Hsa_Circ_0001206 is downregulated and inhibits cell proliferation, migration and invasion in prostate cancer

Zhenyu Song, Zhiyuan Zuo, Zhe Ma, Chuansheng Hou, Gang Chen and Guoxiong Xu

Department of Urology, Jinshan Hospital of Fudan University, Shanghai, China; Center Laboratory, Jinshan Hospital, Fudan University, Shanghai, China

ABSTRACT

Our study is to explore the expression profiles and potential functions of circRNAs in prostate cancer (PCa). A total of 95 circRNAs and 830 mRNAs were screened to be significantly differentially expressed in PCa tissues by microarrays. Co-expression and competitive endogenous RNA (ceRNA) network were constructed to reveal the potential regulatory mechanisms of circRNAs. Three circRNAs, hsa_circ_0001206, hsa_circ_0001633, and hsa_circ_0009061 were validated to be down-regulated in PCa by quantitative real-time PCR (qRT-PCR) and hsa_circ_0001206 as well as hsa_circ_0009061 was significantly associated with clinical features of PCa patients. Meanwhile, Receiver Operating Characteristic (ROC) curves showed their good diagnostic value as biomarkers for PCa. The down-regulation of hsa_circ_0001206 was partly because of the regulation of DExH-Box Helicase 9 (DHX9). Moreover, overexpression of hsa_circ_0001206 inhibited PCa cell proliferation, migration, and invasion in vitro and prevented tumor growth in vivo. Dual-luciferase reporter assays showed hsa_circ_0001206 could directly bind to miR-1285-5p. The expression of Smad4, a well-known suppressive gene in PCa, can be increased by overexpression of hsa_circ_0001206 and this effect could be partly reversed by co-transfection of miR-1285-5p mimic. The study revealed expression profiles and potential functions of circRNAs and demonstrated hsa_circ_0001206 played a suppressive role in the pathogenesis of PCa.

Introduction

Prostate cancer (PCa) is one of the most common malignancies in male. The American Oncology Association estimated 174,650 new cases of prostate cancer (PCa) and 31,620 deaths in 2019, ranking this disease first in male cancer incidence and second in male cancer mortality [1]. At present, treatments of PCa have caused a serious economic burden [2]. Early-stage prostate cancer can be cured by radical surgery or radiotherapy, but the treatment of advanced prostate cancer is still dominated by androgen deprivation therapy (ADT). After 12 months of castration treatments, metastatic prostate cancer can turn into castration-resistant prostate cancer (CRPC) in almost all cases. Nowadays, drugs used to treat CRPC include abiraterone, enzalutamide, docetaxel, cabazitaxel, and others. However, these treatments have limited efficacy and patients may soon progress to the incurable stage of PCa [3]. Although the vast majority of patients with PCa are now detected by PSA (prostate-specific antigen) screening, it is now not recommended by experts due to overdiagnosis and overtreatment [4,5]. Therefore, alternative biomarkers with higher accuracy and specificity for diagnosis and novel therapeutic targets of PCa are urgently needed.

Recently, circular RNA (circRNA), a novel type of non-coding RNA, has attracted much attention. CircRNA has a closed loop structure without 5′–3′ polarity and a polyadenylated tail [6], which renders them more resistant to exonucleases and more stable than linear RNA. Tens of thousands of circRNAs have now been identified in a variety of cells and species, including more than 200,000 different circRNAs in eukaryotes [7,8]. CircRNAs are highly expressed in saliva, blood, and even exosomes [9–11]. In different tumor types, abnormally expressed circRNAs are associated with tumor cell proliferation, migration, and invasion [12–15], suggesting that circRNAs possess great potential as therapeutic targets. However, the regulatory mechanisms of circRNAs remain unclear and the classic model is the microRNA (miRNA) sponge. As competitive endogenous RNAs (ceRNAs), circRNAs play biological roles by combining with their corresponding miRNAs. For example, CDR1as has more than 70 microRNA-7 binding sites that sequester microRNA-7 and inhibit its binding to target genes [7]. To date, the expression profiles and potential functions of circRNAs in PCa remain to be investigated. A previous study revealed that circAMOTL1 was down-regulated in PCa and associated with the metastatic progression of PCa [16]. Moreover, one study used shRNA
libraries to screen 1336 highly abundant circRNAs in prostate cancer and identified 171 circRNAs which affected cell proliferation [17].

In our present study, microarrays were applied to identified circRNAs and mRNAs abnormally expressed between PCa and adjacent normal tissues. In addition, hsa_circ_0001206, hsa_circ_0001633, and hsa_circ_0009061 were validated by quantitative real-time PCR (qRT-PCR) in 50 paired PCa and adjacent normal tissue samples. Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, co-expression, and ceRNA network analyses were carried out to predict their potential roles in PCa. Furthermore, hsa_circ_0001206 was selected for further validation in vitro and vivo experiments to explore its potential functions and regulatory mechanisms.

Materials and methods

Patient samples

PCa tissues and adjacent normal tissues were collected intraoperatively from 50 patients at Jinshan Hospital, Fudan University between February 2017 and October 2018 and validated by two experienced pathologists. None of them received any therapy before surgery. All tissues were snap-frozen and stored in a −80°C freezer. Written informed consent was obtained from each patient and all experiments were approved by the Ethics Committee of Jinshan Hospital, Fudan University.

Culturing and transfection of cell lines

RWPE-1, PC-3, DU145, and LNCaP were purchased from Shanghai Cell Bank (Chinese Academy of Science, Shanghai, China). All cells were cultured at 37°C under a 5% CO2 atmosphere. RWPE-1 cells were maintained in prostate epithelial cell medium (PepiCM, ScienCell, San Diego, USA). PC-3, DU145, and LNCaP cells were maintained in F12K medium, MEM basic medium, and RPMI-1640 medium, respectively, containing 10% fetal bovine serum (FBS, Gibco/Invitrogen, Waltham, MA, USA). To overexpress hsa_circ_0001206, plasmid pLCDH-cir-1206 was generated (Geneseed, Guangzhou, China) and coated with lentivirus (Genomeditech, Shanghai, China). The procedure for lentivirus transfection was conducted according to the manufacturer’s protocol. MIR-1285-5p mimic and miR-negative control (synthesized by Ribobio, Guangzhou, China) were transfected into DU145 cells using X-tremeGENE Transfection Reagent (Roche Applied Science, Indianapolis, IN, USA) in accordance with the manufacturer’s protocol. The negative control-siRNA (siNC) and DHX9-siRNA (GenePharma, Shanghai, China) were used to knockdown DHX9. The sequence of DHX9-si-1 was 5’-3’ GAGCCCAAUCUGAGGAUATT; DHX9-si-2 GCCUCAAGAAUGCUAAU TT; DHX9-si-3 CUGGGAGAUG CUAGAATT.

RNA extraction and quality control

RNA was isolated from tissues and prostate cancer cells using an Axygen Bioscience kit (Axygen Scientific Inc, Silicon Valley, USA). The RNA quality was evaluated by measuring the optical density (OD) at 260/280 nm and 260/230 nm. The integrity of RNA was confirmed by 2% agarose gel electrophoresis.

Microarray analysis

Five pairs of PCa tumors and adjacent normal tissues were used for microarray assays to screen dysregulated circRNAs and mRNAs. Sample preparation and hybridization were performed according to the Arraystar instruction and the R package “limma” was employed for subsequent data processing. CircRNAs and mRNAs exhibiting statistically significant differences between the two groups were elucidated via volcano and scatter plots. Differentially expressed circRNAs and mRNAs were identified based on thresholds of fold change ≥2.0 and p values <.05.

GO and KEGG analysis

Next, differentially expressed mRNAs and host genes of abnormally expressed circRNAs were subjected to functional analysis, including GO and KEGG analyses, which were performed in DAVID website (http://david.abcc.ncifcrf.gov).

Co-expression analysis

Co-expression analysis was performed based on the Pearson correlation coefficient (PCC) between expression levels of differentially expressed circRNAs and mRNAs. Correlation values of PCC ≥ 0.90 and FDR<0.01 were selected for further analysis. Cytoscape software was used for network construction.

qRT-PCR analysis

PrimeScript RT Master Mix (Takara, Shiga, Japan) was used for reverse transcription of total RNA to cDNA, and SYBR (Roche, Basel, Switzerland) was used for qRT-PCR. Each assay was performed at least three times. The 2-ΔΔCt method was used to calculate relative expression levels of selected circRNAs. β-actin was used as an internal control. Student’s t-tests, Mann–Whitney t-tests, paired t-tests, or Wilcoxon paired t-tests were applied as appropriate, and p values <.05 was considered statistically significant.

CeRNA network construction

Homemade microRNA software from Arraystar was used to identify potential microRNA targets; this approach integrates results from TargetScan (http://www.targetscan.org/vert_71/) and miRanda (www.microrna.org/). Cytoscape software was used to construct the network.

Cell viability assessment

Negative control (NC) and hsa_circ_0001206-overexpressing cells were cultured in 96-well plates (5 × 103/well). Cell viability was assessed at 24, 48, 72, and 96 h using a Cell Counting
**Cell migration and invasion assays**

Transwell assays were performed to explore cell migration and invasion abilities. Transwell chambers were used for migration assays and transwell chambers pre-coated with Matrigel were employed for invasion assays according to the manufacturer’s instructions (BD Science, Bedford, MA, USA). Cells were seeded into the upper chamber (5 × 104 cells/100 μl serum-free medium). After 48 h, cells in five random fields were counted under a light microscope (Olympus, Tokyo, Japan; 200× magnification). All assays were repeated at least three times.

**Dual-luciferase reporter assay**

Wild-type and mutant plasmids containing hsa-circ-0001206 were integrated into the pmirGLO vector (Genewiz, Suzhou, China) and 293 T cells were plated into 24-well plates. When cells reached 70–80% confluency, 400 ng of wild-type or mutant luciferase reporter vector was added and co-transfected with 50 nM miR-12855p mimic or NC (Ribobio, Guangzhou, China) and X-tremeGENE Transfection Reagent (Roche Applied Science, Indianapolis, IN, USA) for 24 h. Luciferase activity was measured with the use of a Luc-Pair Duo-Luciferase Assay Kit 2.0 (GeneCopoeia, Rockville, MD, USA).

**Gene expression profiling interaction analysis (GEPIA)**

We utilized GEPIA [18] (http://gepia.cancer-pku.cn/) to compare the mRNA levels of Smad4 and DHX9 between prostate cancer tissue samples and adjacent normal specimens and conduct survival analyses with log-rank test for hypothesis evaluation. In addition, the RNA-sequencing data and clinical data of PCa from TCGA database (https://cancergenome.nih.gov/) were downloaded and utilized in the study.

**Western blot analysis**

Protein was extracted from xenograft tissues using SDS buffer with 1% PMSF (Beyotime Institute of Biotechnology, Haimen, China). The protein concentration was determined using a BCA Protein Assay kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). We used 12% gels separate proteins via SDS-PAGE and transferred the proteins to a PVDF membrane (EMD Millipore, Billerica, MA, USA). We used 12% gels separate proteins via SDS-PAGE and transferred the proteins to a PVDF membrane. After blocking for 1 h with 5% non-fat powdered milk at room temperature, we then incubated the membrane with primary antibody (SMAD4 Rabbit mAb, 1:1000 dilution; β-actin mouse antibody. 1:5000 dilution; Cell Signaling Technology, Inc., Danvers, MA, USA) at 4°C overnight. After washing 3 times with Tris-buffered saline Tween-20 (10 min per time) and incubation with anti-mouse or anti-rabbit IgG HRP-linked antibody (1:5000 dilution, 1 h, Cell Signaling Technology, Inc., Danvers, MA, USA), we then detected the signals of proteins with Immobilon™ Western Chemiluminescent HRP Substrate (EMD Millipore, Billerica, MA, USA) and Tanon-4500 Gel Imaging System (Tanon Science and Technology Co., Ltd., Shanghai, China).

**Xenograft study**

A total of 10 four-week-old BALB/c nude mice were randomly assigned to two groups, with each group containing 5 mice. Negative control and hsa_circ_0001206-overexpressing cells were inoculated subcutaneously (cell suspension concentration: 2E+7 cells/ml, 200ul per mouse). Tumor volumes and body weights were measured and recorded once a week. After 42 days from inoculation (tumor length less than 20 mm), mice were executed and xenografts were excised. Tumors were weighed and stored for subsequent experiments.

**Statistical analyses**

SPSS 22.0 (IBM, Chicago, IL, USA) and Prism 5 (GraphPad, La Jolla, CA, USA) were used for statistical analyses. Student’s t-tests, Mann–Whitney t-tests, and chi-square tests were used to assess differences as appropriate. Receiver Operating Characteristic (ROC) curve analysis was used to estimate the diagnostic values of circRNAs for PCa. p Values <.05 was considered statistically significant.

**Results**

**CircRNA and mRNA expression profile analysis**

Microarray screening yielded 12,532 circRNAs and 14,581 mRNAs across five pairs of PCa and adjacent normal tissues. Differentially expressed circRNAs and mRNAs (fold change ≥2.0 and p values <.05) between the two groups are shown in Figure 1. The most differentially expressed circRNAs were depicted in a heatmap (Figure 1(A)). A total of 95 circRNAs were significantly differentially expressed between the two groups with 71 upregulated and 24 downregulated in PCa, as shown in the scatter plot and volcano plot (Figure 1(B,C)). The 95 circRNAs were listed in Supplementary Table 1. The heatmap illustrated the mRNA expression profiles in Figure 1(D). In total, 830 mRNAs were differentially expressed between the two groups, of which 371 were up-regulated and 459 were down-regulated in PCa. These mRNAs were depicted by a scatter plot and a volcano plot (Figure 1(E,F)). As shown in Figure 1(G,H), the 95 circRNAs are differently distributed among chromosomes. Chromosome 19 contains the most upregulated circRNAs, accounting for 13% of total upregulated circRNAs, while chromosome 14 contains the most down-regulated circRNAs (almost 17% of the total). Based on the association of these circRNAs with their host genes, we found a majority of circRNAs were derived from exons of parental genes (Figure 1(I)).

**GO and KEGG pathway analyses**

We conducted a GO analysis of host genes of differentially expressed circRNAs (Figure 2(A–C)). Many GO terms are associated with the pathogenesis of PCa such as cell-cell adhesion...
According to the ceRNA hypothesis, circRNAs regulate the expression levels of related mRNAs. Thus, we also conducted GO and KEGG pathway analyses of differentially expressed mRNAs. Our data showed that the most significant GO term for up-regulated mRNAs in the biological process category was chromosome segregation (GO:0007059). Other GO terms related to cancer cell proliferation were also identified (Figure 2(D)), such as (GO: 0098609) and extracellular matrix (GO:0031032).
mitotic nuclear division (GO:0140014) and nuclear division (GO:0000280). Regarding down-regulated mRNAs, nervous system development was the most significant GO term associated with biological processes (Figure 2(E)). KEGG pathway analysis showed that circadian entrainment was the most enriched KEGG pathway for up-regulated transcripts, while primary immunodeficiency and p53 signaling were the top pathways for down-regulated mRNAs (Figure 2(F–G)).
Co-expression of circRNAs/mRNAs

To further reveal the potential functions of circRNAs, co-expression analysis of differentially expressed circRNAs and mRNAs was carried out. Twenty significantly up- and down-regulated circRNAs were selected to build the network based on the degree of correlation (Figure 3). The co-expression network indicated that the expression of a single circRNA might be associated with diverse mRNAs.

Validation of circRNAs by qRT-PCR

We selected three significantly differentially expressed circRNAs and confirmed their expression across 50 pairs of samples by qRT-PCR. Primer sequence information was listed in Table 1. The results revealed that expression of three circRNAs (hsa_circ_0001633, hsa_circ_0001206, and hsa_circ_0009061) was significantly down-regulated (Figure 4(A–C)).

Diagnostic value and clinical significance of circRNAs

ROC curves for hsa_circ_0001633, hsa_circ_0001206, and hsa_circ_0009061 were plotted to assess their diagnostic values as biomarkers for PCa. The area under the curve (AUC) for hsa_circ_0001633, hsa_circ_0001206, and hsa_circ_0009061 was 0.809, 0.774, and 0.711, respectively (Figure 4(D)), which indicated that these circRNAs were promising biomarkers for identifying PCa. Analyses of the three circRNAs and clinical features revealed that hsa_circ_0001206 and hsa_circ_0009061 were associated with Gleason score and pathological stage of patients with PCa (Table 2).

CeRNA network construction

CircRNAs and mRNAs may regulate each other if they share the same microRNA response elements (MREs). On the basis of this hypothesis, we built ceRNA network for hsa_circ_0001633, hsa_circ_0001206, and hsa_circ_0009061 (Figure 5). The top
five predicted miRNAs for hsa_circ_0001633, hsa_circ_0001206, and hsa_circ_0009061 were listed in Table 3. This network analysis provided insight into the underlying regulatory mechanisms of the circRNAs in PCA.

Overexpression of hsa_circ_0001206 inhibits PCA cell proliferation, migration, invasion, and colony formation

To examine the functions of hsa_circ_0001206 in PCA, we investigated its expression in RWPE-1, PC-3, DU145, and LNCaP cells.

Figure 4. Validation of three circRNAs in PCA tissues and adjacent normal tissues by qRT-PCR and ROC curves. The probability values were calculated using the Wilcoxon paired t-test or paired t-test, depending on data distributions. (A) Hsa_circ_0001633. (B) Hsa_circ_0001206. (C) Hsa_circ_0009061. (D) ROC curves for hsa_circ_0001633, hsa_circ_0001206, and hsa_circ_0009061. The results are presented as the mean ± SEM (n = 50). **p < 0.01, ***p < 0.001.
Results showed it was significant down-regulation in PCa cell lines (PC-3, DU145, and LNCaP) compared with RWPE-1 (Figure 6(A)). Using hsa_circ_0001206-overexpressing lentivirus, we generated stable hsa_circ_0001206-overexpressing DU145 cells and PC-3 cells as well as negative control cells and validated the expression of hsa_circ_0001206 was dramatically up-regulated in hsa_circ_0001206-overexpressing cells by qRT-PCR (Figure 6(B,C)). Because the lentivirus contained green fluorescent protein (GFP), nearly 90% of cells were green after five passages (Figure 6(D)). In comparison with negative control cells (NC), a

Table 2. Clinicopathological features of PCa patients and the expression of selected circRNAs.

| Parameters               | Group | has_circ_0001206 expression |                   |                   |                   |                   | hsa_circ_0001633 expression |                   |                   |                   | hsa_circ_0009061 expression |                   |                   |
|--------------------------|-------|-----------------------------|-------------------|-------------------|-------------------|-------------------|-----------------------------|-------------------|-------------------|-------------------|-----------------------------|-------------------|-------------------|
|                          |       | Low | High | p Value | Low | High | p Value | Low | High | p Value | Low | High | p Value |
| Age at surgery           |       |     |      |         |     |      |         |     |      |         |     |      |         |
| <65                      |       | 9   | 8    | 0.765   | 9   | 8    | 0.765   | 9   | 9    | 0.765   | 9   | 9    | 0.765   |
| ≥65                      |       | 16  | 17   | 0.371   | 16  | 17   | 0.765   | 16  | 17   | 0.544   | 19  | 19   |         |
| PSA (ng/ml) ≤20          |       | 15  | 18   | 0.045*  | 16  | 17   | 0.775   | 6   | 15   | 0.010** | 19  | 10   | 0.001** |
| >20                      |       | 10  | 7    |         | 9   | 8    |         | 9   | 7    |         | 9   | 7    |         |
| Pathological stage       | pT2   | 7   | 14   | 0.0018** | 10  | 11   | 0.156   | 13  | 14   | 0.777   | 12  | 11   | 0.384   |
|                          | pT3-4 | 18  | 11   |         | 15  | 14   |         | 19  | 10   |         | 19  | 10   |         |
| Gleason                  | G ≤7  | 8   | 19   | 0.0018** | 11  | 16   | 0.156   | 13  | 14   | 0.777   | 12  | 11   | 0.384   |
|                          | G > 7 | 17  | 6    |         | 14  | 9    |         | 12  | 11   |         | 12  | 11   |         |
| Lymph node metastasis    | No    | 22  | 22   |         | 22  | 22   |         | 23  | 21   |         | 23  | 21   |         |
|                          | Yes   | 3   | 3    |         | 3   | 3    |         | 2   | 4    |         | 2   | 4    |         |

*p < .05, **p < .01, ***p < .001. p < .05 represents statistical significance which are in bold font (Chi-square test). * p < .05, ** p < .01.

Figure 5. Competing endogenous RNA network in PCa. The competing endogenous RNA network has been generated based on circRNA/miRNA/mRNA interactions. CircRNA shape: ellipse, color: dark olive-green; microRNA shape: rect, color: gray; mRNA Shape: triangle, color: up-yellow, down-sky-blue.
significant reduction in cell proliferation ability was observed in hsa_circ_0001206-overexpressing DU145 cells at 48, 72, and 96 h (Figure 7(A)) and hsa_circ_0001206-overexpressing PC-3 cells at 24, 48, 72, 96 h (Figure 7(B)). Furthermore, migration assays revealed that the migration ability of overexpressing cells was significantly weaker than that of NC cells at 48 h (Figure 7(C)). Similar results were obtained in invasion assays (Figure 7(D)).

**Hsa_circ_0001206 interacts with miR-1285-5p**

Using home-made microRNA target prediction software from Arraystar, we predicted miR-1285-5p as a potential target of hsa_circ_0001206. A dual-luciferase reporter assay was conducted to demonstrate the circRNA-microRNA interaction. The miR-1285-5p sequence, the wild-type hsa_circ_0001206 sequence containing an miR-1285-5p binding site (position 121–140), and the mutant hsa_circ_0001206 sequence was shown in Figure 8(A). The results showed that miR-1285-5p significantly decreased the activity of wild-type luciferase. However, mutant hsa_circ_0001206 lost the ability to bind miR-1285-5p (Figure 8(B)), which indicated that miR-1285-5p could directly bind to hsa_circ_0001206. Next, we utilized the TargetScan database to identify Smad4 as a potential target of miR-1285-5p and hsa_circ_0001206-overexpressing cells was positively correlated with Smad4 mRNA expression in PCa tissues (Figure 8(C)). Meanwhile, we observed that mRNA expression of Smad4 was increased significantly in hsa_circ_0001206-overexpressing DU145 cells and partially decreased when overexpressing cells were co-transfected with miR-1285-5p mimic (Figure 8(D)). Using the GEPIA web tool, we found the mRNA expression levels of Smad4 were lower in PCa tissue samples than adjacent normal samples and patients with lower Smad4 levels exhibited better disease-free survival (Figure 8(E,F)).

**Overexpression of hsa_circ_0001206 inhibits the growth of PCa cells in vivo**

Negative control and hsa_circ_0001206-overexpressing cells DU145 were planted in the nude mice subcutaneously. As shown in Figure 9(A–D), tumor volumes of hsa_circ_0001206-overexpressing group were significantly smaller than those of NC group at 21, 28, 35, 42 days after inoculation. Comparatively, tumor weights of hsa_circ_0001206-overexpressing group were significantly lighter than those of NC group. Moreover, overexpression of hsa_circ_0001206 significantly increased the expression level of Smad4 protein (Figure 9(E,F)).

**Knocking down of DHX9 increases the expression of hsa_circ_0001206**

DHX9 protein is an abundant intranuclear RNA-binding protein which could bind to the inverted repeat Alu element of RNA to prevent the production of circRNAs [19]. Based on the GEPIA online tool and clinical data from TCGA, we found DHX9 was up-regulated in PCa tissues comparatively (Figure 10(A)) and high expression of DHX9 was associated with poor disease-free survival (Figure 10(B)). Meanwhile, the expression of DHX9 was also positively correlated with clinical features of PCa, such as Gleason score and Tumor grades (Figure 10(C,D)). To explore the effect of DHX9 on the dysregulation of hsa_circ_0001206, we used DHX9-siRNAs to knockdown DHX9 and confirmed their efficiency by qRT-PCT in DU145 cells (Figure 10(E)). Moreover, the expression of hsa_circ_0001206 increased significantly when cells were transfected with DHX9-siRNA2 (the most efficient siRNA to knockdown DHX9) compared with siRNA-negative control.

**Discussion**

Characterized by a closed loop structure, circRNAs draw increasing attention worldwide. High-throughput circRNA sequencing and microarray approaches have facilitated the discovery of numerous circRNAs involved in the pathogenesis of various cancers, including renal carcinoma [20], bladder cancer [15,21], hepatocellular carcinoma [22], gastric cancer [13], breast cancer [23], lung cancer [12], and so forth. Previous studies indicated that some circRNAs, such as circ-102004 [24] and circAMOT1L1 [16] played important roles in the pathogenesis of PCa but the expression profiles and functions of circRNAs in PCa remain to be clarified. In the present study, circRNA and mRNA microarrays were employed to obtain circRNA and mRNA expression profiles in human PCa. We identified 95 significantly differentially expressed circRNAs and 830 mRNAs. GO term analysis showed that differentially expressed mRNAs were enriched in chromosome segregation, mitotic nuclear division, cell cycle process, and some other categories. These GO terms were reported to be closely associated with the pathogenesis of PCa [25,26]. Since circRNAs could regulate the functions of their host genes with pre-mRNA splicing [27], we also performed GO term analysis of host genes of differentially expressed circRNAs in PCa to predict their functions. The results showed that parent genes were enriched in various biological process (BP) terms, such as regulation of cell shape, cell-cell adhesion, and cell morphogenesis. Thus, it is unsurprising that these circRNAs are implicated in the development of PCa.

Co-expression analysis indicated that circRNAs were closely correlated with certain mRNAs, some of which were reported to play vital roles in PCa, such as ZNF217 and STK4 [28,29]. Conceivably, circRNAs might influence PCa carcinogenesis via these mRNAs.

Among these differentially expressed circRNAs, we verified that hsa_circ_0001206, hsa_circ_0001633, and hsa_circ_0009061 were significantly down-regulated in PCa tissues compared with adjacent normal tissues. Hsa_circ_0001206 is a circular transcript derived from the second exon of CRKL.
CRKL, an intracellular signaling adaptor protein, can interact with the AR complex to bypass hormone dependency in advanced PCA [30]. As to hsa_circ_0001633, it is derived from the second exon of the SOBP gene. The SOBP gene is mainly involved in human mental development [31]. In terms of hsa_circ_0009061, it is a circular RNA.
product formed by the transcription of the second exon of KDM1A gene. The encoded protein KDM1A, also known as lysine-specific histone demethylase 1 (LSD1), is a histone demethylase, which has been shown to play a vital role in the development and progression of PCa [32–34]. Up until now, there have been no reports on these three circular RNAs in the pathogenesis of PCa. This study found for the first time that hsa_circ_0001206, hsa_circ_0001633, and hsa_circ_0009061 were significantly down-regulated in PCa tissues and they could act as potential biomarkers for diagnosing PCa.

Figure 7. Overexpression of hsa_circ_0001206 inhibits the proliferation, migration, and invasion of PCa cells. (A) CCK-8 assay in DU145 cells. (B) CCK-8 assay in PC-3 cells. (C) Migration assays. (D) Invasion assays. Effective migrating or invading cells were counted and photographed. Original amplification, ×200. The histogram displays the quantitative analysis of migrating or invading cells. The results are presented as the mean ± SEM. * * * * * p < .05, ** p < .01, *** p < .001.
Furthermore, hsa_circ_0001206 and hsa_circ_9061 were demonstrated to be associated with the Gleason score and tumor grade of PCa, indicating their important roles in PCa.

A circRNA-miRNA-mRNA ceRNA network was also constructed to explore the regulatory mechanisms of circRNAs in PCa. Hsa_circ_0001206 was then selected to explore its biological functions. Figure 8 shows the interaction between hsa_circ_0001206 and miR-1285-5p. The expression of Smad4 mRNA was positively correlated with hsa_circ_0001206. Overexpression of hsa_circ_0001206 induced the expression of Smad4, and this effect was partially reversed by miR-1285-5p mimic. The GEPIA database revealed that the mRNA expression of Smad4 was significantly downregulated in PCa tissues. The disease-free survival of the PCa patients was computed with the GEPIA web tool.
roles in PCa. The results showed that overexpression of hsa_circ_0001206 markedly decreased PCa cell proliferation, migration, and invasion in vitro and significantly inhibited the growth of tumor in vivo. Moreover, hsa_circ_0001206 might function as an miR-1285-5p sponge when exerting its effect on the pathogenesis of PCa. One previous study reported that miR-1285-5p could suppress the proliferation and metastasis of lung cancer cells [35]. Moreover, miR-1285-5p was also reportedly associated

Figure 9. Hsa_circ_0001206 suppressed the growth of PCa in vivo. (A,B) The upper group represented negative control group, the other for hsa_circ_0001206 overexpressing group. (C,D) Tumor volumes and weights of two groups. (E,F) Western blot using xenograft tissues revealed that the protein expression of Smad4 was increased in the hsa_circ_0001206 overexpressing group compared with the control group. The histogram displays the quantitative analysis. The results are presented as the mean ± SEM. *p < .05.
Figure 10. Knocking down of DHX9 could increase the expression of hsa_circ_0001006. (A) The GEPIA database revealed the mRNA expression of DHX9 was significantly up-regulated in PCa tissues. The boxplot analysis showed log2 (TPM + 1) on a log-scale. (B) The disease-free survival of the patients with PCa was computed with the GEPIA web tool. (C) The expression levels of DHX9 in PCa patients with different Gleason scores. (D) The expression levels of DHX9 in PCa patients with different tumor grades. (E) The expression of DHX9 in blank, negative control, and DHX9-siRNA transfected groups detected by qRT-PCR. (F) The expression of hsa_circ_0001206 in blank, negative control, and DHX9-siRNA2 transfected group detected by qRT-PCR. N = 3 independent experiments. The results are presented as the mean ± SEM. *p < .05, **p < .01, ***p < .001.
with overall survival in breast cancer [36] and infiltrative growth of follicular variants of papillary thyroid carcinomas [37]. The accumulating evidence indicated miR-1285-5p might have potentially important roles in the pathogenesis of diverse cancer types. Furthermore, mRNA expression of Smad4 was affected by overexpression of hsa_circ_0001206 and miR-1285-5p. Smad4 was demonstrated to exert a suppressive effect on the growth and metastasis of PCa [38,39]. The results of PCa samples from TCGA also showed that mRNA expression of Smad4 was downregulated in PCa and strongly negatively associated with disease-free survival. Based on the evidence above, we speculated that hsa_circ_0001206 played its suppressive role in the pathogenesis of PCa through hsa_circ_0001206/miR-1285-5p/Smad4 pathway.

Meanwhile, we explored why hsa_circ_0001206 was down-regulated in PCa. Previous studies reported some RNA-binding proteins, such as QKI and DHX9, could regulate the production of circRNAs [40–42]. DHX9 is a member of the DExD/H-box helicase family, which has the ability to unwind DNA and RNA duplexes, regulate DNA replication, transcription, RNA processing, and transport, translation, etc. [43]. DHX9 is closely related to the development of various cancers, such as breast cancer [44], colon cancer [45], lung cancer [46], Ewing sarcoma [47], and so on. DHX9 can bind to the inverted complementary ALU sequences which lie in the flank of circRNAs to reduce the formation of circRNAs [19]. One study showed that DHX9 was up-regulated in hepatocellular carcinoma and inhibited the production of a circular RNA, cSMARCA5 [48]. In the present study, we found DHX9 was highly expressed in PCa tissues and PCa patients with high expression of DHX9 had shorter disease-free survival, suggesting that DHX9 is an independent prognostic factor for the development of PCa. At the same time, knocking down of DHX9 increased the expression of hsa_circ_0001206, indicating that upregulation of DHX9 in PCa was at least one cause of downregulation of hsa_circRNA_0001206.

Conclusion

We revealed the circRNA and mRNA expression profiles in PCa and found hsa_circ_0001633, hsa_circ_0001206, and hsa_circ_0009061 were significantly down-regulated in PCa tissues compared with adjacent normal tissues and all of them had good diagnostic values for PCa. Bioinformatics analysis provided insight into the potential functions of these circRNAs. Furthermore, hsa_circ_0001206 was demonstrated to inhibit the development of PCa in vivo and vitro and might exert its effect on the pathogenesis of PCa via the hsa_circ_0001206/miR-1285-5p/Smad4 pathway. The down-regulation of hsa_circ_0001206 was partly caused by DHX9.

Disclosure statement

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