Original Paper

MiR-221 Promotes Capan-2 Pancreatic Ductal Adenocarcinoma Cells Proliferation by Targeting PTEN-Akt

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Key Words

MiR-221 • Pancreatic ductal adenocarcinoma • Capan-2 • PTEN • Akt

Abstract

Background/Aims: MicroRNAs (miRNAs, miRs) have emerged as critical regulators of cancer cell proliferation. The effect of miR-221 on cancer cell growth could be significantly changeable in different cell lines. Although miR-221 was reported to promote the cell growth of pancreatic ductal adenocarcinoma (PDAC) cells, its role in Capan-2 cell line is largely unknown. **Methods:** Capan-2 cells were transfected with miR-221 mimics, inhibitors, or negative controls. Cell Counting Kit-8 was used to determine cell viability. EdU staining and cell cycle analysis were used to measure cell proliferation. Western blotting was used to detect the expression levels of PTEN and phospho-Akt. The PI3K-Akt pathway activator SC-79 and inhibitor LY294002 were used to perform the rescue experiment in determining cell proliferation. **Results:** Overexpressing miR-221 significantly increased cell vitality and promoted cell proliferation and G1-to-S phase transition of the cell cycle in Capan-2 cells, while inhibition of miR-221 decreased that. The protein level of PTEN in Capan-2 cells was downregulated by overexpressing miR-221, while upregulated by inhibiting miR-221. Consistently, enhanced phosphorylation of Akt^{Ser473} was observed in miR-221 overexpressed Capan-2 cells, and the opposite result was found in miR-221 inhibited cells. LY294002 restored the pro-proliferation effect of miR-221 on Capan-2 cells, while SC-79 had no additional effect on cell proliferation in Capan-2 cells transfected with miR-221 mimics. **Conclusion:** Our study indicates that miR-221 is an oncogenic miRNA which promotes Capan-2 cells proliferation by targeting PTEN-Akt pathway.

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the 13th most commonly diagnosed cancer worldwide and the fifth cause of cancer death in the developed countries [1, 2]. Despite the improvement gained from medical advance, PDAC remains one of the highest mortality rates, and very low survival improvements have been made, a rate essentially unchanged over the course of the years [3, 4]. Additionally, nearly 80% of patients who present with locally advanced or metastatic diseases have an extremely poor prognosis. The treatment of PDAC is primarily a combination of surgery and adjuvant chemotherapy, which is not satisfactory due to its limited efficacy [5]. Consequently, a much deeper understanding of the pathobiology and molecular mechanism of PDAC is essential to develop novel therapeutic strategies.

MicroRNAs (miRNAs, miRs) are a group of small noncoding RNAs with about 20 nucleotides in length [6, 7], which post-transcriptionally regulate message RNA (mRNA), leading to the suppression of target gene expressions [8-10]. To date, microarray technology has revealed that differential miRNA expression patterns were tissue/cell type-specific, and even depended on the developmental stage of tumors. For that reason, studies have highlighted their importance in pathological activity, including tumor development [11-13]. miR-221 belongs to the miR-221/222 family, which locates on the X chromosome and shares some identical seed sequences with its homologous miRNA, miR-222 [14]. Accumulating evidence has indicated that miR-221 was an oncogene, which was up-regulated in a variety of cancers, including hepatocellular carcinoma, prostate adenocarcinoma, and colorectal carcinoma [15-18]. These results indicate that miR-221 may play significant roles in cancer development. Circulating miR-221 level has been detected to be markedly higher in PDAC patients compared to that in patients with benign pancreatic tumors or healthy controls [19]. However, the effect of miR-221 on cancer cell growth could be significantly changeable among cell lines, demonstrating that miR-221 might exhibit either pro- or anti-oncogenic roles in different cancer cell lines [20]. In a previous study on non-small cell lung cancer (NSCLC), miR-221 could promote cell growth in H460, while suppresses cell growth in four other NSCLC cell lines [20]. These data highlight the importance that the effect of miR-221 on cancer cell growth is highly needed to be tested in different cell lines.

In fact, different pancreatic adenocarcinoma cell lines have relative phenotypic differences in adhesion, migration, invasion, angiogenic potential, and tumorigenicity [21]. Recent studies have reported that miR-221 inhibition could attenuate cell proliferation in several human pancreatic adenocarcinoma cell lines, including BxPC-3, CFPAC-1 [22], MIA PaCa-2, PANC-1 [23], and AsPC-1 [24]. However, the effect of miR-221 on the proliferation of Capan-2 PDAC cell line is largely unknown. Indeed, here we aimed to clarify the functional role and the underlying molecular mechanism of miR-221 on the proliferation of Capan-2 cell line *in vitro*.

Material and Methods

Materials and agents

The PI3K-Akt pathway activator SC-79 [25] and inhibitor LY294002 [26] were purchased from Tocris Bioscience, UK. The Capan-2 cells were incubated with either SC-79 (4 µg/ml) or LY294002 (10 µM) in the indicated experiments for 24 h. RPMI-1640 medium and fetal bovine serum (FBS) was from Hyclone, USA. 100X penicillin-streptomycin was purchased from KeyGEN, China. 5-ethynyl-2'-deoxyuridine detection assay (Cell-Light™ EdU Apollo®567 In Vitro Imaging Kit) and miR-221 mimics/inhibitors were purchased from Ribobio, China. The sequences were designed as follows: miR-221 mimics, AGC UAC AUU GUC UGC UGG GUUUC; miR-221 inhibitor, GAA ACC CAG TCT CAA TGT AGC TCC GGA AAC CCA GTC TCA ATG TAGCT. CCK-8 assay was purchased from Dojindo, Japan.

Cell culture

Human PDAC cell line Capan-2 cell was purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Capan-2 cells were maintained in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂. The study was approved by the Ethics Committee of Tongji hospital, Tongji University, Shanghai, China.

Cell transient transfection

Capan-2 cells were seeded at density of 2×10^5 /ml and allowed to adhere overnight. After that, Capan-2 cells were incubated with serum free medium for 6 h, and were then transfected with miR-221 mimics (50 nM), inhibitors (100 nM) or their negative controls (NC) for 48 h using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions.

Real time quantitative PCR assay

Total RNA was isolated from Capan-2 cells using TRIZOL reagent (Invitrogen, USA). miR-221 real time PCR assays and reverse transcription reactions were performed using Bulge-Loop™ miRNA qRT-PCR Primer Set (RiboBio, China) according to instructions.

Cell vitality assay

The effect of miR-221 on cell viability was determined using a Cell Counting Kit-8 (CCK-8) assay, which is based upon WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium]] uptake. Cells (5×10^3 /well) were seeded in 96-well plates and allowed to adhere overnight. After 48 h of miR-221 mimics/inhibitors and their NC transfection, 10 µl CCK-8 solution was added to each well and incubated for 1 h at 37°C, then the absorbance was measured at 450 nm using a spectrophotometer.

EdU Proliferation Assay

To access the effects of miR-221 in proliferative on Capan-2 cells, cells were pre-treated with 50 µM 5-ethynyl-2'-deoxyuridine (EdU) before harvest. Then an Apollo staining was performed according to the instructions to detect the EdU positive cells using a fluorescence microscope [27].

Analysis of cell cycle distribution by flow cytometry

Capan-2 cells were transfected with miR-221 mimics or inhibitors and then incubated for 48 h. After that, cells were collected and washed twice with PBS. Subsequently, cells were fixed in 70% ethanol (pre-cold at -20°C temperature) overnight at -20°C. The fixed cells were then washed twice in ice-cold PBS and then incubated with RNase (50 µg/ml) for 15 min. Subsequently, cells were stained with 50 µg/mL propidium iodide (PI) for 30 min. The stained cells were then analyzed for their DNA content by using a Beckman flow cytometry. The results were analyzed by FlowJo software.

Western blotting

Capan-2 cells were collected and lysed in ice-cold RIPA buffer (Beyondtime, China) with 10nM PMSF. An analytical 10% SDS-PAGE was performed, and 50 µg of protein of each was added into each lane. For immunoblotting, proteins in the SDS gels were transferred to a polyvinylidene difluoride (PVDF) membrane by an electroblot apparatus. Antibodies against Akt (1:1000, Bioworld, USA), phospho-Akt^{Ser473} (1:1000, Bioworld, USA) and PTEN (1:1000, Abcam, USA) were used to detect the protein level. β-actin was used as loading control to equal protein loading. The membrane was developed using enhanced chemiluminescence system (ECL, BioRad, USA).

Statistical analysis

Data were expressed as mean ± SEM. The independent-samples T-test was used for comparisons between two groups. The one-way ANOVA test, followed by Bonferroni's post-hoc test, was performed to analyze difference among more than two groups. All analysis was performed using SPSS 19.0 software. P-value less than 0.05 was considered significant.

Results

miR-221 promotes Capan-2 cells proliferation

miR-221 has been proved to be an oncogenesis miRNA in multiple cancers [28-31], and may as well be an anti-oncogenesis miRNA depending on different cancer cell lines [20]. Still, the role of miR-221 in human PDAC cell line Capan-2 cells remains unknown. To investigate the role of miR-221 in Capan-2 cell growth, we performed miR-221 gain-of-function and loss-of-function experiments.

Effects of miR-221 mimics and inhibitors were confirmed by quantitative real-time PCRs. As shown in Fig. 1, transfection of miR-221 mimic (50 nM) could significantly increase the miR-221 level in Capan-2 cells ($p < 0.01$), while transfection of miR-221 inhibitor (100 nM) could decrease that ($p < 0.01$). We then detected the cell vitality with a CCK-8 assay. As shown in Fig. 2A, overexpressing miR-221 significantly increased cell vitality ($p < 0.01$) and reduction of miR-221 decreased that in Capan-2 cells ($p < 0.01$).

DNA synthesis is another important factor of cancer cell growth. For that reason, we used an EdU assay to assess the effect of miR-221 on Capan-2 cell DNA synthesis. As shown in Fig. 2B, overexpressing miR-221 led to increased EdU-positive cell population compared with NC-mimics treated cells ($p < 0.01$), while inhibiting miR-221 significantly reduced EdU positive-cell population compared with NC-inhibitor group ($p < 0.01$), indicating miR-221 could induce the DNA synthesis of Capan-2 cells.

Malignant cells often show a dysregulated cell cycle, which is a crucial tumorous property that promotes tumor growth and invasion. Here, we used flow cytometry to detect the cell cycle distribution of Capan-2 cells transfected with miR-221 mimics. As shown in Fig. 2C, up-regulation of miR-221 led to an increased S phase cell population ($p < 0.01$) and decreased G1 phase cell population ($p < 0.01$) than that of NC-mimics transfected cells.

miR-221 targets PTEN-Akt pathway in Capan-2 cells

PTEN, p27 and p57, a type of well-established tumor suppressors, as well as PUMA, a pro-apoptosis factor, have been reported as direct target genes of miR-221 [23, 32]. However, whether they could be endogenously regulated by miR-221 in Capan-2 cells remains unclear. Using Western blotting, we found that the protein level of PTEN in Capan-2 cells was down-regulated by overexpressing miR-221, while up-regulated by inhibiting miR-221 (Fig. 3A). Meanwhile, the protein levels of p27, p57, and PUMA remained unchanged (Fig. 3A). These data indicate that PTEN might be a target gene of miR-221 in Capan-2 cells.

Fig. 1. Real time quantitative PCR results of miR-221. (A) The miR-221 level significantly increases in Capan-2 cells transfected with miR-221 mimics than transfected with negative control sequence (NC-mimics). (B) The miR-221 level significantly decreases in Capan-2 cells transfected with miR-221 inhibitors than transfected with negative control sequence (NC-inhibitors). **, $p < 0.01$, $n = 6$.

Fig. 2. miR-221 promotes Capan-2 cells proliferation. (A) CCK-8 assay shows miR-221 mimics enhances cell vitality, and inhibition of miR-221 leads to reduced cell vitality. **, $p < 0.01$, $n = 10$. (B) EdU assay shows overexpression of miR-221 increases cell proliferative rate, and inhibition of miR-221 leads to reduced cell proliferative rate. Red = EdU positive cell, Blue = DAPI. **, $p < 0.01$, $n = 6$. (C) Overexpressing miR-221 increases S phase cell rate and reduces G1 phase cell rate, as demonstrated by flow cytometry. **, $p < 0.01$, $n = 4$.

PTEN is a crucial inhibitor for PI3k-Akt pathway, which dephosphorylates PIP3 to make PIP2 and inactivates Akt [33]. Akt activation is an important contributor to cancer genesis and development, by promoting cell proliferation, reducing cell apoptosis, and enhancing drug-resistance.[34-38]. To investigate the potential link between miR-221 and Akt activation, we detected the phosphorylation level of Akt in Capan-2 cells. As shown in Fig. 3A, we found enhanced phosphorylation of Akt^{Ser473} in miR-221-overexpressed Capan-2 cells, and decreased phosphorylation of Akt^{Ser473} in miR-221-inhibited Capan-2 cells, while total Akt level remained unchanged. These results indicate that miR-221 targets PTEN-Akt pathway in Capan-2 cells.

Akt mediates the proliferative effect of miR-221 on Capan-2 cells

To confirm if Akt mediates the proliferative effect of miR-221 on Capan-2 cells, we firstly upregulated the miR-221 level by transfecting miR-221 mimics in Capan-2 cells, and then we respectively treated the cells with PI3K-Akt activator SC-79 [39] or inhibitor LY294002 [40]. As shown in Fig. 3B, we found that treatment with LY294002 could abolish the proliferative effect of miR-221 on Capan-2 cells. In addition, co-treatment with Akt activator SC-79 and miR-221 mimics had no additional effect on cell proliferation compared with cells transfected with miR-221 mimics alone. These results indicate that the effect of miR-221 on the proliferation of Capan-2 cells is mediated by Akt activation.

Discussion

PDAC is a major cause of cancer deaths all over the world [41, 42]. The prognosis of patients with PDAC is one of the worst in all cancer forms, despite that medical advances have been made in tumor diagnosis and treatment strategies during the past decades [43]. Consequently, it is of great importance to develop novel therapeutic methods to treat patients suffered from PDAC.

Fig. 3. PTEN-Akt pathway mediates the proliferative effect of miR-221 on Capan-2 cells. (A) Western blot assay shows that overexpressing miR-221 can reduce PTEN protein level and increase phospho-Akt^{Ser473} level, while total Akt, PUMA, p27, and p57 levels do not change. Inhibition of miR-221 increases PTEN level and reduces phospho-Akt^{Ser473} level, while total Akt, PUMA, p27, and p57 levels do not change. (B) EdU assay shows both miR-221 mimics (+) group and miR-221 mimics (+) SC-79 (+) group has higher cell proliferative rate than miR-221(-) group. No significant difference is observed between miR-221 mimics (+) group and miR-221 mimics (+) SC-79 (+) group. No significant difference is observed between miR-221 (-) group and miR-221 (+) LY294002 (+) group. MiR-221 (+) LY294002 (+) group has lower cell proliferative rate than miR-221 mimics (+) group. Red = EdU positive cell, Blue = DAPI. **, $p < 0.01$, ##, $p < 0.01$, N.S., No significance, $n = 6$.

Overexpression of miR-221 accompanied by the inhibition of its target gene, PTEN, could significantly suppress tumor cell growth and proliferation in various cancers [44-47]. Our results are consistent with previous studies, implying the proliferative effect of miR-221 on PDAC Capan-2 cells. In the present study, we demonstrated that the elevated expression level of miR-221 could enhance cell vitality, induce proliferation, and promote cell cycle progression from G1 to S phase in human pancreatic cancer cell line Capan-2 cells, whereas suppression of miR-221 had inverse effects *in vitro*. Therefore, it is very likely that inhibition of miR-221 could be a therapeutic way for PDAC.

PTEN is proved as a direct target gene of miR-221 [32, 48], and is a tumor suppressor gene which plays a critical role in the regulation of cell growth. It is well known that PTEN antagonizes the proliferative and prosurvival activity of cells induced by activation of PI3k-Akt signaling [49, 50]. Therefore, we detected the PTEN expression level and Akt phosphorylation level in miR-221 regulated Capan-2 cells. Our results showed that PTEN was downregulated and Akt^{Ser473} phosphorylation was upregulated in Capan-2 cells transfected with miR-221 mimics, while it displayed a contrary tendency in cells transfected with miR-221 inhibitors. We then identified that the proliferative effect of miR-221 on Capan-2 cells could be reversed by Akt inhibition. Meanwhile, overexpression of miR-221 in Capan-2 cells combined with treatment of Akt activator did not show extra effects on cell proliferation. These results indicated that miR-221 may directly target PTEN and thereby activate Akt pathway to induce the proliferation of Capan-2 cells.

In conclusion, our data provide novel insights into how miR-221 functions to promote PDAC development. The method of inhibiting oncogenesis miR-221 may provide a novel therapeutic approach for patients with PDAC.

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Disclosure Statement

The authors declare there are no conflicts of interest.

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