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Original Article

Overexpression of miR-96 promotes cell proliferation by targeting FOXF2 in prostate cancer

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Abstract: Prostate cancer (PC) is one of the most common cancers in males. MicroRNAs (miRNAs) are demonstrated to be involved in prostate cancer development and progression. Recently, miR-96 was identified to play a tumor promoting role in several tumors including PC, however, the underlying function of miR-96 in PC still need to be known. In the study, our results demonstrated that miR-96 was higher in prostate cancer tissues compared with adjacent normal tissues. Higher miR-96 was association with higher PSA level, lymph node metastasis, pathologic stage and distant metastasis in prostate cancer patients. Lose-of-function studies showed that down-regulated expression of miR-96 inhibited cell proliferation and cell cycle by regulating down-regulating CyclinA1, CDK2 and CDK4 expression in PC cells. Furthermore, we found that FOXF2 was a target of miR-96 in PC cells and miR-96 promoted cell proliferation by suppressing FOXF2 expression. Thus, these results showed that inhibition of miR-96 may be a target for prostate cancer treatment.

Keywords: Prostate cancer, miR-96, FOXF2, cell proliferation

Introduction

Prostate cancer (PC) is the leading cause of cancer morbidity and mortality in males in the world [1]. In spite of advance in the therapeutic options including radical prostatectomy and radiation, successfully cure the majority of patients. However, approximately 40% cases will relapse [2, 3]. Most patients deaths from this disease are related to tumor progression and metastases [4]. Thus, to explore the underlying the mechanism of PC development and investigate novel treatment methods was important.

MicroRNAs (miRNAs) are small none-coding RNAs and 18-24 nucleotides in length [5]. MicroRNAs have been identified as regulators of tumor development and progression by regulating their target gene expression and served as a tumor oncogenes or tumor suppressors [6]. MiR-96 had been reported to be participated in some tumors including prostate cancer. Hong et al found that miR-96 promotes cell proliferation, migration and invasion by targeting PTPN9 in breast cancer [7]. Serum miR-96 is a promising biomarker for hepatocellular carcinoma in patients with chronic hepatitis B virus infection [8]. Song et al reported that microRNA-96 plays an oncogenic role by targeting FOXO1 and regulating AKT/FOXO1/Bim pathway in papillary thyroid carcinoma cells [9]. Wu et al revealed that Inhibition of miR-96 expression remarkably decreased cell proliferation and promoted cell apoptosis of BC cell lines [10]. Another study confirmed that miR-96 suppresses renal cell carcinoma invasion via down-regulation of Ezrin expression [11].

In prostate cancer development and progression, up-regulation of miR-96 enhances cellular proliferation of prostate cancer cells through FOXO1 [12]. In the study, we found that miR-96 was higher in prostate cancer tissues. Fur-

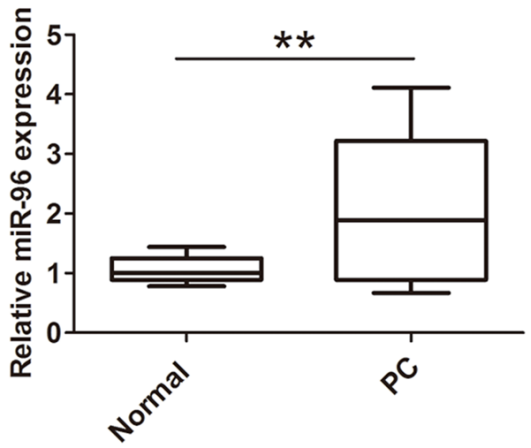

Figure 1. MiR-96 was up-regulated in prostate cancer tissues compared with adjacent normal tissues, the expression of miR-96 was analyzed by qRT-PCR. Data shown are mean ± SD from three independent experiments. **P<0.05.

thermore, we demonstrated that miR-96 promoted cell proliferation by targeting FOXF2 expression in PC. Therefore, these results indicated that inhibition of miR-96 may be a target for prostate cancer treatment.

Materials and methods

Patients and clinical tissue specimens

73 cases pairs of primary PC tissues and adjacent non tumor tissues were obtained from patients undergoing surgery at the Department of Urology, West China Hospital, Sichuan University. The study was approved by the Ethics Committee of West China Hospital, Sichuan University and written informed consent was obtained from all patients. Specimens were immediately snap-frozen in liquid nitrogen and stored at -80°C until further analysis.

Cell culture and transfection

Three human prostate cancer lines, LNCaP, PC-3 and DU-145 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). PC Cells were supplemented with RPMI-1640 medium (GIBCO, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, USA). Cells were cultured in a humidified atmosphere with 5% CO_2 at 37°C. Cells were transfected with miR-96 mimic, miR-96 inhibitor and miR negative control (Ribobio, Guangzhou, China) using Lipofectamine 3000 (Sigma, Palo Alto, CA, USA) based on the manufacturer's protocol.

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from PC cells using the TRIzol reagent, according to the manufacturer's instructions. A total of 20 ng of RNA was reversed into cDNA using the Taqman miRNA reverse transcription kit (Takara, Japan). qRT-PCR was detected by one step SYBR Prime Script plus RT-PCR kit (Takara, Japan) and conducted in an Applied Biosystems 7500 real-time PCR instrument (Life Technologies) using standard conditions. U6 and GAPDH were used as a normalization control, and relative expression was calculated using the using the $2^{-\Delta\Delta Ct}$ method. The following primers were: miR-96-F: 5'-TTTGGCACTAGC ACAT-3'; miR-96-R: 5'-GAGCAGGCTGGAGAA-3'.

Cell proliferation analysis

Cell proliferation was performed using Cell Counting Kit-8 (CCK-8) (Dojindo, Japan) following the manufacturer's recommendation. Briefly, the cells were inoculated into 96-well plates with 2×10^3 cells/per well, and each group had 3 duplicate wells. After cells transfection at 0, 24, 48 and 72 h, 10 μl CCK-8 was added to the culture medium and incubated for 2 h at 37°C. The absorbance at 450 nm was measured.

Cell cycle analysis

Transfected cells were collected and seeded in six-well plates and cultured for 48 h. Briefly, cells were harvested and fixed in ice-cold 70% ethanol overnight at -20°C. The cells were incubated with 50 μg/mL propidium iodide (PI; BD Biosciences San Jose, CA, USA) for 30 min in the dark. The percentage of cells in G0/G1, S and G2/M phases of the cell cycle were determined using a flow cytometer (FACS Calibur, BD Biosciences, San Jose, CA, USA) after propidium iodide (PI) staining.

Western blotting assays

Total protein was extracted from cells using RIPA lysis buffer (Sigma). Proteins were separated by 8%-10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and

Table 1. The correction between expression of miR-96 and clinicopathological variables of patients

Clinical variables	Case number	MiR-96 expression		*p*-value
		Lower (n=35)	Higher (n=38)	
Age				0.492
≤60	45	23	22	
>60	28	12	16	
PSA level (μg/L)				0.001**
≤10	38	25	13	
>10	35	10	25	
Lymph node metastasis				0.014**
Negative	48	28	20	
Positive	25	7	18	
Gleason				0.580
≤7	52	26	26	
>7	21	9	12	
Pathologic stage				0.007**
≤T2	47	28	19	
>T2	26	7	19	
Distant metastasis				0.028**
Negative	54	30	24	
Positive	19	5	14	
Surgical margin status				0.911
Negative	58	28	30	
Positive	15	7	8	

**P<0.05.

then transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was blocked for 2 h in PBST and subsequently probed with primary antibody against FOXF2 (Abcam, USA), CyclinA1 (Cell Signaling, USA), CDK2 (Cell Signaling, USA) and CDK4 (Cell Signaling, USA) at 4°C overnight. GAPDH served as a loading control (Abcam, USA). Membranes were washed and then incubated with HRP-conjugated secondary antibody for 2 h. The proteins blot was visualized using a chemiluminescence kit (Millipore, Germany).

Luciferase reporter assays

The Wild type (Wt) and mutant (Mut) 3'-untranslated regions (UTRs) of FOXF2 mRNA were synthesized by Ribobio (Guangzhou, China), and inserted into psiCHECK-2 vector at the HindIII/SpeI site. Cells were co-transfected with Wt or Mut FOXF2 3'-UTR plasmids and miR-96 mimic or miR-NC using Lipofectamine 2000 according to the manufacturer's protocol. Firefly and Renilla luciferase activities were measured consecutively using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) 48 h post-transfection.

Statistical analysis

All statistical calculations were performed using SPSS 18.0 (version 18.0; SPSS Inc., Chicago, IL, USA). The data are expressed as mean ± standard deviation (SD) of at least three independent experiments. The difference between the mean values was evaluated by one-way analysis of variance. *P*-values were two sided, and P<0.05 was considered to be statistically significant.

Results

Higher miR-96 expression correlates with clinical parameters in PC patients

In the study, we detected the miR-96 expression levels in PC tissues and adjacent normal tissues by qRT-PCR. As shown in the **Figure 1**, the miR-96 expression levels were dramatically higher than that in adjacent normal tissues (P<0.05). Furthermore, we analyzed the association between the expression of miR-96 and clinical parameters. As shown in **Table 1**, the higher miR-96 was association with higher PSA level, lymph node metastasis, pathologic stage and distant metastasis in prostate cancer patients (P<0.05), but no association with age, Gleason, surgical margin status (P>0.05, **Table 1**).

Higher miR-96 expression promotes cell proliferation in PC

To investigate the functional significance of miR-96 expression in PC, we reduced miR-96 expression in PC cells and explored its impacts on cell proliferation and cell cycle. The results showed that down-regulation of miR-96 suppressed the cell proliferation compared with the control group by CCK8 analysis in PC-3 and DU-145 cells (**Figure 2A**, **2B**). Furthermore, the percentage of cells in G0/G1, S and G2/M phases were analyzed by flow cytometry analysis. Compared with the control group, the

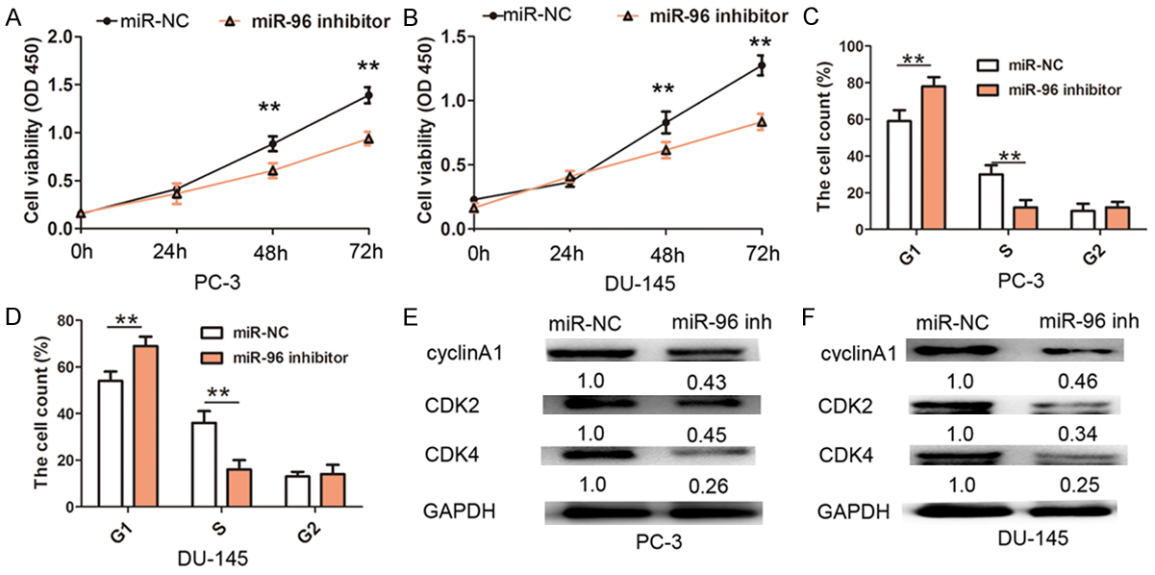

Figure 2. Unregulation of miR-96 promotes cell proliferation in PC cells. A, B. Cell proliferation was evaluated by CCK8 methods after overexpression of miR-96 in PC-3 and DU-145 cells. C, D. Cell cycle results were showed by flow cytometry analysis after overexpression of miR-96 in PC-3 and DU-145 cells. E, F. The expression of Cyclin A, CDK2 and CDK4 were showed by western- blot analysis after overexpression of miR-96 in PC-3 and DU-145 cells. Data shown are mean ± SD from three independent experiments. **P<0.05.

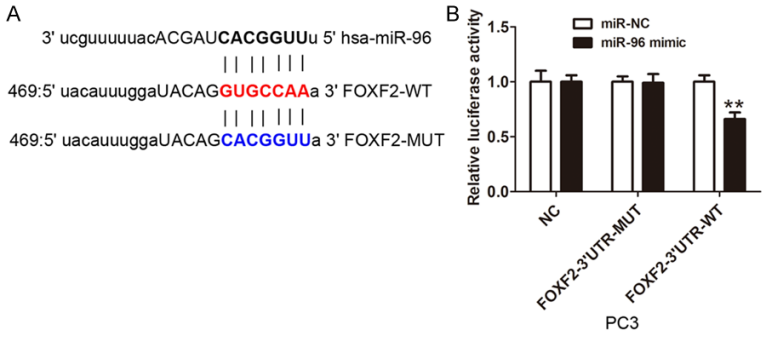

Figure 3. FOXF2 was a target of miR-96 in PC cells. A. MiR-96 binds to the predicted binding site in the 3'UTR region of FOXF2. B. MiR-96 mimic reudced psiCHECK-2-3'-UTR-FOXF2-WT reportor vector luciferase activity, however, luciferase activity in psiCHECK-2-3'-UTR-FOXF2-MUT or NC reporter vector do not be affected. Data shown are mean ± SD from three independent experiments. **P<0.05.

results confirmed that the percentage of cells in G0/G1 phases was up-regulated, but the percentage of cells in S phases significantly reduced in miR-96 silencing group in PC-3 and DU-145 cells (**Figure 2C**, **2D**). We also found that the cell cycle regulated protein Cyclin A1, CDK2 and CDK4 was down-regulated when miR-96 was reduced in PC-3 and DU-145 cells (**Figure 2E**, **2F**). Therefore, these findings provide evidence that miR-96 promoted cell proliferation in PC.

FOXF2 was a target of miR-96 in PC cells

We employed two miRNA target prediction algorithms provided by miRanda, and Targetscan. After integrating the results, we selected the FOXF2 as a potential target due to FOXF2 function as tumor suppressor in some tumors [13]. The binding site for miR-96 at 3'-UTR of FOXF2 was shown in **Figure 3A**. Furthermore, we demonstrated that co-transfection with miR-96 mimic and psiCHECK-2-3'-UTR-FOXF2-WT reporter vector can reduced the luciferase activity, however, luciferase activity in psiCHECK-2-3'-UTR-FOXF2-MUT reporter vector or negative control group do not be affected (**Figure 3B**). Taken together, the results indicated that FOXF2 was a direct target of miR-96. We further performed qRT-PCR and western blot assays to study whether the expression of miR-96 could change FOXF2 expression. We demonstrated that FOXF2 mRNA and protein expression was significantly increased after down-regulation of

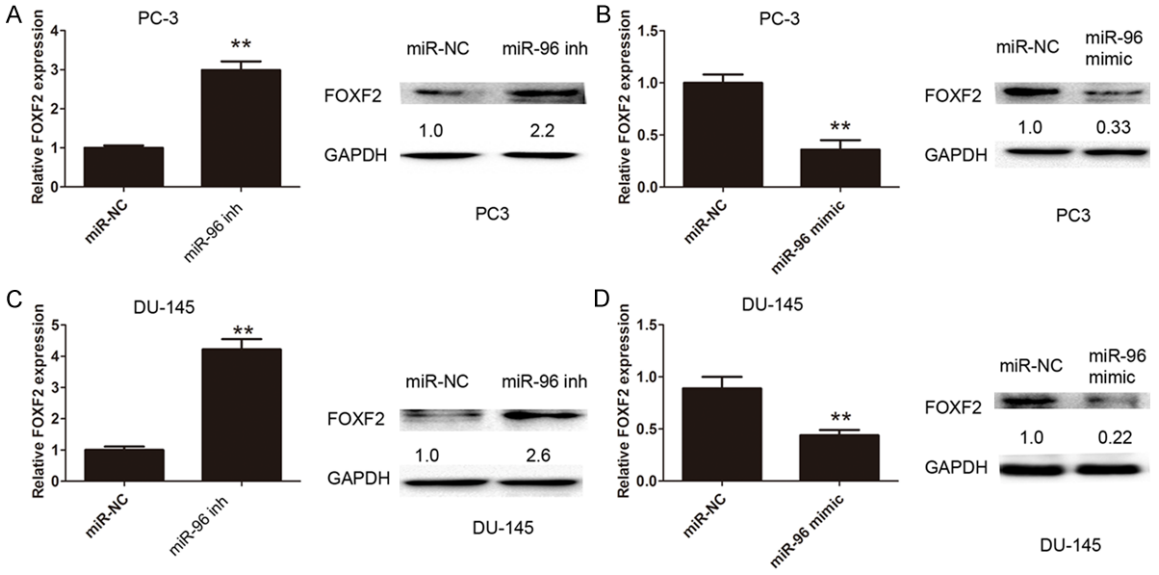

Figure 4. MiR-96 regulated the mRNA and protein expression of FOXF2 in PC cells. A, B. The mRNA and protein expression levels was evaluated by transfected miR-96 mimic, miR-96 inhibitor or miR-NC into PC3 cells by qRT-PCR and western-blot methods. C, D. The mRNA and protein expression levels was evaluated by transfected miR-96 mimic, miR-96 inhibitor or miR-NC into DU-145 cells by qRT-PCR and western-blot methods. Data shown are mean ± SD from three independent experiments. **P<0.05.

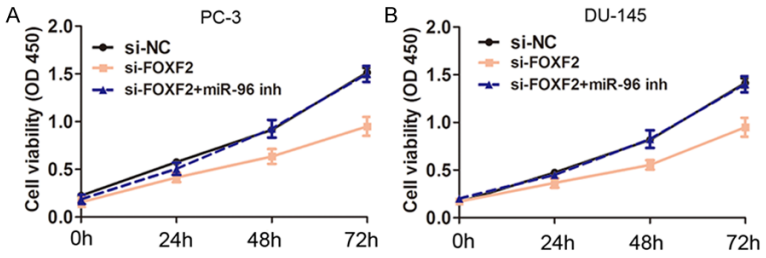

Figure 5. FOXF2 as a downstream mediator of miR-96 in PC cells. A. Cell proliferation was significantly increased after FOXF2 silencing, but was rescued by down-regulation of miR-96 in PC-3 cells. B. Cell proliferation was significantly increased after FOXF2 silencing, but was rescued by down-regulation of miR-96 in DU-145 cells. Data shown are mean ± SD from three independent experiments. **P<0.05.

miR-96, but was significantly decreased after miR-96 overexpression compared with control cells in PC-3 cells (**Figure 4A**, **4B**). We also demonstrated the same results in DU-145 cells (**Figure 4C**, **4D**). Overall, these results showed that miR-96 directly target FOXF2 to regulated its expression in PC.

FOXF2 knockdown influence the effects of miR-96 on PC cells

To further confirm that FOXF2 is a functional target of miR-96, we performed a specific FOXF2 siRNA to transfected into PC-3 and DU-145 cells. We found that cell proliferation was significantly increased after FOXF2 silencing, however, cotransfected with si-FOXF2 and miR-96 inhibitor rescued FOXF2 silencing induced inhibition of cell proliferation (**Figure 5A**, **5B**). These results provide further evidence supporting FOXF2 as a downstream mediator of miR-96 in PC.

Discussion

Increasing evidence indicates that dysregulation of miRNAs is involved in the initiation and progression in PC and miR-96 functions as an oncogene to involved in some tumors [14]. High miR-96 levels in colorectal adenocarcinoma predict poor prognosis, particularly in patients without distant metastasis at the time of initial diagnosis [15]. MiR-96 promotes proliferation and chemo-or radioresistance by down-regulating RECK in esophageal cancer [16]. Unregulated miR-96 induces cell proliferation in human breast cancer by down-regulating transcriptional factor FOXO3a [17]. Hsa-miR-96 up-regulates MAP4K1 and IRS1 and may function as a promising diagnostic marker in human

bladder urothelial carcinomas [18]. In the study, our results showed that miR-96 was higher in prostate cancer tissues compared with adjacent tissue. Higher miR-96 was association with higher PSA level, lymph node metastasis, pathologic stage and distant metastasis in prostate cancer patients. Moreover, we demonstrated decreased miR-96 inhibited cell proliferation and cell cycle progression by inhibiting cell cycle related protein Cyclin A1, CDK2 and CDK4 expression levels, these finding indicated miR-96 acted as promoting oncogene in PC.

MiRNA-regulated gene targets for understanding miRNA biological functions are necessary. Based on target prediction programs and luciferase reporter assays, we identify that FOXF2 was a direct target of miR-96 in PC cells. FOXF2 had been demonstrated to act as a tumor suppressor in tumors. For example, decreased expression of FOXF2 as new predictor of poor prognosis in stage I non-small cell lung cancer [19]. FOXF2 suppresses the FOXC2-mediated epithelial-mesenchymal transition and multidrug resistance of basal-like breast cancer [20]. In prostate development and progression, MicroRNA-182-5p promotes cell invasion and proliferation by down regulating FOXF2, RECK and MTSS1 genes [21]. Furthermore, we demonstrated that FOXF2 mRNA and protein expression was significantly down-regulated when miR-96 was over-expressed. Overall, these results showed that miR-96 directly target FOXK2 to regulate its expression in PC. To further confirm that FOXF2 is a functional target of miR-96, we found that cell proliferation was significantly increased after FOXF2 silencing. Furthermore, FOXF2 knockdown significantly rescued down-regulation of miR-96-induced inhibition of cell proliferation, which indicated FOXF2 as a downstream mediator of miR-96 in PC.

In conclusion, our study showed that miR-96 was higher in PC and function as oncogene to promote cell proliferation and cell cycle progression. Furthermore, we demonstrated that miR-96 promote cell proliferation by target FOXF2 in PC cells, Thus, miR-96 may be potential target of PC treatment.

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Disclosure of conflict of interest

None.

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