molecules and drugs, was predicted for all differential genes. Results of the upstream regulatory factor analysis are presented in Table SI. In the present study, insulin-induced gene 1 (INSIG1) was predicted to be suppressed following *GAB2* knockdown, and there were 18 genes that were consistently suppressed and are related to this gene. The interaction between INSIG1 and its directly associated downstream molecules is shown in Fig. 6. Only the expression level states of INSIG1 and C-X-C motif chemokine ligand 6 (CXCL6) were inconsistent with previously reported results. INSIG1 can inhibit CXCL6 at the mRNA level; in the present study, however, CXCL6 was significantly downregulated when INSIG1 was inhibited.

Disease function and regulation effect analysis. Up- and downregulation of differential gene expression levels are associated with activation or inhibition of functions and diseases. IPA software was used to analyze the changes of 500 functions (Table SII). Z-score >2 means that the function is significantly activated, and Z-score <-2 means that the function is significantly inhibited. A total of 24 functions changed significantly, including 'viral infection' (Z-score=3.176) and 'infection of tumor cell lines' (Z-score=2.934). The functions with significant inhibition were 'morbidity or mortality' (Z-score=-2.906) and 'organismal death' (Z-score=-2.879) (Fig. 7). The regulatory

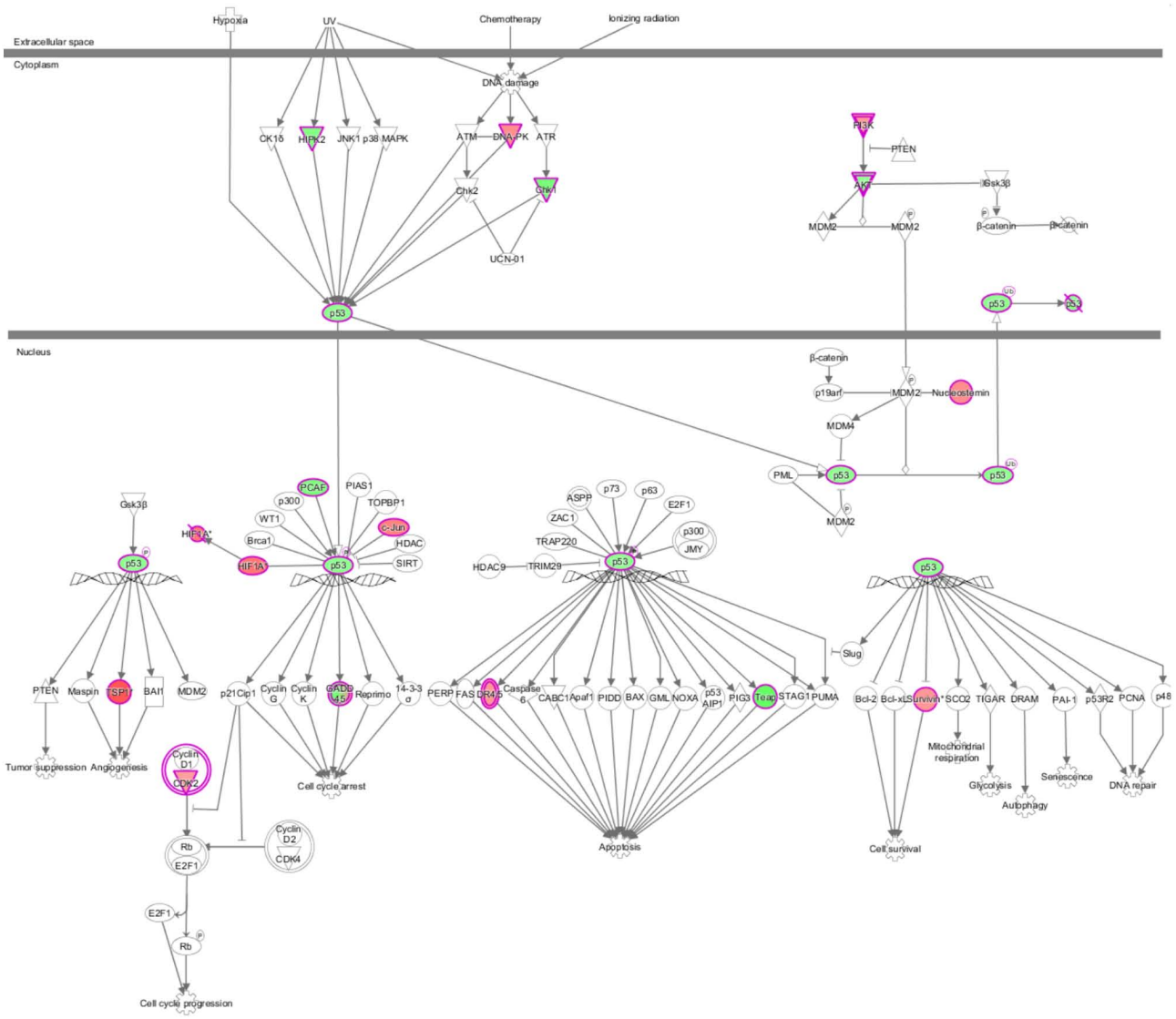

Figure 4. Signaling pathway diagram. Pathway enrichment analysis of differential genes was generated via Ingenuity Pathway Analysis commercial database software.

effect network map shows the interaction between genes and regulators and functions in the data set. In the present study, mouse double minute 2 homolog, methyl-CpG-binding protein 2 and SAM pointed domain containing ETS transcription factor possessed an activating effect on advanced malignant tumor, melanoma cell line viability and muscle cell proliferation, as well as an inhibitory effect on cancer cell death via β-actin, survivin, dipeptidyl peptidase 4, hypoxia inducible factor 1α, homeodomain-interacting protein kinase 2, immunoglobulin heavy constant μ, interleukin-1 receptor-associated kinase 1, lysine acetyltransferase 2B, prostate specific antigen, protein tyrosine phosphatase receptor type F, syndecan 1, SMAD4, thrombospondin 1, TP53, vascular endothelial growth factor A, vimentin and other genes (Fig. 8).

Interaction network analysis. The IPA network generation algorithm was used to split the network map between molecules into multiple networks and to score each network. All networks were sorted by score value. Fig. 9 shows the genetic interaction network map: KCNMA1 (potassium calcium-activated channel subfamily M α 1), cell division cycle 73, nardilysin convertase, insulin-like growth factor 1 and dynein cytoplasmic 1 light intermediate chain 1 are the primary genetic interaction nodes. The interaction network primarily affects 'cellular assembly and organization', 'cellular function and maintenance' and 'connective tissue disorders'.

Discussion

The progression of PCa is complex; abnormal expression levels of a number of tumor-associated genes or the inactivation of numerous tumor suppressor genes may lead to the occurrence and progression of PCa (19). Therefore, by studying the genes and genomic changes related to PCa, new ideas for clinical prevention and treatment of PCa may be found. Analysis of the Oncomine and GEO databases demonstrated that the expression level of the *GAB2* gene was higher in PCa tissues than in benign prostatic hyperplasia tissues, indicating that the *GAB2* gene may possess a regulatory effect on the progression of PCa. Gene chip technology allows rapid, efficient and

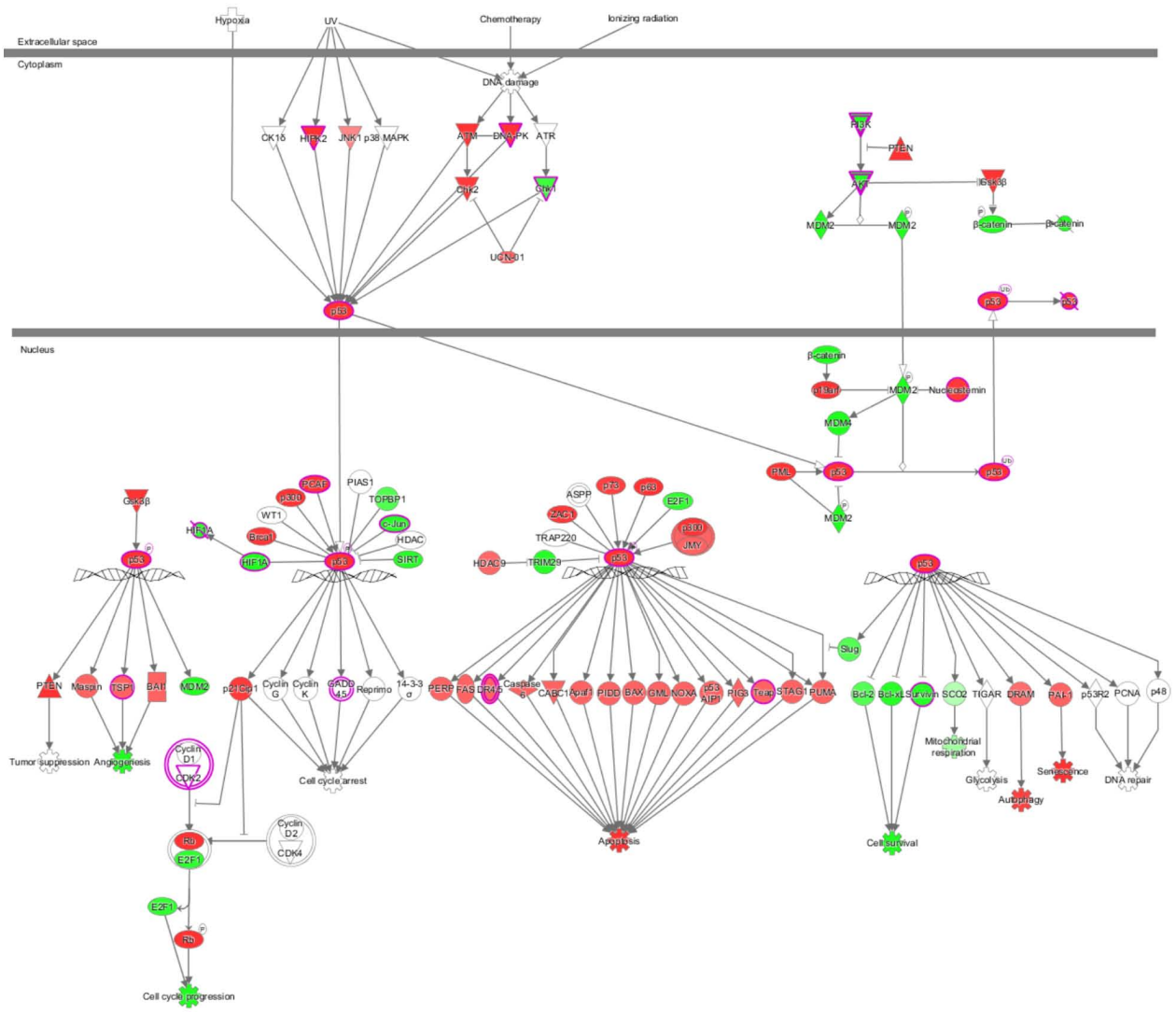

Figure 5. Signaling pathway diagram for activation or suppression states supported by literature. The diagram was generated using Ingenuity Pathway Analysis commercial database software.

parallel detection of large-scale gene expression levels, which facilitates improved understanding of the mechanism of the *GAB2* gene in the development and progression of PCa (20). The present study detected significant differences in the performance of 1,242 genes between the KD and NC groups; 665 genes were upregulated, and 577 were downregulated. These results indicated that the *GAB2* gene serves a regulatory role in PCa that is mediated via a number of pathways acting on numerous genes.

After determining the differential gene expression levels in the KD and NC groups, the differential gene results of the chip analysis, including probe number and fold-change were introduced into the IPA online integration analysis tool. The software further analyzed the molecular pathways activated or inhibited by *GAB2* gene knockdown to predict upstream regulatory molecules, analyze the association between disease and function, and determine the network of gene interactions in order to provide novel insights into the progression of PCa. Previous findings have demonstrated that activation of the p53 signaling pathway leads to cell cycle inhibition and apoptosis in PCa cells (19,20). Singh *et al* (21), reported that targeting the p53 pathway can inhibit PCa cell proliferation and metastasis (22,23). The present study demonstrated that *GAB2* gene expression levels are upregulated in PCa cells, but the association between *GAB2* and the p53 signaling pathway has not yet been reported. In the present study, p53 signaling in PCa cells was suppressed following *GAB2* knockdown, but DNA-PK was abnormally expressed in the p53 signaling pathway. By contrast, Dylgjeri *et al* (24) demonstrated that pharmacological targeting of DNA-PK inhibits tumor growth both *in vitro* and *in vivo*. This difference may be because the complex molecular networks within tumor cells have not yet been elucidated. In addition to DNA-PK, Chk1, AKT, TSP1, and DR4/5 were also abnormally expressed following *GAB2* knockdown; the involvement of these molecules in PCa should be further investigated.

Yang *et al* (22) reported that INSIG1 serves a key role in cholesterol homeostasis. Similarly, in the present classical pathway analysis, *GAB2* was demonstrated to be involved in regulating the superpathway of cholesterol biosynthesis, and INSIG1 was predicted to be strongly suppressed (determined via upstream regulatory analysis). Previous studies have shown

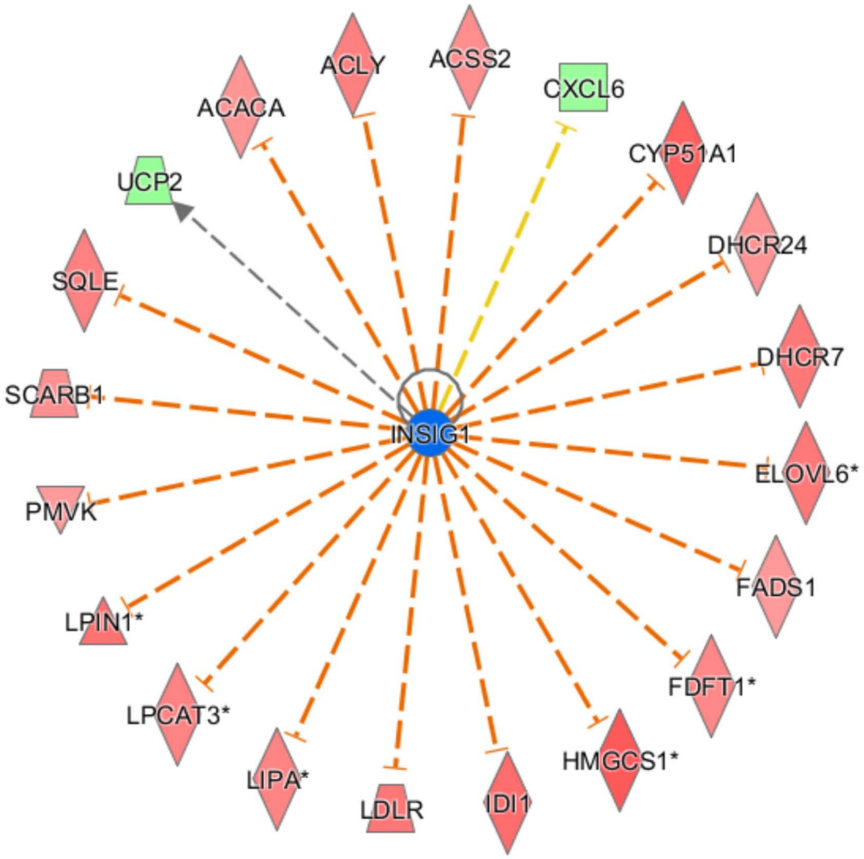

Figure 6. Upstream regulatory factor network map. Orange line indicates consistent activated expression level state between the upstream regulator and the gene; yellow line indicates inconsistent expression level state; gray line indicates there is no predictive information associated with the expression level state in the data set. INSIG1, insulin induced gene 1; ACACA, acetyl-CoA carboxylase α; ACLY, ATP citrate lyase; ACSS2, acyl-CoA synthetase short chain family member 2; CXCL6, C-X-C motif chemokine ligand 6; CYP51A1, cytochrome P450 family 51 subfamily A member 1; DHCR24, 24-dehydrocholesterol reductase; DHCR7, 7-dehydrocholesterol reductase; ELOVL6, ELOVL fatty acid elongase 6; FADS1, fatty acid desaturase 1; FDFT1, farnesyl-diphosphate farnesyltransferase 1; HMGCS1, 3-hydroxy-3-methylglutaryl-CoA synthase 1; IDI1, isopentenyl-diphosphate Δ isomerase 1; LDLR, low density lipoprotein receptor; LIPA, lipase A; LPCAT3, lysophosphatidylcholine acyltransferase 3; LPIN1, lipin 1; PMVK, phosphomevalonate kinase; SCARB1, scavenger receptor class B member 1; SQLE, squalene epoxidase; UCP2, uncoupling protein 2.

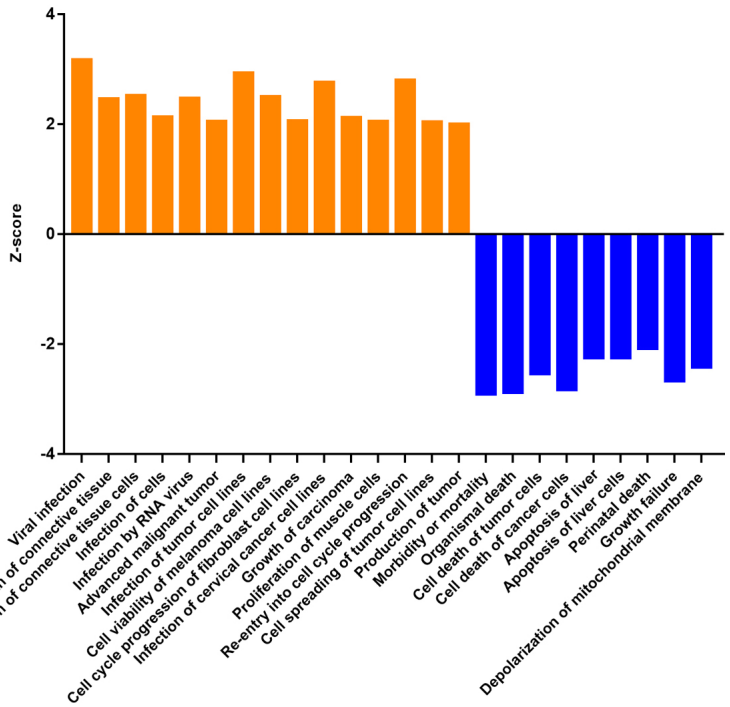

Figure 7. Histogram of disease and function demonstrates the activation and inhibition of each function: 24 functions changed significantly (Z-score>2).

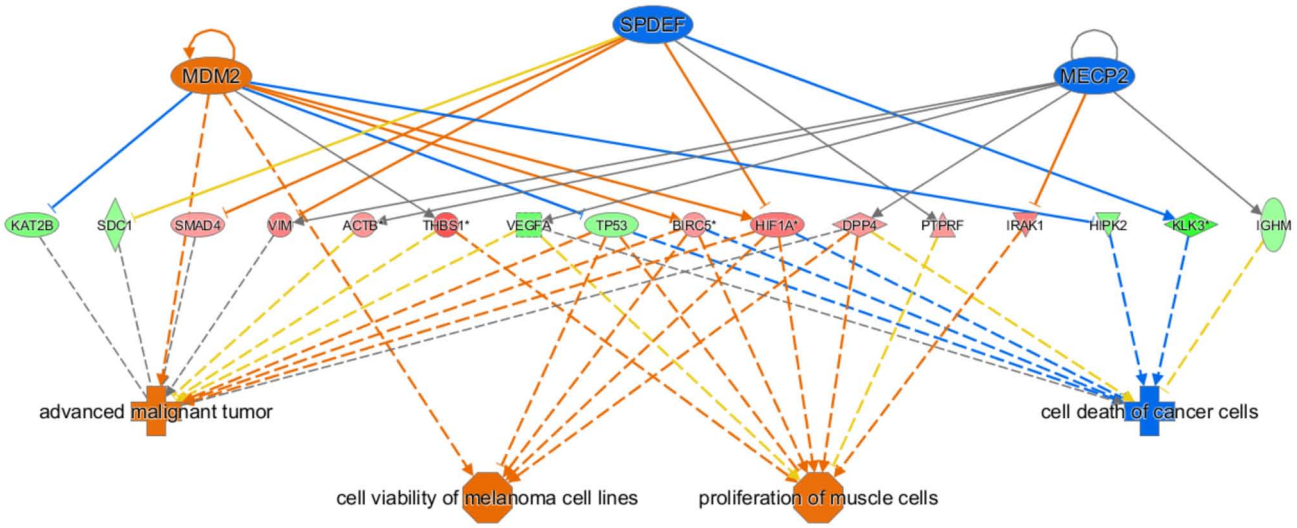

Figure 8. Regulation effect network diagram.

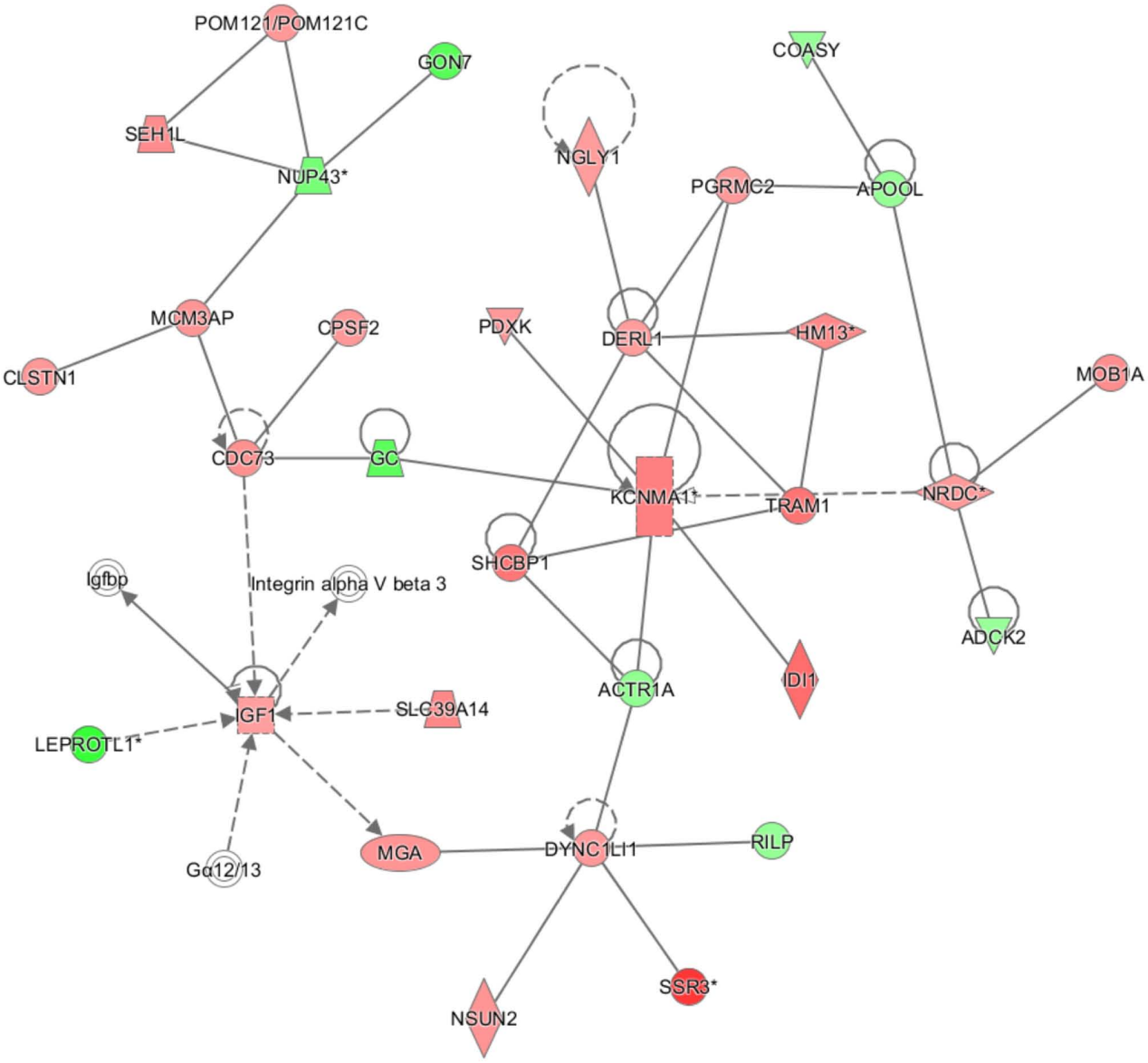

Figure 9. Gene interaction network diagram shows the interaction among KCNMA1, CDC73, IGF1, DYNC1LI1 and molecules related to them, which mainly affect cellular assembly and organization, cellular function and maintenance and are involved in connective tissue disorders. Genes, proteins, and chemicals are represented by different shapes.

that PCa risk factors include chronic inflammation of the prostate (22,25,26) and sexually transmitted infections (27-32). The present study demonstrated that viral infection pathways are significantly activated. These results indicated that PCa may be caused by viral infections that increase the expression level of *GAB2*. Regulatory effects and interaction network analyses have indicated that numerous genes affect tumorigenesis and progression; a number of pathways are involved, such as 'advanced malignant tumors', 'melanoma cell line viability', 'muscle cell proliferation', 'cancer cell death', 'cellular assembly and organization', 'cellular function and maintenance' and 'connective tissue disorders'.

In the present study, the *GAB2* gene was knocked down in PCa cells. Gene chip technology and bioinformatics analyses were then used to investigate the signaling pathways, disease and function, and gene interaction networks associated with *GAB2*. The aforementioned experimental and bioinformatic analysis results demonstrated that *GAB2* regulates pathways such as the superpathway of cholesterol biosynthesis and p53 signaling in cells and serves a role in diseases and functions, such as 'non-melanoma solid tumors', 'viral infections' and 'morbidity or mortality'.

The present study has some limitations, such as the lack of animal experiments and human studies. Therefore, it is not clear whether the GAB2 gene has the same function *in vivo* and whether it can be used as a therapeutic target for patients with PCa. The purpose of the present study was to analyze and summarize the changes of associated pathways following knockdown of the *GAB2* gene, and to provide a novel perspective for the prevention and treatment of PCa by identifying potential research directions and therapeutic targets.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YD and XQ conceived and designed the experiments. YD, XQ, XZ, LM and JT performed the experiments. XQ analyzed the data and wrote the manuscript. All authors have read and approved the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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