Screening and identification of key biomarkers in prostate cancer using bioinformatics

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Abstract. Prostate cancer (PCa) is the second most common cancer amongst males worldwide. In the current study, microarray datasets GSE3325 and GSE6919 from the Gene Expression Omnibus database were screened to identify candidate genes that are associated with the progression of PCa. A total of 273 differentially expressed genes (DEGs) were identified, which included 173 downregulated genes and 100 upregulated genes, and a protein-protein interaction network was constructed using Search Tool for the Retired of Interacting Genes. The enriched functions and pathways of the identified DEGs included cell adhesion, the negative regulation of cell proliferation, protein binding and focal adhesion. A total of 8 hub genes were identified, of which PDZ binding kinase, Krüppel-like factor 4, collagen type XII α-1 chain, RAP1A and RAP39B were indicated to be associated with the progression and recurrence of PCa. In conclusion, the DEGs and hub genes identified in the present study may aid in determining the molecular mechanisms associated with PCa carcinogenesis and progression.

Introduction

Prostate cancer (PCa) is the second most common type of cancer among men globally, and constitutes ~15% of all cancer diagnoses worldwide (1). Digital rectal examination, measurement of the serum level of prostate specific antigen (PSA) and biopsy from a prostate transrectal ultrasonography are the most common diagnostic tools for PCa (2). Additionally, with advances in genetic analysis, alterations have been identified in a number of gene regions in patients with PCa, including prostate antigen 3, androgen-dependent transmembrane serine 2 and S-transferase P1 (3-5). However, genetic analysis exhibits a low specificity and can increase the number of unnecessary biopsies performed without reducing patient mortality (6). Previous studies have associated the tribbles pseudokinase 1 gene with the development of a number of tumors, including colorectal leukemia and hepatocellular cancers (7-9). It has been shown that transmembrane protease, serine 2:ETS-related gene (TMPRSS2:ERG) fusion is associated with diagnosing PCa in urine samples and DNA-based molecular templates (10). However, due to the lack of effective diagnostic methods during the early stages of the disease, the mortality rate of PCa remains high (10). Therefore, it is crucial to understand the molecular mechanisms associated with PCa carcinogenesis, proliferation and recurrence.

Microarray technology and bioinformatics analysis led to the identification of 273 differentially expressed genes (DEGs) and functional pathways in the carcinogenesis and progression of PCa. In the current study, two mRNA microarray datasets from Gene Expression Omnibus (GEO) were analyzed to identify DEGs between PCa tissues and non-cancerous tissues. Subsequently, the molecular mechanisms of PCa carcinogenesis and progression were investigated using Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis and protein-protein interaction (PPI) network analyses. In conclusion, a total of 273 DEGs and 8 hub genes were identified in the current study, and these genes may be candidate biomarkers for PCa.

Materials and methods

Database. GEO (http://www.ncbi.nlm.nih.gov/geo) (11) is a public functional genomics database. GSE3325 (12) and GSE6919 (13) were downloaded from GEO (Affymetrix Human Genome U133 Plus 2.0 Array). The GSE3325 dataset contained 12 PCa tissue samples and 12 non-cancerous samples. GSE6919 contained 8 PCa samples and 8 non-cancerous samples.

Identification of DEGs. The Affy package (version 1.52.0) (14) was used to preprocess the raw expression data in the R statistical software (R x64 3.5.3; https://cran.r-project.org). DEGs were subsequently identified between PCa and normal samples using the limma (version 3.34.7) package of the R statistical software (https://bioconductor.org/packages/release/bioc/html/limma.html). DEGs with log2FC >1 and P<0.01 were selected in the microarray data.

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Enrichment analysis of DEGs. The Database for Annotation, Visualization and Integrated Discovery (DAVID; version 6.7; http://david.ncifcrf.gov) (15), which provides functional annotation information of genes and proteins, was used to perform DEG analysis of KEGG pathway enrichment (16) and GO annotation (17). $P<0.05$ was set as the threshold value.

Module analysis and construction of the PPI network. The Search Tool for the Retrieval of Interacting Genes (version 10.0; http://string-db.org) (18), which offers comprehensive information on PPIs, was used to create the PPI network. The molecular interaction networks were visualized using Cytoscape (version 3.4.0; https://cytoscape.org/) (19). The Molecular Complex Detection (MCODE) (version 1.4.2) of Cytoscape was used to identify densely connected regions (20). The PPI networks were visualized using Cytoscape software. The hub genes and the most significant module in the PPI networks were identified using MCODE.

Hub genes selection and analysis. The hub genes were selected and their co-expression genes were analyzed using cBioPortal (http://www.cbioportal.org) (21). Hierarchical clustering of hub genes was constructed using the University of California, Santa Cruz Cancer Browser (http://genome-cancer.ucsc.edu) (22). The overall survival and disease-free survival analyses of hub genes (the cutoff was the median expression value) were performed using Kaplan-Meier analysis in cBioPortal. The hazard ratio (HR) with 95% confidence intervals and log-rank P-value was also computed. The expression of PDZ binding kinase (PBK) and Krüppel-like factor 4 (KLF4) in cancer tissues were analyzed and presented using the online database Serial Analysis of Gene Expression (SAGE; http://www. ncbi. nlm.nih.gov/SAGE) (23). The relationship between expression patterns and tumor-node-metastasis (TNM) stage, Gleason grade and recurrence status were analyzed using the online database Oncomine (http://www.oncomine.com) (24).

Results

Identification of DEGs in PCa. After standardization of the microarray results, a total of 1,024 DEGs in GSE6919 and 2,371 DEGs in GSE3325 were identified. The overlap between the 2 datasets contained 273 genes, as presented in Fig. 1A, consisting of 173 downregulated genes and 100 upregulated genes in PCa tissues.

PPI network and module analysis. The PPI network of DEGs (Fig. 1B) and the most significant module were identified using Cytoscape (Fig. 1C). The functional analyses of DEGs demonstrated that genes in this module were mainly enriched in nucleotide binding, small molecule binding, focal adhesion and the regulation of the actin cytoskeleton (Table I).

Functional enrichment analyses of DEGs. Functional and pathway enrichment analyses of DEGs were performed using DAVID. GO analysis revealed that the biological processes of DEGs were significantly enriched in cell adhesion, negative regulation of cell proliferation, cell division and extracellular matrix organization (Table II). Molecular functions of DEGs were enriched in protein binding, GTP binding, mannose-binding, chromatin binding and chromatin binding (Table II). Cell components enriched with DEGs included the nucleus, cytoplasm, perinuclear region of the cytoplasm and focal adhesion (Table II). KEGG pathway analysis revealed that DEGs were mainly enriched in focal adhesion, regulation of the actin cytoskeleton, tight junction, coagulation cascades and gap junctions.

Analyses of the 8 hub genes. In the present study, a total of 8 hub genes were identified and these hub genes were presented in Table III. The criteria for selection were as follows: MCODE scores >5, degree cut-off=2, node score cut-off=0.2, Max depth=100 and k-score=2. Among the 8 genes, PBK, RAP1A, GNAS and RAB39B were upregulated, while COPZ2, KLF4, BACE1 and COL12A1 were downregulated A network of the hub genes and their co-expression genes were analyzed using the cBioPortal online platform (Fig. 2A). Hierarchical clustering demonstrated that the hub genes could differentiate PCa samples from noncancerous samples (Fig. 2B). Subsequently, the overall survival analysis of the hub genes was performed using a Kaplan-Meier curve analysis. Patients with PCa and PBK, RAP1A, GNAS, coatomer protein complex subunit ζ 2 (COPZ2), β-secretase 1 (BACE1) and collagen type XII α-1 Chain (COL12A1) upregulation demonstrated decreased overall survival (Fig. 3A). Patients with PCa and PBK, RAP1A, GNAS, COPZ2, BACE1 and COL12A1 upregulation exhibited decreased disease-free survival (Fig. 3B). Additionally, RAB39B and KLF4 upregulation was associated with increased overall survival and disease-free survival. Based on the above survival analysis, PBK and KLF4 were identified to serve important roles in the carcinogenesis or progression of PCa. Oncomine analysis of cancer and normal tissue revealed that PBK and KLF4 were significantly overexpressed in PCa in the different datasets (Fig. 4A and B). In the Taylor prostate of Oncomine dataset, the increased mRNA levels of PBK were associated with TNM stage, Gleason grade and recurrence status (Fig. 5A). In the Tatulippe prostate of Oncomine dataset, decreased KLF4 mRNA levels were associated with TNM stage, Gleason grade and recurrence status (Fig. 5A and B). PBK gene expression in metastatic tissue was higher compared with primary tumor and solid tissue normal via Oncomine (Fig. 6).

Discussion

Previous studies have demonstrated that the TMPRSS2:ERG fusion is significantly associated with the diagnosis of PCa (25-27). Promoter hypermethylation and downregulated expression of glutathione peroxidase 3 have been observed in a variety of cancer types, including thyroid cancer, hepatocellular carcinoma and PCa (10,26,27). Yu *et al* (28) identified an association between Piwi-like protein 2 (PIWIL2) gene expression and metastatic PCa. Potential markers for use in the diagnosis and treatment of PCa, which exhibit high efficiency, are urgently required. To increase understanding of the molecular mechanisms of candidate genes, GO, KEGG and PPI analyses were performed. In the current study, the epigenetic and genetic mechanisms in PCa were assessed using microarray technology.

Table I. GO and KEGG pathway enrichment analysis of DEGs in the most significant module.

Pathway ID	Pathway description	Count in gene set	FDR
GO:0000166	Nucleotide binding	6	0.003
GO:1901265	Nucleoside phosphate binding	6	0.003
GO:0036094	Small molecule binding	6	0.004
GO:0008092	Cytoskeletal protein binding	5	<0.001
hsa04510	Focal adhesion	8	0.007
hsa04810	Regulation of actin cytoskeleton	8	0.008

GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; DEG, differentially expressed genes; FDR, false discovery rate.

Table II. KEGG and GO enrichment analyses of DEGs.

Term	Description	Count in gene set	P-value
GO:0007155	Cell adhesion	12	0.049
GO:0008285	Negative regulation of cell proliferation	11	0.044
GO:0051301	Cell division	10	0.050
GO:0030308	Negative regulation of cell growth	7	0.006
GO:0030198	Extracellular matrix organization	7	0.051
GO:0005634	Nucleus	99	<0.001
GO:0005737	Cytoplasm	93	<0.001
GO:0048471	Perinuclear region of cytoplasm	17	0.010
GO:0005925	Focal adhesion	16	<0.001
GO:0009986	Cell surface	14	0.033
GO:0005515	Protein binding	147	<0.001
GO:0005525	GTP binding	13	0.007
GO:0003682	Chromatin binding	12	0.019
GO:0019901	Protein kinase binding	11	0.035
hsa04510	Focal adhesion	11	0.007
hsa04810	Regulation of actin cytoskeleton	9	0.008
hsa04530	Tight junction	6	0.003
hsa04540	Gap junction	6	0.005

GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; DEG, differentially expressed genes.

A total of two mRNA microarray datasets were selected. A total of 273 DEGs were identified, including 173 down-regulated genes and 100 upregulated genes. The interactions of DEGs were investigated using GO and KEGG analyses. DEGs were found to be enriched in focal adhesion, regulation of the actin cytoskeleton, tight junctions, coagulation cascades and gap junctions. However, other studies (29,30) have demonstrated that DEGs were enriched in a number of functional terms, including cellular response to bone morphogenetic protein (BMP) stimulus, response to BMP, extracellular region and pathways that are associated with transforming growth factor-β signaling. GO enrichment analysis revealed that changes in the most significant modules were enriched in nucleotide binding, nucleoside phosphate binding, small molecule binding and cytoskeletal protein binding, while changes in KEGG were mainly enriched in focal adhesion and regulation of the actin cytoskeleton.

A total of 8 DEGs were selected as hub genes. The criteria for selection were as follows: MCODE scores >5, degree cut-off=2, node score cut-off=0.2, Max depth=100 and k-score=2. Among the 8 genes, PBK, RAP1A, GNAS and RAB39B were upregulated, while COPZ2, KLF4, BACE1 and COL12A1 were downregulated. PBK and KLF4 were identified to be important genes in the present study. PBK is highly homologous to mitogen-activated protein kinase (31,32). By virtue of target utilization, PBK has been revealed to influence growth and differentiation (33-36). PBK is expressed in the outer cell layer of seminiferous tubules in primary spermatocytes (37), and is often increased in a number of human cancer types from different tissue

Table III. Functional roles of 8 hub genes with degree ≥10.

No.	Gene symbol	Full name	Function
1	PBK	PDZ binding kinase	Active lymphoid cells and support testicular functions; over expression of this gene has been implicated in tumorigenesis
2	RAP1A	Member of RAS oncogene family	Affect cell proliferation and adhesion, and may play a role in tumor malignancy
3	GNAS	Member of RAS oncogene family	The encoded protein regulates signaling pathways that affect cell proliferation and adhesion
4	COPZ2	Coatomer protein complex subunit ζ 2	This gene encodes a member of the adaptor complexes small subunit family
5	KLF4	Krüppel-like factor 4	Control the G1-to-S transition of the cell cycle
6	BACE1	β-secretase 1	This gene encodes a member of the peptidase A1 family of aspartic proteases
7	COL12A1	Collagen type XII α 1 chain	Modify the interactions between collagen I fibrils and the surrounding matrix
8	RAB39B	Member RAS oncogene family	Encodes a member of the Rab family of proteins

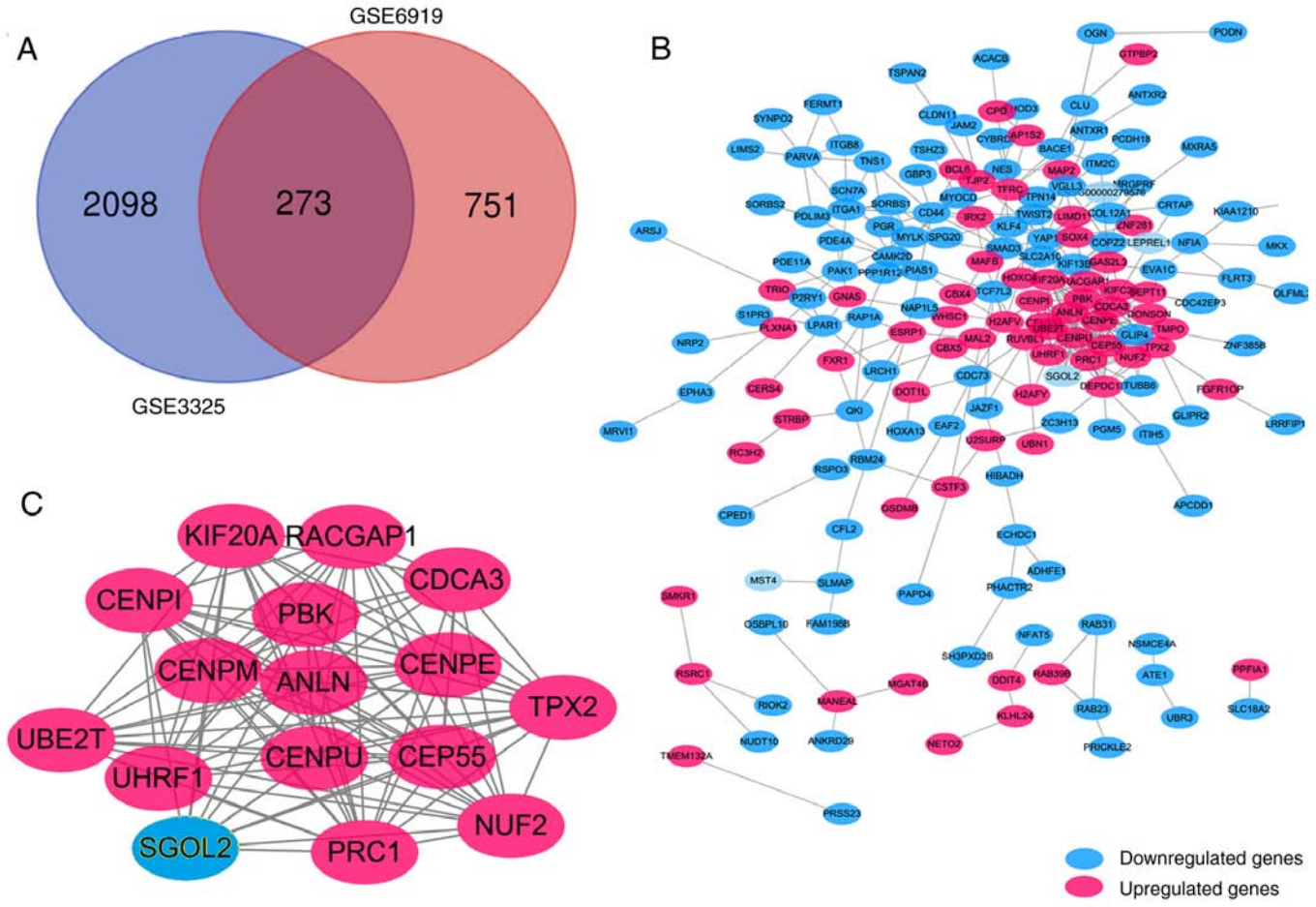

Figure 1. Venn diagram, PPI network and the most significant module of DEGs. (A) DEGs were selected with a fold change >2 and P-value <0.01. The 2 datasets showed an overlap of 273 genes. (B) PPI network of DEGs constructed using Cytoscape. (C) Most significant module with 20 nodes and 100 edges. Upregulated genes are marked in light red; downregulated genes are marked in light blue. DEGs, differentially expressed genes; PPI, protein-protein interaction.

sources (38,39). However, the function of PBK has not yet been fully determined. In a previous study, the immunohistochemical expression of PBK/T-LAK cell-originated protein kinase (TOPK) was revealed to be significantly associated with human bladder cancer, and was identified as a novel diagnostic biomarker for this disease (40). In the present

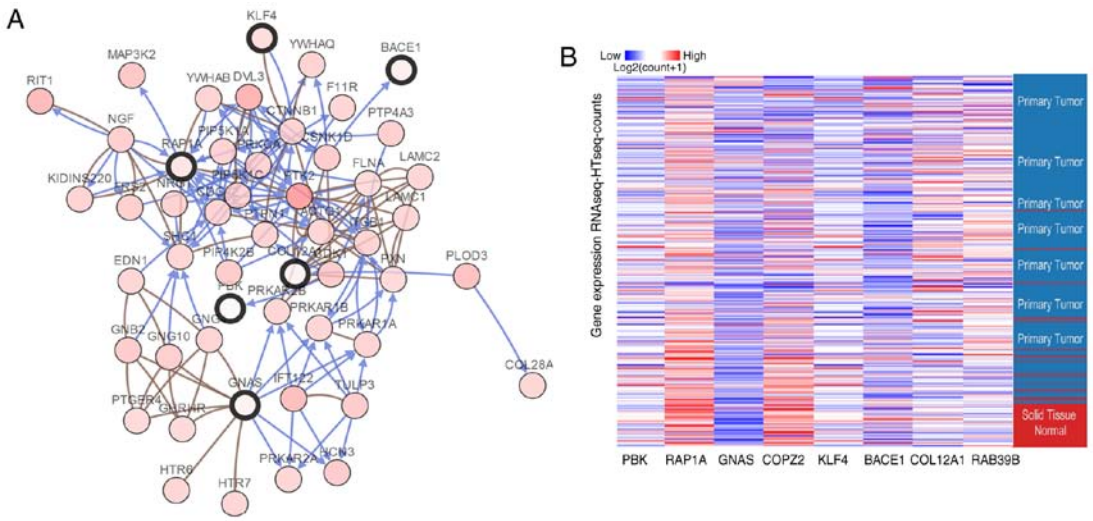

Figure 2. Interaction network and biological process analysis of the hub genes. (A) Hub genes and co-expression genes were analyzed using cBioPortal. Nodes with bold black outline represent hub genes. Nodes with thin black outline represent the co-expression genes. (B) Hierarchical clustering of hub genes. The samples under the red bar are non-cancerous samples and the samples under the blue bar are PCA samples.

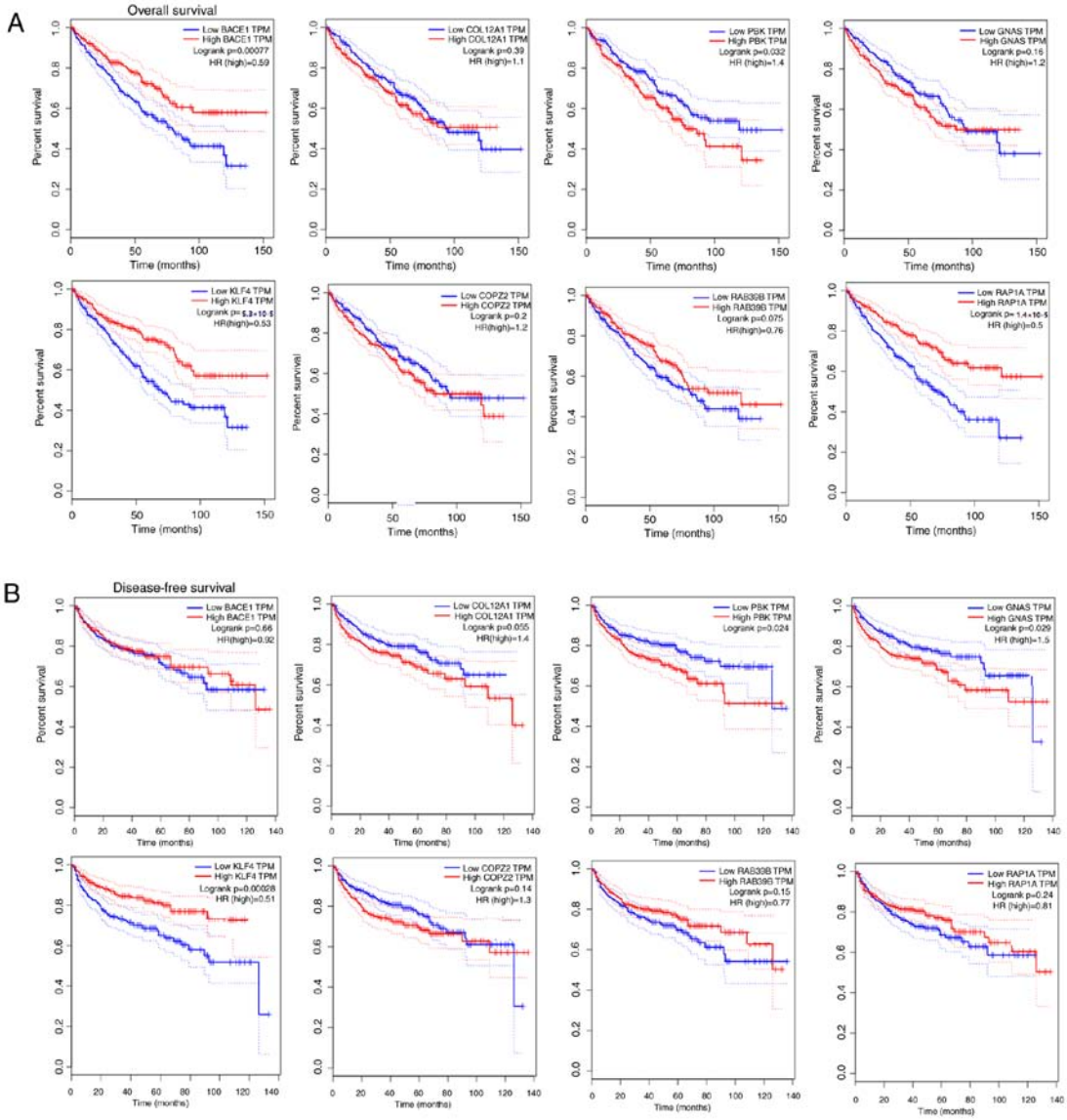

Figure 3. Correlation of expression of individual hubs genes survival. (A) Overall survival and (B) disease-free survival analyses of hub genes were performed using cBioPortal online platform. P<0.05 was considered statistically significant.

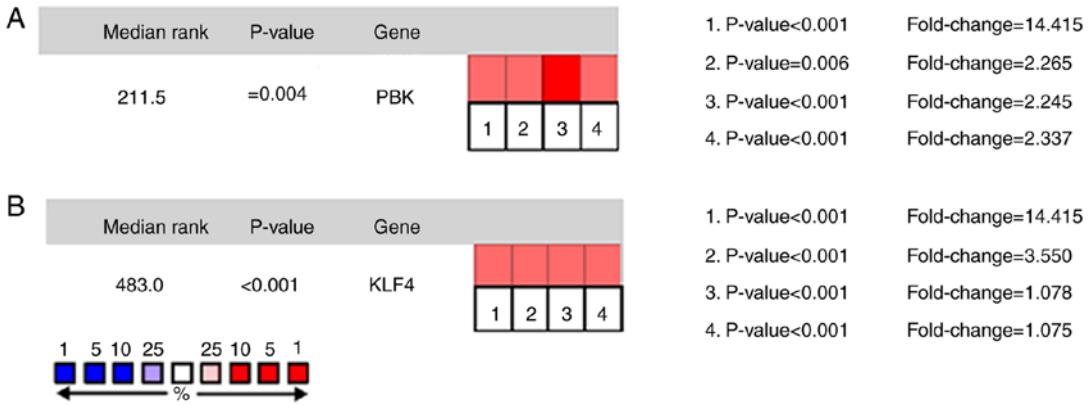

Figure 4. Oncomine analysis of cancer vs. normal tissue of PBK and KLF4. Heat maps of (A) PBK and (B) KLF4 gene expression in clinical PCA carcinoma samples vs. normal tissues. PBK, PDZ binding kinase; KLF4, Krüppel-like factor 4.

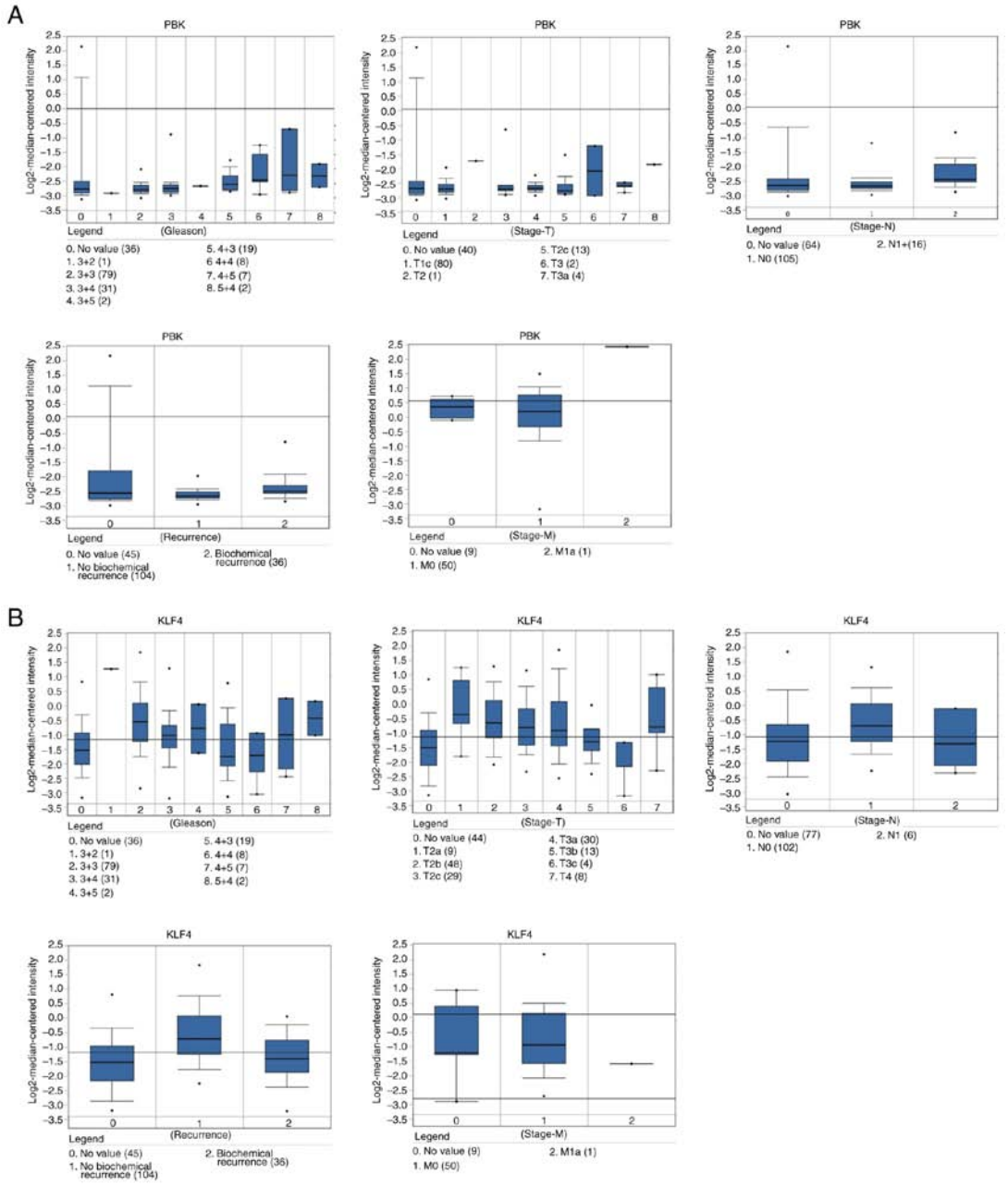

Figure 5. Relationship between the PBK, KLF4 and clinicopathological features. Association between the expression of (A) PBK and (B) KLF4 and TNM stage, Gleason grade and recurrence status. PBK, PDZ binding kinase; KLF4, Krüppel-like factor 4; TNM, tumor-node-mestastasis.

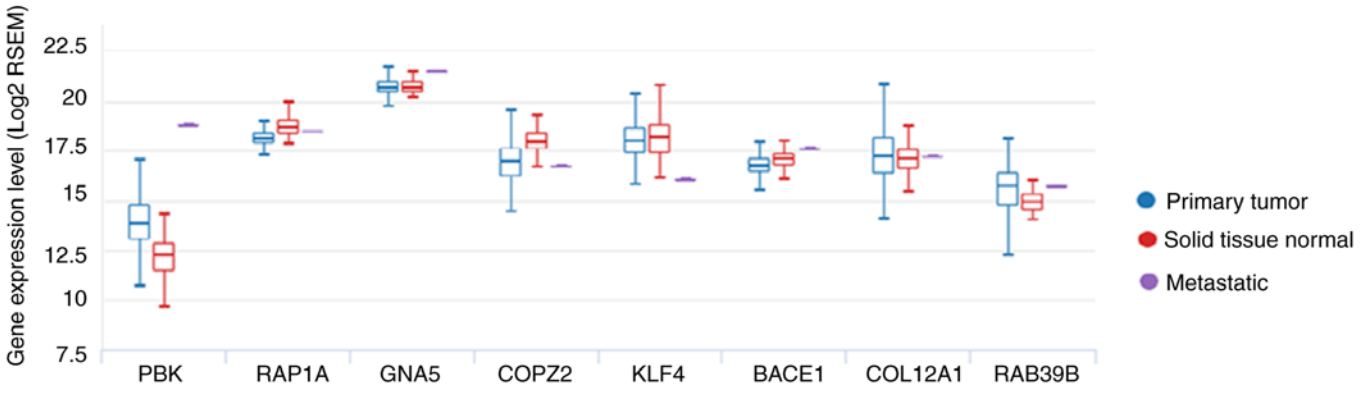

Figure 6. mRNA expression of 8 hub genes between primary tumor, solid tissue normal and metastatic tissue.

study, the PPI network revealed that PBK directly interacted with cyclin-dependent kinase 1, Rac GTPase-activating protein 1, baculoviral IAP repeat containing 5 and protein regulator of cytokinesis 1, indicating a key role for PBK in PCa. The expression of PBK was subsequently assessed. Gene upregulation in PBK were associated with reductions in overall and disease free survival. Additionally, the KLF4 upregulation was significantly associated with increased overall survival and disease-free survival.

High levels of PBK were associated with advanced stages, Gleason score ≥8 and recurrence (41). Additionally, PBK was significantly increased in PCa (P=0.001), and the expression was higher in the Gleason high-scoring group compared with the low-scoring group (P=0.001) (41). PBK, Gleason score and pathological stage are independent predictors of PCa recurrence, and PBK has been indicated to be significantly associated with survival of no biochemical recurrence (42). As an important mitotic kinase, PBK has been reported to exhibit a close association with patient clinical characteristics (43). The current study indicated that higher mRNA levels of PBK were associated with TNM stage, Gleason grade and recurrence status, demonstrating the vital roles of PBK in the carcinogenesis and progression of PCa. PBK gene expression in metastatic tissue was higher compared with primary tumor and solid tissue normal tissue, and PBK has been indicated to serve an important role in mitosis (43). PBK expression and phosphorylation are significantly increased during cell mitosis (44). Previously, a knock-out study of TOPK revealed that PBK can affect spindle formation (36). When PBK is inhibited during mitosis, the spindle (especially the central part) in mitosis and the subsequent cells become blurred (44). The pulp division cannot be completed smoothly and the cells will subsequently split out of the multinucleated cells. Therefore, PBK has been identified to be associated with the regulation of proliferation and cell cycle changes in malignant tumor cells, and has also been revealed to promote tumor cell transformation (33).

KLF4 is a member of the Krüppel-like zinc finger transcription factor family, which serves a role in regulating important processes, including cell proliferation, differentiation and embryo development (36). They are also associated with numerous human cancer types, including gastrointestinal, bladder and lung cancers (33,36,45). A number of KLF4 targeting genes are also biomarker transcription factors in the endothelial-mesenchymal transition (EMT) process (45). Additionally, a previous study indicated that the expression of E-cadherin and α-catenin in the KLF4 overexpression treatment group was significantly higher compared with the control group, while the mesenchymal cell marker vimentin and the expression of vascular endothelial growth was significantly lower compared with the control group (46). It has been shown that KLF4 protein is negatively associated with clinical stages in patients with meningioma, and it promotes or inhibits the EMT process by acting on transcription factors (46). The transcription factor KLF4 in PCa cells promotes the migration and invasion of EMT and tumor cells *in vitro* (47). These results are consistent with the results of the current study, which indicated that lower mRNA levels of PBK were associated with TNM stage, Gleason grade and recurrence status. KLF4 was also indicated to be downregulated in PCa tissue with metastases. Furthermore, the stable knockdown of KLF4 expression in PCa cells has been identified to upregulate the expression of epithelial-related gene E-cadherin and downregulate the expression of a variety of mesenchymal-associated genes *in vitro*, and has been revealed to serve a role in the inhibition of tumor cell migration and invasion (48). Katz *et al* (49) demonstrated that the expression of KLF4 in tumor tissues was significantly decreased in patients with PCa in the USA, and that the upregulation of KLF4 inhibited tumor migration and invasion. Ghaleb *et al* (50) identified a positive feedback loop control between KLF4 and the androgen receptor, and revealed that the inhibition of KLF4 expression in prostatic adenocarcinoma cells can inhibit the occurrence of EMT *in vitro* and serve a role in inhibiting tumor cell migration and invasion. It has also been indicated that KLF4 can serve the role as an oncogene or tumor suppressor gene in a number of cellular environments (50).

In conclusion, a total of 273 DEGs and 8 hub genes were identified as potential novel diagnostic biomarkers for PCa. The current study identified 2 genes associated with PCa progression, including PBK and KLF4. However, the current study is performed based on bioinformatics methods and no experiments were performed to confirm these conclusions. Therefore, further experimental study is required to support the results gained from the current analysis.

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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

SL, WBX and JH conceived and designed the study, and the experiments were performed by SL. SL, JH and WBX analyzed the data and wrote the manuscript. The original text was drafted and modified by SL and WBX. All authors read and approved the final 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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