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Silenced miR-21 inhibits renal interstitial fibrosis via targeting ERK1/2 signaling pathway in mice

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Abstract. – OBJECTIVE: To study the influence of micro ribonucleic acid (miR)-21 on the renal interstitial fibrosis (RIF) model mice, and to preliminarily elucidate the mechanism of action of miR-21 in the development of RIF by studying the influences of miR-21 on the expressions of the proteins related to the extracellular signal-regulated kinase (ERK) 1/2 signaling pathway and its downstream proteins.

MATERIALS AND METHODS: The mouse model of the left unilateral ureteral obstruction (UUO) was established. The experimental mice were divided into the Sham group and UUO group and were normally fed for 3 weeks. Then, they were executed, and their blood was extracted to determine the renal function-related indicators. The left kidney was excised, and the corresponding specimens were reserved for observing the appearance of the kidney and the morphology of the renal tubules and interstitium. The relative expression levels of epithelial (E-)cadherin, α-smooth muscle actin (α-SMA), and ERK1/2 phosphorylated ERK1/2 (p-ERK1/2), the transforming growth factor-β1 (TGF-β1) and the connective tissue growth factor (CTGF) proteins in renal tissues were determined. After the human renal proximal tubular epithelial cell line, the human kidney-2 (HK-2) was treated with high glucose (HG) combined with silenced miR-21 or the ERK1/2 inhibitor PD98059, the relative expression levels of α-SMA and TGF-β1 protein were measured.

RESULTS: UUO group had significantly higher content of blood urea nitrogen (BUN), serum creatinine (SCr), and uric acid (UA) than the Sham group, and exhibited the infiltration of renal interstitial monocytes and lymphocytes, renal tubular podocyte damage, phenotypic transformation and atrophy, the activation and proliferation of interstitial fibroblasts, and excessive deposition of extracellular matrix (ECM). Moreover, the expression level of E-cadherin in the renal tissues was decreased, but the relative expression levels of α-SMA, and TGF-β1, CTGF, and p-ERK1/2 proteins were evidently elevated. Lower relative expression levels of α-SMA and TGF-β1 protein were detected in the human renal proximal tubular epithelial cell line HK-2 after the combined treatment with HG and silenced miR-21 or the ERK1/2 inhibitor PD98059.

CONCLUSIONS: MiR-21 may be related to the occurrence and development of RIF. Silenced miR-21 probably suppresses RIF via the ERK1/2 signaling pathway.

Key Words:
MiR-21, ERK1/2 signaling pathway, Renal interstitial fibrosis.

Introduction

Micro-ribonucleic acid (miRNA), as a short-chain non-coding RNA, has about 22 bases and is highly conservative. It binds to the 3' non-coding region of the target gene to silence or degrade the target gene, thereby regulating its expression at the post-transcriptional level[1]. As miRNA that has been studied thoroughly in the field of diabetic nephropathy, miR-21 activates the protein kinase B (Akt) *via* the target gene phosphatase and tensin homolog (PTEN) to cause the hypertrophy and apoptosis to mesangial cells or renal tubular epithelial cells[2]. Zhang et al[3] also manifested that miR-21 regulates the metabolism-related pathways to accelerate renal fibrosis as well. Additionally, the major pathological pathway of renal interstitial fibrosis (RIF) in patients with chronic

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kidney diseases is renal tubular epithelial-mesenchymal transdifferentiation (EMT)[4-6]. Affected by several pathogenic factors, the renal tubular epithelial cells lose adhesion and marker proteins, such as epithelial (E-)cadherin, but express the marker proteins of mesenchymal cells, ultimately becoming myofibroblasts *via* phenotypic transdifferentiation[7,8].

Therefore, this study aims to establish the model of RIF by means of unilateral ureteral obstruction (UUO) to observe the expression of miR-21 in such a model, explore the influence of its expression on the expressions of extracellular signal-regulated kinase (ERK) 1/2 pathway-related proteins, and search for the action site for the improvement of RIF.

Materials and Methods

Animals and Reagents

The wild-type C57BL/6 mice were provided by the Laboratory Animal Center of Jinan University and adaptively fed for 1 week before being used in experiments. This study was approved by the Animal Ethics Committee of Jinan University Animal Center. The main reagents were: the human renal proximal tubular epithelial cell line human kidney-2 (HK-2; Shanghai Yanjing, Shanghai, China), anti-E-cadherin, anti-α-smooth muscle actin (α-SMA), anti-transforming growth factor-β1 (TGF-β1), anti-connective tissue growth factor (CTGF), anti-phosphorylated ERK1/2 (p-ERK1/2) antibodies (Abcam, Cambridge, MA, USA), fetal bovine serum, Opti-MEM and Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA), ERK1/2 inhibitor PD98059 (MCE), a scale, a cell culture incubator, a super-clean bench, scalpels, Sorvall Evolution RC high-speed refrigerated centrifuge (Thermo Fisher Scientific, Waltham, MA, USA), Roche blood glucose meter (Roche Diagnostics, Basel, Switzerland), and Beckman AU480 automatic biochemistry analyzer (Beckman, Miami, FL, USA).

RIF Animal Models Establishment and Indicators Detection

According to the literature, the mouse model of RIF was established through the left UUO[9]. The mice were subjected to 1 week of adaptive feeding before experiments, and on the 8th day, they underwent the left ureteral ligation. After being anesthetized *via* intraperitoneal injection of the mixture of an equal volume of Ketamine and Diazepam (3 mg × kg^{-1}), the mice were fixed on an operation plate in the supine position and routinely disinfected. Then, an incision was made on the left side of the abdomen to expose the left kidney, bluntly dissect the perinephric fat and search for the ureter along the renal hilus. After that, the ureter was ligated at the upper and lower segments and cut off in the middle. Finally, the incision was sutured layer by layer, and the mice were intraperitoneally injected with 200,000 units of penicillin sodium each for 3 d in a row. According to those procedures in the UUO group, the abdomen of the mice in the Sham group was opened to separate the left ureter without being ligated. A total of 16 mice were divided into the Sham group and UUO group using a random number table and normally fed for 3 weeks. Then, they were executed, and the blood and renal tissues were sampled for the detection of indicators. In addition, Western blotting was performed to detect the expressions of E-cadherin, α-SMA, TGF-β1, CTGF, and p-ERK1/2.

In Vitro Experiments

An appropriate number of HK-2 cells in logarithmic growth phase were obtained in an aseptic operation room and seeded into a sterile 6-well plate. When adhering to the wall and growing overnight to about 60%, the HK-2 cells were transfected with small scrambled interfering (si)RNA and simiR-21 at the concentration of 55 nmol/L for 6.5 h according to the instructions of the Lipofectamine 2000 transfection reagent. Then, a fresh medium was used for other 24 h of culture, and the resulting cells were harvested for the detection *via* Western blotting. Another appropriate number of HK-2 cells were taken and divided into dimethyl sulfoxide (DMSO) + high glucose (HG) group and PD98059 + HG group. Each group of cells and their supernatant were collected and applied to the Western blotting detection for α-SMA and TGF-β1 genes.

Statistical Analysis

The experimental data were expressed as ($\overline{\chi}$±s), and Statistical Product and Service Solutions (SPSS) 19.0 software (IBM Corp., Armonk, NY, USA) was used for statistical processing. The comparison between multiple groups was done using the One-way ANOVA test followed by the post-hoc test (Least Significant Difference). $p<0.05$ suggested that the difference was statistically significant.

Table I. Comparisons of RIF indexes in mice between the two groups ($\overline{\chi}\pm s$).

Group	Proportion of interstitial matrix broadening (%)	Proportion of interstitial collagen deposition (%)
Sham group	5.78±3.25	6.92±3.21
UUO group	18.47±5.92*	32.19±7.28*

Note: *$p<0.05$ *vs*. sham group.

Results

Appearances of Mice

Compared with those in Sham group, the mice in UUO group exhibited evident cachexia, lowered activity, aversion to coldness, huddling up, inappetence, mild diarrhea, dull, dark and messy hairs, weight loss, polyuria and dampness around the urethral orifice, and also showed the infiltration of renal interstitial monocytes and lymphocytes, renal tubular podocyte damage, phenotypic transformation and atrophy, activation and proliferation of interstitial fibroblasts and excessive deposition of extracellular matrix (ECM) (Figure 1 and Table I). In comparison with the Sham group, the UUO group had significantly raised the content of blood urea nitrogen (BUN), serum creatinine (SCr) and uric acid (UA) in mice (Table II).

Expression of MiR-21

A total of 3 samples were randomly selected from the renal tissues of the model mice, and according to the detection results of the Western blotting, the OUU group had significantly up-regulated miR-21 expression compared with the Sham group ($^{a}p<0.05$), indicating that miR-21 may be associated with the occurrence of RIF (Figure 2).

Table II. Statistics of renal function-related indicators.

Group	Sham group	UUO group
BUN [c/(mmol·L^{-1})]	4.7±0.81	15.1±3.31[a]
SCr [c/(μmol·L^{-1})]	14.2±1.23	24.9±3.41[a]
UA (μmol·L^{-1})	109.31±19.54	121.35 ± 18.46[a]
24 h-Pro [ρ/(mg·L^{-1})]	5.3±1.42	14.1±1.52[a]

Note: The above table is the statistics of mouse blood urea nitrogen (BUN) [c/(mmol·L^{-1})], serum creatinine (SCr) [c/(μmol·L^{-1})], uric acid (UA) (μmol·L^{-1}) and 24 h urine protein (24 h-Pro) [ρ/(mg·L^{-1})] in both Sham group and UUO group ($^{a}p<0.05$ represents the significant difference).

Expressions of E-Cadherin, α-SMA, TGF-β1, CTGF, and p-ERK1/2 Genes

The renal tissues were randomly sampled from the model mice, and through the detection of Western blotting, it was found that OUU group exhibited a decreased E-cadherin expression level, but evidently raised the relative expression levels of α-SMA and TGF-β1, CTGF and p-ERK1/2 proteins compared with the Sham group (Figure 3).

Expression of MiR-21 in HG-Treated HK-2 Cells

After the human renal proximal tubular epithelial cell line HK-2 was treated with HG, the

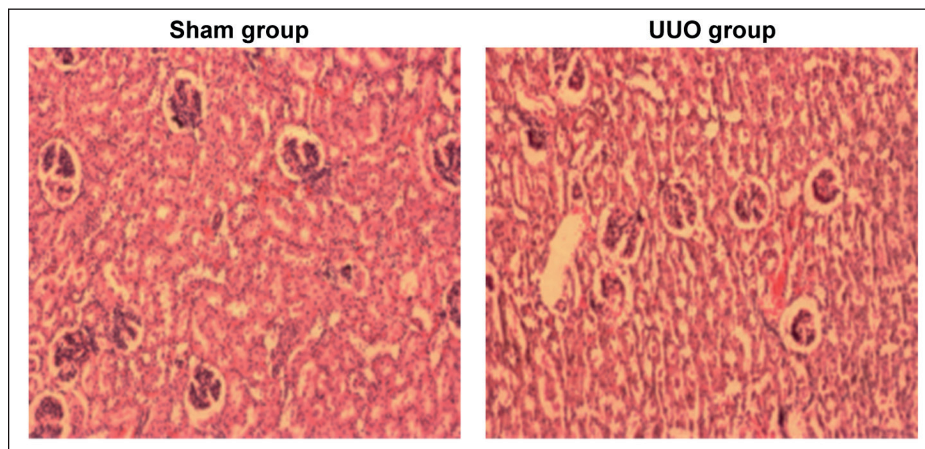

Figure 1. HE staining of renal tissues in individual model mouse (magnification: 100×).

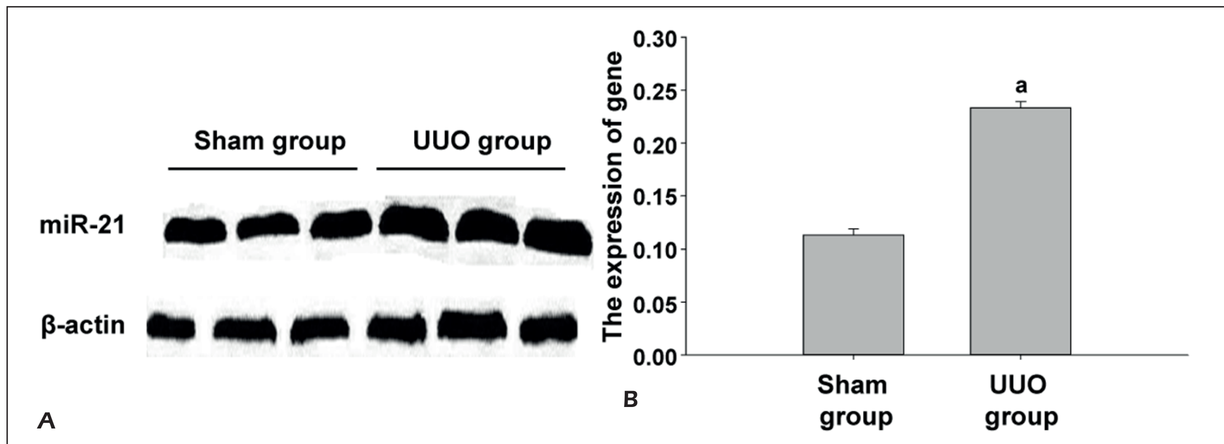

Figure 2. Expression of miR-21 in renal tissues ([a]$p<0.05$ suggests that the difference is significant compared with the Sham group).

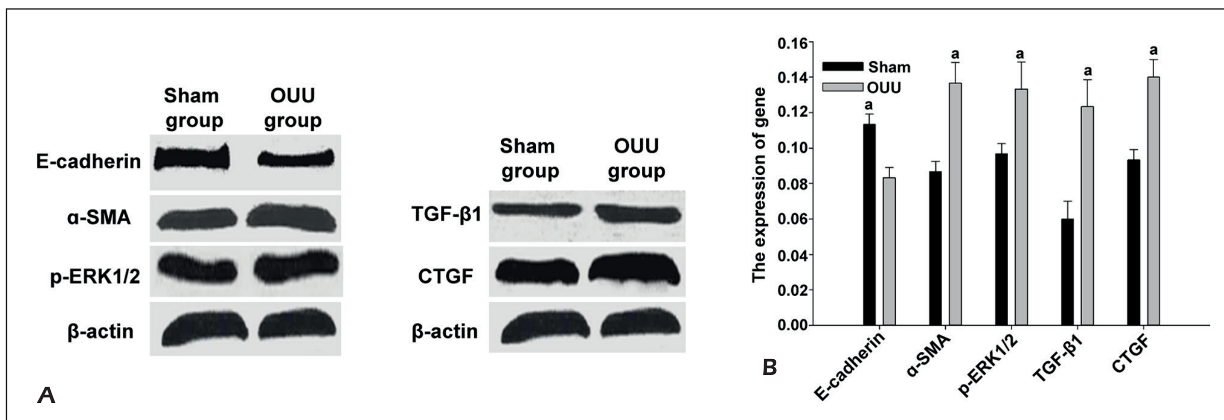

Figure 3. Expressions of relevant genes in renal tissues ([a]$p<0.05$ suggests that the differences in the expression of each gene are significant compared with the Sham group).

expression of miR-21 in the HG group was significantly upregulated compared with those in the Blank control group and Normal glucose group ($p<0.05$) (Figure 4).

Gene Expression of HK-2 Cells Cultured Using HG After Treatment with Silenced MiR-21 and ERK Inhibitor

After the combined treatment with HG and silenced miR-21 or the ERK1/2 inhibitor PD98059, it was detected that the relative expression levels of α-SMA and TGF-β1 protein relatively declined in HK-2 cells ([a]$p<0.05$) (Figure 5).

Discussion

MiR-21, one of the key factors for renal fibrosis, plays an important role in this process. Wang et al[10] proved through an *in-situ* hybridization study that in the KKAy mice model, miR-21 is mainly distributed in cortical glomerular cells and renal tubular cells and regulates the matrix metalloproteinase-9/tissue inhibitor-1 pathway to facilitate RIF. Zhong et al[11] found that in the db/db mouse model of type 2 diabetes, the expression of miR-21 in the kidney is up-regulated to be twice that in db/m (+) mice, while the mice with miR-21 knockout have remarkably lowered the urine protein and alleviated renal fibrosis and inflammation. The expression of miR-21 was decreased while Smad7 was increased in renal tubular epithelial cells cultured with high glucose[12]. Numerous data[13] have suggested that currently, in addition to Smad7, miR-21 can regulate Smad2, PTEN, Smad3/PI3K-Akt[2] to delay the progression of diabetic nephropathy in rats[14,15]. Other studies have suggested that miR-21 can also promote fibrosis in non-diabetic nephropathies such as IgA nephropathy through various pathways.

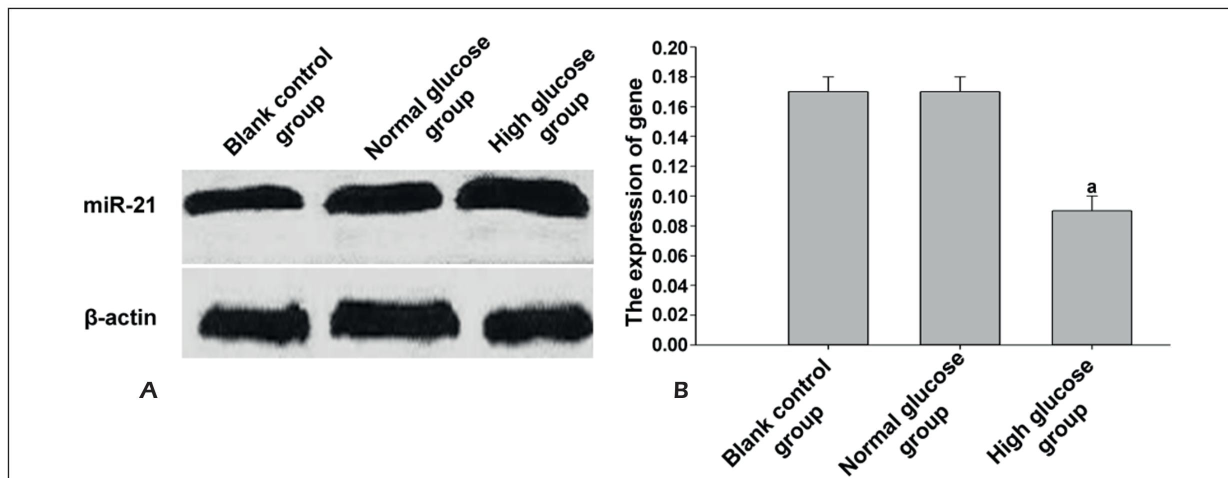

Figure 4. Expression of miR-21 in HG-treated HK-2 ([a]$p<0.05$ represents that the expression of miR-21 in HG group is significantly up-regulated compared with those in the Blank control group and Normal glucose group).

The occurrence and development of RIF are complicated and involve the interactions between numerous cells, cytokines, and ECM. The UUO model established in this study is considered the most widely used in the study of the pathogenesis of RIF for clarifying obstructive nephropathy and explaining the pathogenesis of renal fibrosis[16]. In the process of renal fibrosis, the key links of RIF are the inflammatory cell infiltration, renal tubular EMT, fibroblast proliferation and activation, release of profibrotic cytokines and the imbalance between the synthesis and degradation of ECM[17]. The characteristic signs of EMT include the weakened renal tubular epithelial keratin and E-cadherin expressions and enhanced the expressions of α-SMA and vimentin[7,18]. In the present study, the UUO model was established, and the results of the protein hybridization showed that the expression level of miR-21 was notably raised in the mouse model of RIF. Meanwhile, the content of the relevant calmodulin and vimentin was determined, and it was discovered that the expression level of E-cadherin was lowered, but that of α-SMA was up-regulated, which are the same as the results of previous research. The ERK1/2 signaling pathway is one of the major pathways regulating renal tubular EMT, and the expression of the p-ERK1/2 indicates the activation of this signaling pathway. Xing et al[19] observed the features of TGF-β1 and p-ERK1/2 protein expressions in the UUO model, and the study data revealed that the TGF-β1 and p-ERK1/2 proteins are gradually increased with

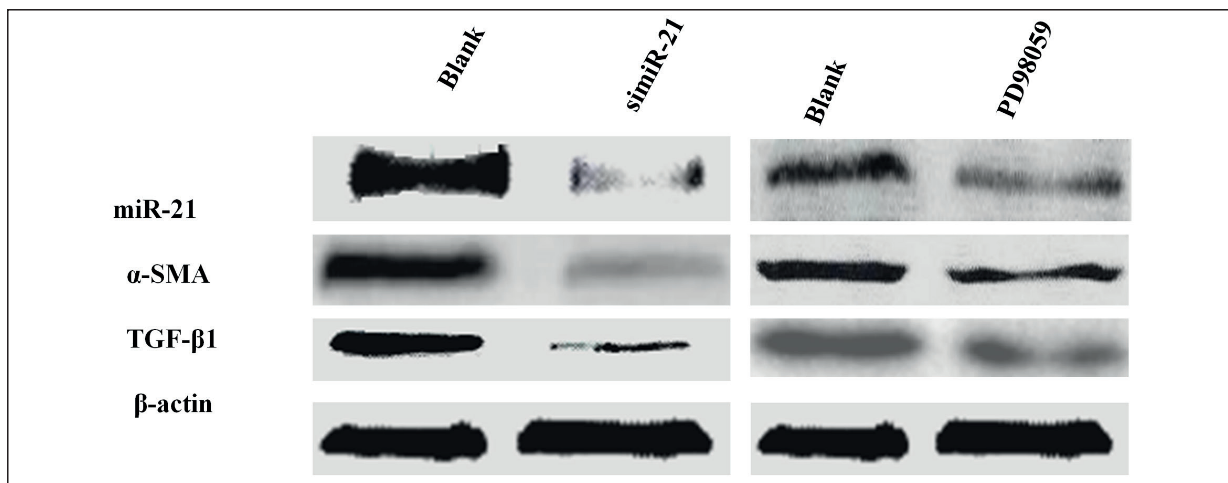

Figure 5. Gene expression of HK-2 cells cultured using HG after treatment with silenced miR-21 and ERK inhibitor.

the time, showing a significant positive correlation. During fibrosis in various organs, multiple cytokines including TGF-β1 and CTGF can activate the ERK1/2 signaling pathway[20]. Additionally, based on the findings in the UUO model and protein hybridization in the present study, the expression level of miR-21 was remarkably elevated in the mouse model of RIF, and those of TGF-β1 and CTGF were significantly increased as well.

In the present study, it was speculated that miR-21 increases the expression levels of TGF-β1 and p-ERK1/2 by regulating the ERK1/2 signaling pathway, thus inducing the production of ECM. The UUO animal model exhibited a raised expression level of miR-21, a significantly weakened E-cadherin expression and evidently enhanced α-SMA, TGF-β1, and p-ERK1/2 expressions. Among the above indicators, the most striking is TGF-β1 that is proven to be upregulated in almost all types of fibrous kidney diseases in animals and humans[21]. Besides, CTGF, a fibrosis-inducing growth factor, serves as the down-stream effector of TGF-β1, and they both play important roles in the regulation of EMT and reflection of fibrosis ECM and can induce the production of ECM[22].

Conclusions

We demonstrated that miR-21 may be related to the occurrence and development of RIF. Silenced miR-21 probably suppresses RIF *via* the ERK1/2 signaling pathway.

Conflict of Interests

The authors declared no that they have no conflict of interests.

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