

MiR-96 expression in prostate cancer and its effect on the target gene regulation

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Abstract. – **OBJECTIVE:** Previous studies showed that miR-96 was associated with a carcinogenic effect. To investigate the expression of miR-96 and related target genes in the regulation of prostatic cancer, we compared the expression of miR-96 in both prostatic cancer and adjacent normal tissues, and explored the role of miR-96 in prostate cancer.

PATIENTS AND METHODS: PC-3 cell line originated from human prostatic cancer tissues was prepared. RNA was extracted for examination of miR-96 expression. The expressional alteration of miR-96 target genes, forkhead box O1 (FOXO1) and forkhead box O3a (FOXO3a), in prostatic cancer, was confirmed by PC-3 cells transfected with miR-96 and anti miR-96.

RESULTS: Compared with control group, levels of FOXO1 and FOXO3a in PC-3 cells treated with anti miR-96 were 1.584 times and 1.637 times higher, respectively. Further, PC-3 cells were transfected with siRNA targeting FOXO1 and FOXO3a, resulting in a significant decrease of FOXO1 and FOXO3a expression, as verified by qPCR and Western blot analyses. Compared with untreated groups, proliferation and cell clonal formation exhibited a marked increase in PC-3 cells under transfection with both siR-FOXO1 and siR-FOXO3a.

CONCLUSIONS: As target genes of miR-96, FOXO1 and FOXO3a confer protection against prostatic cancer, while the inhibition of FOXO1 and FOXO3a enhances cancer proliferation.

Key Words:

miR-96, Prostatic cancer, FOXO, FOXO3.

Introduction

Prostatic cancer (PC) is a type of urogenital malignant tumor, which usually occurs in men

older than 50 years¹. The incidence rate of prostatic cancer gradually increases in China with a rapid development of social economy and changes of dietary habits and the occurrence and development of prostatic cancer is associated with gene-related events, including gene repression, transcription, and expression^{2,3}.

As a polymeric molecule, RNA mainly exists in biological cells and part of virus among which, microRNA, is a kind of small non-coding RNA fragments with a structure of 21 to 23 nucleotides^{4,5}. Primary microRNA was transformed into mature microRNA after a series of RNase cleavages, and the latter binds with mRNA 3'untranslated region of target genes according to complementary base-pairing rule to induce completed decomposition or inhibit translation⁶. MicroRNA was proved to participate in the regulation of multiple tumor cellular events, including tumorigenesis, proliferation, differentiation, apoptosis, and metastasis⁵⁻⁷. A recent study⁸ showed that microRNA had a close relationship with oxidative stress response and drug resistance in tumor.

The coding sequence of miR-96 is located in chromatin 7q32.2, between two protein-coding regions. Studies^{9,10} found miR-96 remarkably increased in a variety of tumors, such as pancreatic cancer, lung cancer, osteosarcoma and gastric carcinoma, suggesting the expression of miR-96 was associated with the progression of tumor. *In silico* study showed that forkhead box O1 (FOXO1) and forkhead box O3a (FOXO3a) were potential target genes of miR-96 based on bioinformatics prediction with TargetScan and PicTar databases. Our study was thus focused on miR-96 expression and regulatory effect of target genes in prostatic cancer.

Patients and Methods

Patients, Cells and Main Reagents

This study enrolled 60 patients with prostatic cancer who were treated at General Hospital of Benxi Iron and Steel CO. LTD between May 2015 and March 2016, including 13 cases with un-differentiation, 17 cases with low differentiation, 19 cases with medium differentiation, 11 cases with high differentiation. Meanwhile, based on the TNM staging assessment, there were 15 cases in stage T1, 14 in stage T2, 16 in stage T3, and 15 in stage T4. Prostatic cancer and para-carcinoma tissues (2 cm distance from cancer tissues) were collected from all patients. None of the patients had any history of surgery, radiotherapy or chemotherapy. Fresh tissues were frozen in liquid nitrogen and stored at -80°C until use. This study was approved by the Research Ethics Committee at General Hospital of Benxi Iron and Steel CO. LTD. All patients have signed the informed consent upon enrollment.

PC-3 cell line and normal prostate cells (RWPE-1) were purchased from ATCC Ltd. in Guangdong, China. mirVana™ PARISTM kit was purchased from Thermo Fisher Scientific (Waltham, MA, USA). The Superscript III First-Strand Synthesis System was purchased from Invitrogen (Carlsbad, CA, USA). The Taqman qRT-PCR kit was purchased from Applied Biosystems (Foster City, CA, USA). 30% H₂O₂, dulbecco's modified eagle medium (DMEM), and fetal bovine serum (FBS) were purchased from North TZ-Biotech. (Beijing, China), Liposome (Lipofectamine TM2000) (Waltham, MA, USA), BCA kit was purchased from Beyotime Institute of Biotechnology (Shanghai, China), microRNA inhibitor, siR-FOXO1 and siR-FOXO3a were purchased from Ambion (Waltham, MA, USA). Mouse anti-human FOXO1 and FOXO3a polyclonal antibodies and horseradish peroxidase (HRP)-conjugated goat anti-human IgG were purchased from Abcam (Cambridge, MA, USA).

Quantitative Reverse Transcription PCR (qRT-PCR)

Total mRNA and protein were extracted from prostatic cancer and para-carcinoma tissues simultaneously with a mirVana™ PARISTM kit. After cell lysis, lysate was divided into mRNA portion and protein portion. An equal volume of 2 × denaturing solution was added into mRNA portion, and then phenol/chloroform (2 × volumes)

was added. The mixture was centrifuged at room temperature, 10 000 g for 5 min to separate the organic and aqueous layers. The aqueous layer was transferred into a new EP tube and mixed with ethanol (1.25 × volumes). The mixture was centrifuged at 10 000 g and washed successively with 800 µL of microRNA Buffer PW and 600 µL of normal Buffer PW. The supernatant was discarded and the precipitate was dissolved in 150 µL of buffer solution. Reverse transcription was performed using the standard protocol¹¹, and cDNA was prepared for amplification. The reaction mixture was prepared: 3.0 µL of dNTP, 3.0 µL of 10 × PCR buffer solution, 1 unit of Taq polymerase, 1.5 µL of PCR primers (F: 5'-GGTTTGG CACTAGCACAT-3', R: 5'-AGTGC GTGTCGT-GGA GTC-3')¹¹, 1.5 µL of cDNA solution. The volume of reaction complex was replenished to 30 µL with ddH₂O. PCR reaction protocol was as follows: 95°C for 5 s, 85°C for 10 s, 75°C for 15 s, and 65°C for 20 s (50 cycles). The experiment was repeated 3 times, and the relative levels of miR-96, FOXO1 and FOXO3a were calculated using the 2^{-ΔΔCt} method.

Examination of FOXO1 and FOXO3a Expression in Tissues

The protein part was analyzed by Western Blot to compare the expression of FOXO1 and FOXO3a in prostatic cancer and para-carcinoma tissues. Briefly, total protein was quantified using a BCA kit. Equal amounts of total protein (20 µg) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis and transferred to polyvinylidene difluoride membranes. The membrane was blocked in tris buffered saline (TBS) containing 5% skim milk at room temperature for 1 h and incubated with the appropriate primary antibody against FOXO1 (catalog no. 2880, Cell Signaling Technology, Danvers, MA, USA) or FOXO3a (catalog no. 2497, Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. The membrane was washed with TBST and incubated with HRP-conjugated goat anti-rabbit secondary antibodies (catalog no. ab97051, Abcam, Cambridge, MA, USA) at 37°C for 1h. The membrane was washed with tris buffered saline-tween (TBST) and treated with ECL detection reagents. The intensity of bands was detected by a Molecular Imager® ChemiDoc™ XRS System (Bio-Rad Laboratories, Hercules, CA, USA), and the gray value of bands was analyzed by Image Lab 2.0 software (Bio-Rad Laboratories, Hercules, CA, USA).

Cells Culture and Quantification of miR-96 Expression

Cell stock was centrifuged at 300 g for 8 min, and collected cells were inoculated into culture flask with DMEM medium containing 10% fetal bovine serum (FBS). Cells were cultured at 37°C in an incubator with 5% CO₂, and the medium was replaced every other day. Cells at 80% confluence were collected. Total microRNA was extracted from cells and miR-96 expression was quantified as described above.

Transfection of miR-96 and Anti miR-96

PC-3 cells were transfected with miR-96, anti miR-96 or anti miR-NC with Lipofectamine 2000 transfection kit. Briefly, PC-3 cells were inoculated into each well on 6-well plates and cultured at 37°C in a 5% CO₂ incubator. miR-96, anti miR-96 or anti miR-NC was mixed with 500 µl of liposomal transfection reagent for a final concentration of 100 nmol/L. The mixture was used to transfect PC-3 cells at 80% confluence. After 8 h of incubation, the medium was replaced with normal medium. Cells were incubated for an additional 24 h and collected. Western blot and qRT-PCR were performed as described above to determine the expression of FOXO1 and FOXO3a protein and mRNA. The sequences of miR-96, anti miR-96 or anti miR-NC were as follows: miR-96: 5'-GCAAAAATGTGCTAGTGCCAAA-3'; anti miR-96: 5'-AGCAAAAUGUGCUAGUGCCAAA-3'; and anti miR-NC: 5'-CAGUACU-UUUGUGUAGUACAA-3'.

Effect of siRNA on FOXO1 and FOXO3a Expression in PC-3 Cells

PC-3 cells were transfected with siRNA (siR-FOXO1 sense: 5'-GCGGGCUGGAAGAAUUCAdTdT-3', antisense: 5'-UUGAAUUCUCCAGCCGd TdT-3'; si-FOXO3a sense: 5'-GCACAGAGUUGGAUGACGd TdT-3', antisense: 5'-ACGUCAUCCAACUCUGUGCd TdT-3'; and siR-NC sense: 5'-UUCUCCGAACGUGUCACGUTT-3', antisense: 5'-ACGUGACACGUUCGAGAAATT-3') as described above. FOXO1 and FOXO3a protein and mRNA expression in transfected PC-3 cells were determined by Western Blot and qRT-PCR analyses as described above.

Effect of FOXO1 and FOXO3a on Proliferation of PC-3 Cells

Transfected PC-3 cells (siRNA, 100 nmol/L final concentration) were cultured with cell population 6×10^3 /well. 20 µL of CellTiter 96 and

aqueous one solution reagent were added into each well, respectively. Transfected PC-3 cells were cultured in an incubator and kept away from light. The proliferation of PC-3 cells was determined by measuring the absorption value of each well with a microplate reader (Bio-Rad 680, Hercules, CA, USA) at the wavelength of 480 nm.

Effect of FOXO1 and FOXO3a on Cell Cloning of PC-3 Cells

Six cultured wells were randomly selected for cell suspension preparation. The cell suspension was centrifuged at 300 g for 8 min. Liquid supernatant was removed, and 20 mL of complete medium was added into each well for further cell culture. Culture collected cells in soft-agar medium till cell proliferation could be detected with naked eyes. The medium was then removed and cell staining was performed. The number of cloning was counted with naked eyes (colony forming efficiency = the number of cloning/the number of inoculated cells $\times$ 100%).

Statistical Analysis

Data were expressed as mean $\pm$ standard deviation and analyzed using SPSS 16.0 (IBM SPSS Inc., Chicago, IL, USA). The difference among groups was compared by one-way ANOVA followed by Fisher's LSD tests when $p < 0.05$ in ANOVA. $p < 0.05$ is considered statistically significant.

Results

miR-96 Expression Was Significantly Increased in Prostatic Cancer

qRT-PCR results showed that the expression of miR-96 in prostatic cancer was 2.151 times higher compared with para-carcinoma tissues ($p = 0.012$, Figure 1A). The expression of FOXO1 and FOXO3a mRNA in prostatic cancer was 0.716 and 0.811 times lower, compared with para-carcinoma tissues ($p = 0.018$ and 0.020 , respectively, Figure 1A). Consistently, Western blot showed that the levels of FOXO1 and FOXO3a protein were significantly lower than that in para-carcinoma tissues ($p = 0.026$ and 0.040 , respectively, Figure 1B).

miR-96 expression Was Significantly Increased in PC-3 Cells

Similar to the *in vivo* result, our *in vitro* study by using qRT-PCR analyses demonstrated that

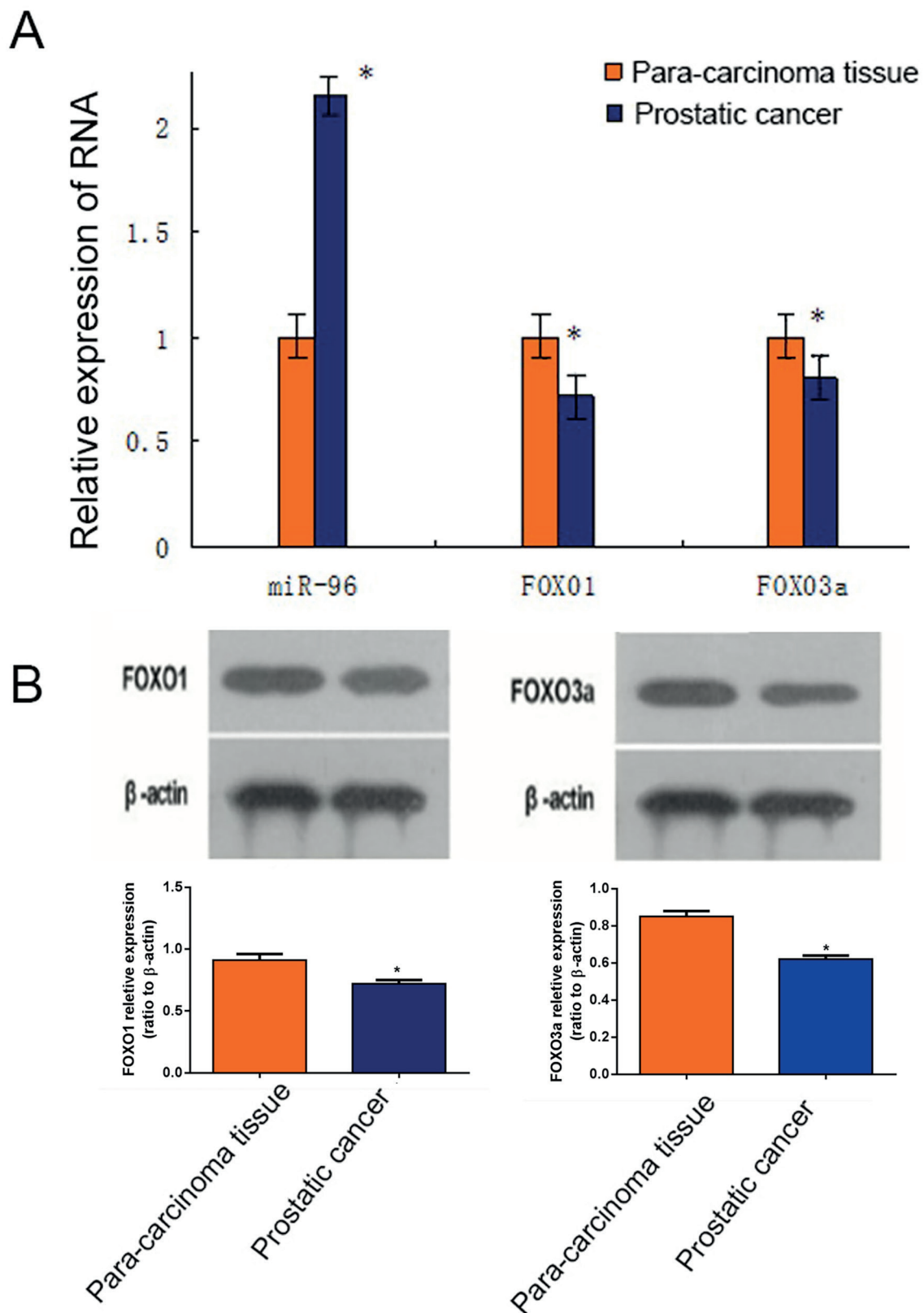


Figure 1. Comparison of the expression of miR-96, FOXO1 and FOXO3a in Prostatic cancer and para-carcinoma tissues. **(A)** qRT-PCR analyses of miR-96, FOXO1 mRNA and FOXO3a mRNA. **(B)** Western blot analyses of FOXO1 and FOXO3a protein. *, $p < 0.05$ compared with para-carcinoma tissue.

compared with normal prostatic cells (RWPE-1), miR-96 expression in PC-3 cells presented a significant higher level of relative level, (PC-3 vs. RWPE-1, 6.95 vs. 1.42, $p = 0.032$), suggesting the level of miR-96 expression in prostatic cancer cells increased by 4.9 times (Figure 2).

miR-96 Targeted FOXO1 and FOXO3a

We then evaluated the impact of miR-96 on the expressions of FOXO1 and FOXO3a by Western blot analyses. Our data validated the *in silico* prediction that FOXO1 and FOXO3a expressions were markedly decreased in miR-96-treated cells, but was substantially elevated in cells after being treated with anti miR-96 (Figure 3A). Further qRT-PCR analyses also demonstrated that the levels of FOXO1 and FOXO3a in miR-96 group were reduced by 0.328 and 0.279, respectively ($p = 0.023$ and 0.026 , respectively), but they were increased by 1.58 and 1.64 times, respectively, in anti miR-96-treated group when compared with anti miR-NC group ($p = 0.025$ and 0.013 , respectively, Table I). These results indicated miR-96 specifically inhibited the expressions of FOXO1 and FOXO3a in prostatic cancer cells.

siRNA Significantly Inhibited the Expression of FOXO1 and FOXO3

Compared with normal PC-3 cells, siRNA-FOXO1 and siRNA-FOXO3a specifically suppressed the expression of FOXO1 and FOXO3a at both in level of mRNA and protein levels,

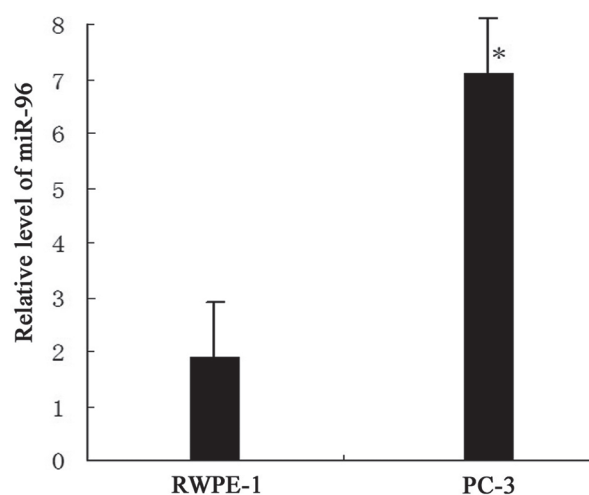


Figure 2. qRT-PCR analyses comparing of miR-96 expression between RWPE-1 and PC-3 cells. *, $p < 0.05$ compared with RWPE-1 cells.

respectively ($p = 0.046$, Figure 3A), suggesting that siRNA model of FOXO1 and FOXO3a were successfully established.

FOXO1 and FOXO3 Inhibition Enhanced Proliferation of PC-3 Cells

Compared with PC-3 cells in control group, proliferation of PC-3 cells after the intervention of siRNA targeting FOXO1 or FOXO3a was remarked enhanced ($p = 0.015$, Figure 3B), suggesting the inhibitory role of FOXO1 and FOXO3a on the development of prostatic cancer.

FOXO1 and FOXO3 Inhibition Promoted Cloning of PC-3 Cells

Both the treatments of siR-FOXO1 and siR-FOXO3a promoted clone formation of PC-3 cells, which was verified by plate colony and soft-agar medium colony (Figure 4A-B). Moreover, compared with siR-FOXO3a treatment, siR-FOXO1 treatment was more likely to accelerate larger clone formation of PC-3 cells (Figure 4C-D).

Discussion

Current diagnostic test for prostatic cancer mainly depends on digital rectal examination (DRE) combined with prostate specific antigen (PSA) examination¹², CT or MRI, while outpatient rate of prostatic cancer was not promising due to insidious onset and asymptomatic characters, which always delayed timely treatment¹². Although prostatectomy presents efficacy for early stage prostatic cancer, surgical treatment is yet unsatisfactory for the therapy of advanced prostatic cancer. As invasion and occlusion of cancer cells occurred in ureter or vesical neck, patients with advanced prostatic cancer suffered from diverse kinds of severe symptoms, such as urethrodynia, osphyalgia, uroschesis, and arthropathy¹²⁻¹⁵, followed by a high recurrence rate of cancer after routine surgical treatment. All limitation of clinical treatment resulted from unclear understanding of prostatic cancer pathogenesis¹⁴. Thus, our study explored possible mechanism of miR-96 on the regulation of prostatic cancer.

microRNA belongs to a group of endogenously expressed, small non-coding single-stranded RNA¹³⁻¹⁵, which is characterized by clustered arrangement, hereditary stability, and high homology. Previous studies^{15,16} showed that microRNA could modulate tumor progress through directly or indirectly influencing oncogenes. Evidence

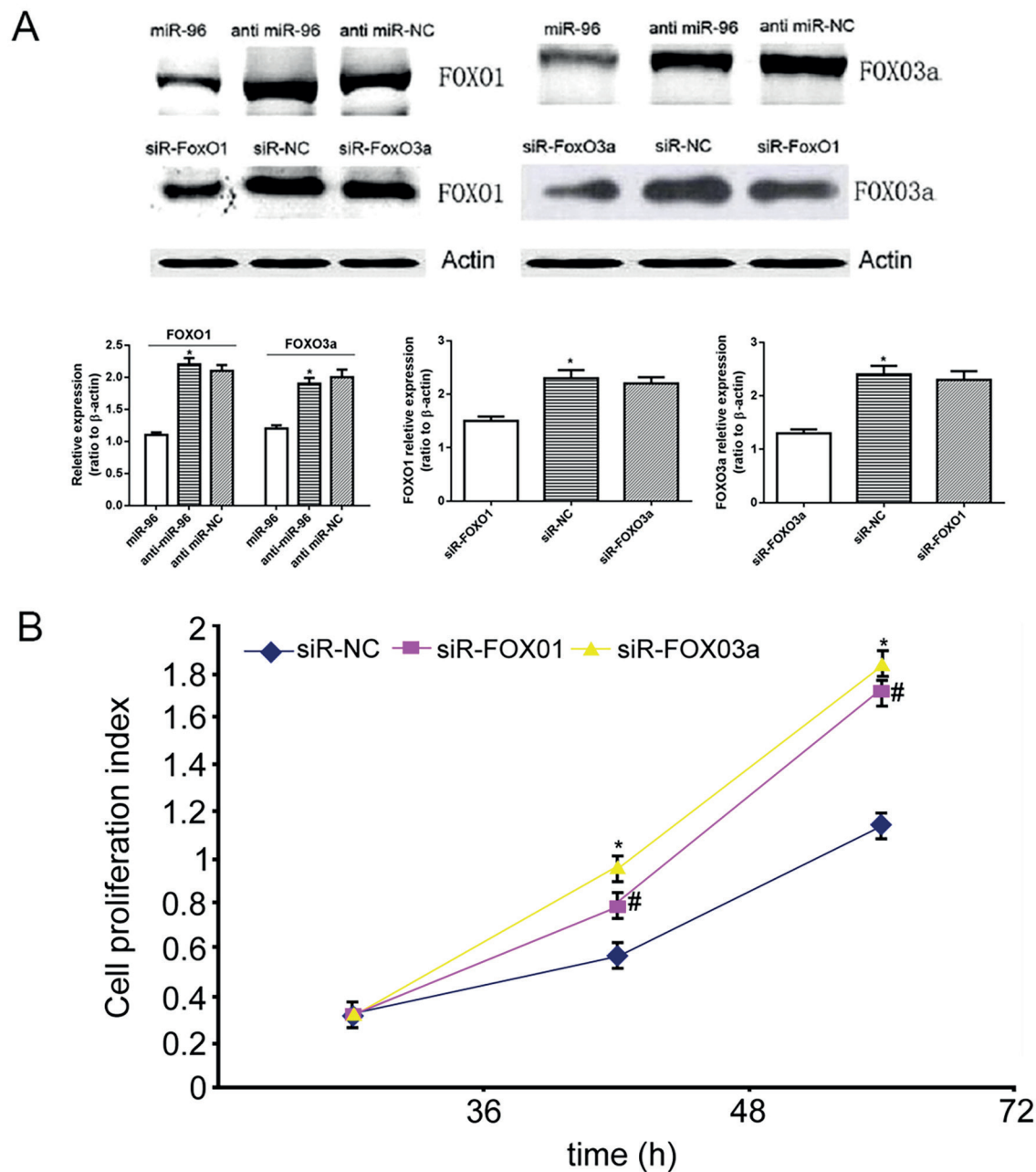


Figure 3. miR-96/si-RNA regulated the expression of FOXO1 and FOXO3a in PC-3 cells **(A)** Western blot analyses comparing the expression of FOXO1 and FOXO3a in PC-3 cells transfected with different miRNAs (miR-NC, miR-96 and anti miR-96) or siRNAs (siR-NC, siR-FOXO1, and siR-FOXO3aa). **(B)** Comparison of cell proliferation index of PC-3 cells in different siRNA-treated groups (siR-NC, siR-FOXO1 and siR-FOXO3a groups).

Table I. Fold-change in FOXO1 and FOXO3a mRNA levels in Pc-3 cells treated with different miRNAs and siRNAs relative to negative controls.

	FOXO1 mRNA ($\bar{x} \pm s$)	FOXO3a mRNA ($\bar{x} \pm s$)
miR-96/miR-NC	0.328 $\pm$ 0.161	0.279 $\pm$ 0.218
anti-miR-96/anti-miR-NC	1.578 $\pm$ 0.334	1.637 $\pm$ 0.269

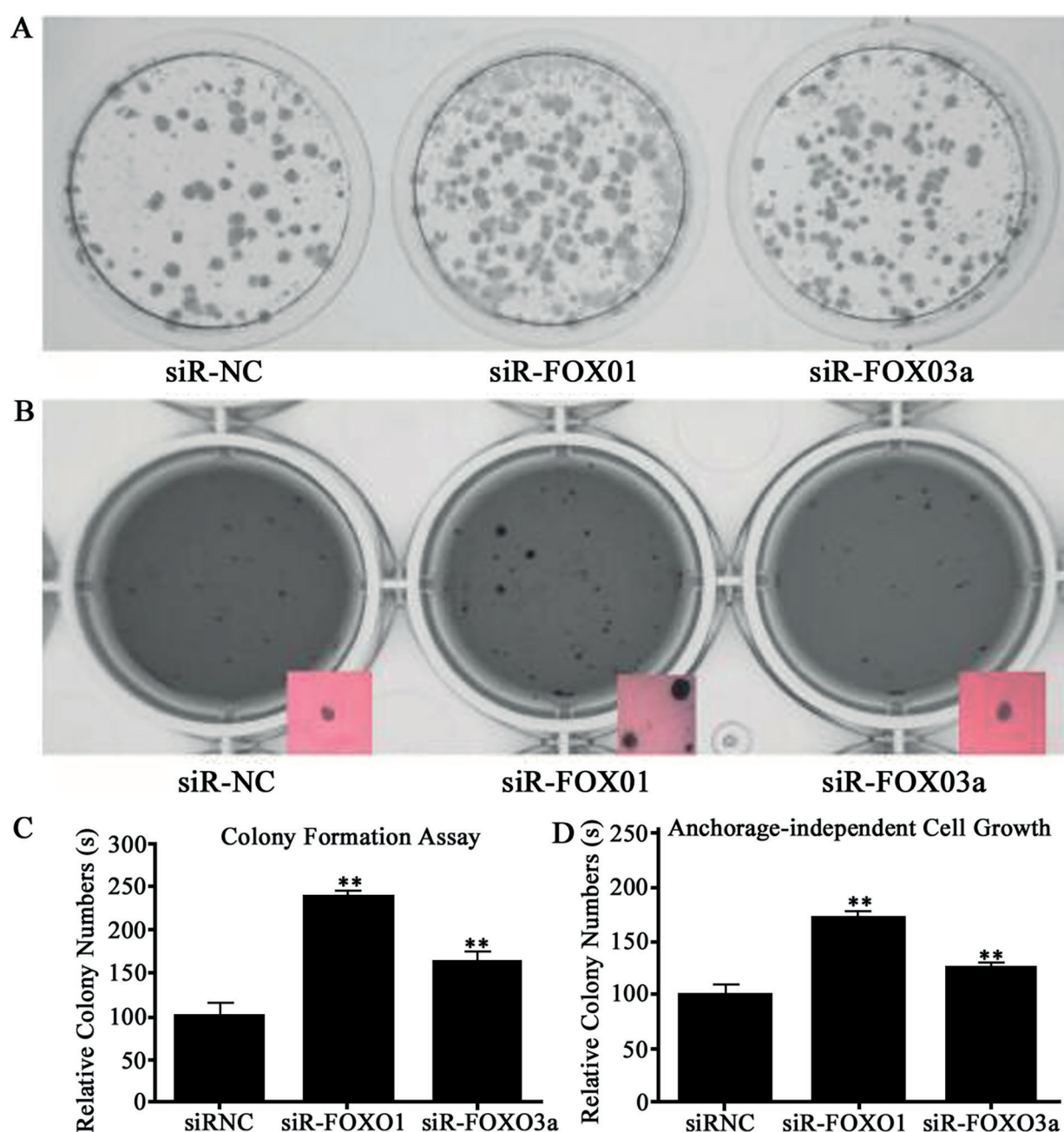


Figure 4. Effect of FOXO1 and FOXO3 inhibition on cloning of PC-3 cells. **(A)** Plate colony in three different groups. **(B)** Soft-agar medium colony in three different groups (siR-NC, siR-FOXO1 and siR-FOXO3a groups). **(C, D)** Analysis of effect on cloning of PC-3 cells.

illustrated that miR-205 played an inhibitory role in prostate cancer cells by targeting TP53INP1¹⁷. As a member of microRNA family, the rise of miR-96 level was found in lung cancer, osteosarcoma, and gastric carcinoma, but reduction in breast cancer¹⁸ suggested miR-96 was involved in tumorigenesis.

Our study elucidated differential expression of miR-96 between prostatic cancer cells and

normal prostatic cells, suggesting miR-96 was associated with prostatic cancer. FOXO protein regulated apoptosis and DNA damage repair¹⁸, and recent findings indicated FOXO attenuated tumor lesion^{18,19}. Further, FOXO1 and FOXO3a were determined to be target genes of miR-96 based on analysis data from *in silico* analysis with TargetScan and PicTar. Accordingly, the expressions of FOXO1 and FOXO3a were chosen to

explore tumor regulatory effect of miR-96. Inhibition of miR-96 increased expression of FOXO1 and FOXO3a by using qPCR and Western blot experiment. Such result proved the inhibition of FOXO1 and FOXO3a by miR-96 increased the proliferation of PC-3 cells, which was consistent with the previous finding of FOXO-mediated cellular events²⁰. Our work demonstrated miR-96 was an early stage biomarker for prostatic cancer prognosis, and inhibition of miR-96 could be a potential therapeutic target via restoring FOXO activities.

Further siRNA experiment showed siR-FOXO1 and siR-FOXO3 significantly restored the proliferation of prostatic cancer, suggesting prostatic cancer growth negative associated with cellular levels of FOXO1 and FOXO3a. Considering the inhibition of FOXO1 promoted cancer cells into S phase, we proposed that inhibition of FOXO1 could further exacerbate prostatic cancer. Both plate colony and soft-agar medium colony experiment showed that compared with inhibition of FOXO3a, inhibition of FOXO1 was more likely to cause large cells mass, which proved FOXO family induced different effects on various kinds of cancer²¹, and suggested FOXO1 was more potential for prostatic cancer treatment.

Conclusions

We showed that miR-96 was a potential predictor for prostatic cancer diagnosis and demonstrated miR-96 enhanced prostatic cancer via inhibiting FOXO1 and FOXO3a. Replenishment of FOXO1 and FOXO3a activities could be a feasible way for the treatment of prostatic cancer.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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