RESEARCH ARTICLE

MicroRNA-122 Promotes Proliferation, Invasion and Migration of Renal Cell Carcinoma Cells Through the PI3K/Akt Signaling Pathway

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Abstract

  Objective: MicroRNAs (miRNAs) are a small class of non-coding, single-stranded RNAs with a critical role in genesis and maintenance of renal cancer mainly through binding to 3'-untranslated regions (3'UTR) of target mRNAs, which causes a block of translation and/or mRNA degradation. The aim of the present study was to investigate the potential effects of miR-122 in human renal cell carcinomas. **Methods:** The expression level of miR-122 was quantified by qRT-PCR. MTT, colony formation, invasion and migration assays were used to explore the potential functions of miR-122 in human renal cell carcinoma cells. **Results:** Cellular growth, invasion and migration in two A498 and 786-O cells were significantly increased after miR-122 transfection. Further experiments demonstrated that overexpression of miR-122 resulted in the increase of phospho-Akt (Ser473) and phospho-mTOR (Ser2448), then activation of mTOR targets, p70S6K and 4E-BP1. **Conclusions:** The up-regulation of miR-122 may play an important role in the progress of renal cancer through activating PI3K/Akt signal pathway and could be a potential molecular target for anti-cancer therapeutics.

  Keywords: miR-122 - renal cell carcinoma - invasion - migration

Introduction

Renal cell carcinoma (RCC) represents the most common kidney malignancy and is the third most common urological cancer after prostate and bladder cancer, but it has the highest mortality rate at over 40%. Among the five subtypes, clear cell carcinoma (CCC) accounts for approximately 70% of the cases (Novick et al., 2007). About 30% of patients with RCC develop invasive disease commonly metastasizing to bone, lung, brain and liver (Milowsky et al., 2003; Rouviere et al., 2006). Therefore, increased understanding of the molecular mechanisms of RCC progression (recurrence, metastasis or drug resistance) is needed to provide a rationale for the effective therapeutic methods of RCC.

MicroRNAs (miRNAs) are short noncoding oligonucleotides with imperfect complementarity predominantly to the 3' untranslated region (UTR) of target mRNAs and cause translational repression or mRNA cleavage (Couzin et al., 2008; Filipowicz et al., 2008; Bartel et al., 2009). miRNAs predominantly act by inhibiting mRNA translation although mRNA degradation and mRNA cleavage may also contribute to downregulation of protein levels and play a role in the pathogenesis of cancer with function as tumor suppressors or oncogenes (Lu et al., 2005). Recently numerous studies have shown aberrant expression of microRNA-122-5p (miR-122) in human cancer tissues, including RCC (Zhou et al., 2010; Heinzelmann et al., 2011; Osanto et al., 2012), suggesting that it is a candidate tumor activator in RCC. miR-122 is one of the most frequent miRNA isolated in the liver and plays important roles in many aspects of liver physiology, such as stress response (Bhattacharyya et al., 2006) and lipid metabolism (Esau et al., 2006). However, up to date, there are no studies of miR-122 in renal cell carcinoma cells.

The present study was undertaken to examine the expression of miR-122 in different RCC cell lines, assesse the impact of miR-122 on cell proliferation, invasion and migration and identify target genes for miR-122 that might mediate their biological effects. In summary, our results suggest that miR-122 contributes to the malignant phenotype of RCC cells and can be a promising therapeutic target against RCC cancer.

Materials and Methods

Cell lines and culture conditions

Human renal cell carcinoma cell lines A498, ACHN, Caki-1, Caki-2 and 786-O cells were purchased from

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the Shanghai Institutes for Biological Sciences or Chinese Academy of Sciences or American Type Culture Collection (ATCC) and grown in RPMI 1640 medium (Life Technologies) containing 10% fetal bovine serum (FBS, Gibco), penicillin (100 IU/mL) and streptomycin (100 μg/mL) in a humidified atmosphere of 5% CO_2 at 37°C.

Transfection assay for miRNA mimics

Pre-miRTM miRNA Precursor Molecule mimicking miR-122, control non-specific miRNA (Pre-miRTM Negative Control) and anti-miR-122 were purchased from Ambion® Life Technologies. A498 and 786-O cells were transfected with Pre-miR miRNA precursor-122, anti-miR-122 or negative control precursor miRNA using LipofectamineTM 2000 Transfection Reagent (Life Technologies) following 24 h of cell seeding. Briefly, Pre-miR miRNA precursor-122, anti-miR-122 or negative control precursor miRNA and LipofectamineTM 2000 were diluted to 100 µL by RPMI 1640 medium without serum and antibiotics, respectively and incubated for 5 min. Then miRNAs-LipofectamineTM 2000 complex were gently mixed for 20 min at room temperature and added to the 6-well plates. After 6 h of incubation, culture medium was replaced by RPMI 1640 medium with 10% FBS, and the cells were incubated at 37°C in a CO_2 incubator for 48 h prior to further testing.

Cell proliferation assay (MTT)

Cell proliferation was determined using the MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide) assay performed according to the manufacturer's instructions. Cells (5,000/well) with various concentrations of miR-122 mimics or negative control miRNA were seeded onto 96-well plates in triplicate and incubated for 72 h. MTT reagent was added and incubated for 4 hours. Then the formazan precipitate was dissolved in 150 µl DMSO and the absorbance rate was measured in a microplate reader at a wavelength of 570 nm. After 48 h transfection, cells were seeded in 24-well plates in triplicate at a density of 1×10^4 cells per well for growth curve assay. The numbers of viable cell from control negative miRNA, miR-122 and anti-miR-122 transfected groups were counted at 24, 48 and 72 h.

Colony formation assay

For colony formation assay, cells were transfected with miR-122 mimics, anti-miR-122 or negative control miRNA for 48 h, and then grown in a 6-well plate in triplicate. After 14 days incubation, the colonies were gently washed with PBS and then fixed with 10% formalin and stained with 0.1% crystal violet for 30 min. The colony containing more than 50 cells was manually counted.

Invasion and migration assays

The invasion and migration assays were performed using the transwell system (24 wells, 8-µm pore size, BD Bioscience, USA). After 48 h transfection with miR-122 or negative control miRNA, 1×10^5 cells were resuspended in RPMI-1640 medium without FBS and seeded into the upper chambers coated with growth factor reduced

Matrigel (BD Bioscience) for invasion assay and collagen IV for migration assay and the lower chamber was filled with 0.4 ml of RPMI 1640 medium containing 10% FBS. Cells were fixed with 10% formalin and stained with 0.1% crystal violet solution after they were incubated for 24 h. Cells on the upper side of the membrane were removed by using a cotton swab. The invaded or migrated cells in triplicate were taken pictures and counted in five randomly fields under a light microscope at ×100 magnification for statistical analysis.

miRNA isolation and quantitative real time RT-PCR

Total RNAs were extracted from the fresh cells using TRIZol reagent (Life technologies). The concentration and quality of RNA were measured by the UV absorbance at 260 nm and 280 nm and guaranteed RNA equal amount from each sample. miRNA isolation was carried out from the total RNA using mirVanaTM miRNA Isolation Kit (Ambion) according to manufacturer's instructions. Quantitative PCR was performed using an initial step of denaturation at 95°C for 5 min, 40 cycles of amplification, denaturation at 95°C for 15 sec, annealing at 60°C for 30 sec, elongation at 72°C for 30 sec on a 7500 Fast Real-time PCR System (Applied Biosystem). All reactions were performed in a 20-µl volume in triplicate. The primer sequences were as below: miR-122, forward primer: 5'-TTGAATTCTAACACCTTCGTGGCTACAGAG-3', reverse primer: 5'-TTAGATCTCATTTATCGAGGGAAG GATTG-3' and U6, forward primer: 5'-CTCGCTTCGGC AGCACA-3', reverse primer: 5'-AACGCTTCACGAAT TTGCGT-3'. U6 snRNA levels were used as the internal control. Data analyses for the miRNA expression were performed using the $2^{-\Delta\Delta Ct}$ method (Chen et al., 2005).

Western blot

Cell lysates were prepared from miR-122 and negative control miRNA-transfected A498 and 786-O cells using a lysis buffer (40 mmol/L Tris-HCl pH 7.4, 10% glycerol, 50 mmol/L BGP, 5 mmol/L EGTA, 2 mmol/L EDTA, 0.35 mmol/L vanadate, 10 mmol/L NaF, and 0.3% Triton X-100) supplemented with protease inhibitors cocktail (Roche Applied Science). The cell extracts were centrifuged at 10,000×g for 20 min at 4°C. Protein concentration was determined by Bradford assay using Coomassie Plus protein reagent. Equivalent amounts of protein were loaded onto SDS-polyacrylamide gel electrophoresis and transferred into polyvinylidene fluoride (PVDF) membrane. Membranes were incubated with the following primary antibodies against Akt ½ (1:500), phospho-Akt (Ser473, 1:500), mTOR (1:500), phospho-mTOR (Ser2448, 1:500), p70S6K (1:1000), p-p70S6K (Ser389, 1:1000), 4E-BP1 (1:1000), p-4E-BP1 (Ser65, 1:1000) and β-Actin (1:2000) overnight at 4°C. The membrane was washed with PBS and then incubated with goat anti-rabbit or anti-mouse IgG (H+L)-HRP conjugate antibodies followed by echochemiluminescence (ECL) reaction.

Statistical analysis

The results were expressed as the means ± stan¬dard deviation from 3 separate experiments. The significance
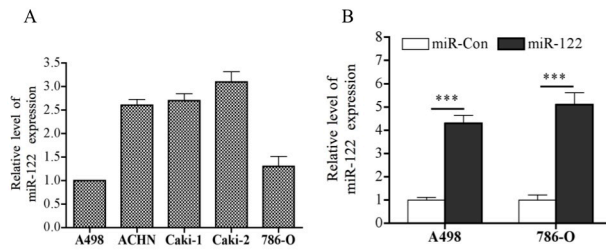
Figure 1. The Expression of miR-122 in Different Renal Cell Carcinoma Cell Lines. (A) miR-122 expression in five renal cell carcinoma cell lines was analyzed by real time RT-PCR. (B) The levels of miR-12 were examined by real time RT-PCR in A498 and 786-O cells after transfecting with miR-122 and negative control miRNA. U6 served as an internal reference. All data are expressed as mean ± SD for three experiments. ***$P < 0.001$
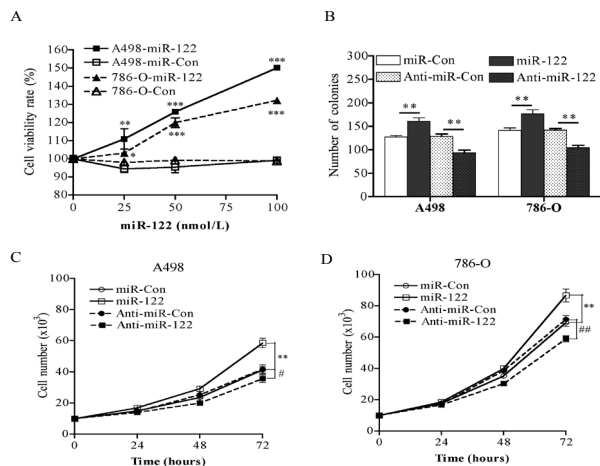

Figure 2. miRNA-122 Promotes Cell Proliferation of Renal Cell Carcinoma Cells. (A) Cell proliferation assay. A498 and 786-O cells were transfected with different concentrations of miR-122 and negative control miRNA for 72 h. Cell viability was determined using the MTT assay. The formazan precipitate was dissolved in 150 µl DMSO and the absorbance rate was measured in a microplate reader at a wavelength of 570 nm. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. (B) Colony formation assay. The number of colony in miR-122, anti-miR-122 or control miRNA transfected groups were counted and showed as the average values, **$P < 0.01$. (C) Growth curve assay. After 48 h transfection with miR-122, anti-miR-122 or control miRNAs, A498 and 786-O cells were seeded in 24-well plates in triplicate at a density of $1×10^4$ cells per well for growth curve assay. The data represent the mean ± SD in triplicate. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

of the data was analyzed using an independent samples Student t-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression levels of miR-122 in different renal cell carcinoma cells

We examined a series of human renal cell carcinoma cell lines, A498, ACHN, Caki-1, Caki-2 and 786-O for miR-122 expression to choose appropriate renal cancer cell models. A498 and 786-O cells have very low expression levels of miR-122, but high levels of miR-122 in other cell lines (Figure 1A). Based on these data and our observation, we chose the two cell lines, A498 and 786-
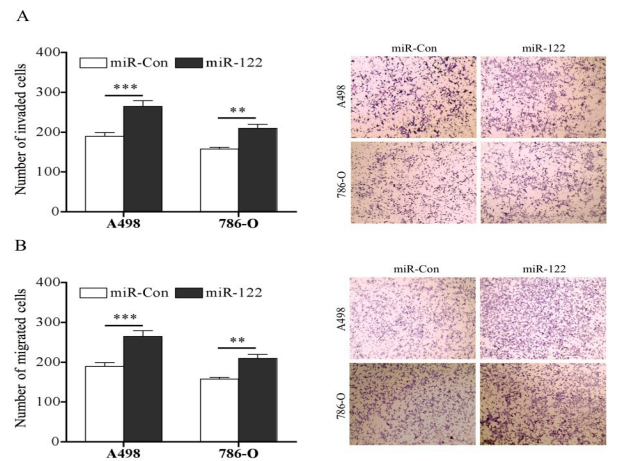

Figure 3. miRNA-122 Enhances the Abilities of Invasion and Migration in Renal Cell Carcinoma Cells. (A) Invasion assay. A498 and 786-O cells transfected with miR-122 or negative control miRNA were seeded on Transwell inserts precoated with Matrigel. Quantification analysis (left) and representative photographs at 100 × magnification (right) were shown. **$P < 0.01$, ***$P < 0.001$. (B) Migration assay. A498 and 786-O cells transfected with miR-122 or negative control miRNA were seeded on Transwell inserts precoated with collagen IV. Quantification analysis (left) and representative photographs at 100 × magnification (right) were shown. **$P < 0.01$

O of lower miR-122 expression for the current study. To investigate the effects of miR-122 restoration on renal cell carcinoma cells, we transfected the A498 and 786-O cells with miR-122 mimics or negative control miRNA mimic. Real time PCR analysis showed that higher expression of miR-122 mimic in the transfected A498 and 786-O cells compared with negative control mimic (Figure 1B).

miRNA-122 promoted proliferation of renal cell carcinoma cells

To study the effects of miR-122 mimics on the proliferation of RCC cells, A498 and 786-O cells were exposed to different concentrations of miR-122 mimics for 72 hours by the MTT assay. Our results showed that a significant increase in cell proliferation of A498 and 786-O in a dose-dependent manner was observed (Figure 2A, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) following miR-122 transfection, compared to cells transfected with miR-Con. For colony formation assay, our results showed that the colony numbers were significantly increased following miR-122 mimics transfection and decreased following anti-miR-122 transfection compared with the control cells (Figure 2B, **$P < 0.01$, ***$P < 0.001$). We also observed that the effect of miR-122 and anti-miR-122 on the growth rate of both cell lines by counting cell numbers in triplicate wells every day for 3 days. The growth of A498 and 786-O was significantly increased by miR-122 and decreased by anti-miR-122 in a time dependent manner (Figure 2C). These results indicated that renal cell carcinoma cell proliferation could be significantly increased by miR-122 and decreased by anti-miR-122.

miRNA-122 enhanced invasion and migration of renal cell carcinoma cells

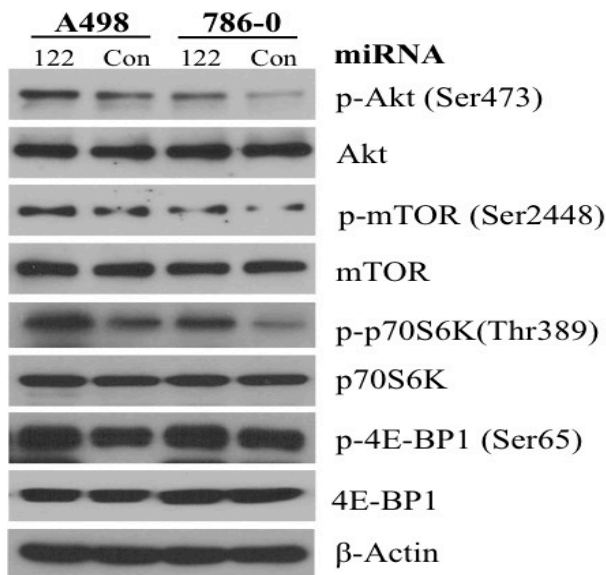We further detected the influence of miR-122 on cell

Figure 4. PI3K/Akt Pathway is Involved in the Regulation of Renal Cell Carcinoma Cell Proliferation, Invasion and Migration by miR-122. A498 and 786-O cells were transfected with miR-122 and negative control miRNA, respectively and then subjected to cell lysates preparation for Western blot. The membranes were blotted by primary antibodies against anti-p-Akt (Ser473), Akt, p-mTOR (Ser2448), mTOR, p-p70S6K (Ser389), p70S6K, p-4E-BP1 (Ser65), 4E-BP1 and β-Actin

invasion and migration, A498 and 786-O cells transfected with miR-122 were applied to the Matrigel pre-coated invasion and collagen IV pre-coated migration assays. As shown in Figure 3A and 3B, invasion of A498 and 786-O cells were increased by miR-122. And the similar effect of migration was also observed in A498 and 786-O cells (**P< 0.01, ***P< 0.001).

PI3K/Akt pathway was involved in the regulation of renal cell carcinoma cell proliferation, invasion and migration by miR-122

To analyze the molecular mechanisms of miR-122 on proliferation, invasion and migration was examined in renal cell carcinoma cells, we transfected miR-122 mimics to investigate the activation of Akt and mTOR by Western blot analysis. Our results indicated that the phosphorylation levels of Akt (p-Akt Ser473) and mTOR (p-mTOR Ser2448) and mTOR downstream targets (p-p70S6K (Ser389) and p-4E-BP1 (Ser65)) were effectively increased after miR-122 mimics transfection in A498 and 786-O cells (Figure 4). Our results suggest that miR-122 promote renal cancer cell growth by activation of Akt and its downstream mTOR.

Discussion

In this study, we provide the first evidence that miR-122 enhances renal cell carcinoma progression by activating PI3K/Akt signaling pathway. Our data indicate that overexpression of miR-122 significantly promoted cell proliferation and colony formation in RCC A498 and 786-O cells. Meanwhile, the invasion and migration potentials of A498 and 786-O cells were also increased compared to control groups. All of these results suggest

that up-regulation of miR-122 may play roles in the occurrence and development of RCC.

Recent studies found that miR-122 is frequently suppressed in hepatocellular carcinomas (Bai et al., 2009; Tsai et al., 2012) and breast cancer specimens (Wang et al., 2012). Overexpression of miR-122 could suppress proliferation and induce apoptosis and cell cycle arrest in cancer cells through reducing the expression of Bcl-W, CCNG1 (Ma et al., 2010), IGF1R/PI3K/Akt (Wang et al., 2012) and Wnt/beta-catenin signal pathways (Xu et al., 2012). These findings suggest that miR-122 may behave as a tumor suppressor in hepatocellular carcinoma and breast cancers. In contrast to what has been observed in the liver and breast cancers, one study for cutaneous T-cell lymphoma showed that miR-122 expression was up-regulated in advanced stage mycosis fungoides (MF) and further induced during chemotherapy-induced apoptosis by regulating p53/Akt signaling (Manfe et al., 2012). Base on these studies, we checked the PI3K/Akt signaling to study the mechanisms of miR-122 on biological effect in RCC cells. Our data showed that overexpression of miR-122 affected the phosphorylation status of Akt but not the amount of Akt protein at the protein levels. We also observed that miR-122 activated mTOR and its downstream targets, p70S6K and 4E-BP1 in A498 and 786-O cells. These results indicate that miR-122 functions as an oncogenic miRNA in RCC, promoting tumor cell growth through activating PI3K/Akt signaling. Our findings are consistent with a previous study by Manfè et al showing that miR-122 regulated PI3K/Akt signaling in human cutaneous T-cell lymphoma. Next, we will analyze the roles and mechanisms of miR-122 in tumorgenesis and progression of renal cell carcinoma in vivo.

In conclusion, for the first time, our study demonstrates that miR-122 promotes renal cell carcinoma proliferation and increases the ability of invasion and migration through activating PI3K/Akt signaling pathway. Our findings suggest that miR-122 might be a promising target for the treatment of renal cell carcinoma in future.

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