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Expression of miR-207 in renal tissue of renal fibrosis rats and its correlation analysis with protein expression of TGF-β1 and Smad3

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Abstract. – OBJECTIVE: This study was designed to analyze the expression of miR-207 in renal tissue of renal fibrosis rats and its correlation with the protein expression of TGF-β1 and Smad3.

MATERIALS AND METHODS: Rat models with renal fibrosis were established via unilateral ureteral obstruction (UUO). Then, the expression levels of miR-207, TGF-β1 and Smad3 in renal tissue of rats were intervened by over-expression vector miR-207 mimic, miR-207 inhibitor and TGF-β/Smad3 signal SIS3 free base, and the effect and mechanism of action of miR-207 on renal fibrosis were analyzed.

RESULTS: In UUO models established in this study, the expression levels of fibrosis related factors TGF-β1, Smad3, Smad2, α-SMA, BMP-7, MMP7 and MMP9 were elevated, and staining results showed that obvious fibrosis occurred in renal tissue of rats. Moreover, we also found that the miR-207 expression increased in UUO model rats. After inhibiting miR-207 expression, their degree of renal fibrosis also reduced significantly, and the expression levels of TGF-β1, Smad3, Smad2, α-SMA, BMP-7, MMP7 and MMP9 were inhibited. Besides, miR-207 had a positive correlation with TGF-β1/Smad3 expression. We designed a group of rats, and found that while miR-207 expression was up-regulated, TGF-β1/Smad3 signals were inhibited, and compared with those with up-regulation of miR-207 expression, the severity of renal fibrosis reduced significantly, and the expression of other fibrosis indicators Smad2, α-SMA, BMP-7, MMP7 and MMP9 also reduced dramatically.

CONCLUSIONS: The miR-207 expression in renal tissue of rats with renal fibrosis increased, which was positively correlated with TGF-β1/Smad3, and miR-207 could promote the progression of renal fibrosis through TGF-β1/Smad3 signals.

Key Words:
MiR-207, Renal fibrosis, TGF-β1, Smad3.

Introduction

Chronic kidney disease (CKD) is a global public health problem, which seriously endangers human health and has a high morbidity[1]. Worldwide, its morbidity is about 10% of the general population[2]. Fibrosis is a manifestation of excessive tissue repair. Continued progression can lead to destruction of organ structure, functional decline, and even failure[3]. Renal tissue fibrosis is a marker of progressive renal disease and one of the most important causes of renal failure[4]. It is quite significant to understand the molecular mechanism of the progression of renal fibrosis and find targets for its treatment.

Short-chain non-coding RNA (miRNAs) is a kind of small-molecule RNA with a length of about 18-25 nucleotides. It combines with messenger RNA (3'-UTR) and induces RNA silencing or degradation through miRNA-induced silencing complexes. It negatively regulates the expression of protein-coding genes and participates in the regulation of various cellular processes including inflammatory reactions[5,6]. Increasing evidence[7-9] has found that miRNAs play a vital role in the progression of fibrosis, such as miR-34a, miR-23, miR-27, miR-24, miR-29 and so on. In the study of miRNAs expression profile, miR-207 expression increased in both pulmonary fibrosis and liver fibrosis[10,11]. There are few studies on the relationship between miR-207 and renal fibrosis, but there is no research on the mechanism of miR-207 in fibrosis. Transforming growth factor -β1 (TGF-β1) can activate collagen accumulation and promote tissue and organ wound repair, but it also triggers the progressive development of fibrosis[12]. It has been reported in many studies that inhibiting TGF-β1 signaling can weaken the

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progression of fibrosis[13,14]. TGF-β combines with its receptor to promote fibrosis, inducing phosphorylation of Smad2 and Smad3, which is the main fibrosis pathway[15]. Feng et al[16] found that lncRNA ERBB 4-IR promoted renal fibrosis by activating TGF-β/Smad 3 pathway.

We speculate that miR-207 also promotes renal fibrosis through TGF-β/Smad 3 pathway, and this study analyzes and provides more experimental basis for clinical search for targets in treating renal fibrosis.

Data and Methods

Objects of Research

A total of 180 healthy Sprague-Dawley (SD) rats aged 8-10 weeks, weighing 250-300 g, were purchased from the Experimental Animal Center of Harbin Medical Sciences University (Harbin, Heilongjiang, China). The drinking water was sterilized and replaced 3-4 times a week, the feeding temperature was 21-27°C, and the relative humidity was 50%±5%. All rats were separately fed in feeding boxes, and the padding was changed regularly every morning and evening; environmental noise was less than 85 decibels, ammonia concentration was not more than 20 ppm, airflow speed was 10-25 cm/s, and ventilation was 8-15 times per hour. We changed nests 1-2 times a week and cleaned and disinfected them; noise shall not exceed 60 dB, ammonia concentration shall not exceed 14 ppm, ventilation shall not be less than 15 times per hour, and fluorescent lamps shall be periodically illuminated at intervals of 12 h. All animal experiments have been approved by the Animal Protection and Use Committee of our hospital and follow the guiding principles of the Council for International Organization of Medical Sciences (CIOMS).

Establishment of Renal Fibrosis Models

Rat models of renal fibrosis were established by unilateral ureteral obstruction (UUO). Rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (350 mg/kg) (Shanghai Winguide Huangpu Pharmaceutical Co., Ltd., SFDA Approval No. H31022494, Shanghai, China). They were fixed on the operating table in the right lateral position, abdominal skin was disinfected after hair was cut off, and we adopted left abdominal incision. Afterwards, they were ligated with line 4 and ureters were cut short. Skin and tissues were sutured layer by layer. No signs of peritonitis were found in the rats during the experiment.

Grouping and Intervention of Rats

The rats were divided into a control group, sham operation group, model group, miR-207 inhibitor group, miR-207 mimic group and rescue group, with 30 rats in each group. The rats in the sham operation group only were processed by cutting the abdominal cavity and freeing their left ureter without ligation and clipping; rats in the control group did not undergo any treatment and were fed normally; those in the other 4 groups were given UUO model rats for the following interventions: rats in the miR-207 inhibitor group were injected with 10 μL miR-207 inhibitor intravenously and pretreated with liposome 2000 before injection; those in the miR-207 mimic group were given 10 μL miR-207 mimic in the same way, while those in the rescue group were given 10 μL miR-207 mimic based on the miR-207 mimic group; SIS3 free base was given 10 mg/kg by gavage, and all intervention methods lasted for 14 days; 350 mg/kg 10% chloral hydrate was injected intraperitoneally, the left kidney of rats was isolated. Rats were sacrificed by cervical dislocation. SIS3 free base is an effective and selective TGF-β1-induced inhibitor of Smad3 phosphorylation, and it was purchased from MCE, China. miR-207 inhibitor and miR-207 mimic were designed and synthesized by Thermo Fisher Scientific (Shanghai, China), so did Lipofectamine 2000 kit.

Analysis of Fibrosis in Rats

After the left kidney of the rat was separated, it was cut into two halves through the midsagittal plane, one half of which was fixed with 4% paraformaldehyde, embedded in paraffin, cut into 4 mm thick slices, and stained with Masson's trichrome stain. We referred to the instruction manual of the kit (Sigma, Shanghai, China) for the specific steps, randomly selected 5 fields of vision, calculated the ratio of fibrosis area to total area by ImageJ (National Institutes of Health, Bethesda, MD, USA), and analyzed the severity of fibrosis.

qRT-PCR

The total RNA was extracted from renal tissue by TRIzol kit (Invitrogen, Shanghai, China). The EasyScript One-Step RT-PCR SuperMix kit was purchased from TransGen Biotech, Beijing (China). We referred to the kit instructions for

the specific detection steps. The reaction system was as follows: RNA Template 1 μg, Forward GSP (10 μM) 0.4 μl, Reverse GSP (10 μM) 0.4 μl, 2*One-Step Reaction Mix 10 μl, EasyScript One-Step Enzyme Mix 0.4 μl, RNase-free Water supplemented to 20 μl; the reaction conditions were as follows: 40°C for 30 min, 94°C for 5 min, 94°C for 30 s, 60°C for 30 s, 72°C for 2 kb/min, 72°C for 10 min, a total of 40 cycles. We calculated $2^{-\Delta CT}$, set up 3 repeat holes and used U6 as internal reference. MiR-207 upstream primer: 5'-ACACTCCAGCTGGGGCTTCTC-CTGGCTCTCC-3', downstream primer: 5'-TG-GTGTCGTGGAGTCG-3'; U6 upstream primer: 5'-CTCGCTTCGGCAGCACA-3', downstream primer: 5'-AACGCTTCACGAATTTGCGT-3'.

Western Blot

The protein in renal tissue was extracted via repeated freeze-thaw method. The protein concentration was detected via bicinchoninic acid assay (BCA) method and adjusted to 4 μg/μL, and the protein was separated by 12% polyacrylamide gel electrophoresis. The initial voltage was 90 V, and then it was increased to 120 V to move the sample to the appropriate position of the separation gel. After electrophoresis was completed and membrane was transferred, it was under 100 V constant voltage for 100 min and closed 60 min at 37°C. Next, the transfer membrane was placed in 5% skim milk for sealing, and then it was under immune reaction and incubated with primary antibody (1:1000) at 4°C all night long. On the next day, it was washed three times with phosphate-buffered saline (PBS), 5 min each time; then, it was incubated 1 h with secondary antibody (1:1000) at room temperature; after that, it was developed and fixed under enhanced chemiluminescence (ECL) luminescent reagent; the strip scanned by the film was statistically analyzed by Quantity One software, and the relative protein expression level = strip gray value/internal reference gray value. BCA protein kit, ECL luminescence kit and trypsin were all purchased from Thermo Scientific™ (Art. No. 23250, 35055, 90058). Rabbit anti-mouse TGF-β1 (monoclonal antibody), Smad2 (monoclonal antibody), Smad3 (monoclonal antibody), α-SMA (monoclonal antibody), matrix metalloproteinase 7 (MMP7) (monoclonal antibody), MMP9 (monoclonal antibody) and goat anti-rabbit IgG secondary antibodies were all bought from Abcam, (Cambridge, UK, Art. No. ab179695, ab33875, ab52903, ab32575, ab205525, ab219372, ab6721).

Detection of Inflammatory Factor Levels

Renal tissue was ground into slurry and centrifuged 15 min at 1000 ×g to take the supernatant. The levels of tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), interleukin 10 (IL-10) in the supernatant were detected by ELISA. We referred to the instruction manual of the kit for the specific steps. The TNF-α, IL-6 and IL-10 ELISA detection kits were purchased from Shanghai Guduo Biotechnology Co., Ltd. (Shanghai, China, Art. No. GD-DS1716, GD-DS1726, GD-DS1731).

Statistical Analysis

SPSS 19.0 (IBM, Armonk, NY, USA) was used for statistical analysis. The measurement data were expressed by mean±SD. Students' *t*-test was used for comparison between the two groups, one-way analysis of variance (ANOVA) was used for comparison between multiple groups, and LSD test and two-tailed test were used for back testing. There were statistical differences when $p<0.05$. The pictures were drawn by Graph-Pad Prism 8.0 (La Jolla, CA, USA).

Results

Establishment of Rat Models With Renal Fibrosis

After UUO, the renal tissue of rats in the model group showed obvious fibrosis, while no fibrosis was found in the kidneys of those in the control group and the sham operation group. The expression levels of TGF-β1, Smad3, Smad2, α-SMA, MMP7 and MMP9 in renal tissue of those in the model group also increased significantly. Moreover, we found that the miR-207 expression in renal tissue of the model group also increased (Figure 1).

Effect of MiR-207 on Renal Fibrosis in Rats

We injected miR-207 inhibitor and found that the expression level of miR-207 in renal tissue of rats in the miR-207 inhibitor group was significantly lower than that in the model group. At the same time, the severity of renal fibrosis in the miR-207 inhibitor group also reduced, and the expression levels of Smad2, α-SMA, MMP7 and MMP9 decreased (Figure 2).

Effect of MiR-207 on Expression of Inflammatory Factors in Kidneys of Rats

After inhibiting the miR-207 expression, we found that the TNF-α and IL-6 levels in renal

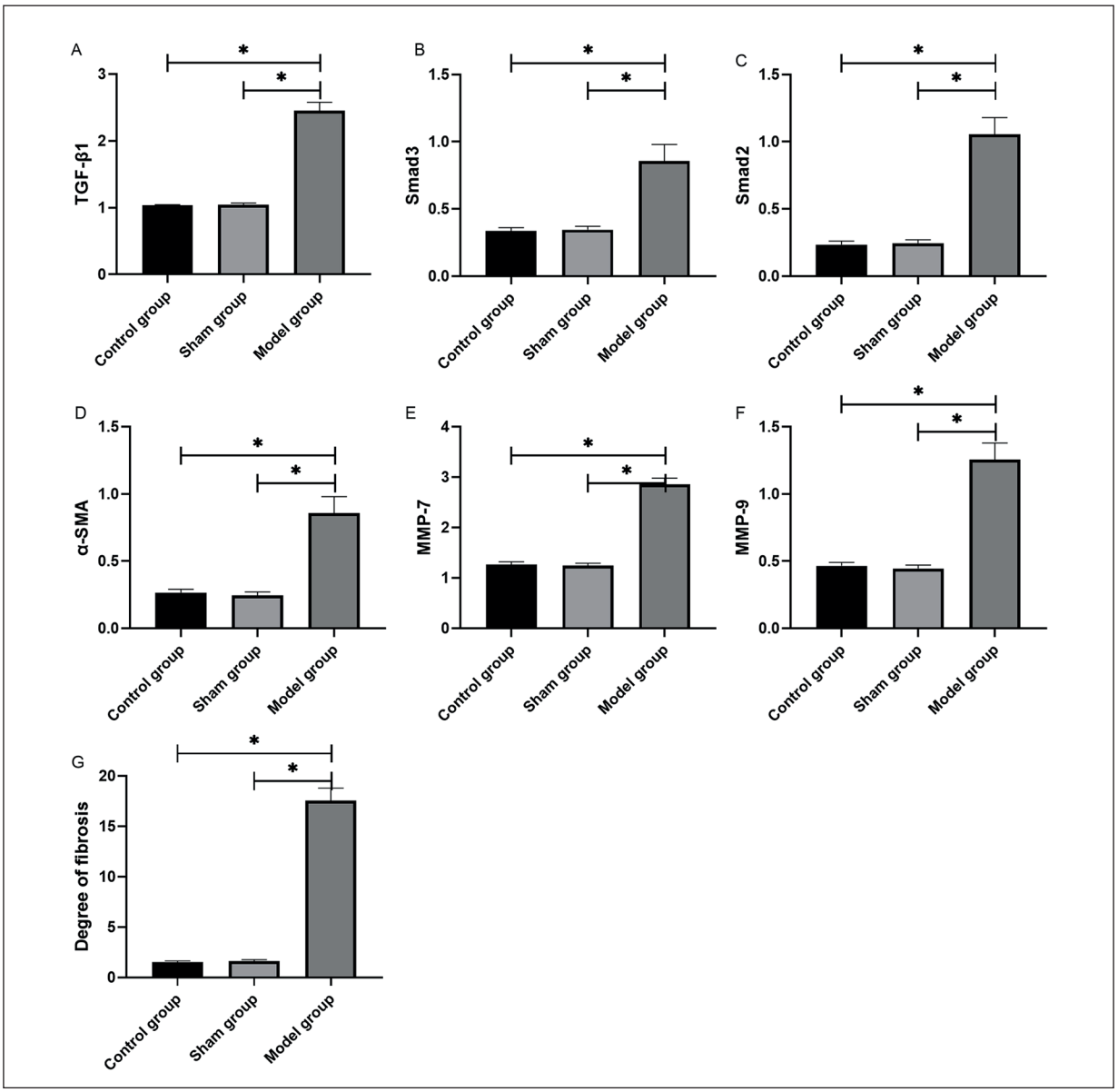

Figure 1. Establishment of rat models with renal fibrosis. **A,** Effect of UUO on TGF-β1 expression in kidneys of rats (**B**) effect of UUO on Smad3 expression in kidneys of rats (**C**) effect of UUO on Smad2 expression in kidneys of rats (**D**) effect of UUO on α-SMA expression in kidneys of rats (**E**) effect of UUO on MMP7 expression in kidneys of rats (**F**) effect of UUO on MMP9 expression in kidneys of rats (**G**), effect of UUO on fibrosis expression in kidneys of rats. *means $p<0.05$.

tissue of rats in the miR-207 inhibitor group also decreased, and the expression of anti-inflammatory factor IL-10 increased (Figure 3).

Effect of MiR-207 on TGF-β1/Smad 3 Signals

We also discovered that the TGF-β1 and Smad3 expression decreased after the miR-207 expression was inhibited. Pearson correlation analysis manifested that miR-207 was positively correlated with the expression levels of TGF-β1 and Smad 3 (Figure 4).

MiR-207 Promotes Renal Fibrosis in Rats by TGF-β1/Smad3 Signals

To verify whether miR-207 promotes renal fibrosis in rats through TGF-β1/Smad 3 signals, we injected miR-207 over-expression vector miR-207 mimic into UUO model rats, and simultaneously

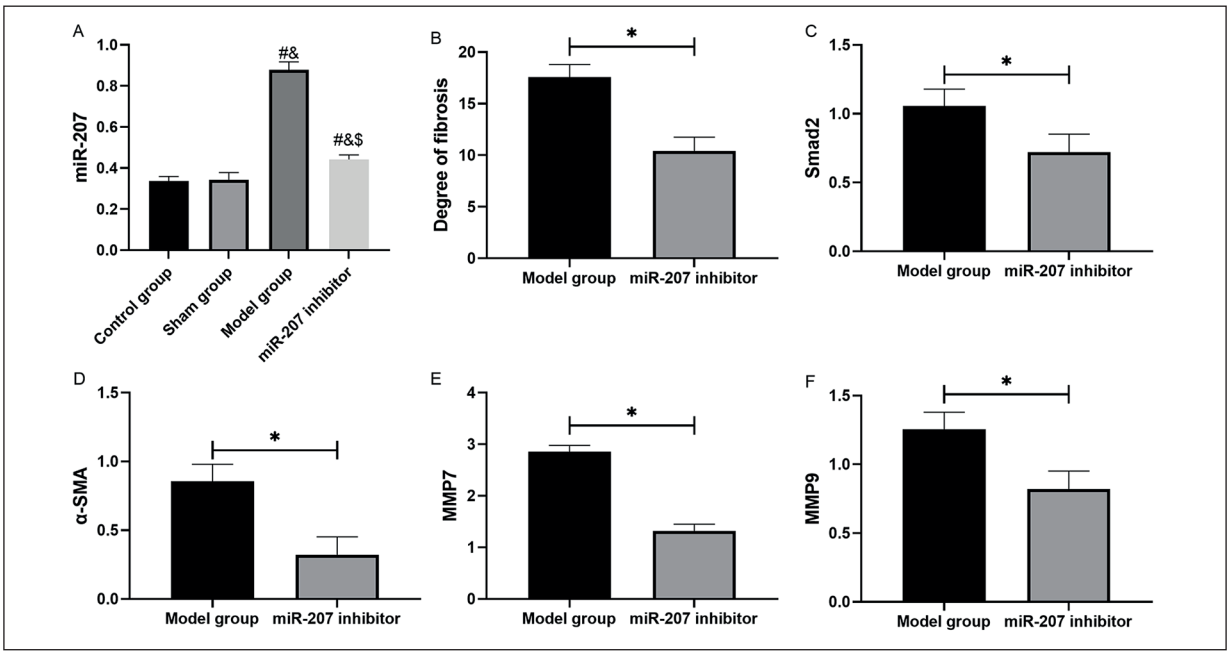

Figure 2. Effect of miR-207 on renal fibrosis in rats. **A,** Results of miR-207 inhibitor intervention (**B**) effect of miR-207 on severity of renal fibrosis in rats (**C**) effect of miR-207 on Smad2 expression in renal tissue of rats (**D**) effect of miR-207 on α-SMA expression in renal tissue of rats (**E**) effect of miR-207 on MMP7 expression in renal tissue of rats (**F**) effect of miR-207 on MMP9 expression in renal tissue of rats. *means $p<0.05$, # means the comparison with the control group ($p<0.05$), & means the comparison with the sham operation group ($p<0.05$) and $ means the comparison with the model group ($p<0.05$).

administered SIS3 free base to inhibit the activation of the signals. We found that compared with rats that only promote miR-207 expression, the severity of renal fibrosis of those in the rescue group reduced significantly, and the expression levels of Smad2, α-SMA, MMP7 and MMP9 decreased (Figure 5).

Discussion

CKD is the third leading cause of premature human death after AIDS and diabetes[17]. Renal fibrosis is the final result of CKD progression to the end stage. Understanding the mechanism of renal fibrosis is quite significant to prevent it or

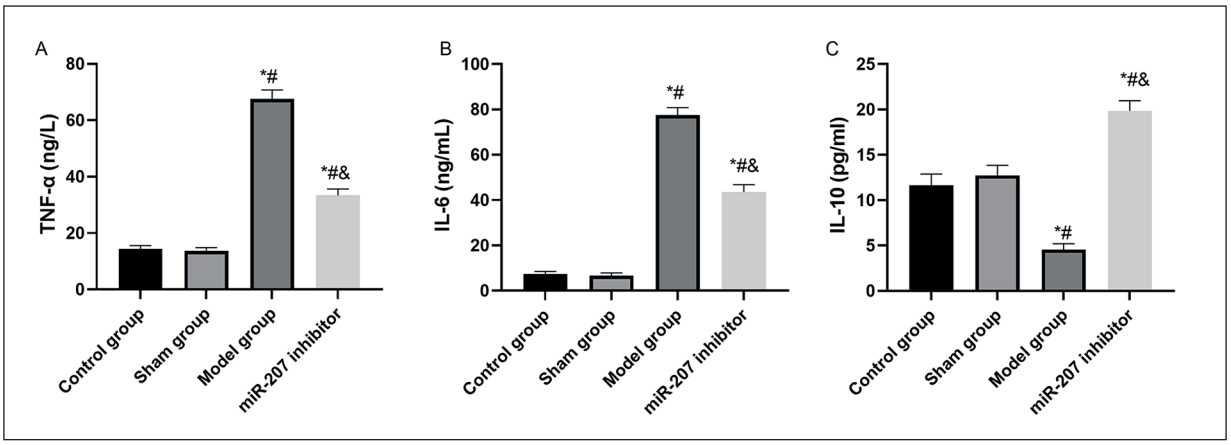

Figure 3. Effect of miR-207 on expression of inflammatory factors in kidneys of rats. **A,** Effect of miR-207 on α-SMA expression in renal tissue of rats (**B**) effect of miR-207 on IL-6 expression in renal tissue of rats (**C**) effect of miR-207 on IL-10 expression in renal tissue of rats. *means the comparison with the control group ($p<0.05$). #means the comparison with the sham operation group ($p<0.05$), and & means the comparison with the model group ($p<0.05$).

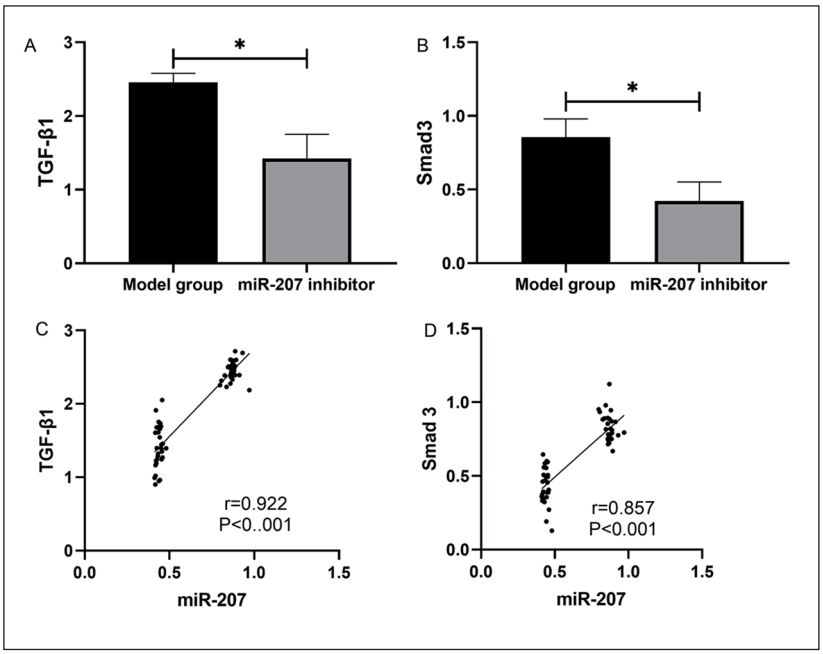

Figure 4. Effect of miR-207 on TGF-β1/Smad3 signals. **A,** Effect of miR-207 on TGF-β1 expression in renal tissue of rats (**B**) effect of miR-207 on Smad3 expression in renal tissue of rats (**C**) correlation analysis of miR-207 and TGF-β1 expression (**D**) correlation analysis of miR-207 and Smad3 expression. *means $p<0.05$.

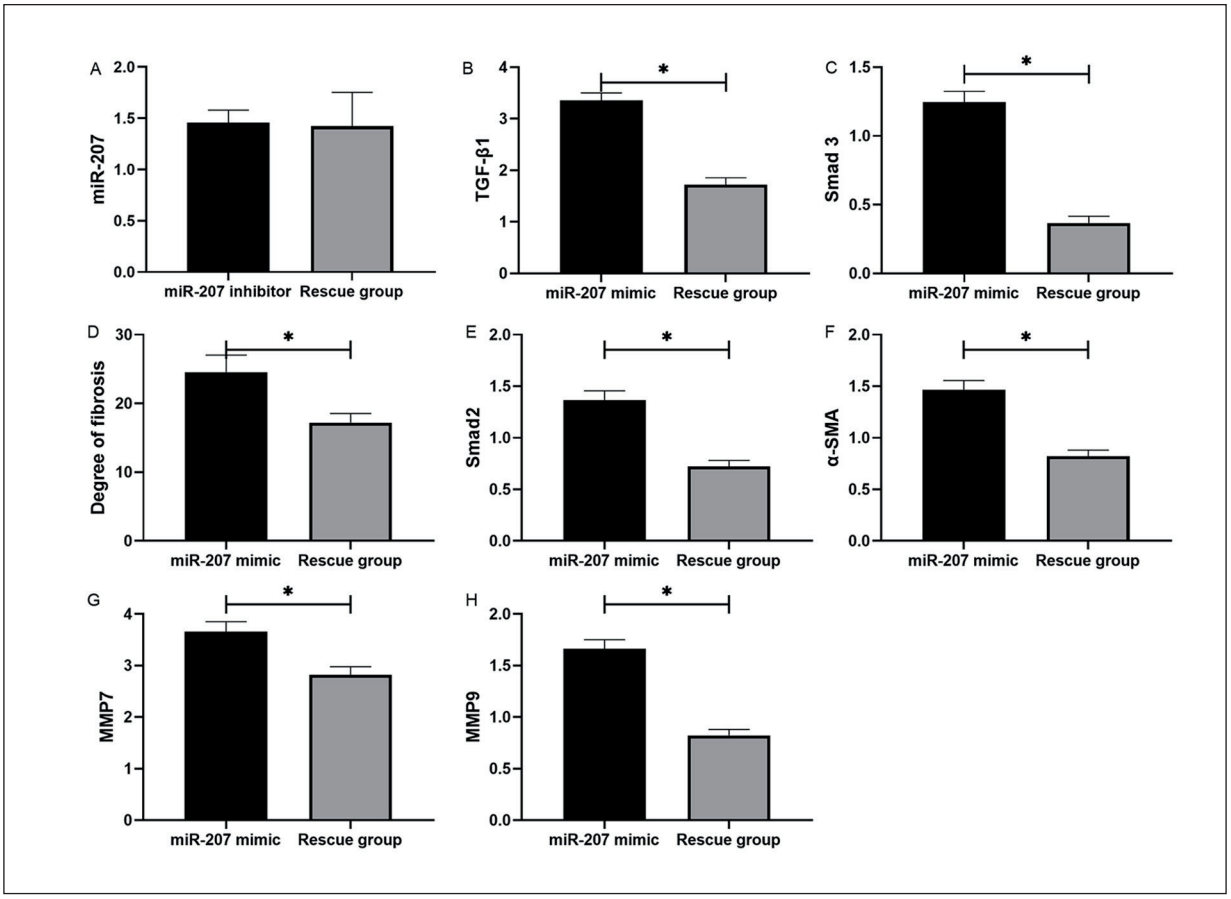

Figure 5. MiR-207 promotes renal fibrosis in rats through TGF-β1/Smad 3 signals. **A,** Analysis of miR-207 expression level (**B**) analysis of TGF-β 1 expression level (**C**) analysis of Smad3 expression level (**D**) analysis of severity of renal fibrosis in rats (**E**) analysis of Smad2 expression level F: analysis of α-SMA expression level (**G**) analysis of MMP7 expression level (**H**) analysis of MMP9 expression level. *means $p<0.05$.

slow it down in CKD patients. This study found for the first time that miR-207 could promote renal fibrosis in rats through TGF-β1/Smad3 signals. It may be a potential therapeutic target for the treatment of CKD renal fibrosis.

UUO is a common experimental model to study the mechanism of renal fibrosis. Transient renal ischemia can induce symptoms similar to CKD during acute kidney injury, including important events leading to renal fibrosis, such as fibroblast activation and epithelial mesenchymal transition. During the progression of renal fibrosis, extracellular matrix deposition and renal tubular atrophy can all promote its occurrence[18,19]. Similar to previous research results[20-22], in UUO model constructed in this study, the expression levels of fibrosis related factors TGF-β1, Smad3, Smad2, α-SMA, MMP7 and MMP9 were elevated, and the staining results showed that obvious fibrosis occurred in renal tissue of rats. Moreover, we found that miR-207 expression increased in UUO model rats, which was similar to the research results of Shi et al[23]. MiR-207 expression was significantly up-regulated in urine and renal tissue of rat renal fibrosis models, and miR-207 up-regulated the expression of fibrosis related genes Col1a1, Col3a1, Ctgf and Fn1 in rat renal tubular epithelial cells. We first verified the effect of miR-207 on renal fibrosis, and found that after inhibiting miR-207 expression, the degree of renal fibrosis in rats reduced significantly, and the expression levels of TGF-β1, Smad3, Smad2, α-SMA, MMP7 and MMP9 were inhibited. We confirmed that miR-207 had a positive correlation with TGF-β1/Smad3 expression. To verify the effect of TGF-β1/Smad3 signals on miR-207 promoting renal fibrosis, we designed an experiment in a group of rats. While up-regulating miR-207 expression, we inhibited TGF-β1/Smad3 signals; and the results showed that compared with rats with up-regulation of miR-207 expression, the severity of renal fibrosis in them reduced markedly, and the expression levels of other fibrosis indicators Smad2, α-SMA, BMP-7, MMP7 and MMP9 also reduced dramatically. Hence, we believe miR-207 can promote the progression of renal fibrosis through TGF-β1/Smad3 signals. A large amount of evidence[24,25] showed that inflammation plays a crucial role in the occurrence and development of renal fibrosis. We found that after miR-207 expression was inhibited, the level of renal tissue inflammation in UUO rats also decreased, which might be another mechanism for miR-207 to participate in renal fibrosis. Roy et al[26] pointed out that miR-207 was found to be related to inflammatory regulation in the process of liver fibrosis.

Conclusions

To sum up, miR-207 is expressed in renal tissue of renal fibrosis rats, which is positively correlated with TGF-β1/Smad3 expression, and it can promote the progression of renal fibrosis through TGF-β1/Smad3 signals.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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