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MiR-182 promotes prostate cancer progression through activating Wnt/β-catenin signal pathway

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ABSTRACT

Although prostate cancer can be surgical excised and effectively treated by androgen-deprivation therapy, radiotherapy, or chemotherapy, management of patients with advanced or drug-resistance prostate cancer stills remains a big trouble. Accumulated evidence indicated that miR-182 and Wnt/β-catenin function as tumor oncogene in the progression of a variety of tumors. However, little is known about how miR-182 regulates β-catenin signal molecular and impacts on the tumorigenesis of human prostate cancer. In this study, employing the analyses of qRT-PCR, we found that prostate cancer tissues expressed much more miR-182 than non-cancer tissues did. In vitro studies revealed that overexpression of miR-182 promoted cell proliferation, colony formation, migration, invasion and inhibited cell apoptosis; in vivo results demonstrated that silencing of miR-182 mediated by inhibitor dramatically reduced prostate cancer xenograft tumor growth. Importantly, through western blotting analysis, we identified that miR-182 dramatically activated Wnt/β-catenin pathway by targeting multiple negative regulators of Wnt/β-catenin signaling, including GSK-3β, APC, CK1 and Axin. Besides, we observed the elevated levels of c-myc and Cyclin D1 when PC-3 and LNCap cells were up-regulated miR-182. Our findings indicate that miR-182 acts as one of oncogenic factor in the progression of prostate cancer by recruiting a mechanism of aberrant activation of Wnt/β-catenin signaling.

1. Introduction

Prostate cancer is one of the most common cancers in men worldwide and is the second leading cause of cancer-related death [1]. According to the estimation from the American Cancer Society, about 1 out of 7 males will be diagnosed with prostate cancer during the lifetime [2]. Despite substantial progress made in therapies of prostate cancer, the prostate cancer patients are also undergoing big suffering. Although factors of both genes and environment are common causes of prostate cancer occurrence and development, the underlying molecular mechanisms are still unclear. Therefore, better understanding the pathogenesis of prostate cancer and exploring novel intervention targets are urgent.

A large number of studies have shown that the Wnt/β-catenin pathway is crucial for the development and progression of prostate cancer [3–6]. Wnt stimulation onsets a series of events enabling the nuclear accumulation of β-catenin and the transcription of some oncogenes such as c-jun, c-myc and cyclin D1 [7]. Normally, tight regulation via a destruction complex comprising Axin, adenomatous polyposis coli protein (APC), glycogen synthase kinase-3β (GSK-3β), and casein kinase 1 (CK1) maintains cytoplasmic β-catenin levels by phosphorylation of β-catenin resulting in its ubiquitination and targeting to the 26S proteasome [8–10]. Excessive activation of the Wnt/β-catenin signaling pathway has been reported to have close relationship with the prognosis of patients with prostate cancer [11].

MicroRNAs (miRs) are short, non-coding RNAs that exert function to repress translation and/or initiate degradation of target messenger RNAs (mRNAs) [12]. Emerging evidences suggest that miRNA served as an oncogene or tumor suppressor, involves in prostate cancer development and progression [13–15]. For example, up-regulation of miR-182 promoted prostate cancer cell proliferation and invasion role by targeting multiple genes [16,17]. Hypoxia-inducible miR-182 enhances HIF1α signaling via targeting PHD2 and FIH1 in prostate cancer [2]. Besides, loss of miR-182 and miR-200a in prostate cancer cells induces GNA13 expression and SDF-1-mediated invasion [18]. However, whether miR-182 could directly target the key components of Wnt/β-catenin pathway and affect prostate tumor progression is not fully elucidated. Understanding the miR-182 regulatory network in Wnt/β-catenin will provide new therapeutic targets for prostate cancer.

In this study, we investigated the molecular mechanism of miR-182/β-catenin signal cascade on cell viability and tumorigenic in prostate cancer cell lines. Firstly, we determined the expression level of miR-182

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in prostate cancer tissues. Then we explored the function of miR-182 on cell viability, apoptosis migration and invasion followed by confirming the related proteins of Wnt/β-catenin pathway. Finally, we elucidated the role of miR-182/β-catenin on prostate cancer progression. Our study aimed to search a new genetic therapeutic target for prostate cancer patients.

2. Materials and methods

2.1. Patient and sample preparation

25 paired samples of fresh prostate cancer and paracarcinoma tissues were obtained from patients with prostate cancer and who received radical prostatectomy without chemotherapy or radiation. The study involved human samples was approved by the ethics committee of Shanghai Jiao Tong University affiliated Ruijin Hospital and had been performed in accordance with the Helsinki Declaration.

2.2. Cell culture

PC-3 and LNCap prostate cancer cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and were routinely maintained in Dulbecco's Modified Eagles Medium (DMEM) containing 10% (v/v) heat inactivated fetal bovine serum (FBS), and 1:1 penicillin/streptomycin (100 U/ml). All cell lines were maintained in a humidified incubator with 5% CO_2 at 37 °C.

2.3. Plasmid, small interfering RNA and transfection

The mimics-miR-182, inhibitor-miR-182 and their controls were synthesis by GenePharma (Suzhou, China). Cells were transfected with inhibitor or mimics using INTERFERin® transfection reagent (Polyplus, France) according to the manufacturer's instructions. A total of 2×10^5 cells were transfected with 2 μg DNA. The transfection effects were detected by western blotting 48 h after transfection.

2.4. Real time PCR detection

The total RNA from prostate cancer tissues and cell lines was extracted with an RNApure Tissue Kit (CWBIO, China), according to the manufacturer's instructions, and reverse-transcribed into firststrand cDNA using the HiFiScript 1st Strand cDNA Synthesis Kit (CWBIO, China). Real time PCR analysis of miR-182, was performed in triplicate using miScript SYBR Green PCR Kit (Qiagen) on the DA7600 Real-time Nucleic Acid Amplification Fluorescence Detection System (Bio-Rad) in a 20 μL reaction system. The expression of miRNAs in the cells and tissues was normalized to U6 levels, while the expression of other mRNAs was normalized by the expression of GAPDH as a reference housekeeping gene. The $2^{-\triangle\triangle Ct}$ method was used to evaluate the relative mRNA expression changes. Primers for each miR-182 as well as for U6 were commercially purchased (miScript Primer Assays, Qiagen).The other primers were synthesized by the Beijing Genomics Institute and are as follows:

β − catenin forward: 5′-GTACGTCCATGGGTGGGACA-3′
Reverse: 5′-GGCTCCGGTACAACCTTCAACTA-3′;
GAPDH forward: 5′-CATCACCATCTTCCAGGAGCG-3′,
Reverse: 5′-TGACCTTGCCCACAGCCTTG-3′.

2.5. Western blotting analysis

Cell lysates were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were probed with antibodies against β-catenin (No.ab32572), Axin (No.ab32197), GSK-3β (No.ab93926), APC (No.ab15270), CK1(No.ab115293), Cyclin D1 (No.ab134175), c-myc (No.ab32072), GAPDH (No.ab8245) (Abcam, Cambridge, MA) overnight at 4 °C, and then incubated with horseradish peroxidase–conjugated secondary antibodies (Cell Signaling Technology) for 1 h at room temperature. The immune complexes were examined by ECL detection (Millipore, USA). For quantification, the western blotting bands were quantified by ImageJ software (National Institutes of Health) after background subtraction.

2.6. -(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2Htetrazolium bromide (MTT) assay

1×10^4 PC-3 and LNCap Cells with different treatments were seeded into 96 well plates and stained at the indicated time point with 100 μl sterile 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich, St Louis, MO) dye (at 0.5 mg/ml) for 3 h at 37 °C, followed by removal of the culture medium and the addition of 150μl dimethyl sulfoxide (Sigma-Aldrich). The absorbance was measured at 570 nm, with 655 nm used as the reference wavelength.

2.7. Colony formation assay

2×10^2 PC-3 and LNCap Cells were plated into six well plates and cultured for 14 days. Colonies were then fixed for 5 min with 10% formaldehyde and stained with 1.0% crystal violet for 30 s.

2.8. Apoptosis assay

Flow cytometry was performed using cells stained with PI and FITC-labeled Annexin V (KeyGen, Shanghai, China) to detect cell apoptosis, as described in the manufacturer's instructions. Briefly, after 72 h of cells were transfected with mimics-miR-182, inhibitor-miR-182 or their controls, cells were washed twice with cold PBS, re-suspended in 1 × binding buffer at 1×10^6 cells/mL, and incubated with 200 μL binding buffer and 10 μL Annexin V-FITC. The samples were gently vortexed and incubated for 15 min at 25 °C in the dark before 300 μL binding buffer and 5 μL PI were added to each tube, and flow cytometry was performed within 1 h.

2.9. Wound healing and transwell assays

To observe the migration of the LNCap and PC-3 cells, we performed a wound healing assay. Various treatments were applied to 70% confluent cells. After 24 h of treatments, scratching was carried out with 20 μL pipette tips. Meanwhile, the medium was replaced by fresh DMEM medium with 1% FBS. The cells were washed several times with PBS to remove the floating cells and were provided with new growth medium. Photographs of the scratches were taken at 0 and 24 h using an inverted microscope (Leica) equipped with a Scion digital camera and built-in software (Leica application suite v 3.0).

For invasion, we coated the upper surface of a transwell chamber (8 μm, BD, USA) with matrigel (BD, USA). LNCap and PC-3 cells (2×10^5) were collected into 1% FBS medium and seeded in an upper chamber after 24 h of treatments. 600 μL medium with 10% FBS was added to the lower chamber. Then cells were cultured at 37 °C in a humidified incubator with 5% CO_2 for 48 h, followed by removal of cells inside the upper chamber with cotton swabs. Invaded cells were fixed with absolute methanol and stained with crystal violet. Fields at 20× magnification were randomly selected and cell numbers were counted.

2.10. In vivo xenograft assay

Four-week-old male BALB/c nude mice were obtained from The Jackson Laboratory (Beijing, China) and housed in a specific pathogen-free facility on our campus. BALB/c mice were implanted 1×10^7 cells treated with different treatments. And control group were subcutaneous