

Original Article

Androgen-regulated miR-26ab suppresses the proliferation of prostate cancer cells by targeting PTEN and androgen receptor signaling

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Abstract: The androgen receptor (AR) signaling pathway plays an important role in the progression of androgen dependent and castration-resistant prostate cancer (CRPC). Only few micro-RNAs (miRNAs) have been found to be androgen regulated. In the present study, we identified six miRNAs, (miR-32, miR-590-5p, miR-21, miR-99a, miR-221 and miR-26ab); they are differentially expressed in CRPC compared with benign prostate hyperplasia (BPH) according to microarray analysis. Transfection of prostate cancer cell line LNCaP with miR-26ab increased PTEN (phosphatase and tensin homolog deleted on chromosome ten) expression both in mRNA and protein level. We further demonstrated that overexpression of miR-26ab significantly reduced the androgen receptor expression in prostate cancer cells. Our findings provide insight into the regulation of PTEN expression through miR-26ab in CRPC, which will lay a foundation for the application of miR-26ab in CRPC treatment.

Keywords: Androgen receptor, CRPC, miRNAs, miRNA-26ab, PTEN

Introduction

Prostate cancer (PCa) is the most common malignancy and a common cause of cancer-related mortality for men all over the world. The incidence rate of PCa shows rising tendency in China. Androgen-ablation therapy (AAT) is the mainstay treatment for PCa patients [1-3]. Most prostate tumors respond to the therapy initially, but often fatal castration-resistant prostate cancer (CRPC) emerges during PCa progression and no effective treatment has been developed for these insensitive cancers [4].

However, the molecular mechanisms involved in androgen-dependent (AD) prostate cancer cells acquire the ability to resist androgen ablation are not well clear. Although androgen receptor (AR) expression in some AIPC raised, but some investigation that AR amplification rate was very low before and after androgen jerk [5, 6]. Also, CRPC retain AR and select AR-regulated gene expression in the absence of or with low levels of circulating androgens, demonstrating that AR signaling continues to

play a significant role in patients with castration-resistant disease [7]. At present, androgen receptor (AR) gene mutations/amplification and methylation are considered the principal cause to the activation of AR signaling [8]. Central to the AR response are the specific downstream target genes, such as kallikrein-related peptidase 3 (KLK3 alias prostate specific antigen, PSA), transmembrane protease, serine 2 (TMPRSS2), phosphatase and tensin homolog deleted on chromosome ten (PTEN) and prostate acid phosphatase (PAP) [9-11]. Recent findings indicated that in the castration-resistant stage of the disease, AR controls a distinct transcriptional program linked to cancer cell proliferation and cell-cycle regulation [12, 13]. Taken together, these results suggest that AR signaling plays a central role in most stages of PCa. Therefore, it is critically important to understand the regulation of its expression and to find novel ways of inhibiting this pathway.

MicroRNAs (miRNAs) are a class of short, endogenous, non-coding RNA molecules that typically inhibit the translation and stability of

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Table 1. Mean expression values of putative androgen-regulated miRNAs in clinical samples according to the microarray

Systematic name of miRNAs	Corrected <i>P</i> -value	Fold change	Regulation	BPH	PC	CRPC
hsa-miR-32	0.00106	2.1	Up	4.12	4.30	5.19
hsa-miR-590-5p	0.00526	3.3	Up	5.02	7.23	6.21
hsa-miR-21	0.03126	2.9	Up	6.39	4.12	5.49
hsa-miR-99a	0.00118	2.6	Down	5.88	3.90	4.98
hsa-miR-221	0.00612	2.5	Down	9.23	6.21	8.69
hsa-miR-26ab	0.02123	3.9	Down	12.32	8.29	9.26

mRNA by binding to the 3'-untranslated regions (3'-UTR) of their target mRNAs [14]. Accumulating evidence has indicated that altered miRNA level resulted from mutation or aberrant expression is correlated with various human cancers [15]. It is suggested that miRNAs can function as oncogenes or tumor suppressors depending on the function of the target genes in malignancy [16]. The recent discovery that AR is a target of miRNAs provides evidence for miRNA-mediated AR regulation that could be crucial for the development of CRPC [17, 18]. It is possible that prostate cancer cells, which are exposed to androgen blockade therapy acquire molecular alterations to promote hypermethylation of cancer cell genome.

In this study, we have performed miRNA expression profiling on a clinical tumor sample set consisting of untreated and castration-resistant tumors and benign controls to identify differentially expressed miRNAs. In our study, we reported that miR-26ab is pathologically down-regulated in CRPC specimens and PC tissues, and that ectopic expression of miR-26ab promoted PC cell growth in vitro. Moreover, AR and PTEN were identified as the direct functional target of miR-26ab in PC.

Materials and methods

Clinical samples

The samples of 56 clinical specimens were collected from the first affiliated hospital of Anhui medical university Hospital. The prostate carcinoma specimens were divided based on their combined Gleason scores (GS) into low ($n = 24$) and high ($n = 52$) grade subgroups, defined as cases with a combined $GS < 7$ (3+4 and/or 4+3), respectively. The patients' clinical information is summarized as shown **Table 1**. The use of clinical material has been approved

by Ethical Committee of the first affiliated hospital of Anhui medical university hospital.

Cell line

LNCaP cell line was purchased from the American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C and 5% CO₂.

MiRNA expression profiling

MiRNA expression was analyzed with Agilent human miRNA microarray v2 chips according to manufacturer's instructions (Agilent Technologies, Santa Clara, CA, USA). 100 ng of total RNA was labeled with pCp-Cy3 and hybridized using miRNA Labeling Reagent and Hybridization Kit, and Agilent SureHyb chambers. The microarray images were scanned with Agilent microarray scanner and analyzed using Agilent Feature Extraction Software and GeneSpringGX7.3.1 software. Microarrays were performed in duplicate, and the results were normalized.

RNA extraction and RT-PCR

Total RNA was extracted using Trizol reagents (Invitrogen) according to the manufacturer's instructions and diluted to 200 ng/mL. Then, quantitative real-time RT-PCR (qRT-PCR) was performed using One Step SYBR® Prime-Script™ RT-PCR Kit II (TaKaRa, China) according to standard protocol. Relative expression was evaluated by comparative 2^{-ΔΔCT} method and normalized to the expression of U6 RNA. For detection of PTEN-mRNA expression, β-actin was used to normalize PTEN-mRNA expression levels. Forward and reverse primers sequences for PTEN were as follows: 5'-GAGGGATAAAAC-ACCATG-3' and 5'-AGGGGTAGGATGTGAACCAG-TA-3'. The primers for β-actin: forward 5'-CAG-AGCCTCGCCTTTGCC-3' and reverse 5'-GTCC-CCCACATAGGAATC-3'. All experiments were performed in triplicates.

Growth curves

LNCaP cells were seeded into 24-well plates after transfection at a density of 1500 cells per well. Growth curve measurements were detected at different time. Resazurin reagent (R&D systems, Minneapolis, MN, USA) was used to analyse cell growth, and the absorbance at

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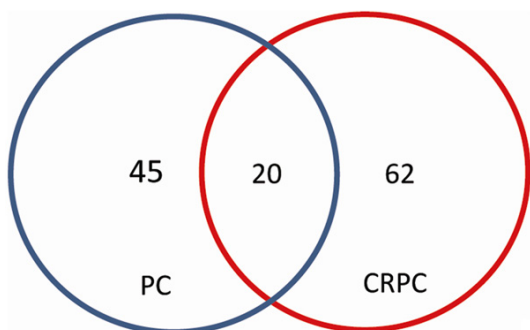


Figure 1. Venn diagram of differentially expressed genes in PC and CRPC according to the miRNA microarray.

450 nm were also measured. All experiments were performed in triplicates.

Cell cycle analysis

LNcaP cells were seeded at a density of approximately 5×10^5 in 100 mm plates and transfection with pre-miRNA for 48 h. Cell cycle was analyzed by flow cytometry with propidium iodide (PI) staining using Cell Cycle Analysis Kit (Beyotime, China). Briefly, cells were harvested and washed with PBS and fixed with 70% ice-cold ethanol at 4°C overnight. Then cells were incubated in a PBS solution containing 10 mg/mL RNase and 1 mg/mL PI for 1 h at room temperature. Finally, the percentage of cells in different phases of the cell cycle was measured by flow cytometry (FACS Calibur, BD Biosciences, NY, US). All samples were examined in triplicate.

Luciferase reporter assay

The 3'-UTR of PTEN containing putative binding site of miR-25ab were amplified and subcloned into pSGG-3UTR plasmid (Promega, Madison, WI, USA). The mutations were made to the miR-26ab binding sites in the 3'-UTR-fragments.

The vector was cotransfected with pre-miRNA into LNcaP cells for 48 h. The cells were harvested and relative luciferase activity was detected using a dual-luciferase reporter assay kit (Promega) according to the manufacturer's instructions [19]. All experiments were performed at least three times.

Western blotting

Total protein extracts were prepared as described [20], resolved on 12% SDS-PAGE

(Invitrogen), and transferred to PVDF membranes (Roche). After transfer, membranes were incubated in blocking solution, probed with various antibodies including anti- β -actin mouse monoclonal antibody, anti-PTEN mouse monoclonal antibody, anti-AKT rabbit monoclonal antibody, anti-phospho-AKT (Ser-473) rabbit monoclonal antibody, anti-AR(H-280) rabbit monoclonal antibody or anti-PSA rabbit polyclonal antibody, washed, and visualized using horseradish peroxidase (HRP)-conjugated secondary antibodies (GE Healthcare) and enhanced chemiluminescence reagents (Amersham).

The pre- and anti-miRNA transfections

Cells were seeded (2×10^5 cells/well) in six-well plates. After incubation for 24 hours, cells were transfected with pre-miRNA-con or pre-miRNA-26ab construct using Lipofectamine 2000 transfection reagent. MiRNA expression levels were analyzed by real time PCR at 3 days after transfection.

Results

MiRNA expression profiling of clinical samples

To evaluate the functional role of miRNAs in human PCa, we performed a comprehensive analysis of miRNA expression in 12 benign prostatic hyperplasia (BPH), 14 PCs obtained from prostatectomies, 10 BPH and 12 CRPCs using miRNA microarrays we found that 339 were considered to be significantly expressed (Figure S1). Moreover, 65 miRNAs were differentially expressed in PC, and 82 miRNAs were in CRPC (Table S1). As shown in Figure 1, 20 miRNAs were identified in both PC and CRPC compared with their controls. In CRPC, miR-32, miR-590-5p and miR-21 were most significantly overexpressed, and miR-99a, miR-221 and miR-26ab were significantly underexpressed (Table 1 and Table S1). However, miR-26ab, miR-21 and miR-221 were found to be differentially expressed in CRPC but not in PC.

From the list of differentially expressed lncRNAs, as previous reports showed that miR-21 and miR-221 was androgen regulated and implicated in CRPC [21, 22], we focused on miR-16 for further studies.

Effects of miR-26ab on prostate cancer cell growth and cell cycle

To determine the effect of miR-26ab on cell growth, miR-26ab or miRNA-26ab inhibitor was

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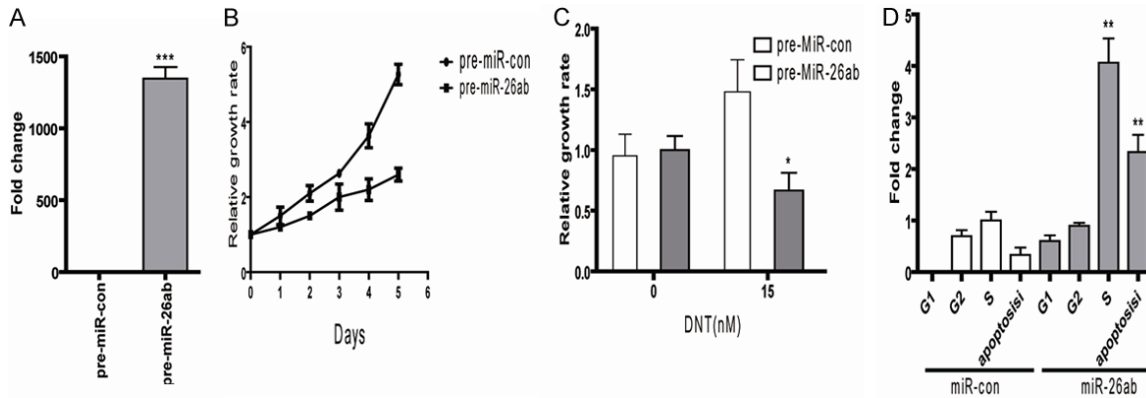


Figure 2. Effects of miR-26ab on prostate cancer cell growth and cell cycle. A. Enhanced expression of miR-26ab by pre-miRNA transfections determined by qRT-PCR analysis at 3 days after transfection. B. Growth curves of LNCaP cells transfected with pre-miR-control, pre-miR-26ab. The mean cell number \pm s. d. on different days is shown. C. Relative growth of DHT-treated (0 or 15 nM) LNCaP cells transfected with pre-miR-26ab at day 5 after transfection. D. Results of PI staining and cell cycle analysis by flow cytometry of LNCaP cells after pre-miR-control or pre-26ab transfections.

transfected into parental LNCaP cells. As shown in **Figure 2A**, the transfection of pre-miRNA increases the levels of miR-26ab in LNCaP cells. miR-26ab significantly reduced the growth rate ($P < 0.05$) of LNCaP cells in a time-dependent manner (**Figure 2B**). Furthermore, double hydrogen testosterone (DHT) was used to determine the androgen regulation on miR-26ab. As shown in **Figure 2C**, the proliferation of miR-26ab-transfected LNCaP cells was significantly inhibited compared with control cells after treated with 15 nM DHT. More important, the effect of miR-26ab transfection on cell growth was abolished upon hormone starvation, indicating that miR-26ab was regulated by DHT. The results of cell cycle analysis showed that apoptosis was significantly enhanced in LNCaP cells transfected with miR-26ab compared with cells transfected with scrambled-oligo at day 5. And the number of cells in phase was significantly increased by miR-26ab expression (**Figure 2D**).

Identification of target genes for miR-26ab by bioinformatics and microarray

To explore the mechanism by which miR-25ab affects PC progression, we employed bioinformatics analysis using TargetScan and attempted to find potential genes which might be involved in miR-25ab mediated PC progression. In our study, five genes, including PTEN, FBN1, REBB, TPM1 and E2F were predicted as potential target genes of miR-25ab. Of these, PTEN was predicted to be the most promising target

for miR-26ab functionally and was selected for further validation.

To confirm that PTEN was the target directly regulated by miR-26ab, we first examined the expression of PTEN in pre-miR-26ab-transfected LNCaP cells. As shown in **Figure 3A**, PTEN mRNA level was significantly up-regulated by pre-miR-26ab transfection. Consistently, PTEN protein expression was also enhanced in pre-miR-26ab-transfected LNCaP cells (**Figure 3B**).

To confirm the direct binding between miR-25ab and PTEN 3'UTR, We inserted the PTEN 3'UTR fragments downstream of luciferase open reading frame (ORF) in a reporter plasmid to test whether PTEN are directly regulated by the miR-26ab. Inhibition of luciferase activity was observed when the PTEN 3'UTR was cotransfected with miR-26ab, but not with miR-control, suggesting that the up-regulation of PTEN mRNA was regulated by miR-26ab (**Figure 3B**). Together with results of mRNA expression microarray, the luciferase results clearly show that PTEN is a direct target of miR-26ab.

MiR-26ab regulates AR signaling in prostate cancer cells

To probe the molecular mechanism by which miRNA-26ab regulates the host gene expression, we examined the expression of AR and phosphorylated(p)-AKT in prostate cancer cells transfected with miR-26ab or the miR-con precursor after 24 h followed treatment with HRG

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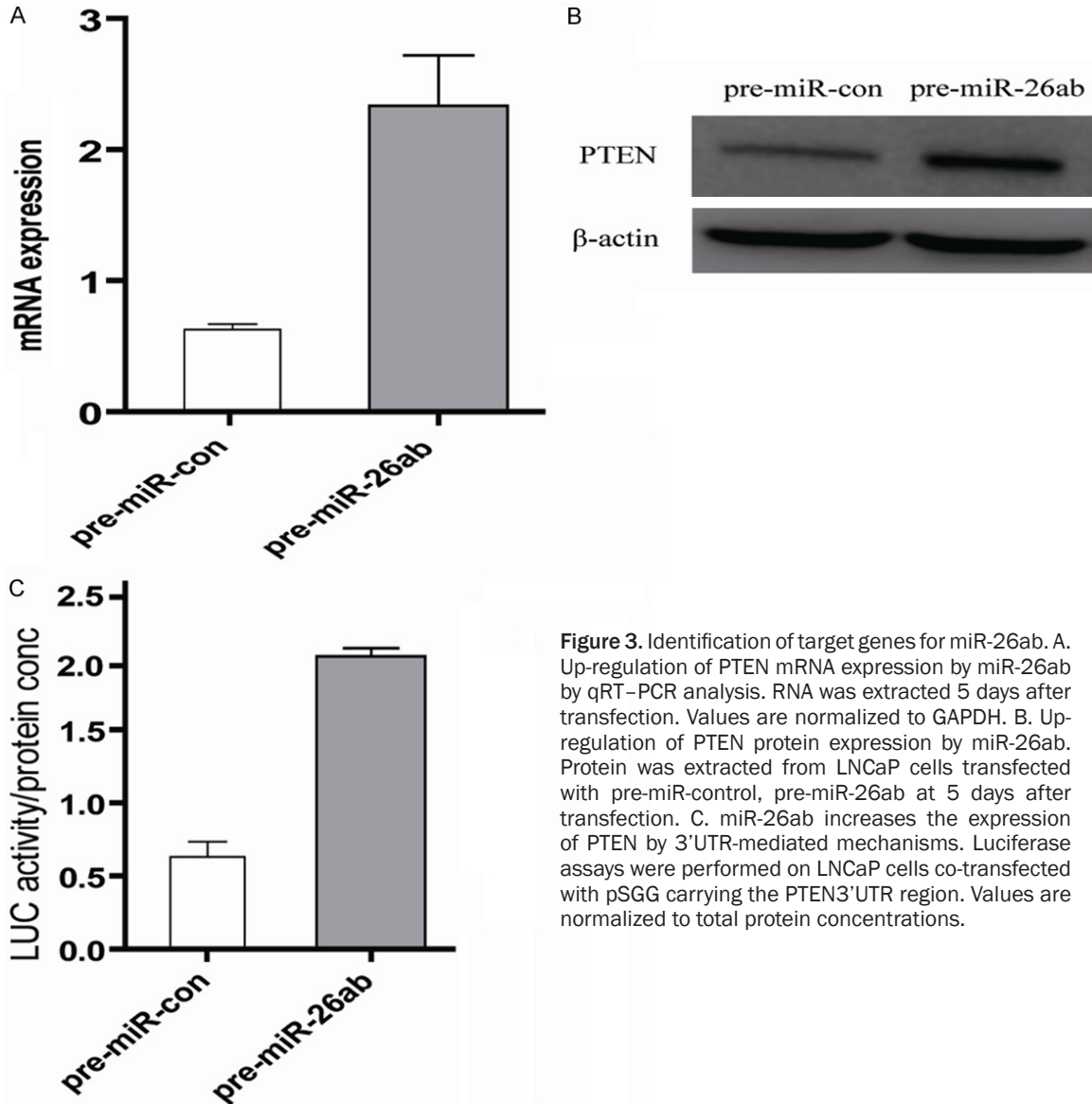


Figure 3. Identification of target genes for miR-26ab. A. Up-regulation of PTEN mRNA expression by miR-26ab by qRT-PCR analysis. RNA was extracted 5 days after transfection. Values are normalized to GAPDH. B. Up-regulation of PTEN protein expression by miR-26ab. Protein was extracted from LNCaP cells transfected with pre-miR-control, pre-miR-26ab at 5 days after transfection. C. miR-26ab increases the expression of PTEN by 3'UTR-mediated mechanisms. Luciferase assays were performed on LNCaP cells co-transfected with pSGG carrying the PTEN3'UTR region. Values are normalized to total protein concentrations.

(50 ng/ml). As shown in **Figure 4A**, p-AKT protein levels was dramatically decreased but AR levels do not be affected. To further conform whether miR-26ab modifies AR, we examined the effect of bicalutamide, an AR antagonist. The effect in LNCaP cells transfected with miR-26ab or the miR-con precursor, followed with DHT after 24 h was determined (**Figure 4B**). Bicalutamide significantly reduced PSA protein expression. The data suggested that miR-26ab repressed the activation of AR signaling pathway.

To define the mechanism by which miR-26ab blocks AR signaling, we co-transfected LNCaP cells with a DHT-responsive PSA-LUC reporter construct and either pre-miR-26ab

or pre-miR-con (**Figure 4C**). Reporter activity was increased following DHT treatment, and this effect was significantly reduced by pre-miR-26ab transfection. Furthermore, the combination of miR-26ab and bicalutamide was more effective at reducing DHT-induced PSA-LUC reporter activity than either treatment alone. The result suggested that pre-miR-26ab indirectly reduces transcription and expression of AR pathway target genes such as PSA.

Discussion

In present study, we identified miRNAs that are differentially expression in CRPC by microarray, and found that miR-26ab significantly under

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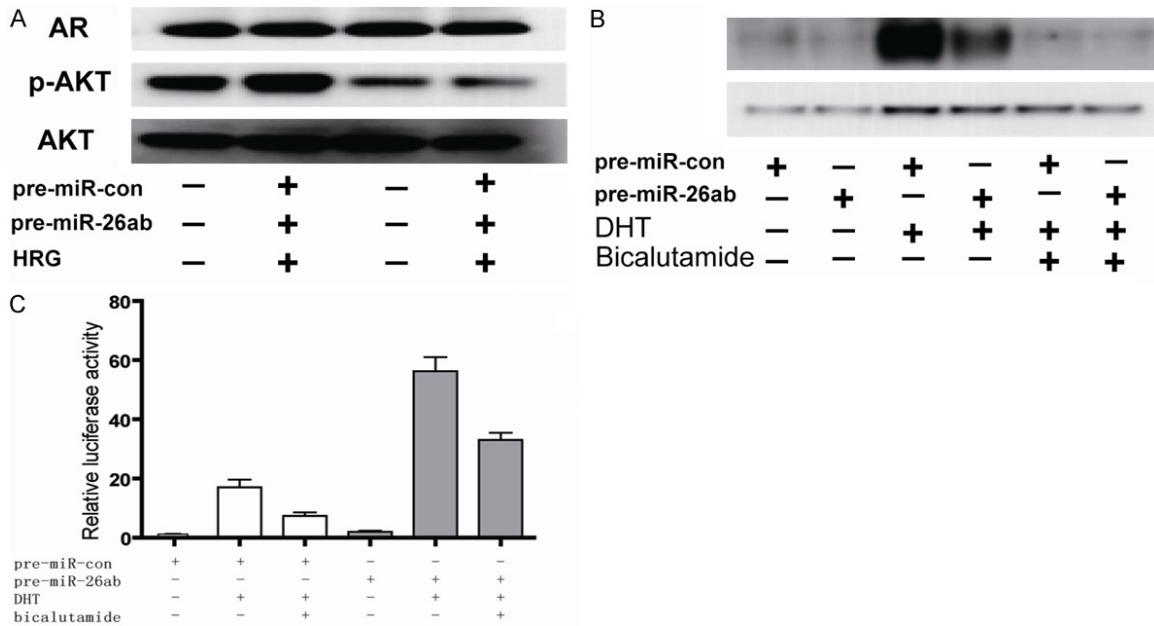


Figure 4. MiR-26ab blocks AR signaling, decreases PSA expression and promoter activity in LNCaP cells. A. LNCaP cells were transfected with pre-miR-con or pre-miR-26ab and serum starved for 24 h thereafter, followed by stimulation heregulin (HRG; 50 ng/ml) for 20 min. Cell lysates were analyzed for AR, total AKT, and phospho-AKT expression by western blot. B. LNCaP cells were transfected with miR-26ab for 48 h and treated with DHT (10 nM) or bicalutamide (10 M). Total PSA expression was determined by western blot. C. LNCaP cells were co-transfected with a PSA-luciferase vector (PSA-LUC) and thymidine kinase-Renilla vector and with miR-con or miR-26ab. Relative luciferase expression (firefly normalized to Renilla) values are expressed as a ratio of miR-con-transfected cells (S. D.).

expressed in CRPC and putatively regulated by androgen. Furthermore, our findings demonstrated that miR-26ab suppressed the proliferation of prostate cancer cells by targeting PTEN and androgen receptor signaling.

In the past several years, increasing evidences have shown that miRNAs play important roles in controlling many fundamental cellular processes including those regulating carcinogenesis. A number of studies have detected a number of miRNA expressions in a variety of human tumors, suggesting that miRNAs may play a role as a novel class of tumor promoters or suppressor [23-25]. In this study, we found that miR-26ab was significantly downregulated in CRPC samples, which suggested that miR-26ab may be involved in the progression of PCa. To further determine the function of miR-26ab in PC cells, we transfect pre-miRNAs into the LNCaP cells. We found that miR-26ab inhibited LNCaP cells proliferation and promoted apoptosis. However, the correlation of miR-26ab with androgen receptor signaling was unclear and remained to be elucidated.

One of the major findings in this study was that low expression of miRNA-26ab dramatically

increased the expression of PTEN at both transcriptional and translational levels. In addition, luciferase reporter assays confirmed that miR-26ab targets the 3'UTR of PTEN. Previous studies reported that PTEN was involved in several cellular processes, including cell cycle and apoptosis [26, 27]. Interestingly, it has recently been suggested that PTEN could be a co-repressor of AR [28]. Thus, miR-26ab/PTEN could form a positive feedback loop of AR signaling in PC.

In conclusion, we findings that interplay between androgen-regulated miR-26ab and PTEN, that will lead to a better understanding of the mechanism mediating the development and progression of AR prostate cancer. This will contribute to the development of more effective treatments that maybe used to prolong the survival of patients with the deadliest form of this disease.

Disclosure of conflict of interest

None.

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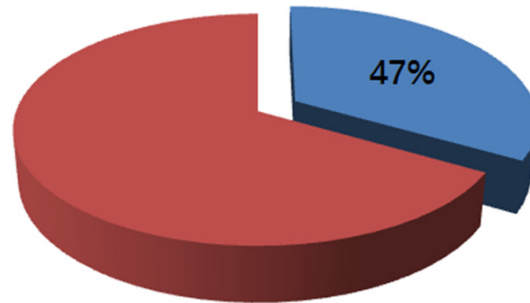


Figure S1. 723 human miRNAs were detected in this study but just 339 (47%) were considered to be significantly expressed. (Blue: significantly expression miRNAs; Red: non-significantly expression miRNAs).