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ORIGINAL ARTICLE

miR-493 Promotes Prostate Cancer Cells Proliferation by Targeting PHLPP2 and Activating Akt Signaling Pathway

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SUMMARY

Background: **MicroRNA-493 (miR-493) was upregulated in prostate cancer (PCa). This study was designed to investigate the mechanism underlying miR-493 mediated pro-proliferation in PCa cells.**
Methods: **Expression of miR-493 in PCa cell lines (DU145 and PC3) and control cells was determined using qRT-PCR. PCa cells were transfected with miR-493 mimics, inhibitor, negative control (NC), PH domain leucine-rich-repeats protein phosphatase 2 (PHLPP2), and Akt expressing plasmids and Akt inhibitor MK-2206. Cell proliferation, quantitative expression of miRNA and mRNA were detected. Protein expression was determined using western blotting analysis.**
Results: **Results showed that miR-493 in PCa cells was up-regulated compared with RWPE-1 cells. Cells transfected with miR-493 mimics or inhibitor significantly reduced or enhanced expression of PHLPP2 ($p < 0.05$), respectively. Cell proliferation was significantly enhanced by miR-493 overexpression, or inhibited by PHLPP2 overexpression. The administration of Akt inhibitor MK 2206 attenuated miR-493-enhanced cell proliferation. PCa cells transfected with Akt express vectors partially enhanced PHLPP2-reduced cell proliferation.**
Conclusions: **These results demonstrated that miR-493 acted as a onco-miR in PCa cells and promoted PCa cell proliferation via inhibiting tumor suppresser PHLPP2 expression and activating Akt signaling pathway.**
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KEY WORDS

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INTRODUCTION

MicroRNAs (miRNAs) are endogenous small non-coding RNAs which act as gene regulators in various biological processes. Growing evidence reveals that miRNAs play significant roles in cell proliferation, differentiation, angiogenesis, and tumorigenesis [1-4]. MiRNAs emerge as critical regulators of carcinogenesis and cancer malignancy by targeting mRNAs, such as oncogenes or tumor suppressor genes [3,5,6]. MiRNAs usually negatively regulate their target mRNAs in a sequence-specific manner to participate into pathogenesis, invasion, and modulation of diseases or tumors. For instance, miR-497 acts as a tumor suppresser miRNA in

breast cancer [7], non-small cell lung cancer [8], pancreatic cancer [9], and prostate cancer [10]. In prostate cancer cells, reports showed that miR-497 could inhibit cell growth and induce cell apoptosis by activating caspase-3 [10]. Tumor suppresser miR-124 could inhibit TGF-α-induced EMT and suppress cell proliferation, aggressiveness, cell motility and adhesion in cancer cells by targeting Slug [11], talin 1 [12] or paired basic amino acid-cleaving enzyme 4 (PACE4) [13].

Prostate cancer (PCa) is a malignancy and a major leading cause of cancer death in men [4]. Cell proliferation and migration are the major causes of malignant cancers including PCa. Studies focused on tumorigenesis or aggressiveness of malignant cancers showed miRNAs played important roles in cell proliferation, migration, or motility [11,13]. However, roles of miRNAs in PCa are still not well understood.

Our previous report showed miR-493 was significantly upregulated in PCa tissues and cell lines compared with control [4]. We also demonstrated that miR-493 could promote PCa cell proliferation and migration. To further investigate the mechanism underlying miR-493 mediated pro-proliferation, we detected the effect of miR-493 target PH domain leucine-rich-repeats protein phosphatase 2 (PHLPP2) and its association with Akt signaling mediated cell proliferation. This study would give us new insights into miR-493-mediated mechanism of cell proliferation in PCa cells.

MATERIALS AND METHODS

Cell line, culture conditions, groups

Human PCa cell lines (PC3 and DU145) and a non-malignant epithelial prostate cell line RWPE-1 were purchased from ATCC (Manassas, VA, USA) and cultured in conditions as previously described [4]. In brief, PCa cell lines were maintained in DMEM (Gbico, USA) supplemented with 10% FBS (Sigma, USA), 100 units/mL penicillin-streptomycin (Invitrogen, CA, USA). RWPE-1 cells were maintained in KSFM (Gbico, USA) supplemented with 50 mg/L bovine pituitary extract, 5% L-glutamine, and 5 μg/L EGF (Sigma, USA). All cells were maintained at 37ºC in 5% CO_2.

Plasmid construction and cell transfection

PHLPP2 and Akt expression plasmids were constructed according to previous methods [4]. The miR-493 mimics, miR-493 inhibitors and negative control (universal scramble, NC; Mock GeneCopoeia Co., Rockville, MD, USA) were transfected into PCa cells using Lipofectamine 2000 reagent (Invitrogen, USA) according to the manufacturer's instructions. Akt inhibitor MK-2206 (Selleck, Houston, TX, USA) were added into PCa cells (1 μm) 2 hours after miR-493 mimic or NC transfection to inhibit Akt activation.

Cell proliferation assay

Cell growth was assessed using MTT assay (Sigma, USA) [4]. Briefly, PCa cells were seeded into 96-well plates and maintained in conditions as above. Cell viability at 24, 48, 72, and 96 hours post transfection were detected using MTT assay [14]. Each experiment was performed in triplicate.

Quantitative real-time polymerase chain reaction (qRT-PCR)

The isolation of total RNA and the detection of miR-493 were performed as we previously described [4]. U6 small nuclear RNA was used as the internal reference gene for miRNA quantitation [4].

Western blotting analysis

Cellular protein was harvested, prepared, and subjected to 10% SDS-PAGE. Then proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Invitrogen Corp., Carlsbad, CA, USA) and then blocked with 5% skimmed milk at room temperature for 1 hour. The membranes were blotted according to the standard methods with specific primary antibodies anti-PHLPP2 (1:1,000 dilution, Abcam, Cambridge, MA, USA), Akt (dilution 1: 5,000, Cell Signal Technology Inc., Danvers, MA, USA), phosphorylated (p)-Akt (dilution 1: 1,000, CST), a-tubulin (dilution 1: 2,000, Sigma), and the corresponding secondary antibodies. Chemiluminescence was used for immunocomplex visualization according to the manufacture's protocol.

Statistical analysis

Statistical analysis was performed using SPSS 19.0 software (IBM). All data were expressed as the mean ± SD. Statistically significant difference between two groups was analyzed using two-tailed Student's *t*-test. A p-value < 0.05 was considered statistically significant.

RESULTS

MiR-493 is upregulated in PCa cells

Using qRT-PCR analysis, we confirmed the upregulation of miR-493 in PCa cells compared with the RWPE-1 cells. The relative expression levels of miR-493 in DU145 and PC3 cells were significantly higher than that in RWPE-1 cells ($p < 0.01$, Figure 1). In addition, miR-493 relative expression level in DU145 was higher than that in PC3 cells ($p < 0.05$).

MiR-493 promotes DU145 cells proliferation

To investigate the influence of miR-493 expression on cell growth, we detected the cell viability of DU145 cells after being transfected with miR-493 mimics, inhibitors, and NC. QRT-PCR analysis showed the relative expression level of miR-493 was significantly up- and down-regulated by mimics and inhibitors, respectively ($p < 0.01$, Figure 2A). MTT assay demonstrated that miR-493 overexpression dramatically enhanced

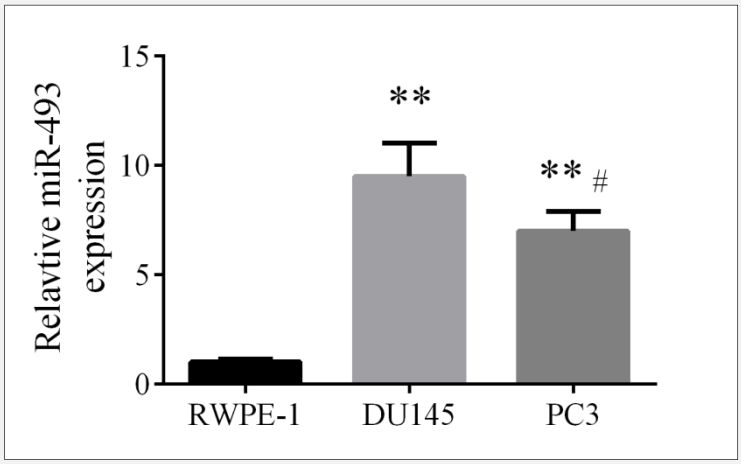

Figure 1. Upregulated expression of miR-493 in PCa cell lines.

Data were expressed as the mean ± SD (bar). ** - p < 0.01 vs. RWPE-1. # - p < 0.05 vs. DU145.

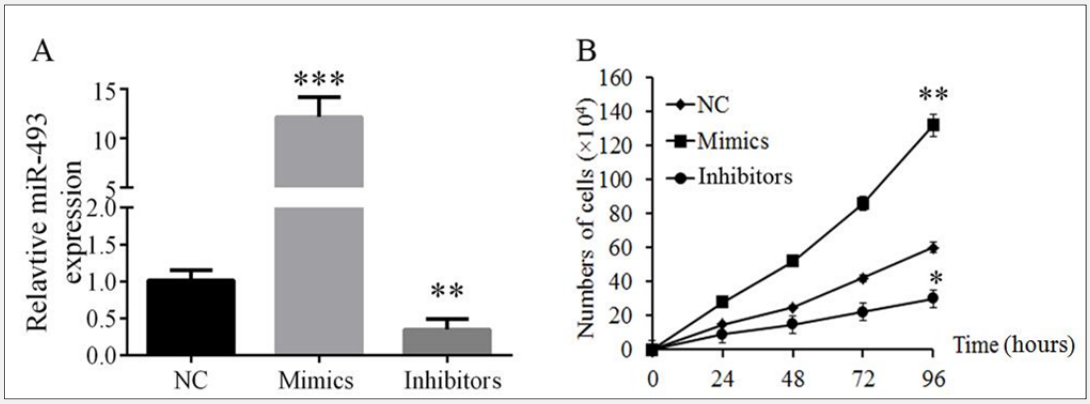

Figure 2. MiR-493 promotes DU145 cell proliferation.

A) qRT-PCR analysis of miR-493 expression levels in transfected DU145 cells. B) cell viability of transfected cells measured by MTT assay. * - p < 0.05 vs. NC. ** - p < 0.01 vs. NC. * - p < 0.001 vs. NC.**

DU145 cell proliferation ($p < 0.01$), and miR-493 inhibition suppressed DU145 cell proliferation ($p < 0.05$, Figure 2B).

MiR-493 suppresses PHLPP2 and activates Akt

In our previous study, we demonstrated the negative correlation between miR-493 and its target PHLPP2 [4]. In order to investigate the association of miR-493 and PHLPP2 expression with Akt activation, we detected the protein expression of PHLPP2, Akt, and p-Akt (Ser 473) in transfected DU145 cells using western blot analysis. Data showed that expression of PHLPP2 and p-Akt was, respectively, inhibited and enhanced by miR-493 mimics compared with NC ($p < 0.01$, Figure 3). On the contrary, the administration of Akt inhibitor MK-2206 upregulated PHLPP2 and significantly reduced

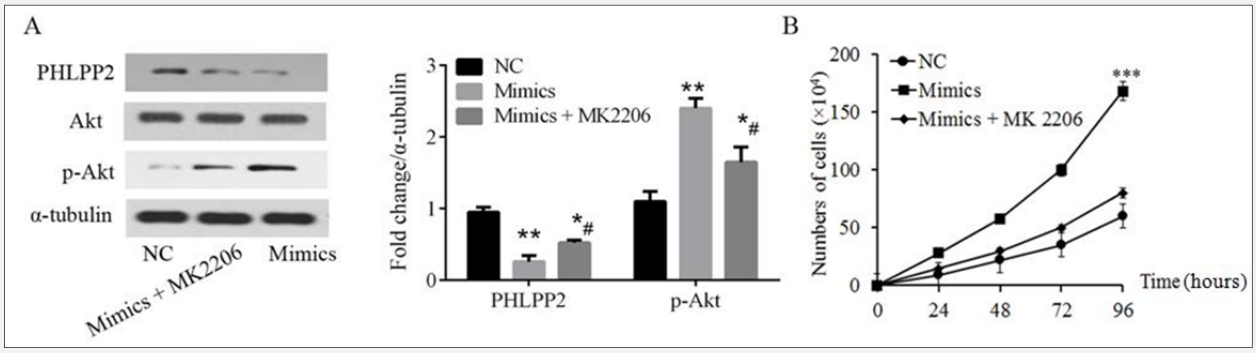

Figure 3. MiR-493 modulates Akt activation and cell proliferation.

A) western blot analysis of PHLPP2 and Akt, p-Akt protein expression in transfected DU145 cells. B) MTT assay for cell viability. Akt inhibitor MK-2206 were added into DU145 cells in cultured medium (1 μm) 2 hours after miR-493 mimic or NC transfection for Akt inactivation. * - p < 0.05 vs. NC. ** - p < 0.01 vs. NC. * - p < 0.001 vs. NC. # - p < 0.05 vs. mimics.**

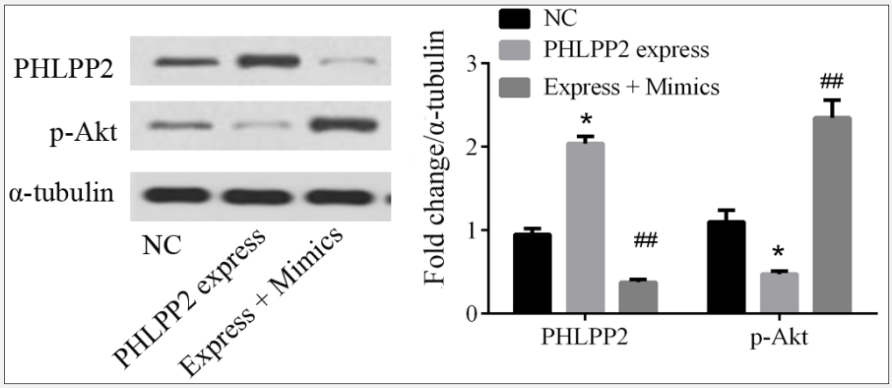

Figure 4. Akt activation is negatively regulated by PHLPP2.

A) western blot analysis of PHLPP2 and p-Akt protein expression in transfected DU145 cells. B) MTT assay for cell viability. DU145 cells were transfected with PHLPP2 express vector, miR-493 mimics, and NC. * - p < 0.05 vs. NC. ## - p < 0.01 vs. PHLPP2 express.

Akt activation compared with cells treated with mimics ($p < 0.05$, Figure 3). These results showed that there was a negative correlation between PHLPP2 expression and Akt activation in PCa cells.

MiR-493 mediated Akt activation promotes DU145 cell proliferation

To investigate cell proliferation, we performed the MTT assay on PCa cells transfected with the above agents. We confirmed that miR-493 overexpression promoted cell proliferation ($p < 0.001$, Figure 3B). DU145 cells additionally treated with MK-2206 showed reduced cell viability to the same level as control ($p > 0.05$ vs. NC). These revealed that Akt activation mediated by miR-493 benefited cell proliferation.

PHLPP2 overexpression inactivates Akt

To investigate whether miR-493 induced Akt activation was mediated by PHLPP2 inhibition, we overexpressed PHLPP2 in DU145 cells. Results showed the overex-

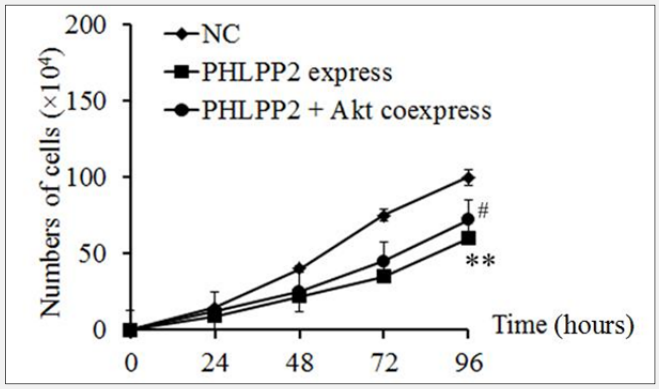

Figure 5. Akt activation rescues PHLPP2 expression-reduced cell proliferation.

** - p < 0.01 vs. NC. # - p < 0.05 vs. PHLPP2.

pression of PHLPP2 in DU145 cells significantly enhanced PHLPP2 protein expression and inhibited p-Akt (Ser473) expression ($p < 0.05$). In addition, cells co-transfected with PHLPP2 expression plasmids and miR-493 mimics cancelled the effect of PHLPP2 expressing plasmids on Akt (Figure 4). These demonstrated that Akt was negatively regulated by miR-493-mediated PHLPP2 inhibition.

Akt rescues cell proliferation reduced by PHLPP2 expression

To investigate the direct effect of PHLPP2 and Akt activation on cell proliferation, we transfected DU145 cells with PHLPP2 and Akt express plasmids. Results revealed that PHLPP2 overexpression dramatically reduced cell proliferation compared with NC ($p < 0.01$, Figure 5). Moreover, additional Akt overexpression plasmid transfection significantly attenuated PHLPP2-inhibited cell proliferation ($p < 0.05$). These suggested that PHLPP2 expression inhibited DU145 cell growth, while Akt expression could rescue it.

DISCUSSION

The dysregulation of miR-493 and its related mechanism had been reported in several tumor types [15,16]. In the present study, we confirmed the upregulation of miR-493 in PCa cells. Overexpression of miR-493 in PCa cells promoted cell proliferation, activated Akt signal, and inhibited the expression of its target PHLPP2. We previously determined the upregulation of miR-493 in PCa tissues and PCa cell lines (DU145 and PC3) [4]. Moreover, we confirmed that ectopic miR-493 expression enhanced PCa cell proliferation by accelerating cell cycle at G1-S phase transition. Our terms had declared the overexpression of miR-493 in PCa cells upregulated the expression of Cyclin D1 (positively control cell cycle progression and proliferation) and downregulated p27 (negatively control cell cycle progression and proliferation). In this present study, we revealed that miR-493 mimics and inhibitors could promote and inhibit cell proliferation of DU145 cells, respectively. These facts were in accordance with those in our previous study [4], suggesting the oncogenic role of miR-493 in PCa tumorigenesis.

PHLPP2 is a direct target of several miRNAs and involves in tumorigenesis [2,17-19]. PHLPP2 acts as a tumor suppresser in various tumor types, such as ovarian cancer [19] and colorectal cancer (CRC) [2]. Liao et al. showed PHLPP2 was a negative target of onco-miR-760, which participated in ovarian cancer carcinogenesis [19]. They also revealed that silencing of PHLPP2 promoted cancer cell proliferation. Li et al. showed the PHLPP2 was a negative target of miR-938, which promoted CRC cell proliferation via c-Myc and Cyclin D1 expression [2]. PHLPP2 had been validated to be a direct target of miR-493 in our previous study [4]. We demonstrated, in the present study, that the expression of PHLPP2 was negatively correlated with PCa cell proliferation. DU145 cells transfected with PHLPP2 overexpression vectors showed reduced cell viability compared with the control cells. Taken together, these results suggested miR-493 might be an onco-miR for PCa, and its target PHLPP2 acted as a tumor suppresser in PCa tumorigenesis.

As we all know, P13K/Akt signaling activation is essential for cell proliferation [20-22]. Akt-dependent cell proliferation is mainly mediated by Cyclin D1/cyclin-dependent kinase (CDK)-4 activity [20,23]. We previ-

ously suggested that miR-493 overexpression enhanced Cyclin D1 and G1-S phase transition, thus promoting cell proliferation and tumorigenesis [4]. In this present study, we demonstrated miR-493 was positively correlated with Akt activation. Overexpression of Akt partially enhanced the PHLPP2-inhibited proliferation of DU145 cells. These might suggest Akt activation was essential for PHLPP2 inhibition-mediated PCa tumorigenesis, or PHLPP2 overexpression might be a potent therapeutic strategy for suppression of PCa tumorigenesis by inactivating the Akt signaling pathway.

CONCLUSION

We confirmed the up-regulation of miR-493 in PCa cells contributed to cell proliferation. MiR-93 mediated cell proliferation was related to PHLPP2 suppression and Akt activation. Moreover, overexpression of PHLPP2 acted as a tumor suppresser by inhibiting cell proliferation and suppressing Akt activation in PCa cells. More experiments should be done regarding the association of miR-493 and PHLPP2 mediated mechanism with Akt signaling.

Declaration of Interest:
None.

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