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Original Article

miR-493 contributes to the proliferation and cell cycle in prostate cancer cells by repressing PHLPP2

Jun Deng[1], Ming Ma[1], Wei Jiang[2], Haifeng Zhang[3], Suping Cui[1]

[1]Department of Urology, The First Affiliated Hospital of Nanchang University, Nanchang 330006, China; [2]Department of Stomatology, The Second Hospital of Nanchang, Nanchang 330006, China; [3]Department of Medicine, Graduate School of Nanchang University, Nanchang 330006, China

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Abstract: Previous studies have shown that miR-493 plays a crucial role in a variety of biological processes in various human cancer cells. The role of this microRNA in prostate cancer (PCa) is presently unclear. In the present study, we investigated the miR-493's role in cell proliferation and cell cycle in PCa. Expression of miR-493 in 8 cancerous, 8 adjacent normal samples from 8 PCa patients undergoing surgical resection and PCa cells was detected by PCR. Luciferase reporter assays were conducted to confirm target gene associations. Effect of miR-493 on cell proliferation and cell cycle were detected by MTT assays, colony formation, soft agar assays and cell cycle analysis. We found that miR-493 is frequently up-regulated in PCa specimens, compared with noncancerous tissues. Bioinformatics analysis further revealed PH domain leucine-rich repeats protein phosphatase-2 (PHLPP2), putative tumor suppressor as a potential target of miR-493. Data from reporter assays showed that miR-493 directly binds to the 3'-untranslated region (3'-UTR) of PHLPP2 mRNA and repressed expression at both transcriptional and translational levels. In functional assays, miR-493 promoted PCa cell proliferation and cell cycle, which could be reversed by inhibitor of miR-493. Taken together, our data provide compelling evidence that miR-493 functions as an oncomiRNA in PCa, which plays important roles in the regulation of cell proliferation and tumor growth in PCa, and its oncogenic effects are mediated chiefly through direct suppression of PHLPP2.

Keywords: miR-493, prostate cancer, PHLPP2, cell proliferation, cell cycle

Introduction

Prostate cancer (PCa) is one of the most common malignancies worldwide, making it the second leading cause of cancer death in men [1]. Proliferation is the major cause of PCa mortality. However, litte is known about the exact molecular mechanisms underlying PCa proliferation. Therefore, a better understanding of the exact mechanisms underlying proliferation is of crucial significance in improving the current therapeutic strategies of PCa.

MicroRNAs (miRNAs), are a class of endogenous small non-coding RNAs (19-22 nucleotides), which lead to mRNA degradation or inhibition of translation through imperfect hybridization to 3'-untranslated region (3'-UTR) in target mRNAs [2]. Recent evidence has shown that miRNAs play essential roles in a variety of biological processes related to cancer, including cell proliferation, differentiation, tumorignesis, angiogenesis, invasion, and metastasis [3-7]. Cell proliferation, which is the most important hallmarks of cancer, are the leading lethal factors for malignant cancer, especially for PCa [8]. Here, we report that upregulation of miR-493 in prostate cancer is associated with development of PCa. Further investigations revealed that miR-493 directly targeted the 3'-UTR of PH domain leucine-rich-repeats protein phosphatase 2 (PHLPP2), a family of novel protein phosphatases, which function as tumor suppressors in prostate cancer, to suppress the expression of these genes, which in turn promoted the proliferation of PCa cell [9, 10].

Materials and methods

Clinical specimens

Eight PCa tissues and adjacent normal tissues were obtained from PCa patients at Huadong Hospital affiliated to Fudan University (Shanghai, People's Republic of China). The study was approved by the ethics committee of

Huadong Hospital affiliated to Fudan University (Shanghai, People's Republic of China). Written informed consent was obtained from all patients.

Cell culture

Human PCa cell lines PC3, DU145, 22RV1, LNCAP and a non-malignant epithelial prostate cell line, RWPE-1 were purchased from the American Type Culture Collection (Manassas, VA, USA). PCa cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gbico, USA) supplemented with 10% fetal bovine serum (FBS, Sigma, USA), 100 units/ml of penicillin-streptomycin (Invitrogen, Carlsbad, CA), and RWPE-1 cells were maintained in keratinocyte serum-free medium (KSFM; GIBCO Laboratories, Grand Island, NY, USA) supplemented with 50 mg/l bovine pituitary extract, 5% L-glutamine and 5 μg/L EGF. Cell lines were cultured in a humidified incubator at 37°C in an atmosphere of 5% CO_2 and 95% air.

Plasmids and transfection

For ectopic expression of PHLPP2, PHLPP2 ORFs with 3'-UTR was amplified using PCR and subcloned into pEGFP-N3 (Invitrogen). To construct a luciferase reporter vector, the PHLPP2 3'-UTR fragment containing putative binding sites for miR-493 was amplified using PCR and cloned downstream of the luciferase gene in the pGL3-luciferase reporter plasmid (Promega). Constructs were verified by sequencing. The miR-493 mimics, negative control, and miR-493 inhibitor were purchased from Genecopoeia (Genecopoeia Co. Ltd.) and transfected into PCa cells using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

RNA extraction and real-time quantitative PCR

For miRNA quantification, total RNA including microRNAs was extracted from culture cells and patient samples using mirVana miRNA Isolation Kit (Ambion) according to the manufacturer's instructions, and then cDNA was synthesized from 5 ng of total RNA using the Taqman miRNA reverse transcription kit (Applied Biosystems). The expression levels of miR-493 were quantified using the miRNA-specific TaqMan MiRNA Assay Kit (Applied Biosystems). The relative miR-493 expression levels after normalization to U6 small nuclear RNA were calculated using 2-[(Ct of miR-493) - (Ct of U6)]. Quantitative PCR was performed by SYBR Kit (Qiagen, China) using a Light Cycler system. The primers selected are as follows: p27: forward 5'-CCGGTGGACCACGAAGAGT-3' and reverse 5'-GCTCGCCTCTTCCATGTCTC-3'; cyclin D1: forward 5'-TCCTCTCCAAAATGCCAGAG-3' and reverse 5'-GGCGGATTGGAAATGAACTT-3'; Expression data were normalized to the geometric mean of GAPDH to control the variability in expression levels (forward primer 5'-GACTCATGACCACAGTCCATGC-3'; reverse primer 3'-AGAGGCAGGGATGATGTTCTG-5') and calculated as 2-[(Ct of p27 and CyclinD1) - (Ct of GAPDH)].

MTT assays and colony formation

Cell growth was assessed by MTT assay, PC3 and DU145 cells were seeded in 96-well plates in medium containing 10% FBS at approximately 2,000 cells/well. For quantitation of cell viability, cultures were stained after 1, 2, 3, 4 and 5 days in MTT assays. In brief, 20 μl of 5 mg/ml MTT solution (Sigma-Aldrich) was added to each well and incubated for 4 h at 37°C. The medium was removed from each well and the resulting MTT formazan was solubilized in 150 μl of DMSO. The absorbance at 490 nm was measured in a Thermo Scientific Multiskan (Thermo Fisher Scientific, USA).

For colony formation assay, PC3 and DU145 cells were plated into three 6-cm cell culture dishes (1,000 per dish) and incubated for 12 days in medium containing 10% FBS. Plates were washed with PBS and stained with 1% crystal violet for 30 s after fixation with 4% paraformaldehyde for 5 minutes. The number of colonies, defined as > 50 cells/colony was counted. Three independent experiments were performed. The data was calculated using paired t test.

Anchorage-independent growth assay

Cells were trypsinized, and 500 cells were resuspended in 2 ml complete medium plus 0.3% agar (Sigma). The agar-cell mixture was plated on top of a bottom layer consisting of 1% agar in complete medium. The plates were incubated at 37°C in a humid atmosphere of 5% CO_2. After 14 days, cell colony numbers were counted under microscope and cell colo-

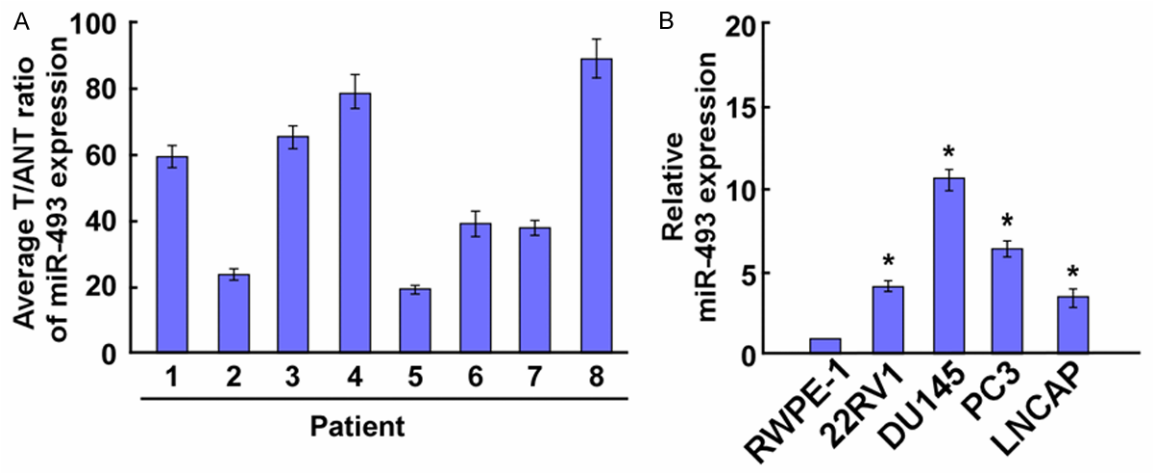

Figure 1. Upregulation of miR-493 in human PCa cell lines and tissues. (A) miR-493 expression in 8 paired primary PCa tissues (T) and the matched adjacent non-tumor tissues (ANT) from the same patient, by PCR analysis. (B) Real-time PCR analysis of miR-493 expression in PCa cell lines, including 22RV1, DU145, PC3, and LNCAP. Experiments were repeated at least three times (A and B). Each bar represents the mean of three independent experiments. *$P < 0.05$.

nies were photographed at an original magnification of 100×. Only cell colonies containing more than 50 cells were counted. The experiment was performed for 3 independent times for each cell line.

Cell cycle analysis

Cells were harvested by trypsinization, washed in ice-cold phosphate-buffered saline (PBS) and fixed in 80% ice-cold ethanol in PBS. Before staining, cells were sedimented in a chilled centrifuge and resuspended in cold PBS. Bovine pancreatic RNase (Sigma-Aldrich) was added to a final concentration of 2 μg/ml, and cells were incubated at 37°C for 30 min, followed by incubation with 20 μg/ml of propidium iodide (Sigma-Aldrich) for 20 min at room temperature. Cell cycle profiles of 5×10^4 cells were analyzed using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Luciferase assays

Cells were seeded in triplicate in 24-well plates (1×10^5/well) and cultured for 24 hours. The pGL3-luciferase reporter gene plasmids pGL3-PHLPP2-3'-UTR, or the control-luciferase plasmid were cotransfected into the cells with the control pRL-TK Renilla plasmid (Promega) using Lipofectamine 2000 Reagent (Invitrogen). Luciferase and Renilla activities were assayed 48 hours after transfection using the Dual Luciferase Reporter Assay Kit (Promega) following the manufacturer's instructions. All experiments were conducted at least 3 times and the data are presented as mean ± SD.

Western blotting

Protein lysates were prepared, subjected to SDS-PAGE, transferred onto PVDF membranes, and blotted according to standard methods using anti-PHLPP2 (Abcam), anti-p27 (Abcam), anti-cyclin D1 (Cell Signaling Technology), anti-Rb (Cell Signaling Technology) and anti-p-Rb (Cell Signaling Technology). To control sample loading, the blotting membranes were stripped and re-probed with an anti-a-tubulin antibody (Sigma-Aldrich). After being washed with TBST and incubation with either anti-rabbit horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich) for 2 h at room temperature, immunocomplexes were visualized using the chemiluminesence (GE, USA) following the manufacturer's protocol.

Statistical analysis

All statistical analyses except for microarray data were performed using the SPSS 19.0 (IBM). The Student's t-test was used to evaluate the statistical significance of differences between two groups of data in all pertinent experiments. A $P < 0.05$ (using a two-tailed paired t-test) was thought to be significantly different for two groups of data.

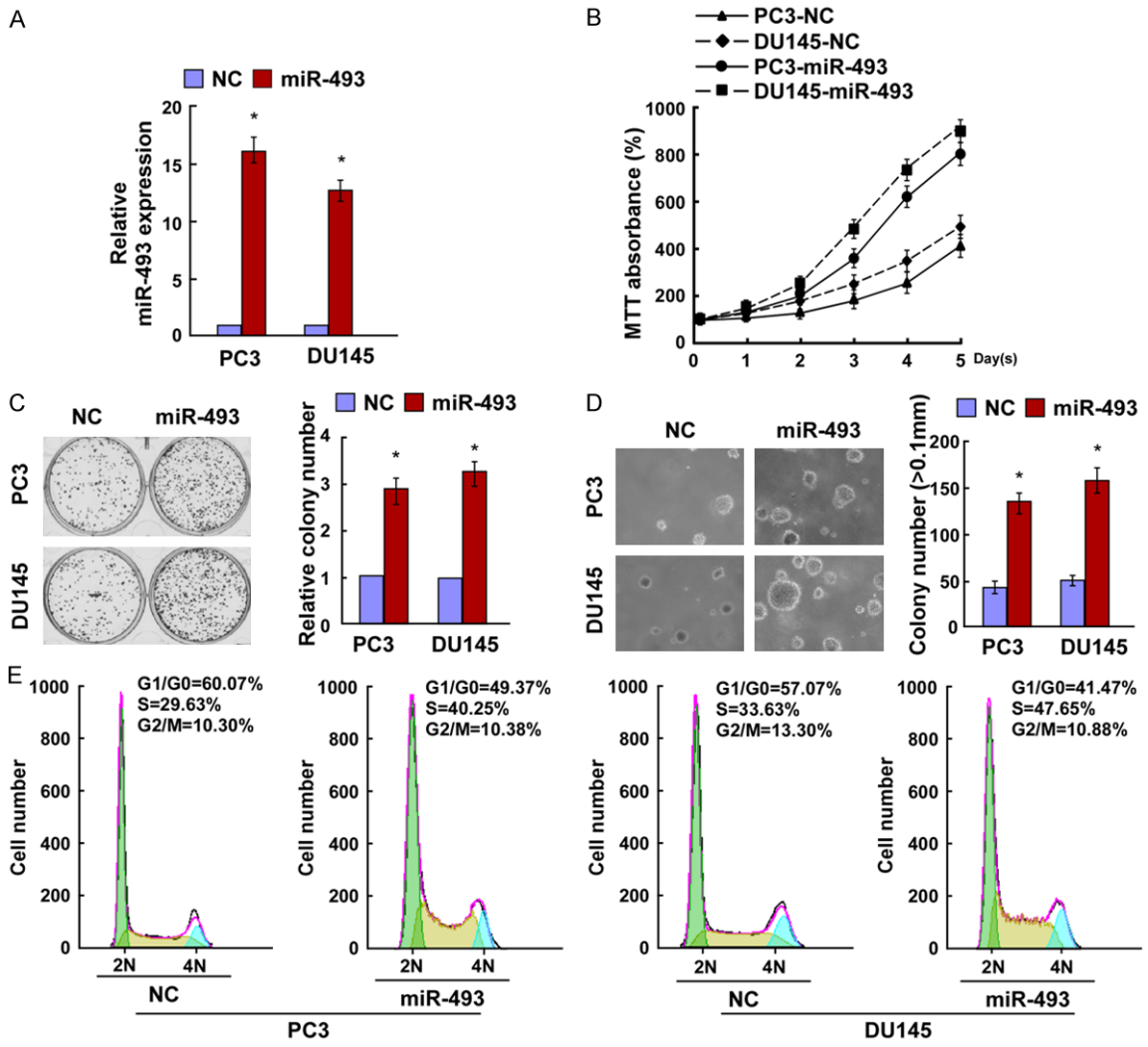

Figure 2. miR-493 upregulation promotes PCa cell proliferation. A. Validation of miR-493 expression levels after transfection by PCR analysis. B. MTT assays revealed that upregulation of miR-493 induced growth of PC3 and DU145 PCa cell lines. C. Representative micrographs (left) and quantification (right) of crystal violet-stained cell colonies. D. Upregulation of miR-493 promoted the anchorage-independent growth of PCa cells. Representative micrographs (left) and quantification of colonies that were > 0.1 mm (right). E. Flow cytometric analysis of the indicated PCa cells transfected with NC or miR-493. Each bar represents the mean of three independent experiments. *$P < 0.05$.

Result

miR-493 is overexpressed in human PCa tissues and PCa cell lines

To investigate the potential roles of miR-493 in PCa development, we examined the expression of miR-493 in human PCa tissues and PCa cell lines. As shown in **Figure 1**, Real-time quantitative PCR demonstrated that miR-493 was differentially upregulated at the mRNA levels in all 4 PCa cell lines compared with a non-malignant epithelial prostate cell line (RWPE-1), and in all 8 PCa patient samples compared with the paired adjacent non-tumor tissues, indicating that miR-493 is overexpressed in PCa. These results in cell lines and patients tissue suggested that miR-493 was gradually increased and may have a crucial role in PCa cancer development and progression.

miR-493 promotes PCa cell proliferation

To investigate whether PCa cell proliferation and cell cycle were regulated by miR-493, we transfected the PCa cell lines, PC3 and DU145, with miR-493 mimics, miR-493 inhibitor or the respective controls. Relative miR-493 expres-

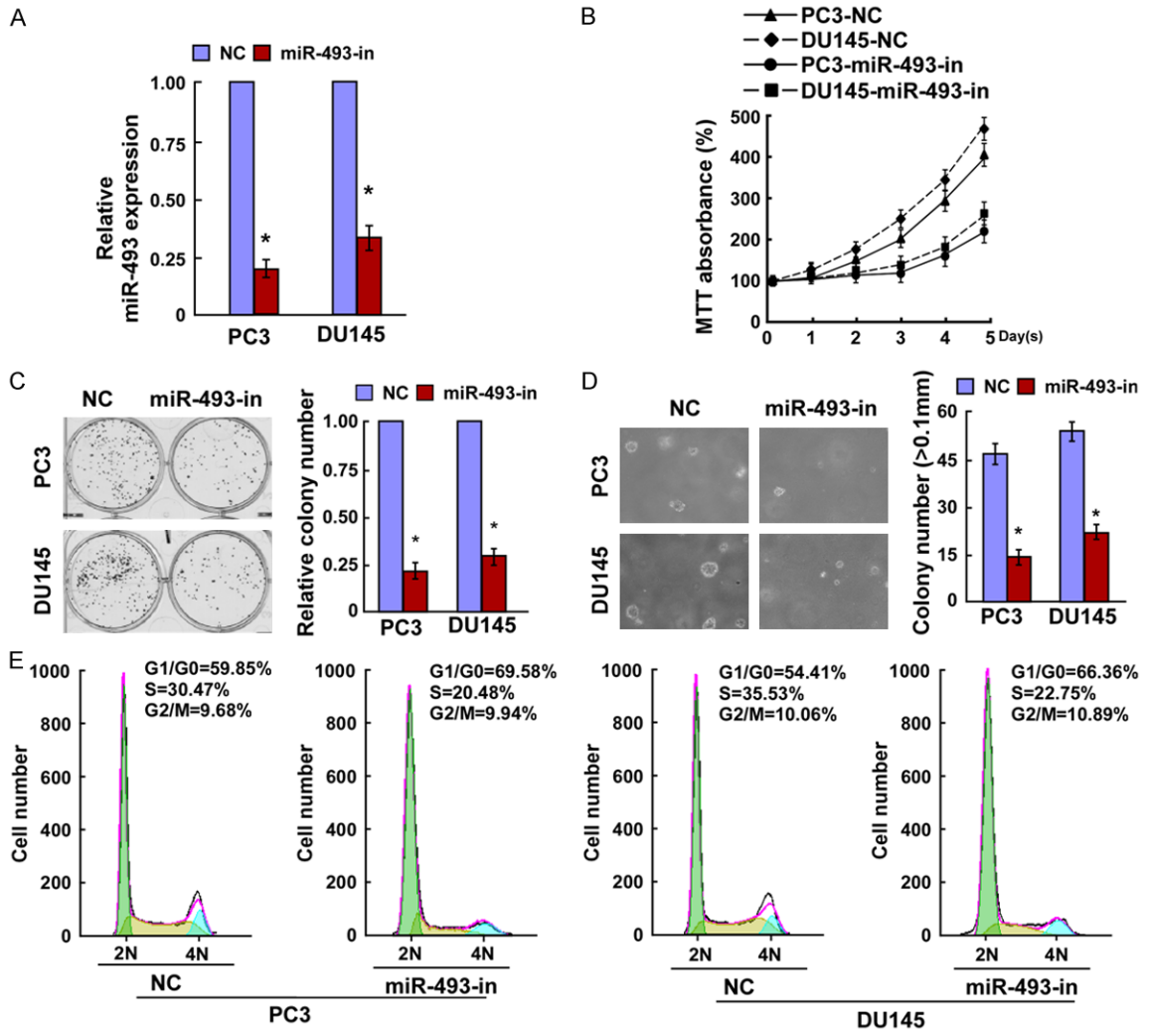

Figure 3. Inhibition of miR-493 suppresses PCa cell proliferation. A. Validation of miR-493 expression levels after transfection by PCR analysis. B. MTT assays revealed that inhibition of miR-493 supressed growth of PC3 and DU145 PCa cell lines. C. Representative micrographs (left) and quantification (right) of crystal violet-stained cell colonies. D. Inhibition of miR-493 impaired the anchorage-independent growth of PCa cells. Representative micrographs (left) and quantification of colonies that were > 0.1 mm (right). E. Flow cytometric analysis of the indicated PCa cells transfected with NC or miR-493 inhibitor. Each bar represents the mean of three independent experiments. *$P < 0.05$.

sion was verified using qRT-PCR (**Figures 2A** and **3A**). We assessed the functional role of miR-493 in PCa cells by determining the effects of miRNA overexpression and inhibition on cell proliferation and cell cycle using MTT, colony formation, anchorage-independent growth and flow cytometry assays, respectively.

To explore the role of miR-493 upregulation in the development and progression of PCa, we next examined its effect on cellular proliferation. MTT assay showed that miR-493 upregulation significantly increased the proliferation rate of PC3 and DU145 cells (**Figure 2B**), and this was further confirmed by a colony formation assay (**Figure 2C**). Strikingly, we found that enforced expression of miR-493 in PC3 and DU145 cells drastically enhanced their anchorage-independent growth ability (**Figure 2D**), as shown by increased colony numbers and sizes, thus suggesting that miR-493 upregulation enhances PCa cell tumorigenicity *in vitro*. Using flow cytometry assays, we found that miR-493 overexpression increased the percentage of cells in S phase and significantly decreased the percentage of cells in G_1/G_0 (**Figure 2E**). Colle-

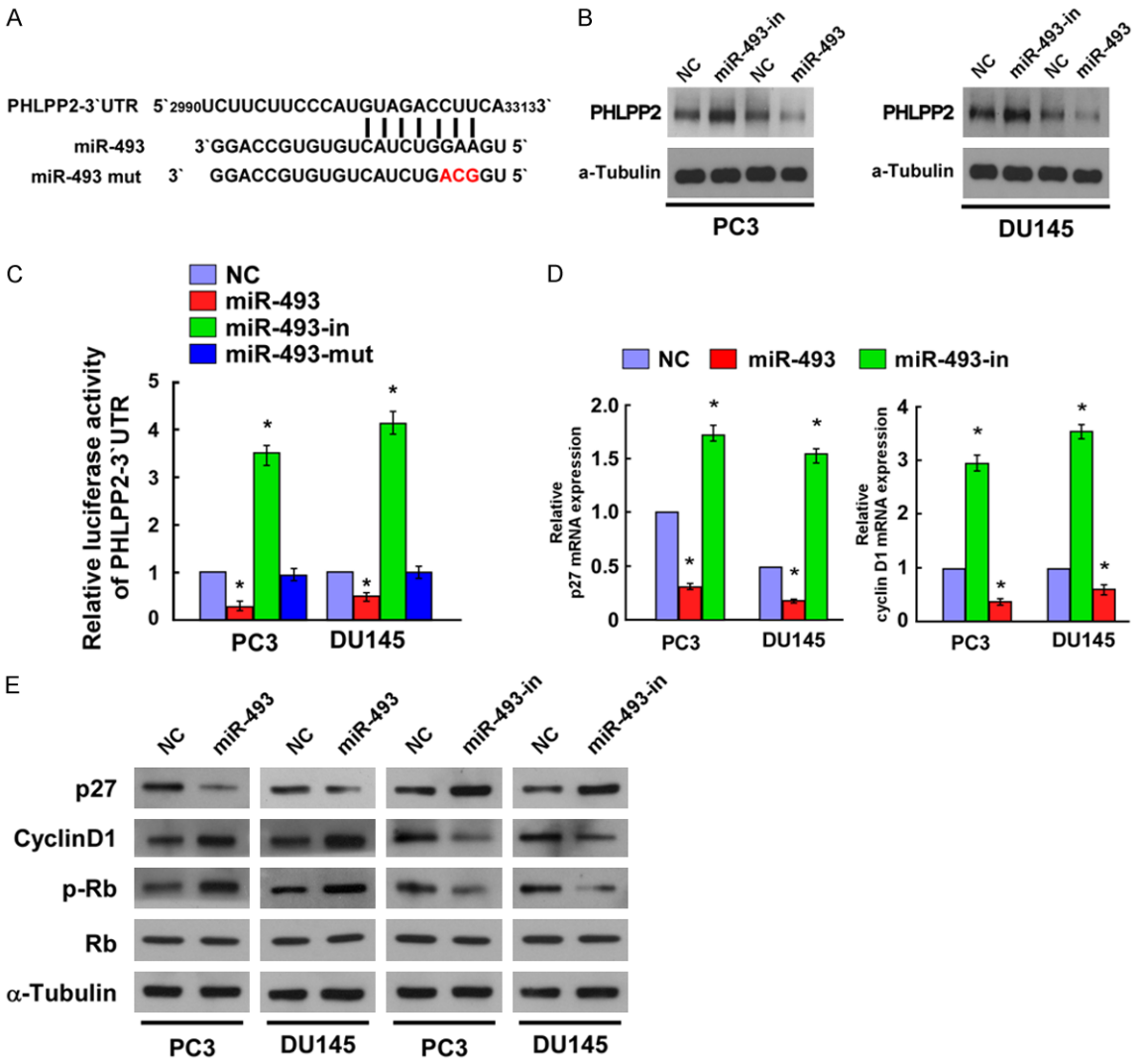

Figure 4. miR-493 suppresses PHLPP2 expression by directly targeting the PHLPP2 3'-UTR and activates the Wnt/β-catenin pathway. A. Predicted miR-493 target sequence in the 3'-UTR of PHLPP2 (PHLPP2-3'-UTR) and positions of three mutated nucleotides (red) in the 3'-UTR of miR-493 (miR-493-mut). B. Western blotting analysis of PHLPP2 expression in cells transfected with miR-493 or the miR-493 inhibitor. α-Tubulin served as the loading control. C. Luciferase reporter assay of the indicated cells transfected with the pGL3-APC-3'-UTR reporter and miR-493 or miR-493 inhibitor oligonucleotides. D. Real-time PCR analysis of expression of p27 and cyclin D1 in indicated PCa cells. E. Western blotting analysis of expression of p27, cyclin D1, phosphorylated pRb (p-pRb) and total pRb protein in indicated PCa cells. α-Tubulin served as the loading control. *$P < 0.05$.

ctively, our results suggest that miR-493 may mediate PCa cell proliferation through regulation of G_1/S transition.

We further examined the effect of miR-493 inhibition (miR-493-in) on PCa cell proliferation. Consistent with the above-mentioned results, MTT and colony formation assays showed that miR-493 suppression significantly inhibited the growth rate of both PC3 and DU145 cells as compared with that of control cells transfected with NC (**Figure 3B** and **3C**). In addition, the anchorage-independent growth ability of PC3 and DU145 PCa cells was significantly decreased in response to miR-493 inhibitor (**Figure 3D**). Furthermore, we found that transfection of the miR-493 inhibitor drastically decreased the percentage of cells in the S peak but increased that in the G_0/G_1 peak (**Figure 3E**), suggesting that the antiproliferative effect of miR-493 inhibition in PCa cells occurs through induction of G_1/S arrest.

miR-493 directly targets PHLPP2 by binding to its 3'-UTR

Potential targets of miR-493 were predicted using bioinformatics methods. PHLPP2, a tumor suppressor gene containing a binding site for miR-493, was selected as the target for further analysis (**Figure 4A**).

To determine whether PHLPP2 is regulated by miR-493, expression of PHLPP2 were detected in the PCa cell lines, PC3 and DU145 cells, which were transfected with miR-493 mimics, miR-493-in or the respective controls. Western blot findings showed that miR-493 mimics significantly inhibit PHLPP2 protein levels in PC3 and DU145 cells, respectively (**Figure 4B**), while miR-493-in clearly promoted PHLPP2 protein expression.

To verify whether PHLPP2 is a direct target of miR-493, PHLPP2 3'-UTR wild type were co-transfected with miR-493, miR-493-in or miR-493-mut into PC3 and DU145 cells, followed by measurement of luciferase activity. As shown in **Figure 4C**, overexpression of miR-493 in both PCa cell lines led to notably reduced luciferase activity for wild-type PHLPP2, whereas inhibition of miR-493 increased wild-type PHLPP2 luciferase activity. The effects of miR-493 mimics and miR-493-in on luciferase activities were completely deprived upon introduction of the 3-nucleotide mutations in PHLPP2 3'-UTR, supporting its identification as a true miR-493 target site. Our data strongly suggest that miR-493 negatively regulates PHLPP2 expression via direct binding to putative binding sites in the 3'-UTR region.

miR-493 modulates cell cycle regulators

The expression levels of a number of critical cell-cycle regulators were also detected. As expected, p27 were strikingly downregulated at both the protein and mRNA levels in miR-493-overexpressing cells (**Figure 4D** and **4E**). Interestingly, both protein and mRNA levels of cyclin D1, a CDK regulator important for regulating the G_1/S transition [11] were upregulated in PC3 and DU145 cells transfected with miR-493 mimic, but decreased in the cells transfected with miR-493-in, relative to control cells (**Figure 4D** and **4E**). pRb phosphorylation was increased in miR-493-overexpressing cells and decreased in the miR-493-inhibited cells, thus providing further evidence that miR-493 plays an important role in PCa cell proliferation (**Figure 4E**). Altogether, our results indicated that miR-493 functionally modulates cell cycle regulators, p27 and cyclin D1, thus relevant to cell proliferation and cell cycle.

Discussion

Growing evidence suggests that miRNAs play an important role in various biological processes including cell proferation, development, and differentiation, angiogenesis, tumorignesis, invasion, and metastasis, which have emerged as critical regulators of carcinogenesis and malignant progression of cancer by targeting oncogenes and tumor suppressor genes [7, 12-15]. miRNAs are a large family of gene regulators that negatively regulate their target mRNAs in a sequence-specific manner. *Wang L et al.* reported that miR-497 inhibited growth and induce apoptosis by caspase-3 activation in prostate cancer [16]. *Qin W et al.* demonstrated that miR-124 inhibits TGF-α-induced EMT in prostate cancer by targeting Slug [17]. Our data expanded the role of miR-493 in PCa. Expression of miR-493 has only been observed in a few tumor types, such as breast carcinoma, colon carcinoma and bladder cancer [3, 18-20]. However, it was uncertain whether dysregulation of miR-493 was associated with the progression of prostate cancer. In this study, we found that miR-493 expression was markedly upregulated in PCa cells and PCa tissues. Furthermore, ectopic expression of miR-493 enhanced the proliferation and anchorage-independent growth of PCa cells, whereas miR-493-in had the opposite effect. More specifically, we showed that the molecular mechanism by which miR-493 promotes prostate cancer cell proliferation was due to acceleration of the G_1-S phase transition, upregulation of cyclin D1 and downregulation of p27 in cells overexpressing miR-493. The cyclin-dependent kinase inhibitor p27 and cyclin D1 are two miR-493 targeted proteins that negatively (p27) and positively (cyclin D1) control cell cycle progression and proliferation [21-23]. This pathway represents a new mechanism which may possibly underlie the development of prostate cancer. Bioinformatic algorithms predicted that PHLPP2, which was bona fide target genes of miR-493. PHLPP2 belongs to a novel family of Ser/Thr protein phosphatases and plays an

important role in maintaining cell survival suppression through negatively regulating the signaling pathways activated by PKC and AKT [24-26]. Evidence has recently emerged to suggest that PHLPP2 acts as a tumor suppressor gene, and its expression is frequently depleted in a variety of human cancers, including breast cancer, lung cancer and colorectal cancer [9, 27-30]. Overexpression of PHLPP2 in cancer cell lines and tumorigenesis [30, 31]. Stable overexpression of PHLPP2 blocked the G_2-M transition or induced G1 cell-cycle arrest, thus decreasing the rate of cell proliferation in colorectal cancer cells [32]. However, precise details of the mechanisms which regulate PHLPP2 remain elusive. Our experimental results further proved that PHLPP2 was direct targets of miR-493 in PCa cells. The activities of PHLPP2 3'UTR luciferase reporter were responsive to miR-493 overexpression. Endogenous PHLPP2 protein expression decreased in miR-493- transfected PCa cells. This study clearly illustrated that miR-493 might directly regulate PHLPP2 expression by inducing translational suppression.

In summary, this study provided an essential link between miR-493-mediated tumor growth and down-regulation of PHLPP2 in PCa cancer. Our findings suggest that the effects of miR-493 on the proliferation and cell cycle of prostate cancer cells may be mediated through down-regulation of PHLPP2 via miR-493 directly targeting the 3'-UTRs of this gene. Understanding the precise roles played by miR-493 in the progression of PCa cancer will not only increase our understanding of the biology of this tumor type, but may also allow the development of a novel therapeutic strategy based on inhibition of miR-493.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Suping Cui, Department of Urology, The First Affiliated Hospital of Nanchang University, Nanchang 330006, China. E-mail: junxush@163.com

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