Circ_0001686 Promotes Prostate Cancer Progression by Up-Regulating SMAD3/TGFBR2 via miR-411-5p

Qiliang Cai (✉ caiqiliang@tmu.edu.cn)

Tianjin Institute of Urology

Jiancheng Pan

Tianjin Institute of Urology

Enli Liang

Tianjin Institute of Urology

Dingrong Zhang

Tianjin Institute of Urology

Cheng Fang

Tianjin Institute of Urology

Zhizhao Yang

Tianjin Institute of Urology

Xiaodong Zhou

Tianjin Institute of Urology

Changyi Quan

Tianjin Institute of Urology

Zhongcheng Xin

Tianjin Institute of Urology

Yegang Chen

Tianjin Institute of Urology

Yuanjie Niu

Tianjin Institute of Urology

Research

Keywords: Prostate cancer, circ_0001686, miR-411-5p, SMAD3/TGFBR2, cell proliferation, migration and invasion

Posted Date: July 17th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-42527/v1>

Abstract

Background: Prostate cancer (PCa) is one of the most common malignancies in men. Circular RNAs (circRNAs) are known to be the important regulators in cancer progression. However, the role of circRNAs in PCa is yet to be investigated. Therefore, this study focuses on investigating the effect and the underlying molecular mechanisms of hsa_circ_0001686 (circ_0001686) in PCa.

Methods: Sample tissues were collected from the PCa patients to carry out the microarray expression profile of the human circRNAs. In addition, the expression levels of circ_0001686, has_miR-411-5p (miR-411-5p), SMAD3, and TGFBR2 were also detected by qRT-PCR. Next, transfection experiments were employed to measure the effect of circ_0001686 on cell proliferation, migration, and invasion in the PCa cell lines (CWR22RV1 and LNCaP). These effects were analyzed using MTT, colony formation, transwell, and scratch wound assays, respectively. The si-circ_0001686 was used as a negative control. Starbase and TargetScan databases were used to predict the putative binding sites among circ_0001686, miR-411-5p, and SMAD3/TGFBR2. The dual-luciferase reporter assays were performed to verify these interactions. Furthermore, the levels of SMAD3 and TGFBR2 in CWR22RV1 and LNCaP cells were measured by western blot. Finally, *in vivo* experiments in the nude mouse model were carried out to strengthen the *in vitro* findings.

Results: The expression of circ_0001686 was markedly up-regulated while the expression of miR-411-5p was down-regulated in PCa cells. Moreover, circ_0001686 promoted cell proliferation, migration, and invasion. Molecular mechanism exploration revealed that circ_0001686 acts as a sponge of miR-411-5p which affects the downstream target gene SMAD3, and TGFBR2. Both the *in vitro* and *in vivo* studies verified that miR-411-5p inhibits cancer growth and metastasis in PCa.

Conclusions: The circ_0001686 sequesters miR-411-5p to increase the expression of SMAD3/TGFBR2 which consequently promotes the proliferation, invasion, and migration in PCa cells.

Background

Worldwide, prostate cancer (PCa) is the most common non-epidermal malignant cancer in men[1]. In China, due to increased aging of the population, PCa associated morbidity and mortality are on the further rise[2]. Despite the significant improvements in the screening, diagnosis, and treatment, the overall survival rates in PCa are still poor [3]. Several genes have been associated with the development of PCa but their molecular mechanism in cancer progression is not yet completely understood[4]. Therefore, it is critical to elucidate the genetic mechanisms that could improve our understanding of the PCa pathogenesis. Additionally, novel biomarkers also need to be developed.

The circular RNAs (circRNAs), having characteristics covalently closed-loop structures, are the new class of non-coding RNAs (ncRNAs) that do not code for the proteins [5,6]. Recently, the circRNAs also gained greater attention due to their role in tumor progression [7]. These have been implicated in regulating tumorigenesis in several cancers, including gastric cancer [8], breast cancer [9], colorectal cancer [10],

malignant melanoma [11], and so on. Multiple evidence now suggests that an increasing number of circRNAs, including circMYLK, circUCK2, and circ_0044516, are associated with the development and progression of PCa [4,12,13]. However, there is a lack of scientific information about their expression specificity and sensitivity to be used as PCa biomarkers. Also, the understanding of their functional networks and mechanisms is inadequate. Therefore, these are the subjects of great interest and must be investigated in detail.

Similar to lncRNAs, circRNAs too act as miRNA sponges to absorb the functional microRNAs (miRNAs) [14,15]. The miRNAs, by directly binding to the 3'-untranslated regions (UTRs) of their target genes, regulate gene expression, cause mRNA cleavage, or lead to translational inhibition of their targets[16]. In this way, miRNAs impart significant effects on cell growth, apoptosis, migration, and carcinogenesis [17]. Thus, elucidating the roles of cancer-specific miRNAs is necessary [18]. A large number of miRNAs have been identified as tumor suppressors. For instance, miR-411-5p inhibits the growth and metastasis of bladder cancer by targeting the zinc transporter 1(ZnT1) protein [19]. Though, the role of miR-411-5p in PCa is yet unknown.

TGFBR2 and SMAD3 proteins, critical for cell proliferation, differentiation, and apoptosis, are the important components of the TGF- β signaling pathway [20]. Using a bioinformatics approach, through the Starbase database, we found that TGFBR2 and SMAD3 are the putative targets of miR-411-5p. However, the roles of TGFBR2 and SMAD3 in PCa are also unknown. It would be interesting to find out if miR-411-5p could function in PCa via targeting TGFBR2/SMAD3.

In this study, we started with the screening of circRNAs expression profiles using microarray and ultimately identified a significantly overexpressed circ_0001686 in the PCa patients. We then demonstrate that circ_0001686 function as a sponge of miR-411-5p which in turn regulates the expression of TGFBR2/SMAD3 and subsequently leads to the PCa tumor growth both *in vitro* and *in vivo* studies. Based on these results, we suggest that circ_0001686 could be used as a potential therapeutic target in PCa patients.

Materials And Methods

Patients and tissue specimens

After having the written informed consent, PCa tissue samples and adjacent normal tissues were collected from 30 PCa patients at the second Hospital of Tianjin Medical University from March 2018 to October 2019. None of these patients received chemotherapy or radiotherapy before the surgery. This study was approved and conducted as per the guidelines of the Ethics Committee of the second Hospital of Tianjin Medical University. All tissue specimens were stored at -80°C, until used for RNA extraction.

CircRNAs Microarray analysis

Microarray assays were carried out to screen the differential expression of the circRNAs between the three pairs of PCa tumors and adjacent normal tissues. The procedure for RNA extraction and microarray hybridization was performed as per the Arraystar's standard protocols, as described previously [4].

Data preprocessing

Preprocessing of the raw data, the original CEL files were carried out using Robust Multi-array Average (RMA) algorithm in affy package within Bioconductor (version 3.6) in R language (version 3.4.3). The process performed the background correction, quantile normalization, and probe summarization of the raw microarray data. Finally, we obtained the expression profile data for 29215 circRNAs.

Differential expression profile analysis of circRNAs

After the preprocessing of the raw datasets, identification of the differentially expressed circRNAs between normal tissue group and PCa tissue group was carried out by the Linear Models for Microarray Data (LIMMA, <http://www.bioconductor.org/packages/release/bioc/html/limma.html>) package in R language. The false positives were removed based on the adjusted P values using Benjamini and Hochberg false discovery rate method. The adjust P value < 0.05 and $|\log FC| \geq 1$ were the cut off criterion.

Cell culture

Human prostate epithelial cell line RWPE1 and prostate cancer cell lines (CWR22RV1 and LNCaP) were obtained from Cosmo Bio (Cosmobio., Tianjin, China). These were cultured in RPMI-1640 (Gibco-BRL, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin/penicillin in a 5% (v/v) CO₂ incubator at 37°C.

RNA transfection

For RNA transfection, CWR22RV1 and LNCaP cells (2×10^5 cells) were cultured with antibiotic-free complete medium in six-well plates. These cells were then transfected with pcDNA3.1/circ_0001686 or pcDNA3.1/NC, and si-circ_0001686 or si-NC, correspondingly. Transfection was carried out using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's protocol. The pcDNA3.1/circ_0001686 and si-circ_0001686 were synthesized by GenePharma (Shanghai, China).

For miRNA transfection, the miR-411-5p mimics (miR-411-5p mimics: 5'-AUCACAUUGCCAGGGAUUUC-3'), miR-411-5p mimics NC (mimics NC: 5'-UUCUCCGAACGUGUCACGUTT-3'), miR-411-5p antisense oligonucleotide (ASO-miR-411-5p: 5'-GUGGUAUCCUGGCAAUGUGAU-3' and ASO-NC (5'-CAGUACUUUUGUGUAGUACAA-3') were obtained from Sangon Biotech Co., Ltd. (Shanghai, China). Cells (1×10^5 cells/well) were seeded in six-well plates and transfected with miR-411-5p mimics, mimics NC, ASO-miR-411-5p, and ASO-NC, correspondingly using Lipofectamine 3000 reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) as per the manufacturer's protocol. After the 48 hours of transfection, the cells were harvested for further experiments.

RNA extraction and Quantitative RT-PCR

Total RNA from the CWR22RV1 and LNCaP cells was extracted using TRIZOL reagent (Invitrogen, USA). Using the Reverse Transcription System Kit (TaKaRa, Dalian, China), RNA was reversed-transcribed into cDNA as per the manufacturer's instructions. The qRT-PCR reactions were performed using the ABI7500 System and SYBR Green PCR Master Mix (Takara). The thermocycling parameters were 94°C for 4 min; 40 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 30 s; and 72°C 2min. The primers sequences used in the experiments were as following: has_circ_0001686: Forward, 5'-CTAGGAGTCACAGGAAGACATC-3'; Reverse, 5'-GTAGATCTCTCAGACTAGGTTG-3'; SMAD3-F: 5'- GCCCAGTGCCTAAGTGAT-3'; SMAD3-R: 5'-ACACTGAGCCAGAAGAGC-3'; TGFBR2-F: 5'-GGAATGTCTTGGGCAAATCT-3'; TGFBR2-R: 5'-ACCTGAATGCTTGTGCTTTTATT-3'; β -ACTIN-F: 5'-CCACATCGCTCAGACACCAT-3'; β -ACTIN-R: 5'-ACCAGGCGCCCAATACG-3'. miR-411-5p-F: 5'-GGGCTTAATGCTAATTGTGAT-3'; miR-411-5p-R: 5'-CAGTGCGTGTGCTGGAGT-3'; hsa-U6-F: 5'-AAAGACCTGTACGCCAACAC-3'; hsa-U6-R: 5'-GTCATACTCCTGCTTGCTGAT-3'. These primers were obtained from GenePharma (GenePharma, Shanghai, China). The U6 was used as a reference gene for miRNA normalization. β -actin was used for normalization in determining the levels of circ_0001686, SMAD3, and TGFBR2. The relative expression levels were measured using the $2^{-\Delta\Delta C_t}$ method [21].

Western blotting

Total protein from the CWR22RV1 and LNCaP cells was extracted using RIPA lysis buffer (Sigma, USA). The protein samples were separated using a 10% SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane. This was followed by membrane blocking with 5% non-fat milk for 2 hours. Then, the membranes were incubated overnight at 4°C with the corresponding primary antibodies: anti-GAPDH (1:1000, ab181602, Abcam, UK), anti-SMAD3 (phospho S467) (1:1000, ab52903, Abcam, UK), anti-SMAD3 (1:1000, ab92547, Abcam, UK), anti-TGFBR2 (1:1000, ab186838, Abcam, UK). After washing three times, membrane incubation with peroxidase-labeled secondary antibody (anti-rabbit IgG, 1:2000, ab6721, Abcam, UK) was carried out for 2 hours. Enhanced chemiluminescence (ECL) (ThermoFisher, USA) was used to visualize the protein bands and analysis was carried out using the Image Lab™ Software (Bio-Rad, USA).

MTT assay

The cell proliferation abilities of the CWR22RV1 and LNCaP cells were tested by MTT assay. Briefly, the transfected cells (1000 cells/well) were plated into 96-well plates, adhered overnight, and cultured at 37°C in 5% CO₂. Then, at 0 and 48 hours, 20 μ l of 5 μ g/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution in PBS was added to each well. After 4 hr of incubation, the formazan crystals were dissolved by adding 100 μ l of 10% SDS (sodium dodecyl sulfate). Finally, absorbance was measured at 490 nm using a microplate reader (Bio-Rad, USA).

Colony formation assay

For the colony formation assay, the transfected CWR22RV1 and LNCaP cells (200 cells/well) were seeded in 12-well plates. After seven days, the cells were stained with crystal violet. The colony formation ability was evaluated by the average colony count.

Transwell assay

For cell invasion analysis, Transwell chambers (Corning, USA) were used to perform the transwell assay. 200 µl of cells (0.1×10^6) were seeded in the upper chamber which was pre-coated with matrigel while 600 µl of DMEM containing 10% FBS was placed in the lower chamber. After a period of 24 hours incubation at 37°C, cells transversed on to the lower chamber were fixed in 1% formaldehyde and stained with 0.1% crystal violet. Both fixing and staining procedures were carried out for 20 min each. Positively stained cells were observed and counted at three randomly selected fields under a microscope (Olympus).

Scratch wound assay

For the cell migration analysis, the transfected cells (CWR22RV1 and LNCaP) were seeded into 12-well plates. Then, a 200 µl pipette tip was used to scratch the cell layers. These were maintained in DMEM with 10% FBS. The cells were photographed to record the wound width, at 0 and 48 hours, in an inverted microscope.

Cell apoptosis analysis

Cell apoptosis was measured using the Annexin V-FITC kit (Biosea Biotechnology Co., Beijing, China). For this, cells (5.0×10^5 cells/mL) resuspended in PBS buffer were subsequently double-stained with Annexin V-Alexa Fluor 647 and propidium iodide (PI). Finally, the cell apoptotic rate was measured using a flow cytometer (BD Biosciences, San Diego, CA, USA).

Flow cytometry analysis of CD44 expression

CD44 expression on prostate cells was evaluated using flow cytometry. Experiments were performed with FITC-conjugated mouse anti-human CD44 antibodies (BD Biosciences, San Diego, CA, USA) using a FACS Canto II instrument (BD Biosciences, San Diego, CA, USA). Data were analyzed with Cell Quest software (BD Biosciences).

Dual-luciferase reporter assay

Binding interaction between the circ_0001686 and miR-411-5p was predicted using the Starbase (<http://starbase.sysu.edu.cn/>) and the complementary target binding sites were established. The pmirGLO vectors with wild type or mutant miR-411-5p binding site in circ_0001686 3'-UTR were constructed. The 3'-UTR sequences of SMAD3 and TGFBR with corresponding binding sites for miR-411-5p were cloned into the pmirGLO3 reporter vectors (Promega, Madison, WI, USA) to generate the wild-type pmirGLO-SMAD3 (pmirGLO-SMAD3 wt) and the wild-type pmirGLO-TGFBR (pmirGLO-TGFBR wt) plasmids. Mutant reporter plasmids, mutant-type pmirGLO-SMAD3 (pmirGLO-SMAD3 mut) and mutant-

type mirGLO-TGFBR reporter (pmirGLO-TGFBR mut) were generated using the GeneArt™ Site-Directed Mutagenesis System (Thermo Fisher Scientific). To perform the luciferase assays, corresponding plasmids were transfected using Lipofectamine 3000 and the luciferase activity was measured using a dual-luciferase kit (Promega, USA).

***In vivo* mice model studies**

6 weeks old BALB/c male nude mice were obtained from the Animal Center of Tianjin Medical University and maintained in a pathogen-free facility. All procedures were performed as per the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of Tianjin Medical University. For the *in vivo* tumor studies, nude mice (9 mice per group) were subcutaneously injected into the right flank with CWR22RV1 cells (3×10^6 , 200 μ L) which were transfected with either miR-411-5p mimics or mimics NC. The growth of the resulting tumors was examined every 7 days. After 4 weeks of inoculation, mice were anesthetized using 1-3% isoflurane. Then, the tumors were excised to measure weight and volume, and photographs were taken. The tumor volume was calculated using the formula: Volume = (length $\times$ width²) / 2(mm³).

Statistical analysis

SPSS 22.0 (Chicago, IL, USA) and GraphPad Prism 7.0 (GraphPad, San Diego, CA, USA) were used to carry out the statistical analyses. The data are presented as the mean $\pm$ SD (standard deviation) from three independent experiments wherever applicable. Student t-test was used to assess the data between the two groups and the differences between various groups were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. $P < 0.05$ was considered to be statistically significant.

Results

Differential profiling of circRNAs reveals that circ_0001686 is upregulated in PCa

To identify the specific circRNAs that could be differentially expressed in PCa patients, three pairs of PCa tissue samples and matched non-tumor tissue samples were obtained from the patients. These were subjected to microarray analysis. The raw data were processed using the affy package in the R language and the differential expression analysis for circRNAs was performed using the LIMMA package.

From this, we obtained a total of 91 differentially expressed circRNAs that comprised of 29 upregulated circRNAs including the circ_0001686, and 62 down-regulated circRNAs. The corresponding heat map is shown in **Fig.1a**. First, we wanted to explore the mechanism for the aberrantly high expression of circRNAs in PCa. Among the 29 circRNAs, circ_0001686 levels showed the most significant difference between the PCa tumor and the healthy tissues. Hence, circ_0001686 was selected as the principal candidate for further analysis. To verify whether circ_0001686 indeed plays a role in PCa progression, we first measured the expression of circ_0001686 in PCa tissues and the PCa cell lines (CWR22RV1 and

LNCaP). As shown in **Fig.1b** and **1c**, the expression level of circ_0001686 was markedly higher both in the PCa tissue and PCA cell lines ($P < 0.05$, $P < 0.01$). These results clearly indicate the upregulation of circ_0001686 in PCa.

Circ_0001686 promotes the malignant progression of PCa cells

To study the biological role of circ_0001686 in PCa cells, respective plasmids pcDNA3.1/circ_0001686, pcDNA3.1/NC, circ_0001686 siRNA (si-circ_0001686) and si-NC were transfected into CWR22RV1 and LNCaP cells. The qRT-PCR results (**Fig.2a**) show that circ_0001686 expression increased in pcDNA3.1/circ_0001686 transfected cells compared with the pcDNA3.1/NC transfected cells ($P < 0.05$; $P < 0.01$). However, in a control experiment, the transfection of si-circ_0001686 in CWR22RV1 and LNCaP cells triggered an obvious abatement of circ_0001686 expression, compared to the si-NC transfected cells ($P < 0.01$). Furthermore, MTT and colony formation assay indicated that within the 48 hours of transfection, circ_0001686 promoted the proliferation of CWR22RV1 and LNCaP cells ($P < 0.05$; $P < 0.01$, **Fig.2b** and **2c**). Here too, control experiments with si-RNAs confirmed that these results were indeed due to the increased levels of circ_0001686. Similarly, in the Transwell assay, we found that pcDNA3.1/circ_0001686 transfection increased the cell migration abilities of CWR22RV1 and LNCaP cells, though it was suppressed upon transfection with si-circ_0001686 (**Fig.2d**).

Circ_0001686 may directly bind to miR-411-5p in PCa cells

Since circRNAs are known to function as miRNA sponges to regulate the target gene expression, we next examined for the potential miRNAs that could associate with circ_0001686. Based on Starbase prediction, we found that circ_0001686 possess putative complementary sequences to the miR-411-5p seed region. To test these interactions, luciferase reporter assays were carried out. As shown in **Fig.3a**, circ_0001686 significantly inhibited luciferase activity of wild type reporter plasmid having miR-411-5p. On the contrary, si-circ_0001686 significantly increased luciferase activity of wild type reporter for miR-411-5p. Besides, miR-411-5p expression was also found to be decreased when tested by qRT-PCR in CWR22RV1 and LNCaP cells (**Fig.3b**). The circ_0001686 noticeably suppressed the expression of miR-411-5p in both cells (**Fig.3c**). Overall, these results indicate that circ_0001686 specifically interacted with miR-411-5p in PCa cells.

miR-411-5p inhibits the proliferation, invasion of PCa cells

The expression analysis of miR-411-5p was carried out using qRT-PCR in the transfected PCa cell lines (CWR22RV1 and LNCaP). As shown in **Fig.4a**, miR-411-5p expression was significantly higher upon transfection with miR-411-5p mimics compared with the mimics NC transfected PCa cells ($P < 0.05$; $P < 0.01$). Moreover, in control experiments with antisense oligonucleotides, compared to the ASO-NC group, ASO-miR-411-5p transfection markedly decreased the expression of miR-411-5p ($P < 0.01$), suggesting for the successful transfection of miR-411-5p mimics or ASO-miR-411-5p.

Furthermore, MTT assay revealed that miR-411-5p significantly repressed the proliferative ability of CWR22RV1 and LNCaP cells within 48 hours ($P < 0.05$; $P < 0.001$, **Fig.4b**). Similarly, colony formation assay also revealed that cell proliferation was significantly decreased by transfection of miR-411-5p mimics while increased by ASO-miR-411-5p (**Fig.4c**). Also, the migration and invasion of CWR22RV1 and LNCaP cells were suppressed by miR-411-5p (**Fig.4d** and **4e**). However, miR-411-5p promoted apoptosis in the CWR22RV1 and LNCaP cells ($P < 0.01$, **Fig.4f**).

CD44 expression has been associated with the migration and invasion of prostate cancer cells (22). Therefore, the expression of CD44 was detected by flow cytometry, post-transfection of miR-411-5p mimics, or ASO-miR-411-5p. In the CWR22RV1 and LNCaP cells, the CD44 population was smaller in the miR-411-5p mimics group when compared to the mimics NC group. In contrast, in the control experiments, the CD44 population increased in the ASO-miR-411-5p group than that in the ASO-NC group (**Fig.4g**). Overall, these results suggest that miR-411-5p inhibits the proliferation, invasion of PCa cells.

miR-411-5p targeted SMAD3 and TGFBR2 in PCa cells

TargetScan, with a significantly high score, predicted that the 3'-UTR of SMAD3 and TGFBR2 could be the target of miR-411-5p (**Fig.5a** and **5b**). These interactions were tested in luciferase reporter assays. Results show that miR-411-5p mimics decreased the luciferase activity of the wild-type reporter vector carrying SMAD3 or TGFBR2 whereas ASO-miR-411-5p showed an increase in the luciferase activity of the reporter vector that contained the mutant binding sites of SMAD3 or TGFBR2 (**Fig.5a** and **5b**). To further analyze the role of miR-411-5p in SMAD3 and TGFBR2 mediated regulation, qRT-PCR and western blot assays were carried out. Results show that miR-411-5p mimics inhibited the levels of SMAD3 and TGFBR2 in CWR22RV1 and LNCaP cells, both at the transcript and protein levels (**Fig.5c** and **5d**). However, TGF- β reversed the lower level of miR-411-5p-induced SMAD3/TGFBR2 expression. These results suggest that SMAD3 and TGFBR2 are the direct target genes of miR-411-5p. Next, we carried out qRT-PCR and western blotting analysis to measure the levels of SMAD3 and TFRBR2. The results show that the both mRNA and protein levels of SMAD3 and TFRBR2 were increased upon pcDNA3.1/circ_0001686 transfection, but decreased in si-circ_0001686 transfected CWR22RV1 and LNCaP cells ($P < 0.05$; $P < 0.01$; $P < 0.001$, **Fig.5e** and **5f**). Interestingly, TGF- β could alter the lower level of SMAD3/TGFBR2 expression which was induced by si-circ_0001686.

miR-411-5p suppresses prostate tumor growth and metastasis

To further investigate whether miR-411-5p would inhibit tumor growth *in vivo*, CWR22RV1 cells transfected with miR-411-5p mimics or mimics NC were inoculated into male nude mice. Xenograft tumors were examined 4 weeks after the inoculation (**Fig.6a** and **6b**). We found that tumor volumes and weights in the miR-411-5p mimics group were significantly lower than those in mimics NC group (**Fig.6c** and **6d**). These data strongly suggest that miR-411-5p could suppress tumorigenesis of PCa *in vivo*.

Discussion

Among the most common cancers, prostate cancer (PCa) is ranked second for the mortality rate in male malignancy [23]. Therefore, finding a novel diagnostic marker is vital for the early screening of the disease. Several previous studies have associated circRNAs with PCa [23]. CircRNAs possess two important properties that they are highly conserved and have a remarkably stable half-life of more than 48 hours [24]. This makes them a promising tumor marker candidate in PCa diagnosis [25]. miRNAs are known to regulate many physiological and pathological processes in cancers [26]. They function by post-transcriptionally modulating the translation of target mRNAs via corresponding miRNA response elements (MRE) [27]. Interestingly, circRNAs are reported to function as a miRNA sponge, their potential role in regulating cancer-related genes by fine-tuning the target miRNAs has been recognized recently. For example, in gastric cancer, miR-411-5p acts as a tumor suppressor by targeting the SETD6/NF- κ B pathway [28]. The miR-411-5p is also associated with non-small cell lung cancer (NSCLC) cells via targeting PUM1 [29].

In this study, using bioinformatics and the high-throughput microarray technology, we identified several differentially expressed circRNAs. Firstly, microarray studies were carried out to analyze the expression profiles of the human circRNAs. We identified a total of 91 differentially expressed circRNAs; among these 29 circRNAs were up-regulated while the other 62 were down-regulated. These circRNAs might participate in PCa tumorigenesis and play an important role in tumor development. Among the up-regulated circRNAs, the circ_0001686 was further validated in PCa tissue. Based on the results, we found that circ_0001686 expression was indeed up-regulated in PCa tissues and PCa cell lines. Interestingly, inhibition of circ_0001686 expression led to decreased cell proliferation and metastasis in PCa cells. Furthermore, we found that circ_0001686 functions as a sponge for miR-411-5p, and by doing so it regulates SMAD3/TGFBR2 to promote PCa.

miR-34a is shown to downregulate CD44 that inhibit cancer stem cells and metastasis in PCa [30]. CD44 leads to increased cell migration and invasion in PCa cells [22]. In this study too, we found that miR-411-5p mimics could suppress CD44 expression in CWR22RV1 and LNCaP cells. These results suggest that similar to gastric cancer and bladder cancer, miR-411-5p could function as a tumor suppressor in PCa too [19,28,29]. The anti-prostate cancer mechanisms elicited by carbon ion radiotherapy (CIRT) also involve exosomal miRNAs including miR-411-5p [31]. Interestingly, we also found that miR-411-5p was down-regulated in PCa and could be a key target for circ_0001686.

Next, using TargetScan we predicted the downstream target for miR-411-5p and found that SMAD3/TGFBR2 could be the potential candidates. These interactions were confirmed in luciferase assays. Moreover, we found that miR-411-5p down-regulated the levels of SMAD3 and TGFBR2. In the TGF- β signaling pathway, TGFBR2 binds to TGF- β which then phosphorylates the downstream targets. The activated kinase subsequently phosphorylates SMAD3 (p-SMAD3) to form a heteromeric complex with SMAD4 and gets translocated into the nucleus to regulate the expression of the target gene. This is critical for cell proliferation, differentiation, and apoptosis [20,32]. Previous research suggests that TR4 might function by down-regulating miR-373-3p to alter the expression of TGFBR2/p-SMAD3 which increases the PCa metastasis [33]. Similarly, in our study too, the TGFBR2 and SMAD3 expression were

up-regulated by the si-circ_0001686 which otherwise supposed to be down-regulated by the miR-411-5p. Furthermore, *in vivo* experiments also showed that miR-411-5p could inhibit the tumor progression in PCa. Thus, our results revealed that circ_0001686 promotes cell growth and metastasis by sponging miR-411-5p, which subsequently up-regulates TGFBR2/SMAD3 expression and leads to tumorigenesis. A hypothetical model to summarize such a regulation is depicted in **Fig. 7**.

Conclusion

Our study explored the circRNAs expression profiles in PCa tissue and identified that circ_0001686 was up-regulated in PCa. Moreover, we demonstrated that circ_0001686 could promote PCa cell proliferation, migration, and invasion both *in vitro* and *in vivo* via specifically sponging miR-411-5p which upregulated SMAD3/TGFBR2. Overall, these results suggest an innovatively regulatory mechanism for the function of circ_0001686 in PCa which might be explored as a potential therapeutic target.

Abbreviations

circRNA: Circular RNA; ncRNAs: non-coding RNAs; miRNAs; microRNAs; PCa: Prostate cancer.

Declarations

Acknowledgements

The authors thank all patients involved in this study.

Funding

This work was supported by The Science&Technology Development Fund of Tianjin Education Commission for Higher Education (No.2018KJ050), Youth Fund of the Second Hospital Tianjin Medical University (No.2018ydey07), Traditional Chinese medicine combined with Western medicine research project (2019137) and Natural Science Foundation of Tianjin (No. 19JCYBJC26900).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

QC, PJ performed experiments, analyzed data and wrote the paper; EL, CF, ZY, GH and XZ performed some experiments and analyzed data; QC, CQ, ZX, YC and YN initiated the study, designed experiments and wrote the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of The Second Hospital of Tianjin Medical University. All written informed consents were received from all patients.

Consent for publication

All authors have read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Author details

Department of Urology, the second hospital of Tianjin Medical University, Tianjin Institute of Urology, Tianjin, 300211, China.

References

1. Mohler JL, Armstrong AJ, Bahnson RR, D'Amico AV, Davis BJ, Eastham JA, et al. Prostate Cancer, Version 1.2016. Journal of the National Comprehensive Cancer Network : JNCCN. 2016;14(1):19-30.
2. Sebesta EM, Anderson CB. The Surgical Management of Prostate Cancer. Seminars in oncology. 2017;44(5):347-57.
3. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. CA: a cancer journal for clinicians. 2020;70(1):7-30.
4. Li T, Sun X, Chen L. Exosome circ_0044516 promotes prostate cancer cell proliferation and metastasis as a potential biomarker. Journal of cellular biochemistry. 2020;121(3):2118-26.
5. Bolha L, Ravnik-Glavač M, Glavač D. Circular RNAs: Biogenesis, Function, and a Role as Possible Cancer Biomarkers. International journal of genomics. 2017;2017:6218353.
6. Meng S, Zhou H, Feng Z, Xu Z, Tang Y, Li P, et al. CircRNA: functions and properties of a novel potential biomarker for cancer. Molecular cancer. 2017;16(1):94.
7. Luan W, Shi Y, Zhou Z, Xia Y, Wang J. circRNA_0084043 promote malignant melanoma progression via miR-153-3p/Snail axis. Biochemical and biophysical research communications. 2018;502(1):22-9.
8. Li P, Chen S, Chen H, Mo X, Li T, Shao Y, et al. Using circular RNA as a novel type of biomarker in the screening of gastric cancer. Clinica chimica acta. 2015;444:132-6.
9. Liang H-F, Zhang X-Z, Liu B-G, Jia G-T, Li W-L. Circular RNA circ-ABCB10 promotes breast cancer proliferation and progression through sponging miR-1271. American journal of cancer research. 2017;7(7):1566.
10. Huang G, Zhu H, Shi Y, Wu W, Cai H, Chen X. cir-ITCH plays an inhibitory role in colorectal cancer by regulating the Wnt/ β -catenin pathway. PloS one. 2015;10(6).

11. Yang Y, Liu G, Yuan D, Li C, Xue M, Chen L. Influence of exosome-derived miR-21 on chemotherapy resistance of esophageal cancer. *European review for medical and pharmacological sciences*. 2019;23(4):1513-9.
12. Dai Y, Li D, Chen X, Tan X, Gu J, Chen M, et al. Circular RNA Myosin Light Chain Kinase (MYLK) Promotes Prostate Cancer Progression through Modulating Mir-29a Expression. *Medical science monitor : international medical journal of experimental and clinical research*. 2018;24:3462-71.
13. Xiang Z, Xu C, Wu G, Liu B, Wu D. CircRNA-UCK2 Increased TET1 Inhibits Proliferation and Invasion of Prostate Cancer Cells Via Sponge MiRNA-767-5p. *Open medicine (Warsaw, Poland)*. 2019;14:833-42.
14. Chen L, Zhang S, Wu J, Cui J, Zhong L, Zeng L, et al. circRNA_100290 plays a role in oral cancer by functioning as a sponge of the miR-29 family. *Oncogene*. 2017;36(32):4551-61.
15. Chen LL. The biogenesis and emerging roles of circular RNAs. *Nature reviews Molecular cell biology*. 2016;17(4):205-11.
16. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *cell*. 2004;116(2):281-97.
17. Bueno MJ, de Castro IP, Malumbres M. Control of cell proliferation pathways by microRNAs. *Cell cycle*. 2008;7(20):3143-8.
18. Cummins J, Velculescu VE. Implications of micro-RNA profiling for cancer diagnosis. *Oncogene*. 2006;25(46):6220-7.
19. Liu Y, Liu T, Jin H, Yin L, Yu H, Bi J. MiR-411 suppresses the development of bladder cancer by regulating ZnT1. *OncoTargets and therapy*. 2018;11:8695-704.
20. Finnson KW, Chi Y, Bou-Gharios G, Leask A, Philip A. TGF- β signaling in cartilage homeostasis and osteoarthritis. *Frontiers in bioscience (Scholar edition)*. 2012;4:251-68.
21. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(Delta Delta C(T)) Method. *Methods (San Diego, Calif)*. 2001;25(4):402-8.
22. Senbanjo LT, Chellaiah MA. CD44: A Multifunctional Cell Surface Adhesion Receptor Is a Regulator of Progression and Metastasis of Cancer Cells. *Frontiers in Cell and Developmental Biology*. 2017;5(18).
23. Xia Q, Ding T, Zhang G, Li Z, Zeng L, Zhu Y, et al. Circular RNA Expression Profiling Identifies Prostate Cancer- Specific circRNAs in Prostate Cancer. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology*. 2018;50(5):1903-15.
24. Hansen TB, Jensen TI, Clausen BH, Bramsen JB, Finsen B, Damgaard CK, et al. Natural RNA circles function as efficient microRNA sponges. *Nature*. 2013;495(7441):384-8.
25. Zhou X, Natino D, Qin Z, Wang D, Tian Z, Cai X, et al. Identification and functional characterization of circRNA-0008717 as an oncogene in osteosarcoma through sponging miR-203. *Oncotarget*. 2018;9(32):22288.
26. Hayes J, Peruzzi PP, Lawler S. MicroRNAs in cancer: biomarkers, functions and therapy. *Trends in molecular medicine*. 2014;20(8):460-9.
27. Bartel DP. MicroRNAs: target recognition and regulatory functions. *cell*. 2009;136(2):215-33.

28. Bai TL, Liu YB, Li BH. MiR-411 inhibits gastric cancer proliferation and migration through targeting SETD6. *European review for medical and pharmacological sciences*. 2019;23(8):3344-50.
29. Xia LH, Yan QH, Sun QD, Gao YP. MiR-411-5p acts as a tumor suppressor in non-small cell lung cancer through targeting PUM1. *European review for medical and pharmacological sciences*. 2018;22(17):5546-53.
30. Liu C, Kelnar K, Liu B, Chen X, Calhoun-Davis T, Li H, et al. The microRNA miR-34a inhibits prostate cancer stem cells and metastasis by directly repressing CD44. *Nature medicine*. 2011;17(2):211-5.
31. Yu Q, Li P, Weng M, Wu S, Zhang Y, Chen X, et al. Nano-Vesicles are a Potential Tool to Monitor Therapeutic Efficacy of Carbon Ion Radiotherapy in Prostate Cancer. *Journal of biomedical nanotechnology*. 2018;14(1):168-78.
32. Bedan M, Grimm D, Wehland M, Simonsen U, Infanger M, Krüger M. A Focus on Macitentan in the Treatment of Pulmonary Arterial Hypertension. *Basic & clinical pharmacology & toxicology*. 2018;123(2):103-13.
33. Qiu X, Zhu J, Sun Y, Fan K, Yang DR, Li G, et al. TR4 nuclear receptor increases prostate cancer invasion via decreasing the miR-373-3p expression to alter TGF β R2/p-Smad3 signals. *Oncotarget*. 2015;6(17):15397-409.

Figures

Figure 1

Differential expression analysis of circRNAs in PCa where circ_0001686 was significantly upregulated. (a) The heat map showed the top fifteen most increased and decreased circRNAs in PCa tissues as compared to that in the matched non-tumor tissues analyzed by circRNAs Arraystar Chip. (d) qRT-PCR expression analysis of circ_0001686 in PCa tissue and normal tissue; N=30. (c) qRT-PCR expression analysis of circ_0001686 in PCa (CWR22RV1 and LNCaP) cells; **P<0.01, ***P<0.001.

Figure 2

Circ_0001686 promotes the malignant progression of PCa cells. (a) The transfection efficiency measurement of circ_0001686 in the CWR22RV1 and LNCaP cells by qRT-PCR. (b) MTT results of the proliferation of transfected cells at the indicated time points (0, 48 h). (c) Knockdown of circ_0001686 significantly suppressed the colony formation capacity of transfected cells. (d) Transwell assay showed that si-circRNA reduced the migration activity in CWR22RV1 and LNCaP cells (200 μ m). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 3

Circ_0001686 directly binds to miR-411-5p in PCa cells. (a) The putative sequences of miR-411-5p and circ_0001686 with binding sites are shown. miR-411-5p significantly inhibited luciferase activity of wild type reporter for circ_0001686. (b) miR-411-5p was down-regulated by qRT-PCR detection in PCa cells (CWR22RV1 and LNCaP). (c) qRT-PCR was conducted to measure miR-411-5p expression in circ_0001686 transfected cells. *P < 0.05, **P<0.01, ***P<0.001

Figure 4

miR-411-5p inhibits the proliferation, invasion of PCa cells. (a) miR-411-5p mimics, ASO-miR-411-5p were transfected into CWR22RV1 and LNCaP cells, and qRT-PCR was conducted to measure miR-411-5p expression. (b) The proliferation of cells transfected with miR-411-5p mimics, ASO-miR-411-5p detected by MTT assay. (c) miR-411-5p significantly suppressed the colony formation capacity of transfected cells. Transwell assay (d) and scratch wound assay (e) showed that miR-411-5p reduced the migration activity in CWR22RV1 and LNCaP cells (200 μm). (f) Apoptosis assay was performed by flow cytometry. (g) Flow cytometry analysis of CD44 expression in CWR22RV1 and LNCaP cells. *P < 0.05, **P<0.01, ***P<0.001.

Figure 5

miR-411-5p targets SMAD3 and TGFB2 in PCa cells. (a,b) The putative sequence of miR-411-5p and SMAD3/TGFB2 with binding sites. miR-411-5p significantly inhibited luciferase activity of wild type reporter for SMAD3/TGFB2 in CWR22RV1 and LNCaP cells. (c) SMAD3 and TGFB2 mRNA expression was down-regulated by miR-411-5p. (d) SMAD3 and TGFB2 protein levels were down-regulated by miR-411-5p mimics. The mRNA (e) and protein (f) levels of SMAD3 and TGFB2 were regulated by circ_0001686. *P < 0.05, **P < 0.01, ***P < 0.001

Figure 6

miR-411-5p suppresses tumor growth of prostate cancer in vivo. (a) Photographs of tumors excised from the nude mice derived 4 weeks after subcutaneous inoculation of CWR22RV1 cells transfected with miR-411-5p mimics or mimics NC. (b) Mice weights were measured every 7 days after inoculation. (c) Tumor volumes were calculated every 7 days after inoculation. (d) Tumor weights of xenograft tumors were measured and analyzed. Bars indicate SD. ** $P < 0.01$.

Figure 7

The hypothetical model to summarize the mechanism of circ_0001686 induced tumor growth via miR-411-5p in PCa.