European Review for Medical and Pharmacological Sciences 2018; 22: 351-357

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.

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 the benign tissue, which was associated with TNM stage. miR-182 expression was markedly decreased 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: Decreasing level of miR-182 plays a role in enhancing mTOR expression and promotes 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.

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Introduction

Renal carcinoma is a sort of common urinary malignancy in urologic area. It most commonly occurs in the adult kidney. 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 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 cancer[2], and was considered to be an important target in the treatment of renal cell[3-5]. 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'-UTR[6]. Based on target genes, microRNAs serve as an oncogene[7,8] or tumor suppressor[9,10] role in tumor. Multiple stud-

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ies[11,12] 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 A489 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). Si-NC and si-mTOR were got from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 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 got from Wuhan Boster Biological Technology, Ltd., (Wuhan, Hubei, China). The BrdU cell proliferation detection kit was purchased from Molecular Probes (Eugene, OR, USA). Luciferase reporter gene vector pGL3 and Dual-Glo Luciferase Assay System were provided by Promega (Madison, WI, USA). Annexin V-FITC/PI Apoptosis detection kit was provided by Yeasen Biotechnology Co., Ltd. (Shanghai, China). Transwell chamber was got from Millipore (Billerica, MA, USA).

Clinical Information

Total of renal carcinoma patients were recruited from Dongyang People's Hospital between Aug 2016 and Feb 2017 were enrolled, including 25 males and 23 females with mean age at 56.6 ± 12.4 years old. No patients received radiotherapy or chemotherapy before surgery. Renal carcinoma tumor tissues were stored at -80°C. The specimens were divided into 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 ± 13.8 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 signed informed consent before the investigation.

Cell Culture

A498 and HK-2 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

PCR products containing the full length of mTOR gene 3'-UTR or mutant segment were cloned to pGL3. Next, it was transformed to 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 H_2O. The

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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 H_2O. 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-182P_R: 5′-TGGTGTCGTGGAGTCG-3′; U6P_F: 5′-ATTGGAACGATACAGAGAAGATT-3′, U6P_R: 5′-GGAACGCTTCACGAATTTG-3′; mTORP_F: 5′-TCCGAGAGATGAGTCAAGAGG-3′, mTORP_R: 5′-CACCTTCCACTCCTATGAGGC-3′; Survivin-P_F: 5′-AGGACCACCGCATCTCTACAT-3′, SurvivinP_R: 5′-AAGTCTGGCTCGTTCTCAGTG-3′; β-actinP_F: 5′-GAACCCTAAGGCCAAC-3′, β-actinP_R: 5′-TGTCACGCACGATTTCC-3′.

Western Blot

Total protein was extracted by radioimmunoprecipitation assay (RIPA) from cells. A[illegible] of 50 μg protein was separated by 10% [illegible] dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to membra[illegible]. Next, the membrane was blocked by 5% skim milk at room temperature for 60 min [illegible] incubated in primary antibody at 4°C ov[illegible]TOR, p-mTOR, Survivin, and β-actin [illegible]500[illegible]1000, 1:3000, and 1:15000, respec[illegible]y). T[illegible] membrane was incubated i[illegible]jugated secondary antibody (1:300[illegible]0) fo[illegible] after washed by phosphate-bu[illegible]d saline [illegible]n 20 (PBST) for three tim[illegible]t last, the prot[illegible] expression was detecte[illegible]nced chemiluminescence (ECL).

Cell Apoptosis [illegible]tection

The cells w[illegible]igest[illegible]d by trypsin and resuspended in bi[illegible]g bu[illegible] after centrifugation at 300 g for 5[illegible] Ne[illegible]he ce[illegible]were incubated in 10 μl Anne[illegible]C an[illegible]l PI. At last, the cells were tested [illegible] Coulter FC 500 MCL f[illegible]metry ([illegible]han Coulter, Fullerton, [illegible] to evaluate cell apoptosis.

F[illegible]ection of Cell Pro[illegible]

Cell p[illegible]ion was assessed by Click-iT EdU Alexa F[illegible]88 Flow Cytometry Assay kit. The cells were added with 10 μM EdU solution for 2 h. After incubated for 4[illegible] were digested and collected. Aft[illegible]ashing, [illegible]on, and penetration, the cells [illegible]e incubated [illegible]e-action liquid tagged by A[illegible]Fluor 488 a[illegible]m temperature avoid of light [illegible]in. Th[illegible]the cells were washed and [illegible]sted by [illegible]ulter FC 500 MCL flow c[illegible]metry (Bec[illegible]oulter, Fullerton, CA, US[illegible]

Transwell As[illegible]

Matrigel w[illegible]s ad[illegible]up[illegible] chamber and incubated a[illegible]C for 3[illegible]μl RPMI-1640 medium c[illegible]ined 10% FB[illegible]ere added to the 24-well [illegible]n the transwell chamber paved 100 μl [illegible]rig[illegible]t onto the plate, added with A498 cells and re[illegible]d in 200 μl serum-free me[illegible]. After 48 h, t[illegible]membrane was fixed in m[illegible]anol and stained by 0.1% crystal violet. At [illegible] the membra[illegible]e was observed under the microscope.

S[illegible]ysis

A[illegible]yses were performed on SPSS 18.0 software (Chicago, IL, USA). The measurement [illegible]were depicted as mean ± standard deviation [illegible]red by *t*-test. $p < 0.05$ was considered [illegible]tatistical 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

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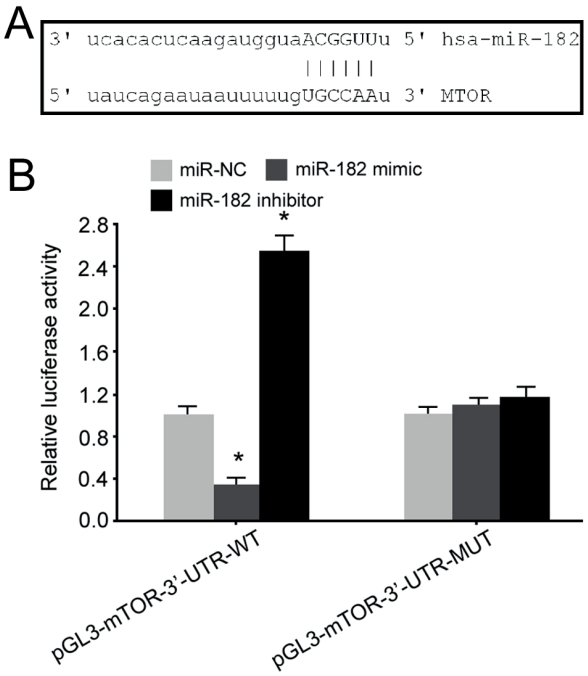

Figure 1. MiR-182 inhibited mTOR expression. ***(A)*** The binding site between miR-182 and the 3'-UTR of mTOR mRNA; ***(B)*** Dual luciferase assay. * $p < 0.05$, compare[d] with mimic NC.

staging dependent manner (Figure 2A). M[o]reover, Western blot revealed that mTOR protei[n] level was markedly increased in re[nal] carcinoma tissue compared with that in cont[rol] (Figure 2B).

MiR-182 Decreased, Whi[le] mTOR Enhanced in Renal Carci[noma]

qRT-PCR demonstrated that [the] mTOR and Survivin mRNA we[re] apparently [up]regulated, whereas the expressio[n] of miR-182 was [sig]nificantly reduced in r[enal] carcinoma A498 cells compared with tha[t in] HK-2 cells (Figure 3A). Western blot validated that th[e] expressions of mTOR, p-mTOR and Survivin prot[eins] were upregulated in A[498] cells compared with HK-2 cells (Figure 3B).

Overexpres[sion] of miR-[182] Attenuated A498 Cell Proli[feration] and Invasion, and Prom[oted] Cell Apo[ptosis]

T[he] treatment of miR-182 mimic or si-mTOR tra[nsfe]ction [both] downregulated mTOR e[xpre]ssion and [inhibited] mTOR phosphorylation and [protein] level (Figure 4A), restrained cell invasion [ability] (Figure 4B) and proliferation by 36.1% (Figure [3C]), as well as enhanced cell apoptosis by 12.7% (Figure 4D).

Discussion

Phosphatidylinositol-3 [kin]ase (PI3K)/protein kinase B (AKT/PKB) [sig]nals a widely expressed signal pathway [in a] variety of tissues and cells that plays a crucial [role] in regulating cell survival, cycle [pro]liferation, [apo]ptosis, migration, and invasion. The [abn]ormality of PI3K/AKT signaling pathway is related to the occurrence, progression, [met]astasis, and drug resistance of various [tum]ors[15]. As an important protein downstream of the PI3K/AKT pathway, mTOR protein regulates the [cell] and organ development, angiogenesis, and tumorigenesis. PI3K can be activated by the growth factor, mitogen, and other factors [via] 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 protein kinase (PDK1) and PDK2[16,17]. Phosphorylated AKT further phosphorylates the critical protein mTOR in PI3K/AKT/mTOR signaling pathway[18]. Phosphorylation-activated mTOR regulates the transcription and expression of a series target genes under the combined action

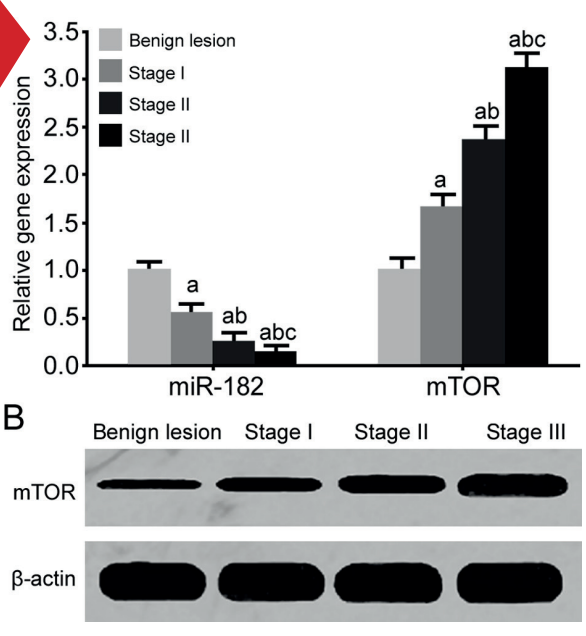

Figure 2. MiR-182 and mTOR expressions in renal carcinoma tissue. ***(A)*** qRT-PCR detection of miR-182 and mTOR mRNA expressions in renal tissue; ***(B)*** Western blot detection of mTOR protein expression in renal tissue. [a]$p < 0.05$, compared with benign renal lesions. [b]$p < 0.05$, compared with stage I. [c]$p < 0.05$, compared with stage II.

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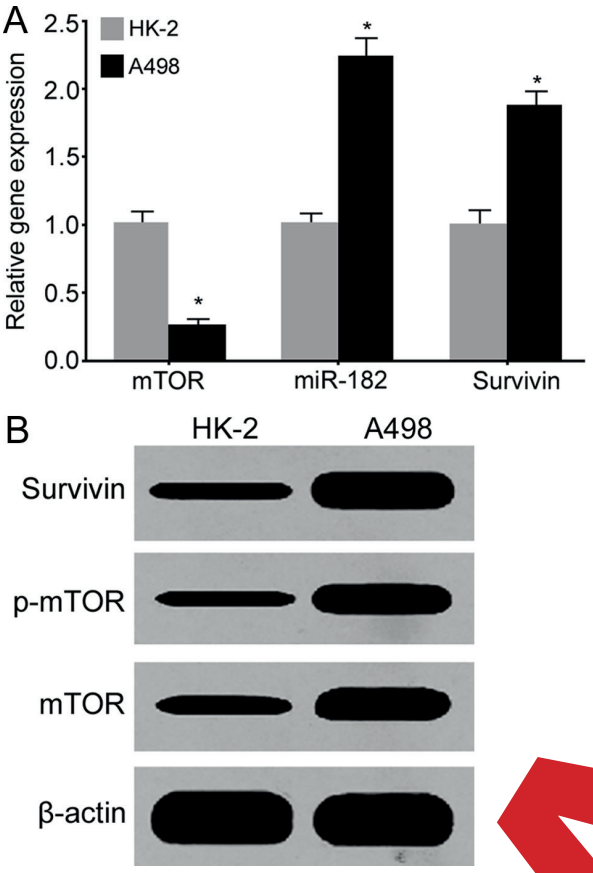

Figure 3. MiR-182 decreased, while mTOR enhanced renal carcinoma cells. ***(A)*** qRT-PCR detection of miR-182, mTOR, and Survivin mRNA expressions; ***(B)*** Western blot detection of mTOR, p-mTOR, and Survivin protein expressions. $^{*}p < 0.05$, compared with HK-2 cells.

of related factors, such as eukaryotic translation initiation factor 1 (4E-BP1)[21]. The overexpression of mTOR plays an important role in multiple tumors, including gastric cancer[22], liver cancer[23], and breast cancer[24]. It is the activation of PI3K/AKT/mTOR signaling pathway. Additionally, it was found that miR-182 expression also impacts the pathogenesis of renal cancer[2]. As some studies revealed that miR-182 expression was significantly decreased in cancer tissue and peripheral blood samples of renal carcinoma patients, this study, therefore, investigated the possible effect of miR-182/mTOR expression and renal carcinoma cell proliferation, invasion, and apoptosis.

Luciferase assay along with the *in silico* prediction analysis revealed regulatory relationship between miR-182 and mTOR mRNA. Our data on mRNA and protein levels also indicated that mTOR was significantly elevated, while miR-182 was downregulated in renal carcinoma tissue compared with that in control tissue. Stage dependent way. Also, we found that the *in vitro* levels of mTOR, p-mTOR and Survivin were apparently increased, while miR-182 was significantly reduced in renal cancer A498 cells compared with that in HK-2 cells. Boulos et al[25] showed that the expression of miR-182 was significantly increased in Caki-1 cells. Wang et al[11] reported that the expression of miR-182 was significantly reduced in tumor tissue and peripheral blood of renal carcinoma patients. Wilflingseder et al[26] found that compared with normal renal tissue, the expression of miR-182 in tumor tissue of renal cell carcinoma patients was markedly decreased. 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 cancer tissues and renal cancer cells, which was consistent with the findings from Wang et al[11] and Wilflingseder et al[26]. Elfiky et al[27] demonstrated that the expression of mTOR protein in tumor tissues 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[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[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[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

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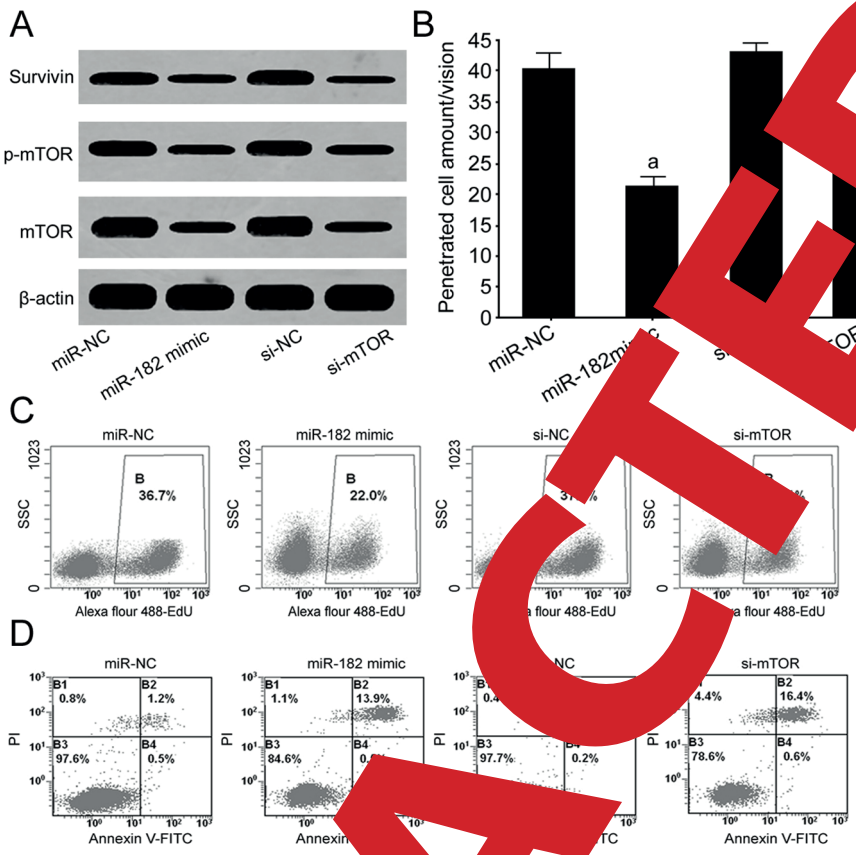

Figure 4. miR-182 overexpression attenuated A498 ce… …f… …n and invasion, and promoted cell apoptosis. ***(A)*** Western blot detection of protein expression. ***(B)*** Transwell assay … of cell invasion; ***(C)*** EdU staining detection of cell proliferation; ***(D)*** Flow cytometry detection of cell apoptosis. [a]p … compared with miR-NC. [b]$p < 0.05$, compared with si-NC.

et al[27] demonstrated that inh… …TOR by rapamycin reduced the gro…th of … …inoma cells. As evidence show… …hat the … …of mTOR suppressed hum… …allbladder car… …oma cell proliferation[28], ou… …onstrated that the tumor cells can be r… …ine… …the suppression of mTOR in the targeting … …miR-182. However, there … …limitation; the re…latory role of miR-182 on … …OR e…pression and renal carcinoma was o… …illustr… …d at *in vitro* cell levels. Further *in* …ve… …ation … …eeded to assess its tumor supp… …le in … …al model of patients with renal c…

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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