miR-19 promotes development of renal fibrosis by targeting PTEN-mediated epithelial-mesenchymal transition

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Abstract: In recent years, it has been found that miRNA may play an important role in the field of gene regulation; miRNAs can participate in the regulation of various physiologic processes such as cell differentiation, proliferation, apoptosis, metabolism, and insulin secretion by regulation of target genes. The purpose of this study is to observe the relationship between the expression of miR-19 and renal fibrosis, to analyze the regulatory effect of miR-19 on renal tubular EMT, and to reveal its role and working mechanism in renal fibrosis. We found that the expression of miR-19 was significantly increased in peripheral blood of patients with renal fibrosis, in renal tissue of unilateral ureteral occlusion (UUO) mice, and in NRK-52E cells treated with TGF-β1. Overexpression of miR-19 could decrease the expression of E-cadherin and increase the expression of α-SMA and fibronectin, while inhibition of miR-19 reverses TGF-β1-induced EMT. Further studies revealed that miR-19 could inhibit its expression by binding to the 3'-UTR of PTEN. MiR-19 inhibitor or Akt inhibitor blocks phospho-Akt by TGF-β1, and Akt inhibitors block miR-19 mimic-induced EMT. In UUO mice, overexpression of miR-19 promoted the development of renal fibrosis, while inhibition of miR-19 expression produced the opposite result. These results indicate that abnormal expression of miR-19 is associated with renal fibrosis. Moreover, miR-19 activates the Akt signaling pathway by targeting PTEN, and induces EMT in renal tubular epithelial cells, thereby promoting renal fibrosis.

Keywords: Renal fibrosis, miR-19, PTEN, EMT

Introduction

Chronic Kidney Disease (CKD) has become one of the major diseases threatening the world’s public health with the aging of the global population and the change in people’s lifestyle [1, 2]. The final common pathologic outcome of CKD is renal fibrosis, which is the destruction and disappearance of renal tissue structures including glomeruli, renal tubules and interstitium, accompanied by excessive accumulation of extracellular matrix (ECM) [3, 4]. Many kidney cells, such as mesangial cells, epithelial cells, and interstitial fibroblasts are involved in this process. Many kidney cells, such as mesangial cells, epithelial cells, and mesenchymal fibroblasts, are involved in this process. Many kidney cells, such as mesangial cells, epithelial cells, and mesenchymal fibroblasts, are involved in this process [7]. Recent studies have confirmed that renal tissue fibrosis characterized by tubulointerstitial fibrosis is the key factor leading to progressive renal failure [8]. Epithelial-mesenchymal transition (EMT) is the key pathogenic mechanism of renal interstitial fibrosis. In recent years, studies have confirmed that EMT is a very important source of mesenchymal myofibroblasts. Renal tubule EMT is a phenotype in which renal tubular epithelial cells lose their epithelial cells and acquire the biologic characteristics of mesenchymal cells (such as myofibroblasts) [9]. The mechanism of tubulointerstitial fibrosis is very complicated. At present, a large number of studies have found that myofibroblasts, which are the main source of extracellular matrix, are a key factor in determining tubulointerstitial fibrosis.
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fibrosis [10]. Therefore, clarifying the origin and biologic characteristics of interstitial myofibroblasts, has attracted the attention of many scholars to explore the mechanism of regulating the function of myofibroblasts, especially the factors that promote the formation and aggregation of myofibroblasts. The regulatory mechanism of renal tubular EMT is still not very clear.

In recent years, miRNA has been a hotspot in medical biology research. As a non-coding small RNA participating in gene transcription and expression regulation, miRNAs are inseparable from the occurrence and development of many life activities and diseases [11, 12]. A large number of studies have shown that miRNA plays an important regulatory role in cell differentiation, proliferation, apoptosis, metabolism, hematopoiesis, cardiac development, morphogenesis, and insulin secretion [13-17], and miRNAs also play an important role in the feedback loop of signal transduction pathways [18-21]. The study of the role of miRNAs in renal system diseases has only just begun. In this study, we found that miR-19 negatively regulates the target gene PTEN level and activates the PKBAkt signaling pathway to induce EMT to affect renal fibrosis, and we observed the effect of miR-19 on renal fibrosis in unilateral ureteral occlusion (UUO) mice, and provide new therapeutic directions and research targets for the diagnosis and treatment of renal fibrosis.

Materials and methods

Peripheral blood collection

We collected 67 patients with renal fibrosis admitted to our hospital and selected 30 healthy volunteers from the same period. All subjects received about 3 ml of peripheral venous blood collection in the early morning following fasting. Blood was placed in an anticoagulation tube containing EDTA, and quickly mixed up and down. All of the above were approved by the ethics committee of the hospital and signed by the family members. The operation is in line with the ethical norms of clinical trials.

Cell culture and transfection

NRK-52E cells were maintained in a DMEM medium supplemented with 10% fetal bovine serum, 100 µg/mL penicillin, 100 µg/mL streptomycin (Thermo Fisher Scientific, Inc.) in an incubator with 5% CO₂ at 37°C. 24 hours before transfection, the cells were plated in 6-well plates at about 5×10⁵ cells/well. When the growth and fusion degree of the cells in 6-well plates reached 60%, NC-mimic, miR-19 mimic, NC-inhibitor or miR-19 inhibitor were transfected into NRK-52E cells according to the instructions of Lipofectamine™ 2000 reagent.

qRT-PCR

Total RNA was extracted from cell and tissue samples using Trizol reagent and reversed into cDNA according to the instructions of the reverse transcription kit. PCR reaction was performed according to qRT-PCR kit instructions. The expression of miR-19 was U6 as the internal reference, the expression of PTEN was GAPDH as the internal reference, and the result of qRT-PCR was obtained as the 2⁻ΔΔCT value. Primer sequences: miR-19-F: 5'-GCA-GTCCCTGTAGGTTTGC-3'; miR-19-R: 5'-GCA-GGGCAACCATCGAGTCTTTT-3'; U6-F: 5'-CTCCTTGTCAGGAAATTTGCGTGTC-3'; U6-R: 5'-ACGCTTCACAGGTTCAATTGTC-3'; PTEN-F: 5'-TGGATTCGAGTCACTCAATGAATTTGCGTGTC-3'; PTEN-R: 5'-TGGATTCGAGTCACTCAATGAATTTGCGTGTC-3'; GAPDH-F: 5'-TGGATTCGAGTCACTCAATGAATTTGCGTGTC-3'; GAPDH-R: 5'-ACACCATGTATCCTGGGTCATAT-3'.

Western blot

Cell or tissue protein was extracted using RIPA Lysis Buffer and the protein concentration was determined in accordance with the BCA Protein Assays (Thermo Fisher Scientific, Inc.). The proteins (50 µg/well) were separated using 12% SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. The membranes were blocked with 5% non-fat dry milk powder for 1 h at 37°C. Primary antibodies: PTEN antibody (1:500), α-SMA antibody (1:500), E-cadherin antibody (1:500), FN antibody (1:500), Akt Antibody (1:500) and internal reference GAPDH (1:1000) were added and incubated overnight at 4°C. Horseradish peroxidase-labeled goat anti-rabbit secondary antibody (1:1000) was added and incubated at room temperature for 1 h. An ECL chemiluminescence detection kit was used to visualize the protein bands.
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Dual-luciferase reporter assay

TargetScan (http://www.targetscan.org) was used to predict the presence of a 3'-UTR region of PTEN to form complementary binding to miR-19. PTEN wild type 3'-UTR luciferase reporter gene plasmid pMIR-PTEN-wt, and mutant reporter gene plasmid pMIR-PTEN-Mut were constructed. NC-mimic, miR-19 mimic, NC- inhibitor, miR-19 inhibitor and pMIR-PTEN-wt, and pMIR-PTEN-Mut were co-transfected into NRK-52E cells. After 24 h of transfection, luciferase activity was detected using a dual luciferase assay kit.

UUO mouse model was established

Twenty-eight C57 male mice, aged about 7 weeks old, weighing about 17-21 g, were housed at 20-23°C, 40-75% humidity, and 12 h light/dark cycle conditions, free access to water and food. All mice were randomly divided into Sham group (n=4), UUO group (n=16), UUO plus miR-19 agomir group (n=4) and UUO plus miR-19 antagonir group (n=4). UUO mice were anesthetized with intraperitoneal injection of 1% sodium pentobarbital (30 mg/kg). The skin, muscle and peritoneal tissues of the abdominal wall were incised in the lower abdomen under sterile conditions. The left ureter was exposed and separated. The ureter was ligated with 4-0 sutures; the 3-0 sutures were sutured to suture the layers to close the abdominal cavity and suture the skin continuously. In Sham group, only the left ureter was bluntly separated without ligation. The other methods were the same as UUO group. Mice in UUO plus miR-19 agomir group were given intravenous injection of 40 mg/kg of miR-19 agomir every three days after model establishment. Mice in UUO plus miR-19 antagonir group were given intravenous injection of 40 mg/kg of microRNA-19 antagonir every three days after model establishment. The UUO group was sacrificed by cervical dislocation on the 1st, 3rd, 7th and 14th day after surgery. The other mice were sacrificed by cervical dislocation on the 14th day after operation. The kidney tissue (the sham-operated group left the left kidney) was taken and placed in liquid nitrogen for quick freezing, and then transferred to -80°C for storage.

Masson staining

The paraffin section of the kidney tissue was dewaxed to water and stained with hematoxylin stain for 10 min. After fully washing, the cells were differentiated with hydrochloric acid and alcohol, and then washed with distilled water. We used Masson lichun red acidic compound solution for 10 min, rinsed with running water and shaked dry. Then 1% phosphomolybdate aqueous solution was applied for 5 min. After the phosphomolybdate was removed, it was directly stained with aniline blue for 5 min and rinsed with water. Then we rinsed in 95% alcohol, anhydrus alcohol, xylene transparent, and sealed with neutral gum.

Sirius red staining

The paraffin sections of kidney tissue were dewaxed and hydrated, stained with Sirius red staining solution for 10 minutes, slightly rinsed, stained with Mayer hematoxylin staining solution for 8 minutes, and rinsed with running water for 10 minutes. Then we rinsed in 95% alcohol, anhydrus alcohol, xylene transparent, and sealed with neutral gum.

Statistical analysis

SPSS Statistics 22.0 statistical software was used for statistical analysis. Data are presented as mean ± standard deviation. A Student’s t-test was used to compare the mean values between the 2 groups; one-way ANOVA (Bonferroni post hoc test) was used to compare the mean among ≥3 groups. P<0.05 was considered asignificant difference.

Results

Expression of miR-19 in kidney tissue of UUO mice and patients peripheral blood

Masson staining results of UUO mice kidney tissue showed that compared with a Sham group, UUO mice had a large number of blue collagen fibers interwoven with each other and deposit ed in the renal interstitium. The fibrosis process increased with the prolongation of UUO operation time. In addition, SiriusRed staining results were consistent with Masson staining (Figure 1A), indicating that the UUO mouse model was successfully constructed. The results of qRT-PCR showed that the expression of miR-19 in
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Peripheral blood of patients with renal fibrosis was significantly higher than that of the control group (Figure 1B). Moreover, the expression of miR-19 in kidney tissue of UUO mice was significantly higher than that of Sham mice, and increased with the time after UUO operation (Figure 1C). These results suggest that there may be a correlation between changes in miR-19 expression levels and renal fibrosis. The results of western blot showed that the expression of E-cadherin protein in the kidney tissue of mice in the UUO group was significantly reduced and the expression of α-SMA protein was significantly increased from the third day after surgery (Figure 1D).

Up-regulation of miR-19 expression induces EMT in NRK-52E cells

It has been reported that TGF-β1 is the strongest fibrotic cytokine currently known, which can induce renal tubular epithelial cells to develop EMT [22]. To investigate the role of miR-19 in renal tubular EMT, TGF-β1 was used
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Figure 2. Up-regulation of miR-19 expression induces EMT in NRK-52E cells. A. The expression of miR-19 was detected by qRT-PCR. B. Protein expression of E-cadherin and α-SMA were detected by western blot. C. Protein expression of FN was detected by western blot. *P<0.05, **P<0.01.

Subsequently, we investigated whether miR-19 mimic has an effect on the production of fibronectin, and the results showed that miR-19 mimic can increase the expression of FN, and the effect was dose-dependent (Figure 2C).

**Down-regulation of miR-19 expression blocks EMT in NRK-52E cells induced by TGF-β1**

We further transfected miR-19 inhibitor into NRK-52E cells and stimulated the cells with TGF-β1 to study the role of miR-19 in renal tubular EMT. Western blot analysis showed that TGF-β1 could decrease the expression of E-cadherin and increase the expression of α-SMA, but transfection of miR-19 inhibitor could reverse the effect of TGF-β1 alone (Figure...
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In addition, down-regulation of miR-19 inhibited TGF-β1-induced FN protein expression, and the inhibitory effect of miR-19 inhibitor was enhanced with increasing transfection dose (Figure 3B).

miR-19 inhibits the expression of PTEN by binding to its 3’-UTR

TargetScan was used to predict potential target genes of miR-19, and the results showed that there may be a complementary pairing with miR-19 in the 3’-UTR region of PTEN (Figure 4A). The prediction was verified by dual luciferase reporter gene assay, and the results showed that luciferase activity was significantly decreased when NRK-52E cells were co-transfected with miR-19 mimic and pMIR-PTEN-wt, while luciferase activity was significantly increased when co-transfected with miR-19 inhibitor and pMIR-PTEN-wt. However, miR-19 mimic or miR-19 inhibitor had no significant effect on luciferase activity of pMIR-PTEN-Mut (Figure 4B). Western blot and qRT-PCR were used to detect the effect of miR-19 on the expression of PTEN. The results showed that the expression of PTEN mRNA and protein was significantly decreased after transfection of miR-19 mimic, and the inhibitory effect was dose-dependent (Figure 4C, 4D). The above results indicate that miR-19 may inhibit the expression of PTEN by binding to the 3’-UTR of PTEN.

Down-regulation of the effect of miR-19 on PTEN expression

Western blot results showed that after TGF-β1 stimulation of NRK-52E cells, the expression of PTEN protein was significantly lower than that of the control group, while the pre-transfection of miR-19 inhibitor could reverse the inhibitory effect of TGF-β1 on PTEN expression, and with the transfection dose, there was increased expression of PTEN protein (Figure 5A). At the same time, qRT-PCR results also showed that transfection of miR-19 inhibitor significantly up-regulated PTEN mRNA expression (Figure 5B).
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After transfection of NC-inhibitor or miR-19 inhibitor, NRK-52E cells were stimulated with TGF-β1, then the protein expression of phosphorylated Akt and total Akt were detected by western blot. The results showed that the cells transfected with NC-inhibitor had increased phosphorylation of Akt after 15 min of TGF-β1

Figure 4. miR-19 inhibits the expression of PTEN by binding to its 3’-UTR. A. TargetScan predicted potential target genes for miR-19. B. Luciferase activity detected by dual luciferase reporter gene assay. C. The expression level of PTEN protein was detected by western blot. D. The mRNA expression of PTEN was detected by qRT-PCR. *P<0.05, **P<0.01.

miR-19 regulates EMT by activating Akt signaling
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To verify whether miR-19 regulates EMT via PTEN/Akt, specific inhibitors of the PI3K/Akt signaling pathway (Wortmannin and LY294-002) were used to pretreat NRK-52E cells for 30 min and then stimulated with TGF-β1 for 30 min, and we found that Akt inhibitors antagonize Akt phosphorylation (Figure 6A). After that, we prolonged the treatment time of TGF-β1 to 48 h; then western blot showed that Akt inhibitor could partially antagonize the increased expression of FN, α-SMA protein, and the decreased expression of E-cadherin protein caused by TGF-β1 (Figure 6B). Further, the inhibitor of Akt was used to treat NRK-52E cells for 30 min and then they were transfected with miR-19 mimic for 48 hours. Western blot analysis showed that Akt inhibitors can reverse miR-19 mimic-induced EMT (Figure 6D).

We further investigated the role of miR-19 in renal fibrosis through the UUO mouse model. Masson staining showed that after miR-19 agomir was injected into the tail vein of UUO mice, collagen deposition increased and the degree of fibrosis worsened. Conversely, injection of miR-19 antagonir slowed the progression of fibrosis in UUO mice and the pathologic changes in the interstitial were alleviated. At the same time, SiriusRed staining results also showed that miR-19 can promote renal fibrosis in UUO mice (Figure 7).

Discussion

MiRNAs are endogenous single-stranded RNA molecules with a length of 19-24 nucleotides, and are the largest family of eukaryotic non-coding regulatory RNA genes ever discovered [23-25]. The miRNAs negatively regulate the expression of the gene after transcription by complementary binding to the target gene [26-28]. Studies have reported that miR-200a can inhibit the development of renal fibrosis by targeting TGF-β2 [29]; miR-29b has anti-fibrotic function by regulating the expression of collagen genes such as COL1A1, COL3A1 and COL4A1 in mouse kidney medullary epithelial cells [30]. Some scholars have detected increased expression of miR-21 in kidney tissue of patients with renal fibrosis [31]. Although a large number of studies have shown that miRNAs may play an important role in renal fibrosis, the relationship between miR-19 and renal fibrosis is not yet clear.

Figure 5. Down-regulation of the effect of miR-19 on PTEN expression. A. The expression of PTEN protein was detected by western blot. B. The mRNA expression of PTEN was detected by qRT-PCR. *P<0.05, **P<0.01.
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fibrosis has rarely been reported. In this study, it was found that the expression of miR-19 in the peripheral blood of patients with renal fibrosis was significantly higher than that of the control group, and the expression of miR-19 in the kidney tissues of UUO mice was also significantly increased, which showed an increasing trend with the increase of fibrosis degree. It suggests that there may be a correlation between changes in the expression of miR-19 and renal fibrosis.

The concept of EMT was proposed by Greenburg and Hay. They found that lens epithelial cells could form pseudopods in collagen gel and transformed into mesenchymal cells. Subsequently, EMT has been reported in the formation of proglott embryo, neural crest cell migration, neural tube, heart valve, craniofacial structure, and skeletal muscle system in many species [32-35]. This study found that there is a high level of epithelial cell marker protein E-cadherin in the kidney tissue of Sham mice, but α-SMA protein was not detected. However, the expression of EMT was significantly decreased but the expression of α-SMA was significantly increased in UUO mice from 3 d postoperatively. It suggests that EMT in renal tubular epithelial cells is closely related to the development of renal fibrosis. It has been reported that TGF-β1 is the strongest fibrotic cytokine currently known, which can induce renal tubular epithelial cells to develop EMT [36]. We stimulated NRK-52E cells with TGF-β1, and found that TGF-β1 can induce increased expression of miR-19. We further observed the effect of changing the expression of miR-19 on EMT of NRK-52E cells, and the results showed that miR-19 mimic can decrease the expression of E-cadherin and induce the expression of α-SMA and FN. But transfection of miR-19 inhibitor produced the opposite effect, reversing the effect of TGF-β1 alone on the NRK-52E cells. These results indicated that miR-19 may promote EMT in renal tubular epithelial cells.

Figure 6. miR-19 regulates EMT by activating Akt signaling. A. Protein expression levels of phosphorylated Akt and total Akt in NRK-52E cells transfected with NC-inhibitor or miR-19 inhibitor were detected by western blot. B. The protein expression of phosphorylated Akt and total Akt in NRK-52E cells pretreated with wortmannin or LY294002 were detected by western blot. C. Western blot was used to detect the protein expression of FN, α-SMA and E-cadherin in Akt inhibitor and TGF-β1 treated NRK-52E cells. D. Western blot was used to detect the protein expression of FN, α-SMA and E-cadherin in NRK-52E cells treated with Akt inhibitor and miR-19 mimic. *P<0.05, **P<0.01.
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PTEN gene, located at 10q23, is the first tumor suppressor gene with phosphatase activity, which plays an important role in cell growth, signal transduction, and apoptosis [37-39]. Stambolic et al. found in vitro that PTEN exerted its role as a tumor suppressor by negatively regulating the PI3K/Akt signaling pathway [40]. White et al. found that in idiopathic pulmonary fibrosis, myofibroblasts have diminished PTEN expression. Inhibition of PTEN in vivo promotes fibrosis, and in vitro PTEN inhibits myofibroblast differentiation [41]. In this study, TargetScan was used to predict PTEN as a potential target gene for miR-19 and dual luciferase reporter gene assay demonstrated that miR-19 can specifically bind to the 3'-UTR of PTEN. We further found that up-regulation of miR-19 mimic inhibited mRNA and protein expression levels of PTEN, while the transfection of miR-19 inhibitor could reverse the inhibitory effect of TGF-β1 on PTEN, and the expression of PTEN increased with the increase of transfection dose. The above results indicate that miR-19 can negatively regulate the expression of the target gene PTEN.

Studies have shown that stimulation of TGF-β1 in mice mammary epithelial cell lines for 30 minutes can induce the phosphorylation of Akt, reduce the expression of E-cadherin and ZO-1, and change the spindle morphology of cells. The specificity of PI3K, LY-294002, a specific inhibitor of PI3K, could block the effect of TGF-β1 [42]. Kattla et al. also demonstrated that TGF-β1 can induce the activation of PI3K and PKB/Akt in NRK-52E cells, and specific inhibitors of PI3K and PKB/Akt can block the decrease of E-cadherin and increase of α-SMA caused by TGF-β1 [43]. These studies suggest that the activation of Akt signaling plays an important role in renal tubular EMT. PTEN is an upstream inhibitor of the PI3K-Akt signaling pathway, and miR-19 can target and...
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regulate PTEN; therefore, this study further investigated the relationship between miR-19 and Akt signaling pathway. We found that Akt inhibitors antagonize Akt phosphorylation by TGF-β1, and transfection of miR-19 inhibitor also leads to TGF-β1, which does not induce an increase in Akt phosphorylation. Akt inhibitors could reverse the down-regulation of E-cadherin expression and the up-regulation of α-SMA and FN expression caused by TGF-β1 or miR-19 mimic. It is suggested that miR-19 is involved in the regulation of EMT by activating Akt signaling pathway. We further investigated the role of miR-19 in renal fibrosis through the UUO mouse model. The results showed that up regulation of miR-19 could increase the degree of renal tubule expansion and fibrosis in UUO mice, while inhibition of miR-19 could alleviate the process of fibrosis. This suggested that miR-19 may promote renal fibrosis.

Conclusions

In summary, the results of this study suggest that changes in miR-19 expression levels are closely related to the development of renal fibrosis and have a profibrotic effect in UUO mice. Furthermore, it was further revealed that miR-19 activates the Akt signaling pathway by targeting PTEN, and induces EMT in renal tubular epithelial cells, thereby promoting renal fibrosis.

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Disclosure of conflict of interest

None.

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