RESEARCH ARTICLE

MicroRNA-497 Suppresses Proliferation and Induces Apoptosis in Prostate Cancer Cells

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Abstract

MicroRNAs (miRNAs) are a class of endogenously expressed small, non-coding, single-stranded RNAs that negatively regulate gene expression, mainly by binding to 3'- untranslated regions (3'UTR) of their target messenger RNAs (mRNAs), which cause blocks of translation and/or mRNA cleavage. Recently, miRNAprofiling studies demonstrated the microRNA-497 (miR-497) level to be down-regulated in all prostate carcinomas compared with BPH samples. The purpose of this study was to investigate the potential role of miR-497 in human prostate cancer. Proliferation, cell cycle and apoptosis assays were conducted to explore the potential function of miR-497 in human prostate cancer cells. Results showed that miR-497 suppressed cellular growth and initiated G0/G1 phase arrest of LNCaP and PC-3 cells. We also observed that miR-497 increased the percentage of apoptotic cells by increasing caspase-3/7 activity. Taken together, our results demonstrated that miR-497 can inhibit growth and induce apoptosis by caspase-3 activation in prostate cancer cells, which suggest its use as a potential therapeutic target in the future.

Keywords: MiR-497 - prostate cancer - apoptosis - growth reduction

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Introduction

MicroRNAs (miRNAs) are a class of endogenously expressed small, non-coding, single-stranded RNAs that negatively regulate gene expression by mainly binding to 3' untranslated region (3'UTR) of their target messenger RNAs (mRNAs) and cause translational repression or mRNA cleavage (Filipwicz et al., 2008). MicroRNAs execute pivotal roles in physiological and pathological process including development, differentiation, metabolism, immunity, cell proliferation and apoptosis (Bartel et al., 2004; He et al., 2004; Vasudevan et al., 2007). Hundreds of altered expressions of miRNAs are found to be closely associated with the formation, invasion and progression of most kinds of human cancers, and about 50% of miRNAs genes are found to be located in cancer-related genomic regions (Calin et al., 2006). In these processes, miRNAs may function as oncogenes or tumor suppressors, and the former ones are often up-regulated, whereas the latter are down-regulated in cancers.

Prostate cancer (PCa) is the most prevalent malignancy in males and the second significant cancer killer of men in America and Europe (Siegel et al., 2012). Up to now, more than 50 miRNAs are abnormally expressed, leading to alteration in the expression and activity of their targets in PCa, such as miR-21, miR-320, miR-29b and miR-34c (Ribas et al., 2009; Hagman et al., 2010; Hsieh et al., 2012; Ru et al., 2012). Recently, miRNAs profiling studies demonstrate that miR-497 level is decreased in all prostate carcinoma samples compared with the BPH samples (Porkka et al., 2007; Watahiki et al., 2011). Several studies showed that the potential functions of miR-497 in human breast cancer (Li et al., 2011; Shen et al., 2012), neuroblastoma (Yadav et al., 2011), cervical cancer (Zheng et al., 2012), gastric cancer (Zhu et al., 2012) and colorectal cancer (Guo et al., 2012). However, no studies about the role of miRNA-497 in prostate cancer have been reported.

In this study, miR-497 mimics were used to determinate the impact of miR-497 on the proliferation, cell cycle and apoptosis of prostate cancer cells.

Materials and Methods

Cell lines and culture conditions

Human prostate cancer cell lines LNCaP (androgen receptor (AR) positive) and PC-3 (AR negative) were obtained from the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences and grown in RPMI 1640 medium (Life Technologies) with 10% fetal bovine serum (FBS, Gibco), 100 IU/mL Penicillin and 100 μg/mL Streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C.

Reagents

MTT Formazan, Ribonuclease A and propidium iodide (PI) were obtained from Sigma-Aldrich. Caspase-3/7 assay kit was purchased from Promega. Annexin V-FITC

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Apoptosis Detection Kit was obtained from Beyotime Institute of Biotechnology (Jiangsu China). Lipofectamine 2000 reagent was obtained from Life Technologies.

Transfection assay for miRNA Mimics

Pre-miRTM miRNA Precursor Molecule mimicking miR-497 and control non-specific miRNA (Pre-miRTM Negative Control) were purchased from Ambion® Life Technologies. LNCaP and PC-3 cells were added into each well of a 6-well plate and cultured with RPMI 1640 medium with 10% FBS. Pre-miR-497 or negative control precursor miRNA and Lipofectamine 2000 reagent were diluted to 100 μL by RPMI 1640 medium without serum and antibiotics, respectively and incubated for 5 minutes at room temperature; then gently combined and incubated for 20 minutes. Subsequently, 200 μL of the complexes were added to each well. After 6 hours of incubation, culture medium was replaced by RPMI-1640 with 10% FBS, and all cells were incubated at 37 ˚C in a CO_2 incubator for 48 hours prior to further experiments.

Cell proliferation assay

After 48 hours transfection, Cells with various concentrations of miR-497 mimics were trypsinized and counted, respectively. Cells (3,000 cells/well) were plated in each well of 96-well plates in triplicate and incubated at 37 ˚C for 72 hours. MTT Formazan was added and used to detect cell proliferation. The spectrophotometric absorbance of each sample was measured at 490 nm. The growth inhibition rate was calculated using the following equation: growth inhibition rate = [the mean optical density (OD) of controls - the mean OD of mimics]/the mean OD of controls. For growth curve assay, Cells were seeded in 24-well plates at a density of $1×10^4$ cells per well. Viable cell numbers in triplicate were determined at 24, 48 and 72 hours.

Colony formation assay

Colony formation assays were performed using LNCaP and PC-3 cells transfected with miRNA-497 mimics and negative control precursor miRNA at 48 hours of post-transfection. Cells were plated and grown at 37 °C in a 5% CO_2 incubator for 14 days. At the end of the experiment, colonies were stained with 0.1% crystal violet and counted under a microscope.

Cell cycle analysis

LNCaP and PC-3 cells were transfected with miRNA-497 or negative control precursor miRNA. At 48 hours of post-transfection, cells were harvested and centrifuged at 1,200 rpm for 5 minutes and washed twice in phosphate buffered saline (PBS). Subsequently, cells were fixed in 70% ethanol at -20oC for overnight. After washing twice with PBS, cells were digested by Ribonuclease A (25 μg/mL) at 37oC for 30 minutes, and stained with PI (25 μg/mL) at 4oC for 1 hour. Cell cycle distribution was analyzed by FACScan flow cytometer (Becton Dickinson).
Caspase-3/7 activity assay

LNCaP and PC-3 cells were transfected with miRNA-497 or negative control precursor miRNA. At 48 hours of post-transfection, cells were trypsinized and plated in 96-well plate for 48 hours incubation. 100 μL of Caspase-Glo 3/7 substrate reagents were added and incubated for 1 hour in the dark. The luminescence of each sample was measured by a plate-reading luminometer.

Apoptosis assay

At 72 hours after miR-497 mimics transfection, cells were trypsinized and washed with PBS, then resuspended in 100 μL of binding buffer. Annexin V staining was accomplished following the product instruction. In brief, 5 μL Annexin V-FITC (2 mg/mL) and 5 μL PI (2 mg/mL) were added and stained for 15 minutes in the dark. The percentage of early and late apoptotic cells was analyzed by flow cytometry.

Statistical analysis

Data were expressed as the means ± stan¬dard deviation from at least 3 separate experiments. Statistical analyses were performed using Student's two-tailed t-test. Difference with P values of < 0.05 are considered significant.

Results

miRNA-497 inhibited cell proliferation of prostate cancer cells

To explore the potential role of miR-497 on the proliferation of prostate cancer cells, miR-497 mimics were used to treat with tumor cells at the concentrations of 25, 50 and 100 nmol/L for 72 hours. Cell viability was measured by MTT assay. Compared to negative control, miR-497 significantly inhibited the growth of LNCaP and

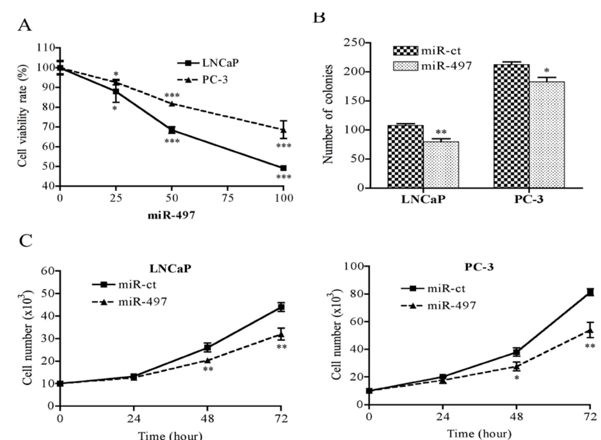

Figure 1. miRNA 497 Inhibited Proliferation of LNCaP and PC-3 Cells. A, LNCaP and PC-3 cells were transfected with different concentrations of miR-497 mimics or negative control precursors. Cell viability of LNCaP and PC-3 was measured by MTT assay. B, LNCaP and PC-3 cells were transfected with negative control or miR-497 mimics. After 48 hours, 200 cells were seeded in 6-well plate and changed with fresh complete medium every 3 days. After 3 weeks incubation, cells were fixed and stained with 1% crystal violet. The colony numbers of LNCaP and PC-3 cells were counted. Each bar showed the mean and standard derivation of triplicate samples. C, after transfection with negative control or miR-497 mimics for 48 hours, the growth of LNCaP and PC-3 cells was determined by counting cell numbers. Cell numbers in triplicate wells were counted every day for 3 days. *P<0.05, **P<0.01, ***P<0.001, compared with negative control group

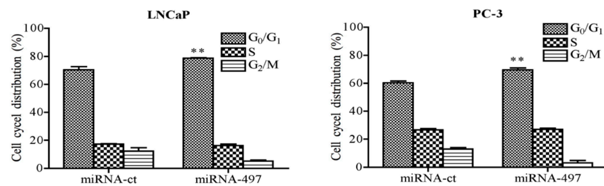

Figure 2. miRNA-497 Caused Cell Cycle Arrest of LNCaP and PC-3 Cells. The cell cycle progression of LNCaP and PC-3 cells were arrested at G_0/G_1 phase after transfection with miR-497 mimics. LNCaP and PC-3 cells were plated and transfected with negative control or miR-497 mimics for 48 hours. Cells were fixed in 70% cold ethanol and stained with PI solution. Cell cycle distribution of prostate cancer cells was assessed by flow cytometer. $**P<0.01$, compared with negative control group

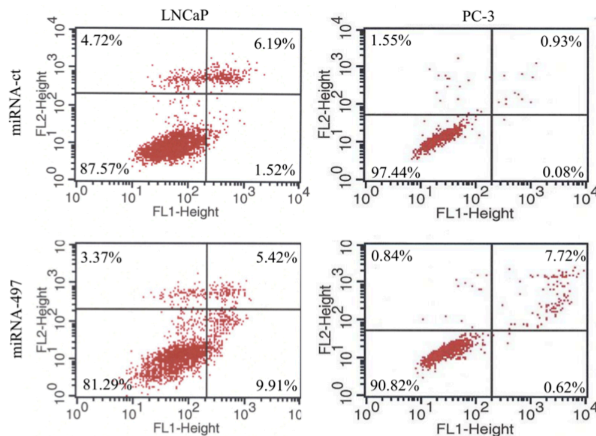

Figure 3. miRNA-497 Induced Apoptosis of LNCaP and PC-3 Cells. LNCaP and PC-3 cells were plated and transfected with negative control or miRNA-497 mimics. After transfection for 72 hours, cells were collected and stained with Annexin-V/PI. The percentages of early (low right quadrant) and late apoptotic cells (upper right quadrant) were assessed by flow cytometry

PC-3 prostate cancer cells in the dose-dependent manner (Figure 1A). The results of colony formation assay showed that overexpression of miR-497 in both LNCaP and PC-3 cells exhibited a significant reduction of colony numbers when compared with the cells transfected with the negative control (Figure 1B). We also observed that the effect of miR-497 on the growth rate of both cell lines by counting cell numbers in triplicate wells every day for 3 days. The growth of LNCaP and PC-3 was significantly inhibited by miR-497 in the time dependent manner (Figure 1C). These results indicated that prostate cancer cell proliferation could be significantly inhibited by miR-497.

miRNA-497 caused cell cycle arrest of prostate cancer cells

The prostate cancer cells were transfected with miR-497 or negative control precursors to study their effects on cell cycle distribution. After the transfection of miR-497 mimics at the concentration of 100 nmol/L for 48 hours, cell cycle distribution analysis revealed that the percentage of G_0/G_1 phase cells dramatically increased in the miR-497 treatment group (78.70 ± 0.45% in LNCaP cells, 69.55 ± 1.40% in PC-3 cells), which was higher than those in the negative control group (70.41 ± 2.18% in LNCaP cells,

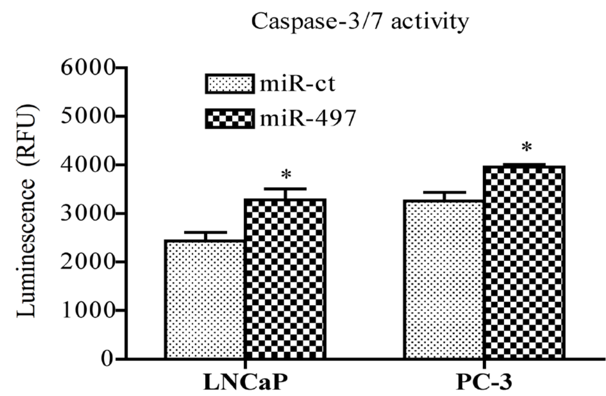

Figure 4. miRNA-497 Increased Caspase-3/7 Activities of LNCaP and PC-3 Cells. After transfection with negative control or miR-497 mimics for 48 hours, caspase-3/7 activities of LNCaP and PC-3 cells were detected by a Caspase-Glo 3/7 assay kit. $*P<0.05$, compared with negative control group

60.28 ± 1.37% in PC-3 cells), and while the proportion of G2/M phase cells decreased, the percentage of S-phase cells was not significantly altered (Figure 2). These data suggested that miR-497 could initiate G_0/G_1 phase arrest in prostate cancer cells.

miRNA-497 induced apoptosis and caspase-3/7 activities in prostate cancer cells

We further investigated the effect of miR-497 on apoptosis. LNCaP and PC-3 cells were transfected with 100 nmol/L of miR-497 mimics for 72 hours. Flow cytometry analysis demonstrated that the percentage of early apoptosis (1.52% for negative control and 9.91% for miR-497 mimics) in LNCaP cells and the percentage of late apoptosis (0.93% for negative control and 7.72% for miR-497 mimics) in PC-3 cells were increased by miR-497 mimics, compared to the negative control. These results suggested that miR-497 could act as an apoptosis inducer in prostate cancer cells in vitro (Figure 3). To analyze the molecular mechanism of miR-497 mimics on apoptosis, caspase-3/7 activities were examined in prostate cancer cells. The results showed that caspase-3/7 activities were significantly increased in the miR-497 mimics-transfected group, compared with negative control. These data demonstrate that miRNA-497 could induce apoptosis through increasing caspase-3/7 activities (Figure 4).

Discussion

Increasing evidence suggests that miRNAs play a critical role in carcinogenesis and cancer progression (Lu et al., 2005). miRNAs, a class small regulatory RNA molecules that repress gene expression of their mRNA targets in a sequence specific manner, have been identified as key regulators in a wide variety of oncogenic processes, such as cell proliferation, angiogenesis, cellular differentiation, invasion and metastasis, and can function as either tumor suppressors or oncogenes (Kefas et al., 2008; Sun et al., 2010; Zhang et al., 2010).

In this study, our data for the first time have revealed that miR-497 greatly inhibited cellular growth and induced apoptosis and caused G_0/G_1 cell cycle arrest of prostate cancer cells.

The previous studies provided several set of information on the effect of miR-497 on inhibiting cell viability and inducing apoptosis in several different other cancer cell lines. Shen et al reported that miR-497 induced apoptosis of breast cancer cells by targeting Bcl-w (Shen et al., 2012). Zheng et al also reported that miR-497 simultaneously inhibits protein levels of three members of the ERK pathway, RAF1, MEK1 and ERK1 in Hela cells (Zheng et al., 2012). Guo, et al and Luo, et al showed that miR-497 could target the insulin-like growth factor 1 receptor (IGF-1R) and had a tumor-suppressive role in human colorectal and cervical cancer (Guo et al., 2012; Luo et al., 2013). miR-497 maybe have a tumor suppressive role in prostate cancer cells by targeting MAPK, IGF-1R or other signaling pathways, which we will prove them next step.

In this present study, miR-497 caused G_0/G_1 cell cycle arrest in LNCaP and PC-3 cells. The previous data showed that miR-497 have been reported to cooperatively regulate a number of targets, including CDK6, CARD10 and CDC27 leading to regulation of cell proliferation in colon cancer cells (Linsley et al., 2007). We will validate the cell cycle effects induced by miR-497 to explain its mechanism as a tumor suppressor. Caspase-3 protein is a member of the cysteine-aspartic acid protease family. Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis. Caspase-3 cleaves and activates caspases-6 and -7; and the protein itself is processed and activated by caspases-8, -9 and -10. Our results showed miR-497 increased the caspase-3/7 activities in LNCaP and PC-3 cells. We should analyze the exact mechanisms of miR-497 on apoptosis by Real time PCR and Western blot analysis in the future.

In summary, our results demonstrated that miR-497 induced cell growth inhibition, cell cycle arrest and apoptosis in LNCaP and PC-3 cells. In addition, these effects were associated with increased caspase-3/7 activities in both cells. It may be concluded that miR-497 acts as a tumor suppressor by activating caspase-3/7 activities. However, given the fact that each miRNA may regulate many target genes, more studies are needed to investigate targets by miR-497. Therefore, miR-497 could be a promising therapeutic target for prostate cancer treatment in the future.

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