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MiR-506-3p acts as a novel tumor suppressor in prostate cancer through targeting GALNT4

C.-Y. HU[1], P. YOU[2], J. ZHANG[1], H. ZHANG[1], N. JIANG[1]

[1]Department of Urology, Gongli Hospital Affiliated to The Second Military Medical University, Shanghai, China
[2]Graduate School, Ningxia Medical University, Ningxia, China

Abstract. – **OBJECTIVE:** Researches have indicated that microRNA-506-3p (miR-506-3p) was downregulated and functioned as tumor suppressor in cancers. However, the biological role of miR-506-3p in prostate cancer (PCa) remains to be elucidated.

MATERIALS AND METHODS: Expression of miR-506-3p in PCa cell lines was measured by qRT-PCR. Effects of miR-506-3p expression on PCa cell behaviors were investigated with MTT assay, colony formation assay, and transwell invasion assay. Connection of miR-506-3p and N-Acetylgalactosaminyltransferase-4 (GALNT4) was analyzed with luciferase activity reporter assay and Western blot assay.

RESULTS: miR-506-3p expression was downregulated in PCa cell lines. Function studies demonstrated that overexpression of miR-506-3p inhibits PCa tumor progression in vitro. Mechanistic investigations found GALNT4 was a direct target of miR-506-3p. Overexpression of GALNT4 reversed the tumor-suppressive effects of miR-506-3p on PCa cell.

CONCLUSIONS: Our results elucidated genetic silencing of miR-506-3p enhances GALNT4 oncogene expression to accelerate PCa progression.

Key Words:
MiR-506-3p, GALNT4, Prostate cancer, Tumor suppressor.

Introduction

Prostate cancer (PCa) is estimated to be the third leading-cause of newly diagnosed cancer type worldwide with 1,276,106 new cases in 2018[1]. The incidence and mortality rate of PCa in Asia countries have steadily increased in recent years[2]. Therefore, it is imperative to develop novel molecular targets that can regulate the progression of PCa.

MicroRNAs (miRNAs) are non-coding RNAs with the length of 18-24 nucleotides that are able to regulate gene expression through 3'-untranslated region (3'-UTR) binding[3]. A sizeable amount of evidence has demonstrated miRNAs are crucial players in the initiation and progression of human cancers including PCa[4,5]. Targeting miRNAs have shown to be promising strategy against cancer.

MiR-506-3p has been demonstrated to be downregulated in several human cancers. miR-506-3p was shown to be downregulated in nasopharyngeal carcinoma and its overexpression was able to inhibit tumor growth and metastasis through targeting LIM Homeobox 2 expression[6]. MiR-506-3p was found to be reduced in osteosarcoma, and force miR-506-3p expression was shown to inhibit mesenchymal-to-epithelial transition and autophagy via targeting the expression of sphingosine kinase 1[7]. In non-small cell lung cancer, miR-506-3p expression was found downregulated in cancer tissues and correlated with large tumor size, late tumor stage, and advance lymph node metastasis[8]. In addition, aberrant expression of miR-506-3p inhibits cancer cell growth, migration, and invasion *in vitro* and *in vivo* via targeting the expression of coactosin-like protein 1[8]. These results illustrated the tumor suppressive role of miR-506-3p; however, its role in PCa remains to be elucidated.

N-Acetylgalactosaminyltransferase-4 (GALNT4) belongs to the family of N-Acetylgalactosaminyltransferases whose role was to transfer GalNAc from UDP-GalNAc to serine or threonine residues[9]. GALNT4 has been demonstrated to have an oncogenic role in human cancers. In colon cancer, GALNT4 expression was increased in cancer and negatively regulated by miR-4262[10,11]. Importantly, miR-4262 overexpression was able to decrease GALNT4 expression and results in growth inhibition[10,11].

Corresponding Author: Chuanyi Hu, MD; e-mail: sh_huchuanyi@tom.com

Therefore, we aimed at investigating the role of miR-506-3p in PCa progression. Bioinformatics analysis software predicted that GALNT4 was a putative target of miR-506-3p. The synthetic miRNAs were transferred to PCa cell lines for the purpose to evaluate the biological roles of miR-506-3p. This work provided evidence regarding the critical role of miR-506-3p in PCa.

Materials and Methods

Cell Culture

Normal prostate epithelial cell line (RWPE-1) obtained at Cell Bank of Chinese Academy of Sciences was incubated in keratinocyte-SFM medium (Thermo Fisher Scientific, Waltham, MA, USA). PCa cell lines (DU145 and PC-3) purchased at American Type Culture Collection (Manassas, VA, USA) were incubated at Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific). These cells were incubated at 37°C in a humified incubator containing 5% CO_2 / 95% air.

Cell Transfection

MiR-506-3p mimic and corresponding negative control (miR-con) were synthesized by GeneChem (Shanghai, China). Open reading frame of GALNT4 cloned into pcDNA3.1 (pGALNT4) was established by GenScript (Nanjing, China). Cell transfection was conducted by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

RNA Isolation and qRT-PCR

RNA from cultured cells was isolated using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Inc.) according to the provided protocols. After quantified with NanoDrop-1000 (Thermo Fisher Scientific, Inc.), equal amount of RNA was reverse transcribed into complementary DNA using Superscriptase II (Invitrogen, Carlsbad, CA, USA). qRT-PCR was performed using SYBR Green Mix (TaKaRa, Dalian, China) at ABI7500 system (Applied Biosystems, Foster City, CA, USA). The primers were as follows: miR-506-3p: F: 5'-TAAGGCACCCTTCTGAGTAGA-3' and R: 5'-GCGAGCACAGAATTAATACGAC-3'; U6 snRNA: F: 5'-TGACACGCAAATTCGTGAAGCGTTC-3' and R: 5'-CCAGTCTCAGGGTCCGAGGTATTC-3'. The RT-qPCR condition was 95°C for 10 min followed by 40 cycles at 95°C for 15 s, 60°C for 25 s, and 72°C for 35 s. Expression levels were measured using the $2^{-\Delta\Delta Cq}$ method.

Protein Isolation and Western Blot

Protein was isolated using RIPA lysis buffer supplemented with protease inhibitor (Beyotime, Haimen, Jiangsu, China) from cultured cells according to the manufacturer's protocols. After quantified with BCA kit (Beyotime), equal amount of protein sample was separated at 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membrane (Beyotime). Membranes were blocked with fat-free milk and then incubated with primary antibodies against GALNT4 (ab80676, Abcam, Cambridge, MA, USA), MMP-9 (ab194314, Abcam) and GAPDH (ab181602, Abcam) at 4°C for overnight. After washing, the membranes were incubated with secondary antibody (ab6721, Abcam) at 37°C for 4 h. Band signals were developed using BeyoECL kit (Beyotime). Relative expression level was analyzed using Image J 1.42 software (NIH, Bethesda, MD, USA).

MTT Assay

Cells (2000 cells/well) were plated at 96-well plates and incubated for indicated time. 10 µl of MTT solution (5 mg/ml, Beyotime) was added to each well at the indicated time. 200 µl dimethylsulphoxide was added to each well after incubation for 4 h. Optical density at 570 nm was measured using microplate reader (Bio-Tek, Winooski, VT, USA).

Colony Formation Assay

Cells (500 cells/well) were seeded in 6-well plates and incubated for two weeks. Next, colonies were fixed with methanol, stained with crystal violet for 20 min, and counted under microscope from 5 independent fields.

Transwell Invasion Assay

Cell invasion was analyzed using Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) pre-coated chamber (Corning Costar Co., Corning, NY, USA). 5×10^4 cells were cultured to the upper chamber in serum-free medium, and Dulbecco's Modified Eagle's Medium (DMEM) containing fetal bovine serum (FBS) was added to the lower chamber. 48 h after incubation, the invasive cells were fixed with paraformaldehyde, stained with crystal violet, and then counted under microscope.

Bioinformatics Analysis

Targets for miR-506-3p were analyzed at TargetScan (http://www.targetscan.org/vert_71/) and miRDB (http://mirdb.org/miRDB/index.html). Prediction results from these two algorithms were analyzed to select the target of miR-506-3p.

Luciferase Reporter Gene Assay

The 3'-UTR of GALNT4 contains putative miR-506-3p binding site was cloned from genome and inserted into psiCHECK-2™ vector (Promega, Madison, WI, USA) to obtain wt-GALNT4. The mutant type luciferase activity vector (mt-GALNT4) was established based on wt-GALNT4. Cells were co-transfected with either miR-506-3p mimics or miR-con and wt-GALNT4 or mt-GALNT4 using Lipofectamine 2000. Relative luciferase activity was analyzed using Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) after 48 h of transfection.

Statistical Analysis

Data were analyzed at SPSS version 22.0 (Armonk, NY, USA). Paired Student's *t*-test and one-way ANOVA analysis with Tukey post-hoc test were conducted to compare significance in groups. Data were presented as means ± standard deviation (SD). *p*-value less than 0.05 was considered statistically significant.

Results

MiR-506-3p Expression Was Downregulated in PCa Cell Lines

To verify miR-506-3p expression level in PCa, PCa cell lines and normal cell line were used. As presented in Figure 1, PCa cell lines demonstrated a significantly lower miR-506-3p expression level when compared with normal cell line RWPE-1.

MiR-506-3p Inhibits PCa Cell Proliferation, Colony Formation and Invasion In Vitro

qRT-PCR results revealed that miR-506-3p expression levels were significantly elevated in PCa cell lines by miR-506-3p mimic (Figure 2A). Subsequently, cell growth was analyzed by MTT assay and colony formation assay. Transfecting of miR-506-3p mimic showed a significantly lower rate in cell proliferation rate and colony formation ability compared with the cells transfected with miR-con (Figure 2B and 2C). Transwell invasion assay revealed that cell invasion was significantly

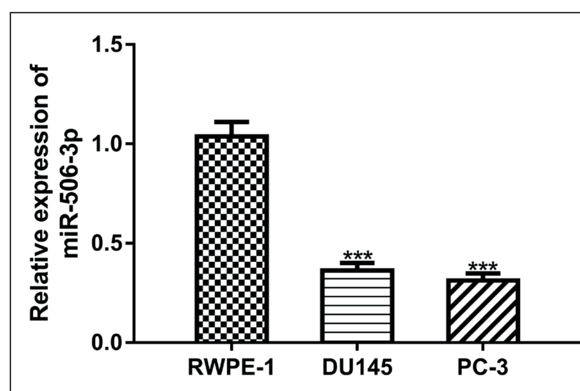

Figure 1. MiR-506-3p expression was reduced in PCa cell lines (DU145 ad PC-3) compared with normal prostate epithelial cell line (RWPE-1). MiR-506-3p: microRNA-506-3p; PCa: prostate cancer.

suppressed by miR-506-3p in PCa cells (Figure 2D). We also detected MMP-9 expression in miRNAs transfected cells. It was found that MMP-9 expression was suppressed by miR-506-3p mimic in PCa cells (Figure 2E). In summary, these results indicated miR-506-3p inhibits PCa cell growth *in vitro*.

GALNT4 Was a Direct Target of MiR-506-3p

After bioinformatics algorithm analyses, GALNT4 was identified as a putative target of miR-506-3p (Figure 3A). To verify whether GALNT4 was a direct target of miR-506-3p, luciferase reporter assay was carried out. In this assay, relative luciferase activity was reduced in PCa cells co-transfected with wt-GALNT4 and miR-506-3p mimics (Figure 3B). Western blotting further indicated GALNT4 expression in PCa cells was decreased by miR-506-3p mimic transfection (Figure 3C).

GALNT4 Can Reverse the MiR-506-3p Mediated PCa Cell Biological Events

We investigated whether GALNT4 could reverse the miR-506-3p mediated PCa cell biological events. PCa cells with miR-506-3p mimic, were further co-transfected with pGALNT4 vector. These results demonstrated that, in comparison to pcDNA3.1 group, introduction of pGALNT4 increased GALNT4 expression level, cell proliferation rate, colony formation ability, and cell invasion ability (Figure 4A-4D). Additionally, overexpression of GALNT4 reversed the inhibitory effects of miR-506-3p on GALNT4

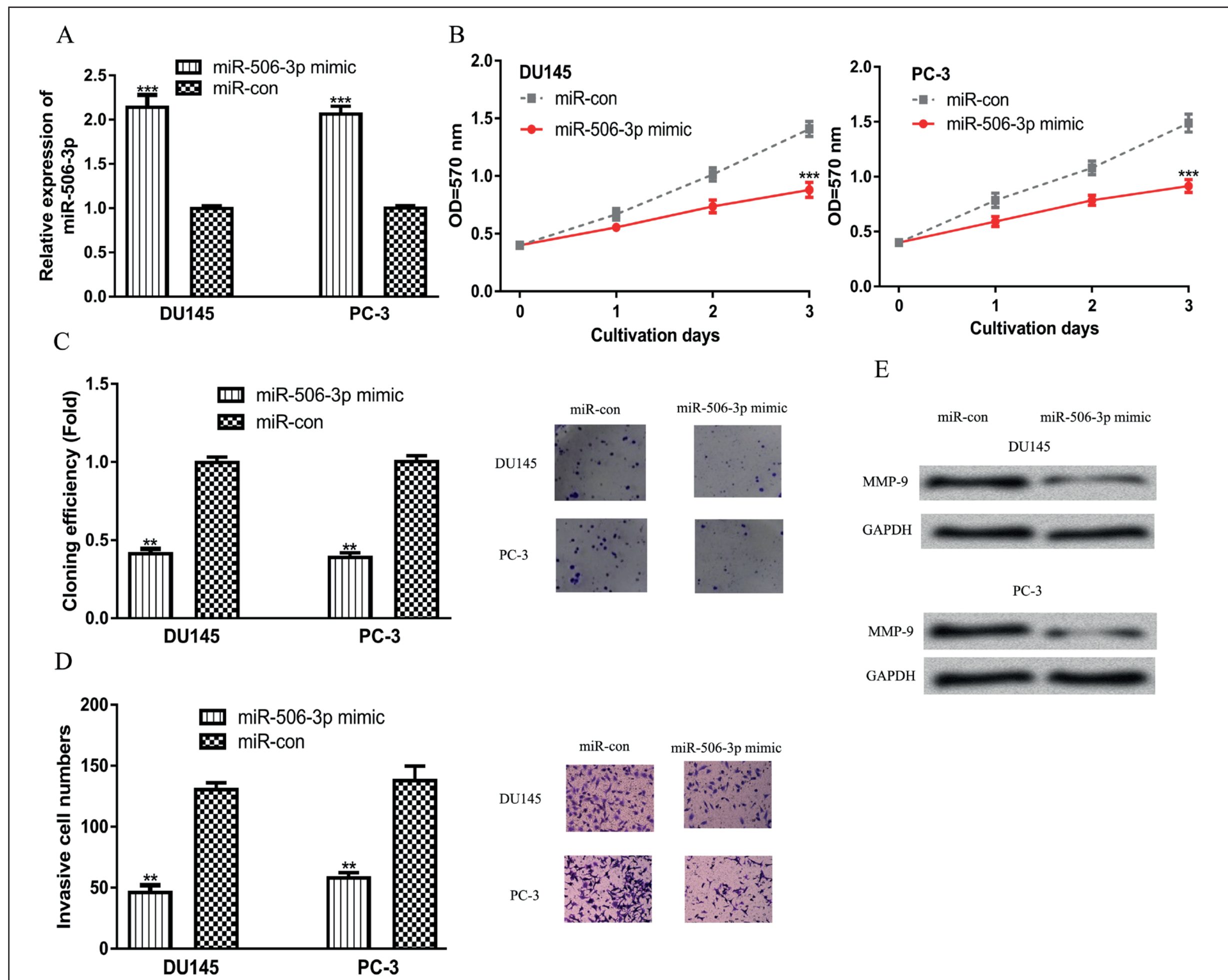

Figure 2. Overexpression of miR-506-3p inhibits PCa cell growth. ***(A)*** MiR-506-3p expression, ***(B)*** cell proliferation, ***(C)*** colony formation, ***(D)*** cell invasion, and ***(E)*** MMP-9 expression of PCa cells transfected with synthetic miRNAs. MiR-506-3p: microRNA-506-3p; PCa: prostate cancer; miR-con: negative control miRNA; MMP-9: matrix metalloprotein-9.

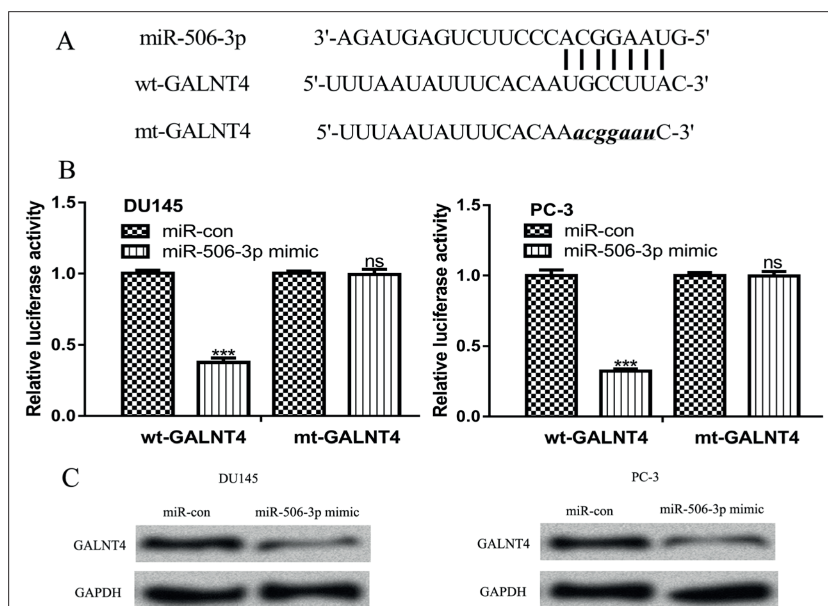

Figure 3. GALNT4 was a target of miR-506-3p. ***A,*** Binding site between miR-506-3p and the 3'-UTR of GALNT4. ***B,*** Relative luciferase activity in PCa cells transfected with synthetic miRNAs and the luciferase activity vectors. ***C,*** GALNT4 expression in PCa cells transfected with synthetic miRNA. miR-506-3p: microRNA-506-3p; PCa: prostate cancer; miR-con: negative control miRNA; UTR: untranslated region; wt: wild-type: mt: mutant; GALNT4: N-Acetylgalactosaminyltransferase-4.

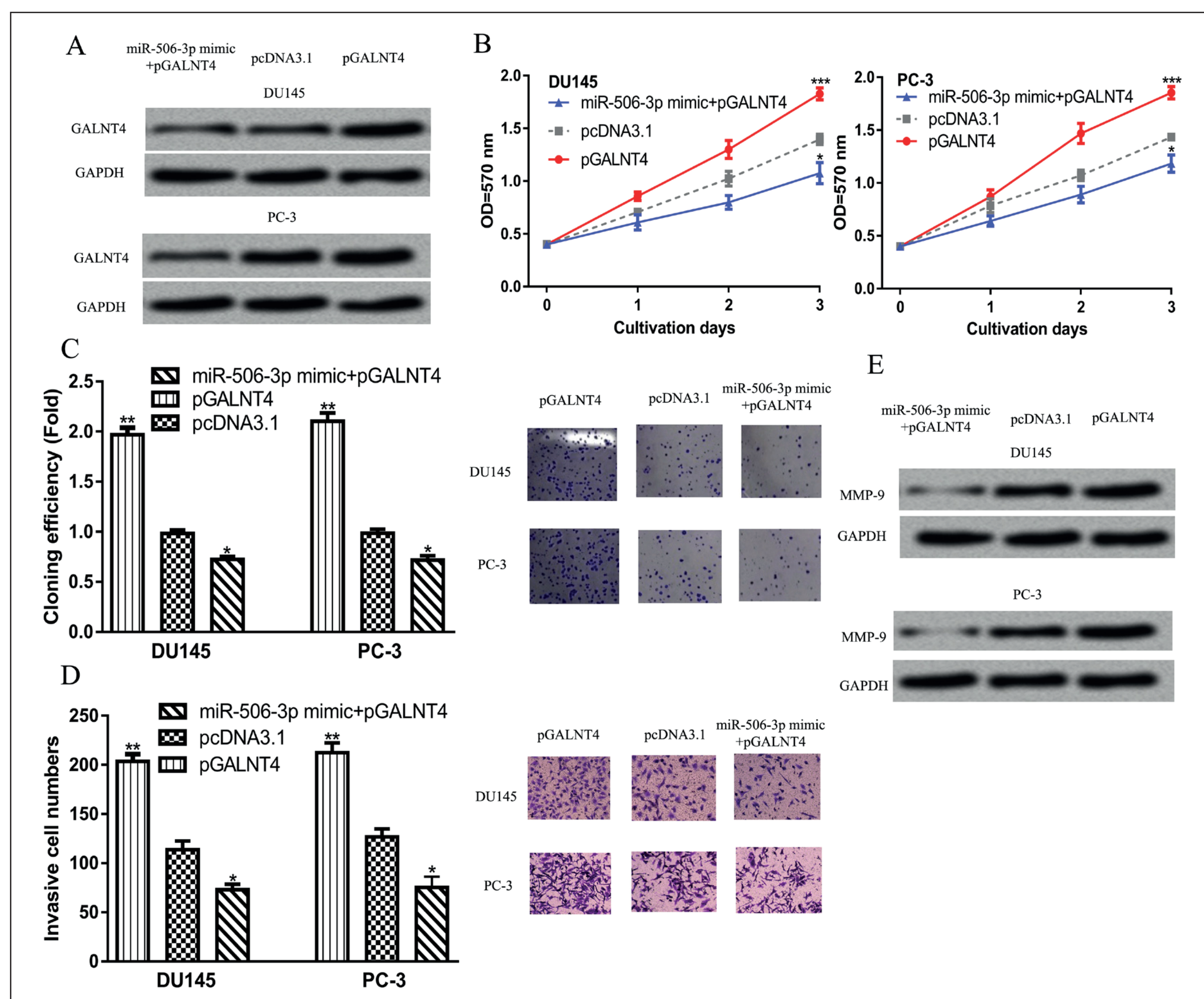

Figure 4. Overexpression of GALNT4 promotes PCa cell growth. ***(A)*** GALNT4 expression, ***(B)*** cell proliferation, ***(C)*** colony formation, ***(D)*** cell invasion, and ***(E)*** MMP-9 expression of PCa cells transfected with pGALNT4, pcDNA3.1, pGALNT4 and miR-506-3p mimic, or pcDNA3.1 and miR-506-3p mimic. MiR-506-3p: microRNA-506-3p; PCa: prostate cancer; GALNT4: N-Acetylgalactosaminyltransferase-4; MMP-9: matrix metalloprotein-9.

expression, cell proliferation, colony formation, and cell invasion (Figure 4A-4D). In addition, we found pGALNT4 transfection increased MMP-9 expression and partially reversed the effects of miR-506-3p on MMP-9 expression (Figure 4E).

Discussion

Non-coding RNAs, especially miRNAs, have been demonstrated to be crucial regulators in cancer progression[12,13]. Aberrantly expression of miRNAs can induce complex RNA regulatory networks in cancers[14]. For example, the upregulation of miR-875-3p leads to the suppression on PCa cell proliferation and metastasis through regulating platelet factor 4 variant 1[15]. MiR-582-3p and miR-582-5p overexpressions were able to inhibit PCa cell migration, invasion, and metastasis to bone through targeting transforming growth factor β-signaling pathway[16]. However, the role of multiple miRNAs in PCa remains unclear.

The role of miR-506-3p in PCa remains to be elucidated even though it has been demonstrated to function as tumor suppressor in several human cancers[6-8]. In our study, we found miR-506-3p expression was reduced in PCa cell lines (DU145 and PC-3) compared with normal prostate epithelial cell line (RWPE-1). These data suggested that mR-506-3p may also function as tumor suppressor in PCa.

To further investigate the biological roles of miR-506-3p in PCa, synthetic miRNAs including miR-506-3p mimic and miR-con were transferred into PCa cell lines. The introduction of

miR-506-3p mimic significantly increased the levels of miR-506-3p in PCa cell lines compared with miR-con. The results of MTT assay, colony formation assay, and transwell invasion assay revealed that miR-506-3p overexpression inhibits PCa cell proliferation rate, colony formation ability, and cell invasion ability. These results validated that miR-506-3p exerts crucial roles in PCa progression.

Targets regulated by miR-506-3p are responsible for the effects of miR-506-3p in PCa. We employed two miRNA target prediction software to identify putative target of miR-506-3p. GALNT4 was selected as a target of miR-506-3p as it was previously reported to have a crucial role in human cancers[10,11]. The luciferase activity reporter assay confirmed the interaction between miR-506-3p and the 3'-UTR of GALNT4. Functional assays revealed that overexpression of GALNT4 promoted PCa cell growth and partially reversed the inhibitory effects of miR-506-3p on PCa cell behaviors. These results indicated that GALNT4 was a functional target for miR-506-3p.

Conclusions

We showed the tumor suppressive role of miR-506-3p in PCa. Most importantly, we identified GALNT4 as a direct target of miR-506-3p. Therefore, the identification of miR-506-3p/GALNT4 axis will help us to understand the mechanisms underlying PCa progression.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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