Downregulation of microRNA-429 inhibits cell proliferation by targeting $p27^{Kip1}$ in human prostate cancer cells

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Abstract. MicroRNAs (miRNAs) are closely associated with cell proliferation, invasion and metastasis in various types of cancer, including prostate cancer. In this study, the role of miR-429 in the regulation of cell proliferation was investigated in prostate cancer cells. miR-429 expression levels were measured in the IF11 and IA8 prostate cancer cell lines and normal prostate epithelial tissues by quantitative polymerase chain reaction. miR-429 mimics or an miR-429 inhibitor were then transfected into the human prostate cancer cell lines. MTT and fluorescence-activated cell sorting were used to detect the effect of miR-429 on cell proliferation. A luciferase reporter system was employed to verify the potential target of miR-429. The results revealed that miR-429 was significantly upregulated in the human prostate cancer cell lines, compared with the normal prostate epithelial tissue. Downregulation of miR-429 expression in IF11 and IA8 cells inhibited cell proliferation and arrested the cells in the G1 phase of the cell cycle. The luciferase assay demonstrated that $p27^{Kip1}$ was a direct target of miR-429. Furthermore, overexpression of $p27^{Kip1}$ was observed to partially rescue the proliferation-promoting effect of miR-429 on IA8 cells. In conclusion, to the best of our knowledge this study was the first to show that miR-429 is involved in the oncogenesis of prostate cancer and thus may be a novel prognostic biomarker in prostate cancer.

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Introduction

Prostate cancer has the second highest cancer-related mortality rate in males (1). In the early stages of the disease, the most effective treatment is surgical castration and hormonal manipulation using gonadotropin-releasing hormone agonists or androgen receptor antagonists. However, numerous prostate cancer patients eventually experience recurrence and androgen independence, which commonly results in accelerated disease progression and fatality (2,3). Thus, novel molecular targets for effective prostate cancer treatment strategies and chemopreventative interventions are urgently required.

MicroRNAs (miRNAs) are a novel class of endogenous, small, non-coding, single-stranded RNAs that regulate gene expression at the post-transcriptional level by targeting the 3' untranslated region (3'UTR) of target mRNAs (4-7). miRNAs have been implicated in a wide range of physiological processes, including cell proliferation, apoptosis and cell differentiation (8,9). Aberrant miRNA expression has been demonstrated to be correlated with cell proliferation, invasion, metastasis and prognosis in various types of cancer, including prostate cancer (10,11). miR-429, a member of the miR-200 miRNA family, has been shown to be downregulated in gastric carcinoma and may act as a tumor suppressor by targeting c-myc (12). Other studies have reported that miR-429 is upregulated in bladder and endometrial carcinoma (13,14). In addition, higher expression levels of miR-429 have been correlated with poor prognosis in patients with serous ovarian carcinoma (15). These results suggest that miR-429 is correlated with tumorigenesis and may exert different effects in distinct types of cancer. Whether miR-429 is involved in the genesis and development of prostate cancer remains to be elucidated.

In the present study, the expression levels of miR-429 in two prostate cancer cell lines and six normal prostate epithelial tissues were analyzed in order to explore the function of miR-429 in the oncogenesis of prostate cancer.

Materials and methods

Cell culture. The IF11 and IA8 human prostate cancer cell lines (American Type Culture Collection, Manassas, VA, USA) were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen),

100 U/ml penicillin and 100 μg/ml streptomycin (Shanghai Genebase Gen-Tech Co., Ltd., Shanghai, China) at 37˚C in a 5% CO_2 incubator.

Tissue collection. Normal prostate epithelial tissues were obtained from patients at the Department of Pathology of Tangdu Hospital (Xi'an, China). All patients provided written informed consent for the use of the excess pathological specimens for research purposes. The use of human tissues in the present study was approved by the Institutional Review Board of The Fourth Military Medical University (Xi'an, China) and was conducted in accordance with the International Guidelines for the Use of Human Tissues.

miRNA mimics and inhibitor. The hsa-miR-429 mimics, hsa-miR-429 inhibitor, negative control miRNA mimics (Mock/mimics NC) and negative control miRNA inhibitor (inhibitor NC) were chemically synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China).

Cell transfection. Prior to transfection, the logarithmically growing cells were harvested and seeded in 6-well plates ($4x10^5$ cells per well), 24-well plates ($1x10^5$ cells per well) or 96-well plates ($1x10^4$ cells per well). Following overnight proliferation, RNA oligonucleotides (mimics NC/inhibitor NC or hsa-miR-429 mimics/inhibitor) were transfected into the adherent cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Luciferase assay. A luciferase reporter assay was conducted using pMIR-REPORT™ vectors (Guangzhou RiboBio Co., Ltd., Guangzhou, China). For recombinant vector construction, a full-length 3'UTR of the $p27^{Kip1}$ gene was cloned and inserted downstream of the firefly luciferase gene in the pMIR-REPORT plasmid as follows: cDNA from the IA8 cells was amplified by polymerase chain reaction (PCR) using $p27^{Kip1}$-3'UTR-wild-type (wt) primer for $p27^{Kip1}$-3'UTR-wt cloning. The PCR products were then digested with *Mlu*I and *Sac*I (Takara Bio, Inc., Shiga, Japan), and inserted into the multiple cloning site of the pMIR-REPORT Luciferase vector (Ambion®; Thermo Fisher Scientific, Waltham, MA, USA). The recombinant vector was designated as pMIR-$p27^{Kip1}$-3'UTR-wt. Using this as a template, the pMIR-$p27^{Kip1}$-3'UTR-mutant (mut) plasmid, which carried the mutated $p27^{Kip1}$ 3'UTR sequence in the complementary site for the seed region of miRNA-429, was generated by overlap PCR using $p27^{Kip1}$-3'UTR-mut-1 and $p27^{Kip1}$-3'UTR-mut-2 primers. The primers used in the PCR are shown in Table I. The cells were transiently cotransfected with miR-429 mimics or miR-NC mimics and the pMIR-$p27^{Kip1}$-3'UTR-wt/pMIR-$p27^{Kip1}$-3'UTR-mut vector. Luciferase activity was measured 48 h after transfection with a Dual-Luciferase assay kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions.

RNA isolation and quantitative (q)PCR. Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA (2 μg) from each sample was used for cDNA synthesis using RT primers for miR-429 and U6 small nuclear RNA (Guangzhou Ribobio Co., Ltd). qPCR was performed using the SYBRII Premix Ex Taq™ (Takara Bio, Inc., Shiga, Japan). The amplification was performed under the following thermal program: Initial denaturation (95˚C, 20 sec), 40 cycles of 95˚C for 10 sec, 60˚C for 20 secs and 70˚C for 10 sec. The qPCR primer sets for miRNA-429 and U6 small nuclear were purchased from Guangzhou Ribobio Co., Ltd. miR-429 and U6 small nuclear RNA were quantified according to a standard curve and this was performed in triplicate. U6 small nuclear RNA was used for normalization. The relative expression levels of miRNA-429 were calculated using the following equation: Copies miR-429/copies U6. The quantitative analysis of the change in expression levels was calculated by qPCR analysis software (Bio-Rad CFX manager 2.0; Bio-Rad, Hercules, CA, USA).

Western blotting. Total proteins were isolated 24 h after transfection. The protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad). Equal quantities of total protein (20 μg) were separated on a 12% SDS-polyacrylamide gel, then transferred to a polyvinylidene membrane (Millipore, Billerica, MA, USA). Subsequent to blocking, the membrane was incubated with mouse monoclonal anti-$p27^{Kip1}$ antibody (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or rabbit polyclonal anti-β-actin antibody (1:1,000; Bioss Biotechnology, China) followed by incubation with polyclonal goat anti-mouse or polyclonal goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc.). Signals were determined with a chemiluminescence detection kit (NEN™ Life Science Products, Inc., Boston, MA, USA).

Cell proliferation and colony formation assays. The cells were seeded in 96-well plates at ~$1x10^4$ cells/well and cultured in growth medium. At 0, 24, 48 and 72 h after miR-429 mimic/inhibitor transfection, the effect of miR-429 overexpression/knockdown on cell viability was determined by an MTT assay. Each experiment was performed in triplicate. The absorbance value of each well was measured with a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at 570 nm. All proliferation assays were repeated as independent experiments at least three times. For the colony formation assay, the transfected cells and control cells were plated on 10-cm plates (500 cells/plate), respectively, cultured for another 14 days, fixed with 10% formaldehyde for 5 min, stained with 1.0% crystal violet for 30 sec and counted without microscopy.

Flow cytometric analysis. The cells were harvested by trypsinization, washed with ice-cold phosphate-buffered saline, fixed in 75% ice-cold ethanol and stained with propidium iodide (10 mg/ml; 15 min; Invitrogen). A total of $2x10^4$ cells were analyzed with a FACSCalibur Flow Cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA). The experiments were performed in triplicate.

Statistical analysis. All experiments were repeated at least three times and data are expressed as the mean ± standard deviation. The statistical significance between groups was determined using Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Table I. Primer sequences used in qPCR and site-directed mutagenesis cloning.

Primer	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
$p27^{kip1}$	ACCCAAAGACTGATCCGTC	TTGGGGAACCGTCTGAAAC
β-actin	CAGAAGGAGATTACTGCTCTGGCT	TACTCCTGCTTGCTGATCCACATC
$p27^{kip1}$-3'UTR-wt	GCACGCGTACAGCTCGAATTAATAA	GCGAGCTCACAATAATTGGCATC
$p27^{kip1}$-3'UTR-mut-1	GCACGCGTACAGCTCGAATTAATAA	CAATGATTATGAGTTTAAAG
$p27^{kip1}$-3'UTR-mut-2	CTTTAAACTCATAATCATTG	GCGAGCTCACAATAATTGGCATC

qPCR, quantitative polymerase chain reaction; 3'UTR, 3' untranslated region; wt, wild type; mut, mutant.

Results

miR-429 is upregulated in prostate cancer cell lines. The expression levels of miR-429 were examined in two prostate cancer cell lines and six normal prostate epithelial tissues by qPCR. The results revealed that miR-429 expression was upregulated in the IF11 and IA8 prostate cancer cell lines, compared with the normal prostate epithelial tissue (Fig. 1), indicating a potential role for miR-429 in the tumorigenesis of prostate cancer.

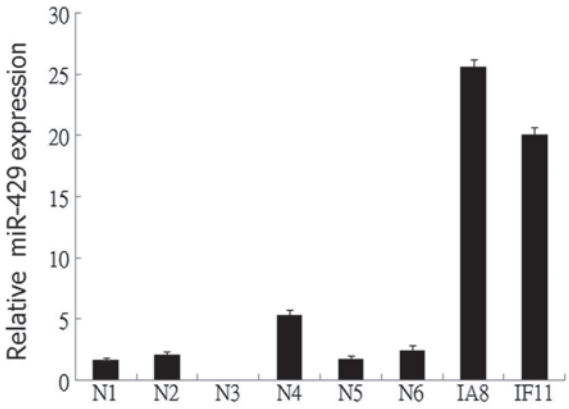

Figure 1. MicroRNA (miR)-429 expression levels in IF11 and IA8 human prostate cancer cells and normal prostate tissues. Quantitative-polymerase chain reaction analysis of miR-429 expression levels in normal prostate epithelial cells (shown as N1, N2, N3, N4, N5 and N6) and the prostate cancer cell lines. Bars signify the mean ± standard deviation from at least three independent experiments.

Downregulation of miR-429 inhibits the proliferation of prostate cancer cells. In order to investigate the effect of miR-429 on prostate cancer cell proliferation, the has-miR-429 inhibitor was transfected into the IF11 and IA8 prostate cancer cells. Colony formation assays and MTT were employed to analyze cell proliferation. As shown in Fig. 2A and B, downregulation of miR-429 in IF11 and IA8 cells significantly inhibited cell proliferation ($P<0.05$), compared with the NC transfection. To further demonstrate the effect of miR-429 on cell proliferation, has-miR-429 mimics was transfected into IF11 and IA8 prostate cancer cells; compared with the mock group, the proliferation of tumor cells in the miR-429 mimics transfected group was significantly increased ($P<0.05$; Fig. 2C and D).

Downregulation of miR-429 arrests IF11 and IA8 cell division in the G1 phase. An important characteristic of tumor cells is the increased proliferative capability, which commonly results from impaired regulation of the cell cycle. Thus, the effect of miR-429 on the prostate cancer cell cycle was investigated using flow cytometry. As shown in Fig. 3, miR-429 mimic-transfected IF11 and IA8 cells exhibited lower percentages of cells in the G1 phase (IF11, 51.39%; IA8, 54.37%) and increased percentages of cells in the S (IF11, 42.47%; IA8, 40.04%) and G2/M phases (IF11, 6.14%; IA8, 5.59%), compared with the mock control group cells (G1: IF11, 62.57%; IA8, 63.55%; S: IF11, 28.69%; IA8, 27.25%; and G2/M: IF11, 8.74%; IA8, 9.21%). However, the miR-429 inhibitor-transfected IF11 and IA8 cells exhibited higher percentages of cells in the G1 phase (IF11, 72.39%; IA8, 74.63%) and reduced percentages of cells in the S (IF11, 21.72%; IA8, 18.95%) and G2/M phases (IF11, 5.89%; IA8, 6.42%), compared with the mock control groups. These results indicated that downregulation of miR-429 mainly arrested IF11 and IA8 cells in the G1 phase, which may result in cell proliferation inhibition.

$p27^{Kip1}$ is a direct target of miR-429. miRNAs usually exert effects by binding to the 3'UTR of target genes; thus, the targets of miR-429 mimics were investigated to elucidate the underlying mechanism of this effect in IF11 and IA8 prostate cancer cells. Bioinformatic analysis using the miRanda algorithm (http://www.microrna.org) and target scan (http://www.targetscan.org/) indicated that the 3'UTR of $p27^{Kip1}$ contains a predicted binding site for miR-429 (Fig. 4A). To verify whether $p27^{Kip1}$ is a direct target of miR-429, a Dual-luciferase reporter system using pMIR-REPORT™ luciferase vectors containing wt or mutant $p27^{Kip1}$ 3'UTR was employed (Fig. 4A). Cotransfection with miR-429 significantly suppressed the luciferase activity of the reporter containing wt 3'UTR but did not suppress the mutant reporter ($P<0.05$; Fig. 4B). Consistent with these results, transfection with miR-429 mimics significantly reduced the endogenous $p27^{Kip1}$ mRNA and protein expression levels in IF11 and IA8 cells ($P<0.05$; Fig. 4C and D). In conclusion, these data suggest that $p27^{Kip1}$ is a direct target gene of miR-429.

Overexpression of $p27^{Kip1}$ partially rescues the proliferation-promoting effect of miR-429 on prostate cancer cells. To further confirm whether miR-429 mediates tumorigenic effects through $p27^{Kip1}$ in prostate cancer cells, IA8 cells were cotransfected with miR-429 mimics and the $p27^{kip1}$ expres-

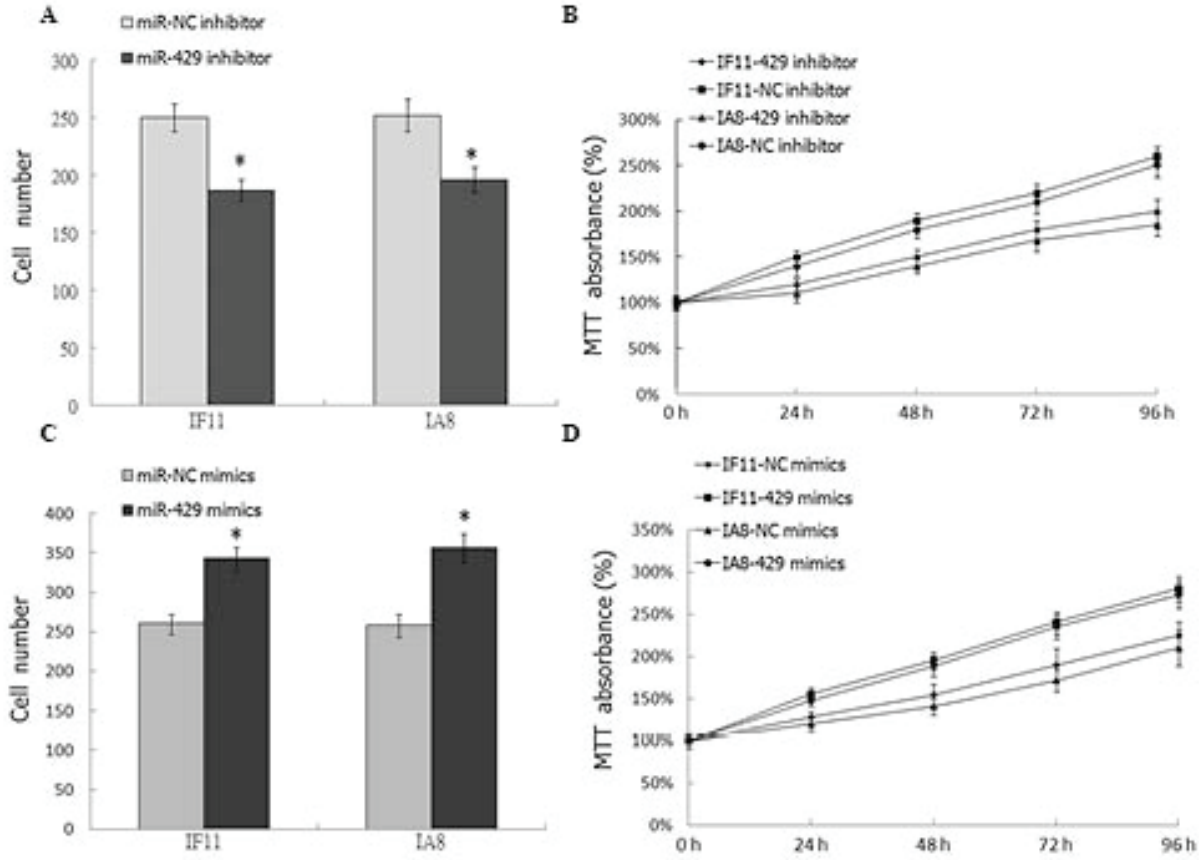

Figure 2. Downregulation of microRNA (miR)-429 inhibits proliferation of IF11 and IA8 human prostate cancer cells. Colony formation assay following (A) miR-429 downregulation or (B) upregulation in the cells; representative quantification of the crystal violet-stained cell colonies. An MTT assay indicated that miR-429 inhibitor transfection inhibited cell proliferation, compared with the negative control (NC)-transfected cells (C). However, miR-429 mimic transfection promoted cell proliferation, compared with the NC-transfected cells (D). Bars indicate the mean ± standard deviation from at least three independent experiments. *P<0.05, compared with the NC.

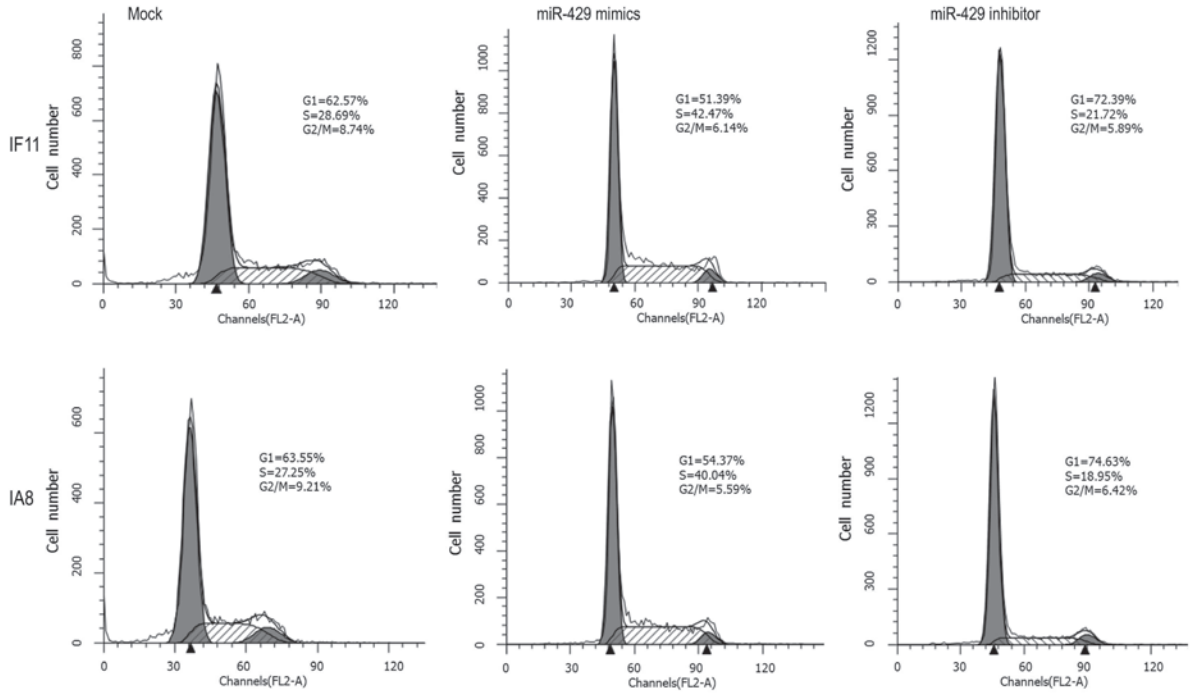

Figure 3. MicroRNA (miR)-429 modulates cell cycle progression in IF11 and IA8 human prostate cancer cells. The cell cycles of miR-429 inhibitor or miR-429 mimic-transfected cells were detected by flow cytometry. The miR-429 mimic-transfected groups exhibited lower percentages of cells in the G1 phase and increased percentages of cells in the S and G2/M phases, compared with the control groups. However, the miR-429 inhibitor-transfected groups exhibited higher percentages of cells in the G1 phase and reduced percentages of cells in the S and G2/M phases, compared with the control group.

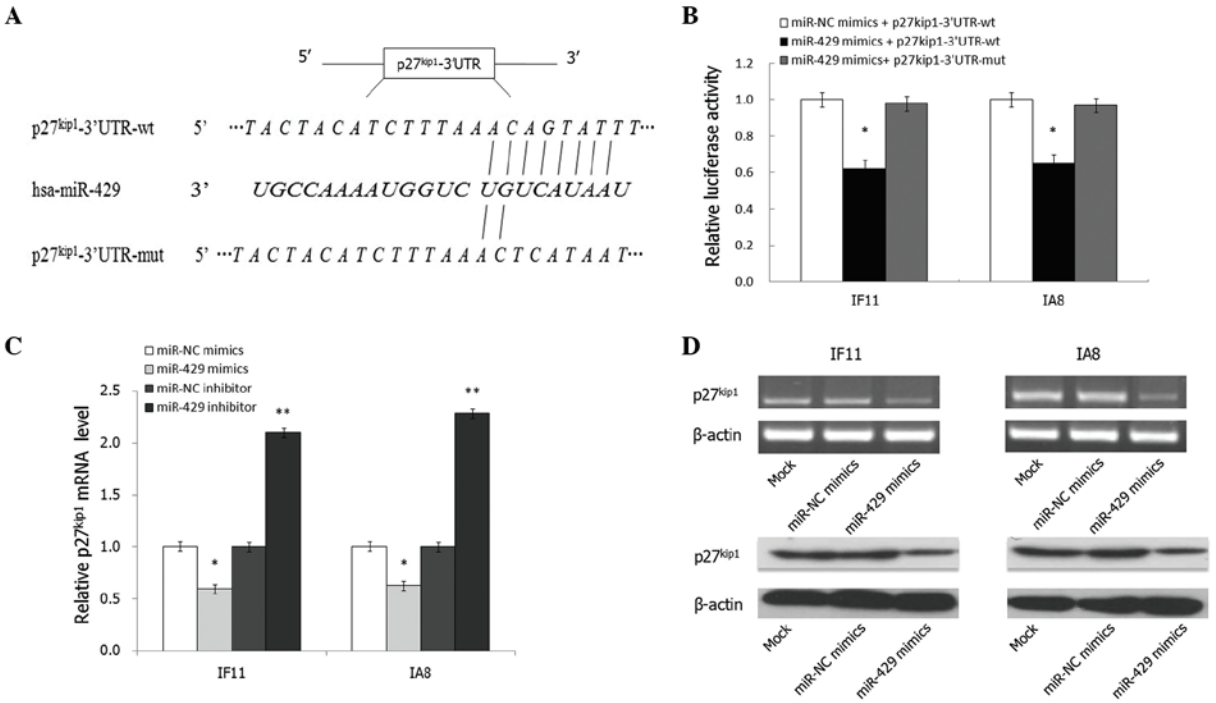

Figure 4. $p27^{Kip1}$ is a direct target of microRNA (miR)-429. (A) Putative binding sites for miR-429 in the 3' untranslated region (3'UTR) of $p27^{Kip1}$. A mutation was generated in the 3'UTR of the $p27^{Kip1}$ sequence in the complementary site for the seed region of miRNA-429 as indicated. (B) IF11 and IA8 human prostate cancer cells were co-transfected with miR-429 mimics/negative control (NC) mimics, pMIR-$p27^{Kip1}$-3'UTR-wild type (wt) reporter vector or pMIR-$p27^{Kip1}$-3'UTR-mutant (mut) reporter vector. Relative luciferase activities are expressed as the relative values to those of the miR-NC mimics-transfected group. (C) Quantitative polymerase chain reaction (qPCR) analysis of $p27^{Kip1}$ mRNA expression levels in miR-429 mimics/inhibitor and NC-transfected IF11 and IA8 cells. (D) qPCR and western blot analyses of $p27^{Kip1}$ mRNA and protein expression levels in miR-429 mimics/inhibitor or NC-transfected IF11 and IA8 cells at 24 h after transfection. For western blotting, total protein was isolated from cells transfected with miR-429 mimics/inhibitor or NC for 24 h. The $p27^{Kip1}$ protein expression levels were analyzed by western blotting using antibodies against $p27^{Kip1}$. Bars signify the mean ± standard deviation from at least three independent experiments. $^{*}P<0.05$ and $^{**}P<0.01$, compared with the respective NC group.

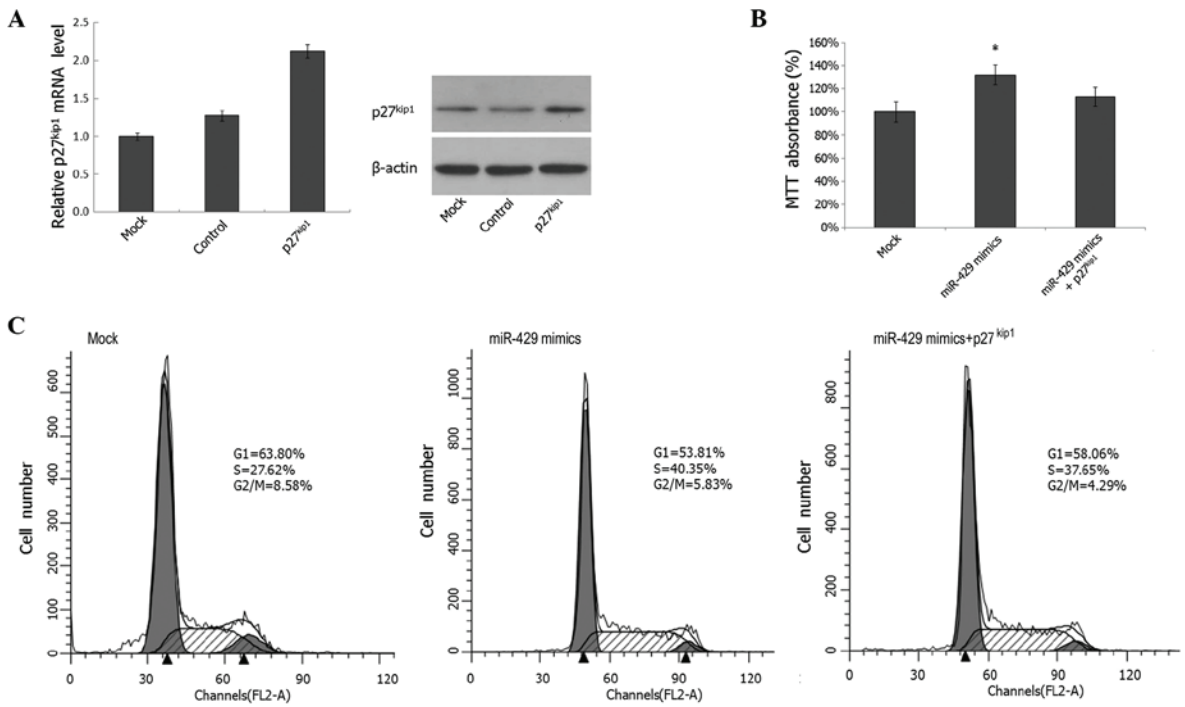

Figure 5. Overexpression of $p27^{Kip1}$ partially reverses the proliferation-promoting effect of microRNA (miR)-429 in IA8 prostate cancer cells. $p27^{Kip1}$ expression plasmid was co-transfected with miR-429 mimics into the cells. (A) At 48 h after transfection, $p27^{Kip1}$ mRNA and protein expression levels in the cells were measured by quantitative polymerase chain reaction and western blot analysis. β-actin served as a loading control. (B) At 48 h after transfection, cell proliferation was measured by an MTT assay. (C) At 48 h after transfection, cell cycles were analyzed by flow cytometry. Bars indicate the mean ± standard deviation values of three independent experiments; $^{*}P<0.05$, compared with the mock group.

sion plasmid; the increased $p27^{Kip1}$ expression levels were confirmed by PCR and western blotting, as shown in Fig. 5A. MTT analysis revealed that cotransfection of the $p27^{Kip1}$ expression plasmid partially reversed the effect of miR-429 on cell proliferation (Fig. 5B). Furthermore, cotransfection of the $p27^{Kip1}$ expression plasmid also partially abrogated the effect of the miR-429 mimics on G1 phase arrest (Fig. 5C). These findings demonstrate that the cell cycle arrest effect of miR-429 is achieved, at least in part, by the direct downregulation of $p27^{Kip1}$ expression. Consequently, cell cycle arrest results in proliferation inhibition in prostate cancer cells.

Discussion

miRNAs are endogenous small non-coding RNA molecules that regulate gene expression at the posttranscriptional level by targeting the 3'UTR (4-7). Aberrant expression of miRNAs is involved in the development of cancer (16). Notably, certain miRNAs may be either down- or upregulated in different types of cancer. miR-429 is a member of the miR-200 family and is located on chromosome 1p36. miR-429 has been demonstrated to be downregulated in particular types of cancer and may function as a tumor suppressor (12,17-19). However, certain studies have shown that miR-429 may act as oncogene in other types of cancer (13-15,20). The discrepancies in the function of miR-429 in different types of cancer may reflect the differences of cellular types or differences in the targeted genes.

Prostate cancer, the second leading cause of fatalities in males, continues to be a problem (1). Several studies have observed that certain miRNAs regulate the expression of cancer-related genes in prostate cancer, affecting the phenotype of these cells (8,10,21,22). However, the relevance of miRNAs in the development, progression and prognosis of prostate cancer is not fully understood. In the present study, miR-429 was, to the best of our knowledge, found for the first time to be upregulated in the IF11 and IA8 human prostate cancer cell lines, compared with normal prostate epithelial tissues. Downregulation of miR-429 arrested the prostate cancer cell cycle in the G1 phase. The progression of the cell cycle in eukaryotes is governed by complex-containing cyclins and cyclin-dependent kinase (CDK). Deregulation of G0/G1 phase cell cycle regulators is hypothesized to promote the aberrant proliferation of cancer cells (9,23,24). The data from the present study indicate that miR-429 regulated the proliferation of prostate cancer cells through targeting the progression of the cell cycle.

Since miRNAs exert effects by regulating the expression of other target genes, several algorithms were employed to determine these genes, and $p27^{Kip1}$ was identified as a potential target of miR-429. As shown in the luciferase assay and western blot analysis in the present study, the expression of the potential target gene $p27^{Kip1}$ was directly regulated by miR-429 in prostate cancer cells. Upregulation of $p27^{Kip1}$ by transfection with an exogenous expression vector partially reversed the effects exerted by miR-429 on the cell cycle and cell proliferation. The cyclin/CDK inhibitor $p27^{Kip1}$ has been established as important in the regulation of cell cycle progression. Studies have shown that $p27^{Kip1}$ interacts with cyclin E or cyclin A/CDK2 or other cyclin/CDK binary complexes to inhibit the respective kinase activities. Thus, $p27^{Kip1}$ blocks cell cycle progression by inhibiting the activity of cyclin E/CDK2 complexes that normally promote G1/S phase progression (25,26). The results obtained in the present study indicate that miR-429 regulates cell cycle progression primarily through targeting the $p27^{Kip1}$ cyclin/CDK inhibitor.

In conclusion, to the best of our knowledge, this is the first study documenting that miR-429 is overexpressed in IF11 and IA8 prostate cancer cells. Overexpression of miR-429 was associated with cell cycle progression and cell proliferation in prostate cancer. Additional results demonstrated that the effect of miR-429 in prostate cancer cells was executed through targeting the $p27^{Kip1}$ CDK inhibitor; $p27^{Kip1}$ was demonstrated to be directly regulated by miR-429 in the cells.

Acknowledgements

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