MiR-182 affects renal cancer cell proliferation, apoptosis, and invasion by regulating PI3K/AKT/mTOR signaling pathway

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Abstract. – OBJECTIVE: PI3K/AKT/mTOR signaling pathway plays a crucial role in tumorigenesis and development. It was shown that mTOR overexpression was associated with the pathogenesis of renal cancer. Down-regulation of MiR-182 was found in renal carcinoma tissue. This study thus aims to investigate the influence of miR-182 in regulating mTOR expression and renal carcinoma cell proliferation, invasion, and apoptosis.

PATIENTS AND METHODS: The targeted regulatory relationship between miR-182 and mTOR was tested by dual luciferase assay. Renal carcinoma tissue and benign renal tissue were collected to detect miR-182 and mTOR expressions. MiR-182, mTOR, p-mTOR, and Survivin levels were compared between HK-2 and A498 cells. Renal carcinoma A498 cells were divided into four groups, including miR-NC, anti-miR-182 mimic, si-NC, and si-mTOR groups. Cell apoptosis and proliferation were evaluated by flow cytometry. Cell invasion was determined by transwell assay.

RESULTS: Bioinformatics analysis revealed the complementary relationship between miR-182 and the 3'-UTR of mTOR mRNA. The level of miR-182 was significantly reduced, while mTOR expression was upregulated in renal carcinoma tissue compared with that in benign lesion, which was associated with TNM stage. MiR-182 expression was markedly declined, whereas mTOR, p-mTOR, and Survivin levels were apparently upregulated in A498 cells compared with that in HK-2 cells. The treatment of miR-182 mimic or si-mTOR transfection significantly downregulated mTOR, p-mTOR, and Survivin expressions, restrained cell proliferation and invasion, and enhanced cell apoptosis.

CONCLUSIONS: The decreasing level of miR-182 plays a role in enhancing mTOR expression and promoting renal carcinoma pathogenesis. Overexpression of miR-182 inhibited mTOR expression and weakened cell proliferation and invasion, which provides leads to the future therapy of renal cancer.

Key Words: miR-182, PI3K/AKT/mTOR, renal carcinoma, proliferation, apoptosis, invasion.

Introduction

Renal carcinoma is a sort of common urinary malignancy in clinic and most commonly occurs in the adult kidney1. Renal carcinoma is characterized as high metastatic potential as well as low sensitivity to radiotherapy and chemotherapy. Therefore, the detection of the abnormally regulated signaling molecules in renal carcinoma is of great significance to early diagnosis, treatment, survival, and prognosis.

Mammalian target of rapamycin (mTOR) represents a serine/threonine protein kinase that interacts with a variety of signaling molecules, such as mitogen, cytokine, nutritional status, and ATP levels. However, its activity and function are mainly regulated by phosphatidylinositol-3 kinase (PI3K)/protein kinase B (AKT/PKB). Moreover, it exerts to signal transduction function at the downstream of PI3K/AKT signaling pathway. The overexpression of mTOR can enhance the activity of PI3K/AKT/mTOR signaling pathway and can be detected in a variety of tumor tissues. It was found that mTOR contributed to the regulatory role in the pathogenesis of renal cancer2, and was considered to be an important target in the treatment of renal cell1,3. MiRNA is a type of endogenous single-stranded noncoding RNA with the length of 22-25 nt. It plays a degrading or inhibiting role on more than 30% of mRNA by binding with the 3'-UTR4. Based on target genes, microRNAs serve as an oncogene5,6 or tumor suppressor7 role in tumor. Multiple stud-
ies demonstrated that the expression of miR-182 in tumor tissue and peripheral blood samples of renal cell carcinoma patients was significantly decreased, suggesting that miR-182 may be a tumor suppressor in the pathogenesis of renal cell carcinoma. Therefore, this work focuses to determine the impact of miR-182 in regulating mTOR expression, PI3K/AKT/mTOR signaling pathway activity, and renal carcinoma cell proliferation, invasion, and apoptosis.

**Patients and Methods**

**Main Reagents and Materials**

Human renal carcinoma cell A498 and normal renal proximal tubule epithelial cell HK-2 were purchased from Shanghai Cellular Library, Chinese Academy of Sciences. Roswell Park Memorial Institute 1640 (RPMI 1640) medium, fetal bovine serum (FBS), and penicillin-streptomycin were purchased from Gibco (Waltham, MA, USA). TRIzol and Lip2000 were bought from Invitrogen (Waltham, MA, USA). Real-time PCR reagent PrimeScript™ RT reagent Kit and SYBR Fast qPCR Mix were obtained from Takara (Otsu, Shiga, Japan). MiR-182 mimic, miR-182 inhibitor, and miR-NC were bought from Ribobio (Guangzhou, Guangdong, China). Mouse anti-human mTOR and p-mTOR antibodies were provided by Abcam (Cambridge, MA, USA). Rabbit anti-human Survivin and β-actin antibodies were obtained from GeneTex (Irvine, CA, USA). HRP conjugated secondary antibody was gotten from Wuhan Boster Biological Technology, Ltd., (Wuhan, Hubei, China). Transwell chamber was got from Millipore (Billerica, MA, USA).

**Clinical Information**

A total of 48 cases of renal carcinoma patients who received surgery in Dongyang People’s Hospital between Aug 2016 and Feb 2017 were enrolled, including 25 males and 23 females with mean age at 58.3 ± 12.4 years old. No patients received radiotherapy or chemotherapy before surgery. Renal carcinoma tumor tissue was stored at -80°C. The specimens were divided in three groups, 16 in stage I, 20 in stage II, and 12 in stage III, according to TNM staging. Another 18 cases of renal tissue were obtained from benign renal lesion, including 10 males and 8 females with average age at 56.6 ± 11.8 years old. No statistical difference on age and gender were observed between the two groups.

The study protocol was approved by the Research Ethics Committee of Dongyang People’s Hospital, and all patients gave their informed consent before the investigation.

**Cell Culture**

A498 and HK-2 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) medium containing 20% FBS and 1% penicillin-streptomycin. The cells were passed at 1:4 and used for experiments during logarithmic phase.

**Dual-Luciferase Reporter Gene Assay**

The PCR products containing the full length of mTOR gene 3’-UTR or mutant segment were cloned to pGL3. Next, it was transformed to DH5α competent cells. Then, pGL3-mTOR-3’-UTR-wt (or pGL3-mTOR—3’-UTR-mut) and miR-182 mimic (or miR-182 inhibitor, or miR-NC) were co-transfected to HEK293T cells using FuGENE6 together with miR-155 mimic (or anti-miR-155, or miR-NC). After 48 h incubation, the cells were lysed by Passive Lysis Buffer on ice for 20 minutes and detected using the Stop & Glo solution (Promega, Madison, WI, USA). At last, the sample was analyzed at 560 nm.

**Cell Transfection and Grouping**

A498 cells were divided into four groups, including miR-NC, miR-182 mimic, si-NC, and si-mTOR groups. MiR-NC, miR-182 mimic, si-NC, or si-mTOR at 30 nmol/L and Lip2000 at 5 μL were diluted in serum-free DMEM medium at room temperature for 5 min. Then, they were added to the cells and incubated for 72 h for the following experiments.

**qRT-PCR**

Total RNA was extracted using TRIzol and was reversed transcribed to cDNA by PrimeScript™ RT reagent kit. The reaction system contained 1.0 μg RNA, 0.5 μL oligo dT Primer at 50 μM, 0.5 μL Random 6 mers at 100 μM, 0.5 μL PrimeScript RT Enzyme Mix, 2 μL 5×PrimeScript Buffer, and RNase Free H₂O. The
reverse transcription was performed at 37°C for 15 min and 85°C for 5 s. The PCR reaction system contained 10.0 μL SYBR Fast qPCR Mix, 0.8 μL Reverse Primer at 10 μM, 2.0 μL cDNA, and 6.4 μL RNase Free H₂O. The PCR reaction was composed of 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 60°C for 20 s, and 75°C for 15 s. Real-time PCR was performed on Bio-Rad CFX96 Real-time PCR Detection System to test the relative expression. The primer sequences were listed as follows. miR-182P F: 5′-ACACTCCAGCTGGGTTTGGCAATGGTAGAACT-3′, miR-182PR: 5′-TGGTGTCGTGGAGTCG-3′; U6P F: 5′-ATTGGAACGATACAGAGAAGATT-3′, U6P R: 5′-GGAACGCTTCACGAATTTG-3′; mTORP F: 5′-TCCGAGAGATGAGTCAAGAGG-3′, mTORPR: 5′-CACCTTCCACTCTATGAGGC-3′; SurvivinP F: 5′-AGGACCACCTCTCAGTG-3′, SurvivinPR: 5′-AAGTCTGGCTCGTTCTCAGTG-3′; β-actinP F: 5′-GAACCCTAAGGCCAAC-3′, β-actinPR: 5′-TGTCACGCACGATTTCC-3′.

Western Blot
Total protein was extracted by radioimmunoprecipitation assay (RIPA) from cells. A total of 50 μg protein was separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to membrane. Next, the membrane was blocked by 5% skim milk at room temperature for 60 min and incubated in primary antibody at 4°C overnight (mTOR, p-mTOR, Survivin, and β-actin at 1:3000, 1:1000, 1:3000, and 1:15000, respectively). Then, the membrane was incubated in HRP conjugated secondary antibody (1:30000) for 60 min after washed by phosphate-buffered saline with Tween 20 (PBST) for three times. At last, the protein expression was detected by enhanced chemiluminescence (ECL).

Cell Apoptosis Detection
The cells were digested by trypsin and resuspended in binding buffer after centrifugation at 300 g for 5 min. Next, the cells were incubated in 10 μl Annexin V-FITC and 5 μl PI. At last, the cells were tested on Beckman Coulter FC 500 MCL flow cytometry (Beckman Coulter, Fullerton, CA, USA) to evaluate cell apoptosis.

Flow Cytometry Detection of Cell Proliferation
Cell proliferation was assessed by Click-iT EdU Alexa Fluor 488 Flow Cytometry Assay kit. The cells were added with 10 μM EdU solution for 2 h. After incubated for 48 h, the cells were digested and collected. After washing, fixation, and penetration, the cells were incubated in reaction liquid tagged by Alexa Fluor 488 at room temperature avoid of light for 30 min. Then, the cells were washed and tested by Beckman Coulter FC 500 MCL flow cytometry (Beckman Coulter, Fullerton, CA, USA).

Transwell Assay
Matrigel was added to the upper chamber and incubated at 37°C for 30 min. 500 μl RPMI-1640 medium contained 10% FBS were added to the 24-well plate. Then, the transwell chamber paved 100 μl Matrigel was put onto the plate, added with A498 cells and resuspended in 200 μl serum-free medium. After 48 h, the membrane was fixed in methanol and stained by 0.1% crystal violet. At last, the membrane was observed under the microscope.

Statistical Analysis
All data analyses were performed on SPSS 18.0 software (Chicago, IL, USA). The measurement data were depicted as mean ± standard deviation and compared by t-test. p < 0.05 was considered as statistical significance.

Results
The Targeted Regulatory Relationship Between miR-182 and mTOR
MicroRNA.org online prediction showed the targeted binding site between miR-182 and 3′-UTR of mTOR mRNA (Figure 1A). Dual luciferase assay revealed that miR-182 mimics or miR-182 inhibitor transfection significantly declined or elevated the relative luciferase activity of HEK293T cells transfected by pGL3-mTOR-3′-UTR-WT, respectively (Figure 1B), while no change of relative luciferase activity was observed in HEK293T cells transfected by pGL3-mTOR-3′-UTR-MUT, indicating the regulatory relationship between miR-182 and mTOR mRNA.

MiR-182 and mTOR Expressions in Renal Carcinoma Tissue
qRT-PCR demonstrated that the level of mTOR mRNA was significantly elevated, while miR-182 level was downregulated in renal carcinoma tissue compared with that in control. The reduction of miR-182 expression was presented in a TNM.
staging dependent manner (Figure 2A). Moreover, Western blot revealed that mTOR protein level was markedly increased in renal carcinoma tissue compared with that in control (Figure 2B).

**MiR-182 Decreased, While mTOR Enhanced in Renal Carcinoma Cells**

qRT-PCR demonstrated that levels of mTOR and Survivin mRNA were apparently increased, whereas the expression of miR-182 was significantly reduced in renal carcinoma A498 cells compared with that in HK-2 cells (Figure 3A). Western blot validated that the expressions of mTOR, p-mTOR, and Survivin proteins were up-regulated in A498 cells compared with HK-2 cells (Figure 3B).

**Overexpression of miR-182 Attenuated A498 Cell Proliferation and Invasion, and Promoted Cell Apoptosis**

The treatment of miR-182 mimic or si-mTOR transfection significantly downregulated mTOR expression, attenuated mTOR phosphorylation and Survivin level (Figure 4A), restrained cell invasion by 50% (Figure 4B) and proliferation by 36.1% (Figure 4C), as well as enhanced cell apoptosis by 12.7% (Figure 4D).

**Discussion**

Phosphatidylinositol-3 kinase (PI3K)/protein kinase B (AKT/PKB) represents a widely expressed signal pathway in a variety of tissues and cells that plays a crucial role in regulating cell survival, cycle, proliferation, apoptosis, migration, and invasion. The abnormality of PI3K/AKT signaling pathway is related to the occurrence, progression, metastasis, and drug resistance of various tumors. As an important protein downstream of the PI3K/AKT pathway, mTOR protein regulates tissue and organ development, angiogenesis, and tumorigenesis. PI3K can be activated by the growth factor, mitogen, and other factors for conformational change, thus promoting the conversion of phosphatidylinositol (4,5)-bisphosphate (PIP2) to phosphatidyl (3, 4, 5)-trisphosphate (PIP3), which phosphorylates AKT in the effect of 3-phosphoinositide kinase 1 Protein kinase-1 (PDK1) and PDK2. Phosphorylated AKT further phosphorylates the critical effector mTOR in PI3K/AKT/mTOR signaling pathway. Phosphorylation-activated mTOR regulates the transcription and expression of a variety of target genes under the combined action...
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compared with that in control in a TNM stage dependent way. Also, we found that the in vitro levels of mTOR, p-mTOR, and Survivin were apparently increased, whereas miR-182 was significantly reduced in renal carcinoma A498 cells compared with that in HK-2 cells. Papadopoulos et al\textsuperscript{25} showed that the expression of miR-182 was significantly increased in Caki-1 cells. Wang et al\textsuperscript{11} reported that the expression of miR-182 was significantly reduced in the tumor tissue and peripheral blood of renal cell carcinoma patients. Wilflingseder et al\textsuperscript{26} found that compared with normal renal tissue, the expression of miR-182 in tumor tissue of renal cell carcinoma patients was markedly declined. Moreover, the methylation of promoter region in miR-182 gene from 780-O and Caki-1 cells was also markedly increased compared with that in normal renal cells, suggesting that miR-182 expression reduction was related to the pathogenesis of renal carcinoma. In this work, miR-182 expression was abnormally reduced in renal carcinoma and renal cancer cells, which was consistent with the findings from Wang et al\textsuperscript{11} and Wilflingseder et al\textsuperscript{26}. Elfiky et al\textsuperscript{27} demonstrated that the expression of mTOR protein in tumor tissue of patients with renal carcinoma was significantly increased, and was associated with poor survival and prognosis, which was in agreement with our results that mTOR protein expression was apparently lower in renal carcinoma tissue than in benign lesions.

Further analysis exhibited that miR-182 mimic or si-mTOR transfection significantly reduced mTOR expression, mTOR phosphorylation, and Survivin level, along with the inhibition of cell invasion and proliferation, suggesting that miR-182 regulated renal carcinoma cell proliferation, invasion, and apoptosis through targeting mTOR. Wang et al\textsuperscript{11} observed that miR-182 suppressed renal carcinoma Caki-1 cell viability, migration, invasion, and colony formation by specific inhibition of IGF1R. Wilflingseder et al\textsuperscript{26} revealed that miR-182 attenuated the proliferation and colony formation of renal carcinoma 780-O and Caki-1 cells, arrested cell cycle in G1 phase, and weakened cancer cell growth and tumorigenic ability in nude mice by targeted suppression of FLOT1 gene expression. This study also observed that miR-182 served as a tumor suppressor role in renal carcinoma, which was in accordance with previous reports. Fang et al\textsuperscript{28} showed that simvastatin treatment significantly inhibited the functional activity of mTOR and restrained the proliferation of renal carcinoma A498 and 786-O cells. Elfiky
et al. demonstrated that inhibition of mTOR by rapamycin reduced the growth of renal carcinoma cells. As evidence showed that the inhibition of mTOR suppressed human gallbladder carcinoma cell proliferation, our data demonstrated that the tumor cells can be restrained through the suppression of mTOR in the targeting effect of miR-182. However, there is a limitation; the regulatory role of miR-182 on mTOR expression and renal carcinoma was only illustrated at in vitro cell levels. Further in vivo investigation is needed to assess its tumor suppressor role in animal model of patients with renal carcinoma.

**Conclusions**

Overexpression of miR-182 weakened cell proliferation and invasion, and facilitated cell apoptosis via targeted suppression of mTOR expression.

**Conflict of Interest**

The Authors declare that they have no conflict of interest.

**References**


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