EXCLI Journal 2019;18:1049-1061 – ISSN 1611-2156
Received: June 05, 2019, accepted: October 09, 2019, published: November 07, 2019

Original article:

MICRORNA-9-5P FUNCTIONS AS A TUMOR SUPPRESSOR IN PROSTATE CANCER VIA TARGETING UTRN

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http://dx.doi.org/10.17179/excli2019-1512

ABSTRACT

Accumulating evidence indicates that miR-9-5p plays an important role in several diseases, especially tumor progression. In this study, we investigated the clinical significance and biological function of miR-9-5p in prostate cancer (PCa). Using quantitative real time PCR (qRT-PCR) analysis, we found miR-9-5p level was significantly down-regulated in PCa tissues and cell lines. The decreased miR-9-5p expression was associated with tumor size, preoperative PSA, Gleason score and lymph node metastasis. Kaplan-Meier survival analysis showed patients with low level of miR-9-5p had significantly decreased rates of overall survival (OS). Multivariate analyses showed that miR-9-5p was an independent predictor of PCa patients' prognosis. Through CCK-8 and Transwell assays, miR-9-5p overexpression by miR-9-5p mimics transfection was demonstrated to suppress the proliferation, migration and invasion of PCa cells. Mechanistically, luciferase reporter assay, qRT-PCR and Western blot demonstrated that Utrophin (UTRN) is a direct target of miR-9-5p in PCa cells. The status of UTRN protein in PCa tissues was much higher than that in adjacent tissues by immunohistochemical staining and its mRNA levels were inversely correlated with miR-9-5p in PCa tissues. Importantly, UTRN knockdown by siUTRN imitated the suppressive effects of miR-9-5p on cell proliferation, migration and invasion in PCa. In summary, miR-9-5p might novel prognostic biomarker in and targeting UTRN by miR-9-5p could be potential therapeutic candidates for PCa.

Keywords: Prostate cancer, miR-9-5p, UTRN, prognosis, cell proliferation, migration

INTRODUCTION

Prostate cancer (PCa) has long been one of the most dangerous malignant tumors among males and has surpassed lung cancer as the most frequent cancer entity in many countries (Mohler et al., 2016). It is estimated that PCa will be projected to occur in 161,360 men in the United States and that 26,730 will die from their disease in 2017 (Siegel et al., 2017). In East Asia, there were approximately

122,000 incidences and 42,000 deaths (Baade et al., 2013). The growth of PCa is androgen-dependent, hormone-ablation therapy with anti-androgens and/or androgen-deprivation therapies has become an important component of the treatment (Otsuki et al., 2017). However, most PCa patients ultimately develop a recurrent independent form (castration-resistant PCa) after a median time of 18 months (Ko et al., 2018). In recent years, multiple studies have identified the involvement of microRNAs (miRNAs) in the malignant transformation and progression of PCa, thus, understanding the clinical and pathological implications of miRNAs in PCa may provide therapeutic guidance.

MiRNAs represent a class of small, noncoding RNAs that regulate gene expression in many cellular processes (Balacescu et al., 2018). Despite the first miRNA, lin-4 was discovered in the worm *Caenorhabdities elegans* (*Ce*), it didn't draw much attention until the identification and characterization of let-7, a novel miRNA that is required for developmental timing in *Ce* (Reinhart et al., 2000). To date, more than 2000 genes that encode miRNAs have been found in human genome (Acunzo et al., 2015). These molecules typically degrade or translationally inhibit their target mRNAs, including those of genes that involved in carcinogenesis, including cell proliferation, apoptosis, differentiation, and cell cycle progression (Shenoy and Blelloch, 2014). miRNA-target chimeras is initiated by incomplementary base paring interaction between the 5'-untranslated region (UTR) of the miRNA and the binding sequence within the coding region or the 3'UTR of their mRNAs (Hoffman et al., 2016). miR-9-5p is a member of the miRNA family that has been reported to be primarily expressed in the central nervous system (Barbano et al., 2017). It was found to inhibit the transformation of fibroblasts into myofibroblasts through targeting TGBR2 and NOX4 (Fierro-Fernandez et al., 2015). Sun et al. (2016) showed that miR-9-5p impairs proliferation, differentiation and adhesion of skeletal cells. The involvement and the role played by miR-9-5p in breast cancer revealed that miR-9-5p is associated with hormone receptor status and patients' survival (Barbano et al., 2017). However, biologic and clinical significance of miR-9-5p remains unknown in PCa.

Utrophin (UTRN) encodes a dystrophin-related protein that shows protein sequence homology, size, and functional properties with dystrophin, and overexpression of UTRN enables functional of dystrophin-deficient skeletal muscle (Perkins and Davies, 2018). Integrated genomic and functional analyses revealed that UTRN is located at 6q24 and capable of inducing cancer cell tansformation when expressed at a low level, implying an antioncogenic role in multiple tumors (Li et al., 2007). Whereas, Lomnytska et al. (2006) observed significantly increased expression of UTRN in plasma of ovarian and breast cancer patients. Recently, knockdown of UTRN by using lentivirus-mediated system was found to reduce the cellular proliferation and survival in human glioma (Shen et al., 2016). The previous meta-analysis by Wang et al. (2013) identified UTRN as an overlapped differentially expressed gene in PCa and suggesting the importance of UTRN in tumor progression.

In the current study, the expression of miR-9-5p in PCa was examined and its clinical significance and correlation to overall survival were analyzed. Functional assays were employed to reveal the effects of miR-9-5p on PCa proliferation, migration and invasion *in vitro*. Using computational molecular biology and luciferase reporter assay, we identified the PCa-related gene UTRN as a direct target of miR-9-5p. This study on the role of miR-9-5p/UTRN network in PCa progression will no doubt enrich of knowledge of PCa etiology and treatment.

MATERIALS AND METHODS

Patients' and tissues' samples

A total of 78 pairs of fresh surgical resected tumor tissues and adjacent matched tissues were collected from patients with PCa,

EXCLI Journal 2019;18:1049-1061 – ISSN 1611-2156
Received: June 05, 2019, accepted: October 09, 2019, published: November 07, 2019

who were undergone a radical prostatectomy, and received no chemotherapy, radiation therapy or androgen-deprivation treatment in The Fifth Affiliated Hospital of Sun Yat-sen University. Collected fresh tissues were immediately frozen in liquid nitrogen and stored at -80 °C for RNA extraction. Some clinicopathological characteristics for all patients, including tumor size, Gleason score and biochemical recurrences were summarized in Table 1. The beginning of the follow-up period was defined as the date of prostatectomy and the follow-up period lasted for five years. For immunohistochemistry, paraffin-embedded tissue sections (n= 45), including 26 PCa tissues and 19 adjacent normal prostate tissues were obtained from The Fifth Affiliated Hospital of Sun Yat-sen University. All patients signed the informed consent before enrolled in this research. The study was performed according to the principles of the Helsinki Declaration, which was approved by the Ethical Committee of The Fifth Affiliated Hospital of Sun Yat-sen University (SYU-2017-42).

Cell lines and culture condition

Human PCa cell lines (LNCaP, DU145, 22RV1, and PC-3), normal prostate epithelial cell line RWPE-1 and 293T cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (GIBCO, Grand Island, NY, USA) with 10 % fetal bovine serum (FBS, GIBCO) and maintained in a humidified atmosphere containing 5 % CO_2 at 37 °C.

Table 1: Association between miR-9-5p expression and clinicopathological variables of patients with prostate cancer

Variable	Cases (n=78)	Expression of miR-9-5p		*P* value
		Low (n=44)	High (n=34)	(chi-square test)
Age				0.605
< 60	47	24	23	
≥ 60	31	20	11	
Tumor size (cm)				**0.003**
< 4	43	17	26	
≥ 4	35	27	8	
Preoperative PSA				**0.004**
< 4 ng/ml	3	1	2	
4-10 ng/ml	20	4	16	
>10 ng/ml	55	39	16	
Pathological stage				0.054
I-II	51	21	30	
III-IV	27	23	4	
Gleason score				**< 0.001**
< 7	24	7	17	
7	33	17	16	
≥ 8	21	20	1	
Lymph node metastasis				**0.002**
No	68	35	33	
Yes	10	9	1	
BCR				0.061
Negative	59	28	31	
Positive	19	16	3	

PSA, prostate-specific antigen; BCR, biochemical recurrence; BCR was described as postoperative serum PSA ≥ 0.2 ng/ml

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Oligonucleotide transfection

The miR-9-5p mimics (5′-CGAGCTCT-GTGTGTGTGTGTGTGTG-3′), negative control (miR-NC: 5′-UUCUCCGAACGUG-UCACGUTT-3′), small interfering RNA that targeted UTRN (siUTRN: 5′-CUCCAU-UAGCUGAGUUCUAACAAG-3′) and scrambled siNC (5′-UGACCUCAAC-UACAUGGUUTT-3′) were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). For cell transfection, LNCaP and DU145 were seeded in six-well plates at a density of 2×10^5 cells per well and cultured until 60–80 % confluency, followed by transfection with the above oligonucleotides at a final concentration of 50 nM using Lipofectamine^{TM} 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

Quantitative real time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from surgically resected fresh tissues and cell lines using TRIZOL reagent and then reversely transcribed by using Superscript III kit (all from Invitrogen) according to the manufacturer's instructions. For the quantification of miR-9-5p, the PCR amplification was performed with TaqMan miRNA Assay kit (Applied Biosystems, Foster City, CA, USA) using the primers (forward: 5′-GTGCAGGGTCCGAGGT-3′ and reverse: 5′-GCGCTCTTTGGTTATCTAGC-3′) on ABI Prism 7700 system (Applied Biosystems, USA). For UTRN mRNA, the PCR amplification was conducted using SYBR Green Real time PCR Master Mix (Takara Bio, Inc., Shiga, Japan) with the primers (forward: 5′-CAAACACCCTCGACTTGGTT-3′ and reverse: 5′-TGGTGGAGCTGCTATCA-GTG-3′) on ABI Prism 7700 system (Applied Biosystems, USA) according to the manufacturer's protocols. Each sample was analyzed in triplicates and the expression of miR-9-5p and UTRN relative to U6 and GAPDH, respectively was calculated using the $2^{-\Delta\Delta Ct}$ method.

Cell proliferation assay

Cell proliferation rate was determined using Cell Counting Kit-8 (CCK-8, Beyotime Institute of Biotechnology, Haimen, China) following the instructions of manufacturer. In brief, PCa cells were seeded at a density of 3,000/well in 96-well plates and incubated with 10 µl CCK-8 reagent at the indicated time points (24, 48, 96 and 120 h, respectively). The absorbance value was measured at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA). The experiments were independently performed in triplicate.

Cell migration and invasion assays

The Transwell chambers (pore size, 8.0 µm, EMD Millipore, Billerica, MA, USA) were used to assess cell migration and invasion ability without or with 10 % Matrigel (BD Biosciences), respectively. Briefly, approximately 1.5×10^4 cells in 200 uL of serum-free medium were added to in the upper chamber of the Transwell chamber, while 500 µl medium containing 10 % FBS used as an attractant was added to the lower chamber. After 24 h incubation at 37 °C, the cells that had migrated or invaded in the lower side of the chamber were fixed in 4 % paraformaldehyde and stained with 0.2 % crystal violet (Sigma-Aldrich Co.) at room temperature. Finally, stained cells in five random high-power microscopic fields (200× magnification) were photographed and counted under a light microscope (Olympus Corporation). The experiments were independently performed in triplicate.

Target prediction and luciferase reporter assay

The online algorithm TargetScan (http://www.targetscan.org) and miRanda (http://www.microrna.org) were used to predict the targets of miR-9-5p. For luciferase reporter assay, a wild-type 3′-untranslated region UTRN (WT-3'UTR-UTRN) containing the putative miR-9-5p binding sequence and a mutant UTRN (MUT-3′-UTR-UTRN) specific for miR-9-5p binding site were amplified and inserted into the psi-CHECK2 vector

(Promega Corporation, Madison, WI, USA) to generate WT-UTRN and MUT-UTRN reporter vectors, respectively. The 293T cells at a density of 1×10^4 cells/well were seeded in 96-well plates and cultured overnight, followed by co-transfection with miR-9-5p mimics or miR-NC (40 nM) and reporter vectors (0.3 μg/mL) using Lipofectamine™ 2000. After 48 h transfection, the cells were harvested and relative luciferase activity was calculated using the Dual-Luciferase Reporter Assay System (Promega Corporation). The experiments were independently performed in triplicate.

Western blot

Total protein was extracted using RIPA buffer (Cell Signaling Technology, Inc., Danvers, MA, USA) and quantified by BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). Equal amounts of protein (30 μg) were separated by 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes (EMD Millipore, Billerica, MA, USA) in semi-dry Trans-Blot Turbo system (Bio-Rad). Membranes were blocked 5 % non-fat milk in PBS containing 0.5 % Tween-20 (PBST) for 1 h and subsequently incubated overnight at 4 °C with specific primary antibodies against UTRN (1:500, ab159789, Abcam, Cambridge, UK) and GAPDH (1:5000, ab9482, Abcam) served as a loading control. After being washed with PBST, the membranes were incubated with horse radish peroxidase (HRP)-coupled secondary antibodies (1:5000, Santa Cruz, CA, USA) and visualized by enhanced chemiluminescence (Thermo Scientific).

Immunohistochemical (IHC) staining

IHC staining was performed to detect the expression of UTRN in PCa and adjacent tissues according to a previous report (Wu et al., 2013). Briefly, the paraffin-embedded sections were cut into 4-μm thickness slides and deparaffinized with xylene. After rehydration with gradient concentrations of ethanol, microwave antigen retrieval was performed with EDTA buffer (pH = 8.0) for 30 min. Subsequently, endogenous peroxidase was blocked in 0.2 % H_2O_2 and the slides were incubated with primary antibody against UTRN (1:500, ab159789, Abcam) overnight at 4 °C, followed by incubation with HRP-conjugated secondary antibody for 30 min at 37 °C. The IHC staining of UTRN was independently evaluated by two experienced pathologists. The staining percentage with a score of 0–4 was assigned (0, 0-5 %; 1, 6–20 %; 2, 21–60 %; 3, 61–75 %; or 4, 76–100 %). The staining intensity was graded as 0 (no staining), 1 (weak), 2 (moderate) and 3 (strong staining). The sum of the two scores of 0-1, 2-4 and 5-6 were considered to be weak staining (-+), moderate staining (+) and strong staining (+ +), respectively according to a previous report (Schuster et al., 2012).

Statistical analysis

Statistical analysis was performed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). The unpaired *t* test was used to compare tumor and healthy tissues. Box plots and Shapiro-Wilk (W test) were used to assure our data were normally distributed. The Bartlett test was used for homogeneous variances. The *t* test checks for equality of the means requiring normality (tested by Shapiro-Wilk test) and homogeneity of the variances (tested by Bartlett test). The Welch *t* tests can be used in case non-homogeneous variances. The association between miR-9-5p expression and the categorical variables was assessed by using $\chi 2$ tests. Survival analysis was conducted with the Kaplan-Meier method with the log-rank test. The independent prognostic value of miR-9-5p expression was evaluated using univariate and multivariate (covariable includes tumor size, Preoperative PSA, Gleason score) Cox regression models. The differences between two groups were assessed by 2-tailed Student *t* test and multiple groups using one-way or two-way analysis of variance followed by a post-hoc Bonferroni test (factors include different transfections, cell lines and luciferase constructs). Values of $p < 0.05$ were considered statistically significant.

EXCLI Journal 2019;18:1049-1061 – ISSN 1611-2156
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RESULTS

Down-regulation of miR-9-5p was closely associated with worse clinical outcomes in human PCa

To investigate the biological function of miR-9-5p in PCa, qRT-PCR analysis was first performed to determine the expression of miR-9-5p in PCa tissues and adjacent prostate tissues. As shown in Figure 1A, miR-9-5p levels were found to be significantly down-regulated in 78 pairs of PCa tissues compared with matched adjacent tissues. At the same time, we compared the expression of miR-9-5p in PCa with different Gleason Scores and found lower levels of miR-9-5p were mostly observed in PCa tissues with higher Gleason score (Figure 1B). Subsequently, total 78 PCa tissues were divided into high- (n = 34) and low-miR-9-5p (n = 44) expression group based on the median value of miR-9-5p levels to investigate the clinical significance of miR-9-5p in PCa patients. As shown in Table 1, down-regulated miR-9-5p was significantly correlated with tumor size (p = 0.003), pre-operative PSA (p = 0.004), Gleason score (p < 0.001) and lymph node metastasis (p = 0.002). Kaplan–Meier analyses revealed that PCa patients with higher levels of miR-9-5p had longer overall survival rate than those with lower levels of miR-9-5p (Figure 1C, p = 0.0035). Importantly, multivariate analyses showed that miR-9-5p (HR = 3.05, 95 % CI: 2.97-3.55; p = 0.004) was an independent predictor of PCa patients' prognosis (Table 2). Taken together, these results suggest that decreased expression of miR-9-5p indicates a poor prognosis and might be a potential biomarker for PCa patients.

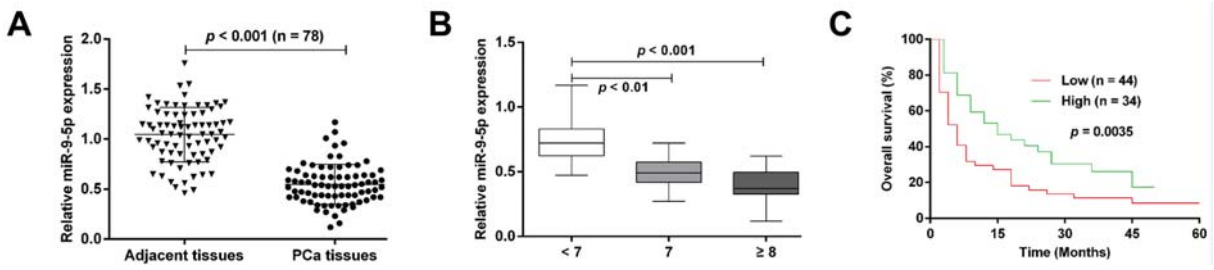

Figure 1: Down-regulated expression level of miR-9-5p was correlated with PCa progression. (**A**) The expression levels of miR-9-5p were determined in 78 pairs of PCa tissues and matched adjacent tissues using qRT-PCR analysis. (**B**) MiR-9-5p expression was evaluated in PCa tissues with low, moderate and high Gleason Score, respectively. (**C**) Kaplan-Meier analysis of overall survival rate for patients with PCa with low and high serum miR-9-5p levels.

Table 2: Univariate and multivariate analysis using Cox proportional hazard regression model for five-year survival rate in PCa patients

Variable	HR (95 % CI)	P-value
Univariate		
Age	2.35 (1.86-2.86)	0.532
Tumor size (cm)	1.02 (0.86-1.86)	0.022
Preoperative PSA	2.67 (2.16-2.98)	0.004
Pathological stage	4.02 (3.75-5.01)	0.083
Gleason score	2.89 (2.46-3.16)	0.021
Lymph node metastasis	1.65 (1.16-2.24)	0.103
BCR	2.75 (1.98-3.26)	0.062
Multivariate		
Tumor size (cm)	3.15 (2.96-3.47)	0.051
Preoperative PSA	2.46 (1.96-2.99)	**0.014**
Gleason score	1.59 (1.26-2.34)	**0.002**
Tissue miR-9-5p expression	3.05 (2.97-3.55)	**0.006**

HR: hazard ratio; CI: confidence interval

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miR-9-5p overexpression inhibited the proliferation, migration and invasion of PCa cells

To better perform the *in vitro* experiments, the expression of miR-9-5p was analyzed in several PCa cell lines using qRT-PCR analysis. As depicted in Figure 2A, the expression of miR-9-5p was significantly decreased in PCa cell lines (LNCaP, DU145, 22RV1, and PC-3), in comparison with normal prostate epithelial cell line RWPE-1 ($p < 0.01$, $p < 0.001$). As the miR-9-5p level was lowest in LNCaP and DU145 cells, we thus chose them for gain-of-function experiments. As demonstrated in Figure 2B, miR-9-5p mimics transfection significantly increased the expression level of miR-9-5p in both LNCaP and DU145 cells compared with miR-NC transfection ($p < 0.001$). The CCK-8 assay showed that miR-9-5p overexpression significantly suppressed cell proliferation of LNCaP ($p < 0.001$) and DU145 ($p < 0.01$, $p < 0.001$) cells at the indicated time points (Figure 2C). The relentless PCa cell invasion is the major cause resulting in poor outcome and death of the patients. Next, we examined whether miR-9-5p affected cell migration and invasion in PCa using Transwell assays. As shown in Figure 2D, miR-9-5p overexpression significantly decreased the migratory cells from 68.3 ± 3.5 to 22.0 ± 3.6 in LNCaP cells and from 50.7 ± 4.9 to 7.0 ± 1.6 in DU145 cells ($p < 0.001$). Consistently, transfection with the miR-9-5p mimics markedly decreased invasion ability of LNCaP (15.7 ± 2.5 *vs.* 56.3 ± 2.5, $p < 0.001$) and DU145 (10.0 ± 2.6 *vs.* 41.7 ± 2.9, $p < 0.001$) cells compared to transfection with miR-NC (Figure 2E). These data indicate that miR-9-5p might be an effective inhibitor of cell proliferation, migration and invasion in PCa cells.

UTRN might be a direct target of miR-9-5p in PCa

Based on the TargetScan and miRanda software, potential binding sites of miR-9-5p at positions 160-166 in the 3'UTR of UTRN were predicted and shown in Figure 3A. Meanwhile, luciferase reporter assay was used to confirm this prediction and the results showed that the luciferase activity of the WT UTRN 3'UTR was significantly inhibited by miR-9-5p mimics transfection, whereas the

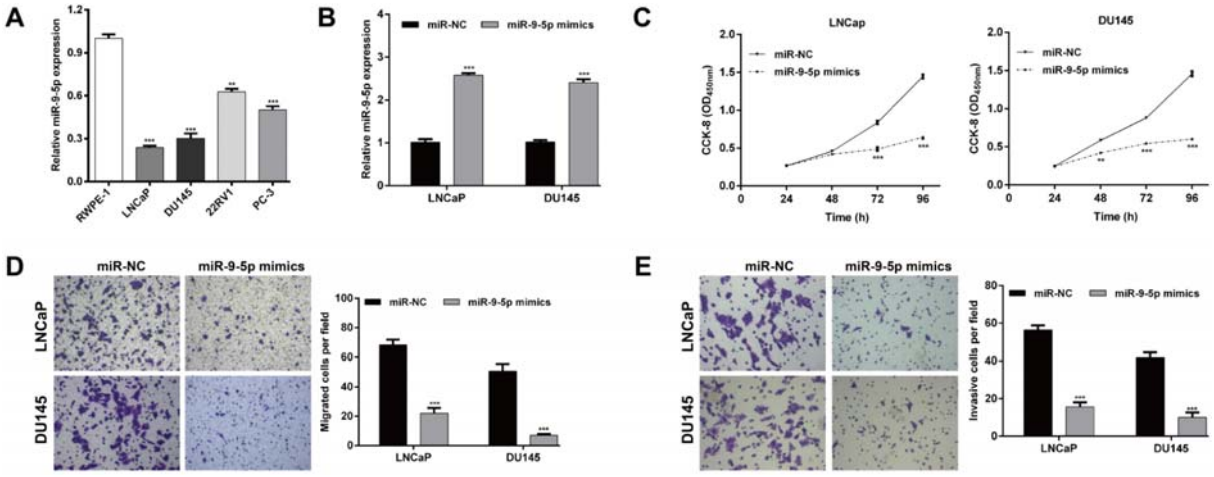

Figure 2: miR-9-5p suppressed cell proliferation, migration and invasion in PCa cells. (**A**) The expression levels of miR-9-5p were determined in PCa cell lines (LNCaP, DU145, 22RV1, and PC-3) and normal prostate epithelial cell line RWPE-1 using qRT-PCR analysis. (**B**) The significantly elevated expression of miR-9-5p in LNCaP and DU145 cells transfected with miR-9-5p mimics. (**C**) Relative cell proliferation rate was assessed in LNCaP and DU145 cells using CCK-8 assay. Representative images (left panel) and statistical analysis (right panel) of the (**D**) migratory and (**E**) invasive cell number of LNCaP and DU145 cells transfected with miR-9-5p mimics are presented (magnification, ×200). Data are presented as the mean ± SD from at least three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$

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luciferase activity of the mutant UTRN 3'UTR was not affected (Figure 3B, $p < 0.01$). To illustrate whether the endogenous miR-9-5p can directly regulate the expression of UTRN, the changes of UTRN was monitored in LNCaP and DU145 cells after miR-9-5p mimics transfection. Using qRT-PCR (Figure 3C, $p < 0.001$) and Western blot analysis (Figure 3D), we found the mRNA and protein of UTRN were significantly decreased in LNCaP and DU145 cell transfected with miR-9-5p mimics, as compared with miR-NC. These results revealed that miR-9-5p directly regulated UTRN expression in PCa cells by targeting its 3'UTR.

UTRN was highly expressed in PCa tissues and negatively correlated with miR-9-5p

The expression of UTRN protein was examined in 26 paraffin-embedded PCa samples and 19 normal prostate samples by IHC staining analysis. As shown in Figure 4A, three degrees of UTRN expression intensity were described by representative photomicrographs. Further statistical analysis demonstrated that most of paraffin-embedded PCa tissues showed strong and moderate UTRN staining, while weak UTRN staining was mainly observed in adjacent normal tissues (Figure 4B, $p < 0.001$). In addition, we analyzed the expression of UTRN mRNA in 78 pairs of PCa tissues and matched adjacent tissues using qRT-PCR. In agreement with the above results, PCa tissues displayed remarkably higher levels of UTRN mRNA compared with those in adjacent prostate tissues (Figure 4C, $p < 0.001$). Moreover, Spearman correlation analysis demonstrated the expression of UTRN was negatively correlated with miR-9-5p expression levels in PCa tissues (Figure 4D, r = -0.2711, $p = 0.0163$).

UTRN knockdown imitated the suppressive effects of miR-9-5p on cell proliferation, migration and invasion in PCa cells

Next, we explored the biological function of UTRN in PCa cells by performing loss-of-function assays. As shown in Figure 5A, the expression of UTRN at mRNA and protein levels was both significantly down-regulated in LNCaP cells after siUTRN transfection, in comparison with siNC transfection, as determined by qRT-PCR ($p < 0.001$) and Western blot analysis. Cell proliferation was assessed via a CCK-8 assay. The data suggested that

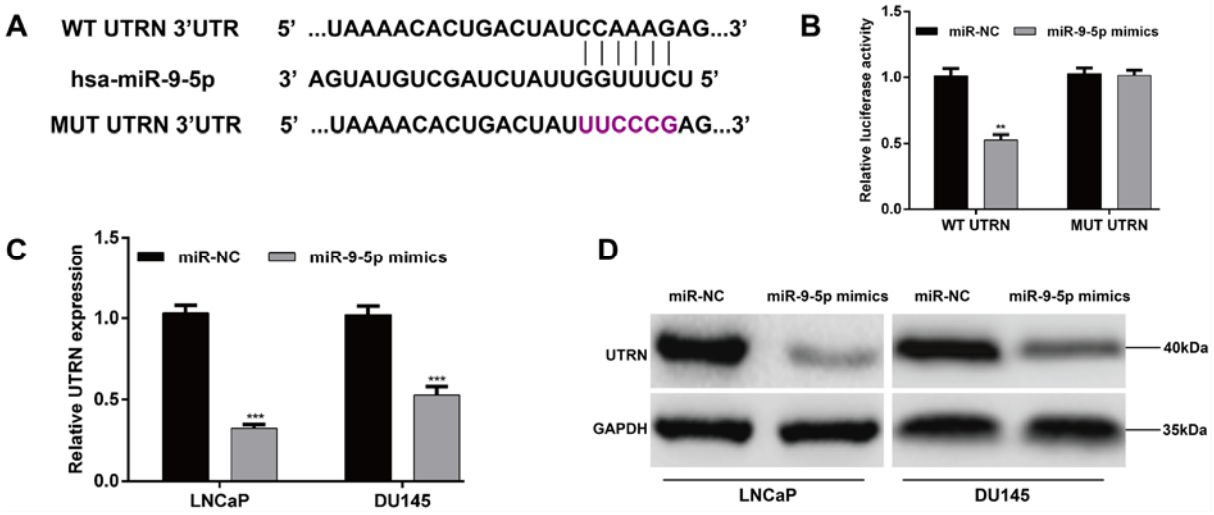

Figure 3: miR-9-5p down-regulated the expression of UTRN by targeting its 3'UTR. (**A**) The seed sequences for miR-9-5p in the 3'UTR of UTRN were displayed. (**B**) Luciferase reporter assay was performed 48 h after co-transfection in 293T cells with WT UTRN or MUT UTRN vectors together with miR-9-5p mimics or miR-NC. (**C**) QRT-PCR revealed the effects of miR-9-5p mimics on the expression level of UTRN mRNA in LNCaP and DU145 cells. (**D**) Western blot analysis revealed the effects of miR-9-5p mimics on the expression level of UTRN protein in LNCaP and DU145 cells. Data are presented as the mean ± SD from at least three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$

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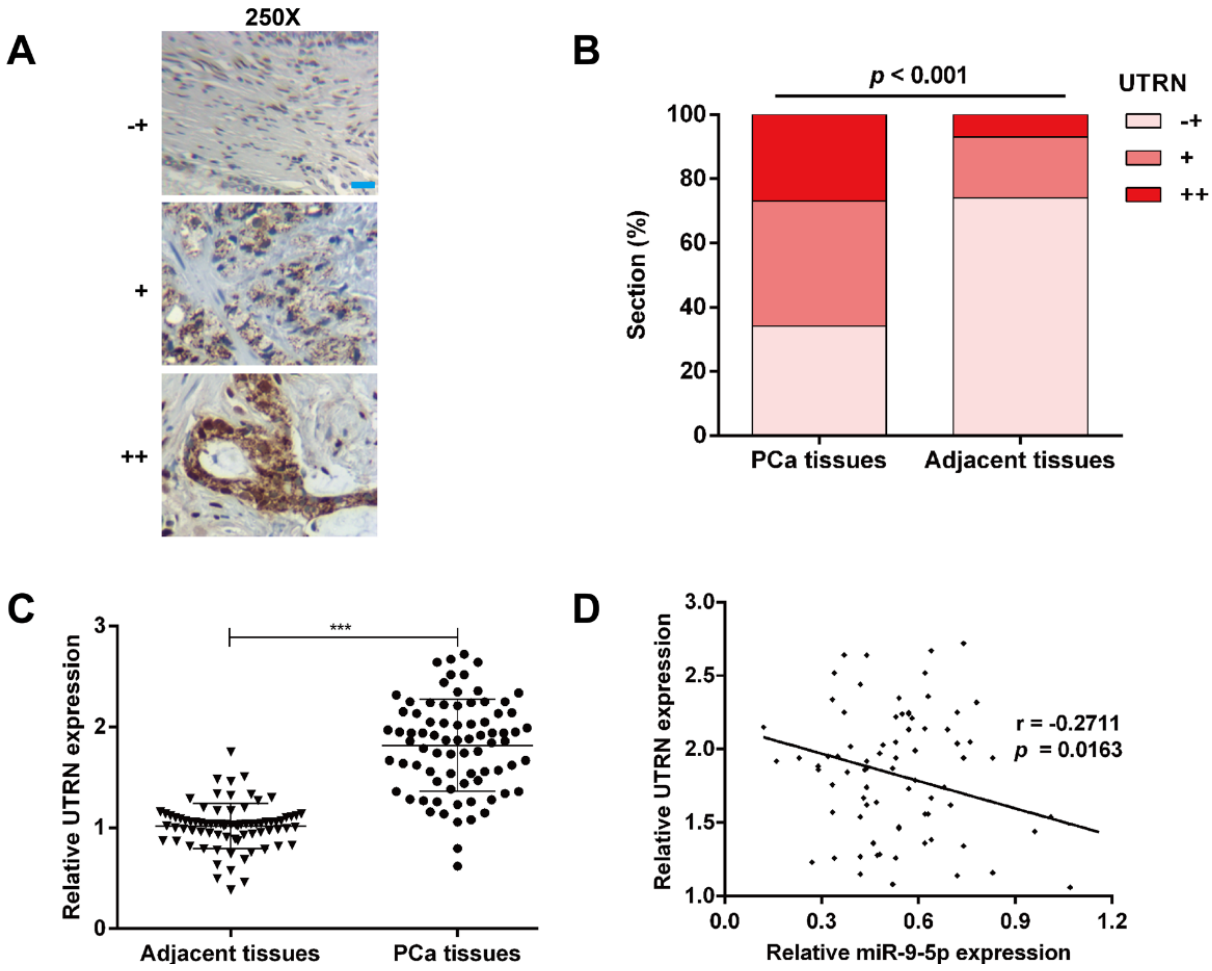

Figure 4: Upregulated UTRN levels were negatively correlated with miR-9-5p in PCa tissues. (**A**) Representative images of immunohistochemistry staining of UTRN in PCa and adjacent tissues (-+, weak staining, + moderate staining, ++ strong staining); (**B**) Statistical analysis of UTRN protein expression by immunohistochemistry; $p < 0.001$; (**C**) Relative UTRN mRNA levels in PCa tissues and matched adjacent tissues were measured using qRT-PCR. $^{***}p < 0.001$ compared with adjacent tissues; (**D**) The association between UTRN mRNA and miR-9-5p expression was evaluated using Spearman correlation analysis in PCa tissues. r = −0.2711, $p = 0.0163$

UTRN knockdown significantly attenuated cell proliferation of LNCAP cells (Figure 5B, $p < 0.001$). Additionally, transwell assay (Figure 5C, $p < 0.001$) revealed a significant decrease in migratory and invasive cells transfected with siUTRN compared with in the corresponding siNC in LNCaP cells. These findings indicated that UTRN might be a functional regulator in the effects of miR-9-5p on PCa cells.

DISCUSSION

MiR-9 has been found to be expressed in the nervous system and originally defined as a versatile regulator of neurogenesis (Coolen et al., 2013). In recent years, miR-9 in humans was reported to target genes involved in microglial activation (Yao et al., 2014), fibroblasts transformation (Fierro-Fernandez et al., 2015), and tumorigenesis (Wang et al., 2015). In this study, we observed that miR-9-5p was significantly decreased in PCa tissues compared with adjacent tissues, and this reduction was obviously related to poor overall survival

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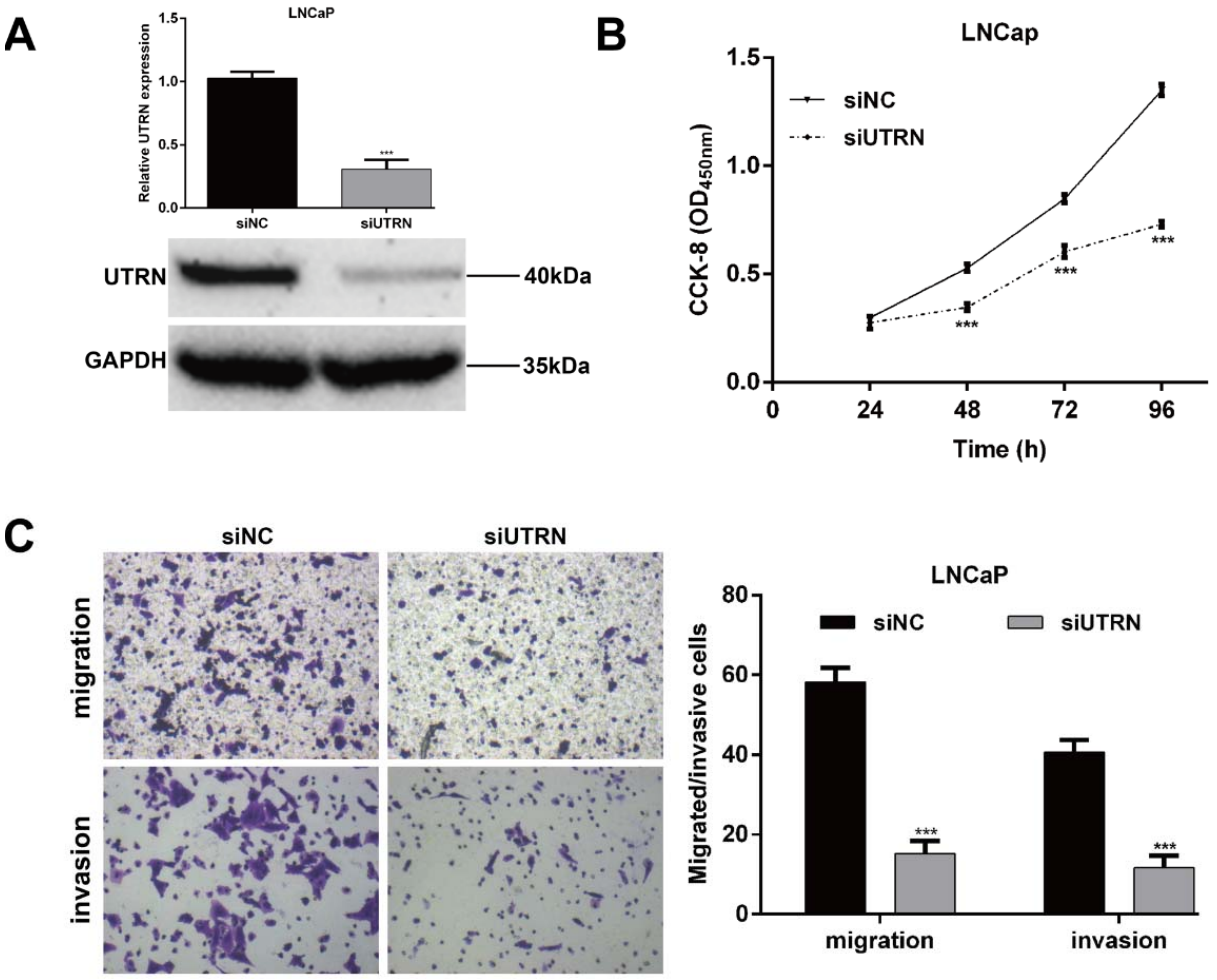

Figure 5: UTRN knockdown presented similar effects in miR-9-5p-overpressing PCa cells. (**A**) QRT-PCR and western blot analysis of UTRN expression in LNCaP cells after transfection with siUTRN or siNC, respectively. (**B**) The cell proliferation rate of LNCaP cells after UTRN knockdown was analyzed by the CCK-8 assay. (**C**) Representative images (left panel) and statistical analysis (right panel) of the migratory and invasive cell number of LNCaP cells transfected with siUTRN are presented (magnification, ×200). Data are presented as the mean ± SD from at least three independent experiments. $^{***}p < 0.001$

in patients with PCa. Uncontrolled cell proliferation, invasion and migration: all these properties of a cancer cell lead to its tumorigenic phenotype and impact patients prognosis (Musa et al., 2017). Here, enforced expression of miR-9-5p inhibited these properties in vitro by gain-of-function experiments through directly binding and inhibiting the expression of UTRN. MiR-9-5p and its downstream target UTRN can be used as predictive and/or prognostic biomarkers in PCa.

The association between miR-9-5p and malignant biological behaviors of tumor cells have already been reported in several studies. A growing literature supports the role of miR-9-5p in the modulation of biological processes are mainly mediated by network of miRNA-mRNA target interactions. Wang et al. (2015) showed that miR-9-5p is low-expressed in bladder cancer tissues, and treatment of BIU-87 cells with miR-9-5p inhibitor *in vitro* suggested that the proliferative and invasive effects of miR-9-5p were due to specific targeting of LASS2. In esophageal squamous cell carcinoma (ESCC), miR-9-5p plays a critical role in migration and metastasis by activating β-catenin pathway and inducing epithelial-mesenchymal-transition via binding to target

sites in the 3'UTR region of E-cadherin (Song et al., 2014). Particularly, Sohn et al. (2015) revealed that overexpression of miR-9-5p in ESCC significantly correlated with clinical progression, lymph node metastasis, and poor overall survival. Similarly, a previous study indicates that miR-9-5p induces an aggressive and invasive phenotype in non-small cell lung cancer (NSCLC) by targeting TGFBR2 (Li et al., 2017). Therefore, the transcriptional regulation by miR-9-5p seems to be dependent on human tissues and cell types: In PCa miR-9-5p appears to have anti-growth functions and predicts a good prognosis. However, it mainly has pro-metastasis functions and/or indicates a poor prognosis in many cancers, including bladder cancer, ESCC, and NSCLC. However, the capability of miR-9-5p to act as a prognostic biomarker in PCa will require further exploration.

UTRN is present as a high-molecular-weight protein that has substantial structural and sequence homology to dystrophin (Keefe and Kardon, 2015). It is known that loss of dystrophin is a major risk factor for Duchenne muscular dystrophy, a fatal X-linked muscle-wasting disorder (Dumont et al., 2015). UTRN can functionally compensate for dystrophin deficiency when it is upregulated in the myofibre (Gordon et al., 2013). In recent years, behavior and effects of UTRN in multiple biological processes has attracted much attention. In this study, qRT-PCR and immunohistochemistry staining revealed that UTRN is amplified in PCa tissues, indicating that UTRN is an oncogene in PCa. In a further, inhibition of UTRN expression was found to attenuate the capabilities of PCa cells proliferation, migration and invasion. Interestingly, in pancreatic endocrine tumors (PETs) cells, UTRN was shown to be localized to cell membranes, and the membranous UTRN localization correlated with short survival of PETs patients (Chang et al., 2007). Therefore, negative regulation of UTRN by miR-9-5p may not be at all but at least partially responsible for poor prognosis of PCa patients with low serum miR-9-5p levels. In a previous study, the proliferation of muscle-derived stem cells (MDSCs) was shown to be significantly decreased in double dystrphin/UTRN deficient mice compared to that isolated from mice with dystrophin deficient but UTRN expressing (Sohn et al., 2015). What's more, Shen et al. (2016) demonstrated that knockdown of UTRN suppressed glioma cells proliferation through regulation of cell cycle distribution. Ntantie et al. (2018) showed that TAPP2 links phosphoinositide 3-kinase signaling and interaction with UTRN to promote the leukemia B-cell adhesion. Importantly, focal adhesion formation is known to play a pivotal role in migration, invasion and growth of malignant tumor cells. Despite the role of UTRN is poorly understood and rarely investigated directly, however, the molecular biologic feature of miR-9-5p/UTRN axis in PCa will enrich this field. The molecular mechanisms downstream of miR-9-5p/UTRN axis still need further investigation.

Our findings revealed that miR-9-5p may be useful as a novel prognostic biomarker in PCa and play a critical role in proliferation, migration and invasion though targeting UTRN. Restoration of miR-9-5p and down-regulation of UTRN may represent a potential therapeutic for the treatment of PCa.

Competing interests

The authors declare that there is no conflict of interest.

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