Let-7a-5p may participate in the pathogenesis of diabetic nephropathy through targeting HMGA2

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Abstract. Diabetic nephropathy (DN) is one of the most common complications of diabetes mellitus (DM), and has been demonstrated as one of the major causes of renal failure. In a previous study, it was noted that microRNA let-7a-5p was downregulated in DN; however, the underlying mechanism requires additional investigation. The aim of the present study was to investigate the roles of let-7a-5p in the pathogenesis of DN and its associated mechanism. The renal tissues of db/db and db/m mice, and renal mesangial cells treated with high concentrations of glucose were obtained; reverse transcription-quantitative polymerase chain reaction, and western blot analysis were applied to detect the expression of let-7a-5p and high-mobility group AT-hook 2 (HMGA2) in vivo and in vitro. In addition, renal mesangial cells cultured under high-glucose conditions (20 and 30 mmol/l) were transfected with either let-7a-5p mimics or let-7a-5p inhibitors. The effects of let-7a-5p on the proliferation and apoptosis of renal mesangial cells were examined using CCK-8 and flow cytometry methods. Additionally, cells were collected and the expression of phosphoinositide 3-kinase (PI3K), phosphorylated protein kinase B (p-AKT) and HMGA2 was analyzed with western blot analysis. Finally, a dual luciferase reporter assay was performed to confirm whether HMGA2 was a direct target of let-7a-5p. Let-7a-5p was downregulated in DN and may participate in the pathogenesis of DN via regulating HMGA2 expression and the PI3K-AKT signaling pathway.

Introduction

Diabetic nephropathy (DN) is one of the most common complications of diabetes mellitus (DM), and is known as one of the major causes of renal failure (1-3). Previously, the incidence of DN was demonstrated to be increasing yearly; according to the data from the World Health Organization, the population of patients with diabetes will reach 370 million by the year of 2025, and 30% of these patients will develop DN (4). Generally, the distinct pathological changes of DN include aberrant mesangial cell proliferation, which causes the widening of the mesangial region and abnormal accumulation of the extracellular matrix, which results in glomerular sclerosis and tubulointerstitial fibrosis. DN eventually leads to the development of chronic renal failure, which may seriously affect the quality of life of patients (5-7). At present, anti-DN therapies include the control of blood glucose and blood pressure, and the inhibition of the renin-angiotensin system; however, these methods only delay the progression of DN; such therapies are not able to reverse the progression of DN to the stages of renal function failure (7-9). Therefore, there is an urgent requirement to identify novel therapeutic targets for the management and prevention of DN.

The pathogenesis of DN remains unclear. In previous years, increasing evidence has indicated that microRNA (miRNAs) are involved in the progression of a number of diseases. miRNAs are a family of small non-coding RNAs with a length of 18-25 nucleotides. miRNAs bind to the 3'-untranslated region (3'UTR) of their target mRNAs, thereby suppressing the expression of their target genes (10-12). A variety of miRNAs have been demonstrated to participate in the pathogenesis of a number of types of disease, including cancer, autoimmune diseases, cardiovascular diseases, and acute and chronic renal disease (13-15). However, the underlying mechanism of miRNAs in renal-associated disease requires additional study.

In a previous study, it was suggested that miRNA let-7a-5p was aberrantly expressed in DN (16). The present study aimed to confirm whether HMGA2 was a direct target of let-7a-5p. Let-7a-5p was downregulated in DN and may participate in the pathogenesis of DN via regulating HMGA2 expression and the PI3K-AKT signaling pathway.
to identify the roles of let-7a-5p in the pathogenesis of DN using in vivo and in vitro models. The present study may provide novel evidence for the diagnosis and treatment of DN.

Materials and methods

Establishment of DM animal models. A total of 32 4-week-old male C57BL/6J-db/db mice with type II DM and an additional 32 4-week-old male db/db mice were purchased from the Animal Center of Nanjing Medical University (Nanjing, China) and included in the present study. Mice were maintained under conventional conditions with 12 h light/dark cycle at 20-22°C and were provided with standard chow and water ad libitum. Prior to sacrifice, mice underwent fasting for 12 h. The bilateral kidneys were then collected following laparotomy in each mouse; the connective tissues and blood vessels on the renal hilum were removed. Next, the renal specimens of one kidney from each bilateral pair were fixed in 10% neutral formalin at room temperature for 48 h, embedded in paraffin and sliced into 2-μm sections. Then, the tissue sections underwent H&E staining and were analyzed under a light microscope (magnification, x200) to determine pathological changes within the renal tissues. Simultaneously, renal specimens of the other kidney of each bilateral pair were preserved in liquid nitrogen for RNA extraction. The present study as approved by the Ethical Committee of Taixing City Second People's Hospital.

High-glucose-stimulated renal mesangial cell model. Mouse renal mesangial cells were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China; cat. no., GNM21). The renal mesangial cells were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), and glucose at a concentration of 20 mmol/l, in an atmosphere containing 5% CO2. After 24 h, the cells were collected for subsequent analysis.

Cell transfection. The mmu-let-7a-5p inhibitors and mmu-let-7a-5p mimics oligonucleotides were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). Renal mesangial cells treated with 0, 20 or 30 mmol/l glucose were transfected with 50 nM mmu-let-7a-5p inhibitors or mmu-let-7a-5p mimics mixed with Lipofectamine RNAi Max (Thermo Fisher Scientific, Inc.). Cells were then cultured at 37°C in DMEM supplemented with 10% FBS in an atmosphere containing 5% CO2 for 48 h. Cells were harvested at 48 h for the subsequent analysis. The sequences of the oligonucleotides were:

- mmu-let-7a-5p inhibitors
- mmu-let-7a-5p mimics

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from cells using TRIZol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and the reverse transcription was performed with the PrimeScript™ RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China) with the temperature of 37°C for 15 min and 85°C for 5 sec. RT-qPCR was conducted with a SYBR ExScript RT-PCR kit (Takara Biotechnology Co., Ltd., Dalian, China) on an ABI 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were: Initial denaturation, 95°C for 30 sec; followed by 40 cycles of denaturation at 95°C for 5 sec and annealing at 60°C for 30 sec. The primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The relative expression of high-mobility group AT-hook 2 (HMGA2) in each sample was normalized to that of GAPDH using the 2ΔΔCq method (17). The expression of let-7a-5p was determined using the Hairpin-it™ miRNAs qPCR Quantitation kit (Shanghai GenePharma Co., Ltd.). U6 was applied for the normalization of miRNA expression. The primers of the sequences used were: Let-7a-5p, forward 5'-GCC GCTGAGGTAGTAGGTTGTA-3', reverse 5'-GTGCAGGGT CCGAGGT-3'; HMGA2, forward 5'-CAGCAGCAAGAACCA ACCG-3', reverse 5'-TGTTGTTGCCCCATTTTCTAGGT-3'; PI3K, forward 5'-GAA ATCTCCCTGGATGTGTCGT-3', reverse 5'-ATCTGTGTCCTCGGATGATA-3'; AKT, forward 5'-GATGGAGCCAGGTTACAAA-3', reverse 5'-GACAGC AACCACAAAAAATGA-3'; GAPDH, forward 5'-TCAACGG ATTTTGCTGCTATG-3', reverse 5'-TGGTTGGAATCA TATTGGAAC-3'; U6, forward 5'-AACGCTTCAGCAATT TGCGT-3', reverse 5'-AACGCTTCCAGAATTTGGCT-3'.

Cell proliferation analysis. Renal mesangial cells were plated at a density of 5,000 cells/well in 96-well plates. A Cell Counting Kit-8 (CCK-8) assay was performed at 48 h after transfection to determine cell viability using a CCK-8 proliferation assay kit (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) according to the manufacturer's protocol.

Cell apoptosis analysis. For the analysis of apoptosis, 48 h after transfection or following treatment with the protein kinase B (AKT) inhibitor, MK2206 (10 μmol/l), cells were stained using a propidium iodide/Annexin V-fluorescein isothiocyanate apoptosis detection kit (BD Biosciences, San Jose, CA, USA). Briefly, cells were collected, and 5 μl Annexin V-FITC and propidium iodide (PI) solutions were mixed with the cells at room temperature, and incubated on ice in the dark for 30 min. Then the apoptotic rate and cell cycle in each sample were analyzed with a BD FACsVerse flow cytometer (BD Biosciences) according to the manufacturer's protocol. The data was analyzed by FlowJo version 8.7 (FlowJo LLC, Ashland, OR, USA).

Western blot analysis. Cells were lysed with radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China) and then concentration of the proteins were quantified by Enhanced BCA Protein Assay kit (Beyotime Institute of Biotechnology). Then SDS-PAGE was performed to separate the extracted proteins using 8% gel, and 30 μg protein loaded into each lane. Then, the proteins were transferred onto polyvinylidene fluoride membranes, which were then blocked with 5% non-fat milk at room temperature for 1 h and incubated with primary antibodies [anti-phosphoinositide 3-kinase (PI3K, ab151549; 1:1,000), anti-AKT (ab8805; 1:1,000), anti-phosphorylated (p)-AKT (ab38449; 1:1,000), anti-HMGA2 (ab97276; 1:500), anti-GAPDH (ab9485; 1:1,000), anti-phosphorylated (p)-AKT (ab38449; 1:1,000), anti-HMGA2 (ab97276; 1:500) and anti-GAPDH (ab9485; 1:1,000).
In the present study, the expression of HMGA2 in DN mouse tissue and control was examined. It was observed that the expression of HMGA2 was significantly increased at the mRNA and protein levels, as detected by RT-qPCR and western blot analysis (Fig. 1B and C). Finally, the present study identified that the expression levels of let-7a-5p in DN mice were negatively correlated with that of HMGA2 (Fig. 1D; P=0.0088; r=-0.4555).

Effects of high-glucose conditions on the expression of let-7a-5p and HMGA2 in renal mesangial cells. Renal mesangial cells were cultured under high-glucose conditions for 24 h in the present study; the effects of high-glucose conditions on the expression of let-7a-5p and HMGA2 were then examined by RT-qPCR. It was observed that high-glucose conditions induced significant decreases in the expression levels of let-7a-5p (Fig. 2A) and markedly increased the expression of HMGA2 (Fig. 2B), in a dose-dependent manner.

Let-7a-5p may affect the proliferation and apoptosis of renal mesangial cells under high-glucose conditions in vitro. To additionally explore the roles of let-7a-5p in DN, renal mesangial cells were transfected with let-7a-5p mimics or inhibitors; the effects of let-7a-5p on the proliferation and apoptosis of renal mesangial cells under high-glucose conditions were examined using CCK-8 and flow cytometry analyses. As indicated in Fig. 3A, the expression of let-7a-5p was significantly upregulated in let-7a-5p mimic-transfected cells, and markedly downregulated in let-7a-5p inhibitor-transfected cells; in addition, transfection with let-7a-5p mimics induced a significant decrease in the proliferation and an increase in the apoptosis of renal mesangial cells cultured under high-glucose conditions; transfection with let-7a-5p inhibitors exhibited opposing effects (Fig. 3B-D; P<0.05).

Let-7a-5p may affect the expression of HMGA2, PI3K and p-AKT in renal mesangial cells under high-glucose conditions. To additionally explore the underlying mechanism of let-7a-5p in DN, the effects of let-7a-5p mimics or inhibitors on the expression of HMGA2, PI3K, AKT and p-AKT were examined. As demonstrated in Fig. 4, transfection with let-7a-5p mimics resulted in decreased expression levels of HMGA2 and inhibition of the PI3K/AKT signaling pathway, which was indicated by decreased expression levels of PI3K, AKT and p-AKT. Conversely, transfection with let-7a-5p inhibitors appeared to activate the PI3K-AKT signaling pathway.

HMGA2 is a direct target of let-7a-5p in vitro. As aforementioned, using the online computational prediction tools TargetScan and miRanda, HMGA2 was predicted as a target gene of let-7a-5p (Fig. 5A). Finally, a dual luciferase reporter assay was performed to investigate whether let-7a-5p may directly target HMGA2. As demonstrated in Fig. 5, transfection of let-7a-5p mimics induced significant decreases in the luciferase activities within wild-type HMGA2 3’-UTR (HMGA2-3’-UTR)-transfected 293 cells, while transfection with let-7a-5p mimics exhibited no significant effects on mutant HMGA2 3’-UTR (HMGA2-MUT)-transfected cells (Fig. 5B; P<0.01). These results indicated that HMGA2 is a direct target of let-7a-5p.
Discussion

In previous years, increasing evidence has indicated that miRNAs may participate in the pathogenesis of DN. For example, it has been observed that miR-29c may promote the development of DN via targeting tristetraprolin (21). In addition,
miR-27a has been observed to regulate renal function through the activation of β-catenin in DN (22). Furthermore, suppression of miR-217 may ameliorate high-glucose-induced podocyte injury by inhibiting the apoptosis of cells (23). The present study focused on the roles of miRNA let-7a-5p in DN by performing in vitro and in vivo analyses. It was observed that the expression of let-7a-5p was significantly downregulated in the kidney tissues of DN mice and renal mesangial cells treated cultured under high-glucose conditions compared with the controls, which was consistent with previous data (24). Taken together, these results indicated that let-7a-5p was downregulated in DN, suggesting that let-7a-5p may serve as a biomarker for the diagnosis of DN.

At present, the majority of studies investigating let-7a are cancer-associated; let-7a-5p has been described previously in a number of studies, either as tumor suppressor or as an oncogenic miRNA. In the field of renal diseases, let-7a has been described as a tumor suppressor in renal cell carcinoma via the targeting of v-myc avian myelocytomatosis viral oncogene homolog expression (25). In addition, it has also been observed that the detection of let-7 miRNAs in urine-derived supernatant samples may be a potential approach for the diagnosis of renal cell carcinoma. In the present study, renal mesangial cells were treated with high concentrations of glucose in vitro and it was observed that let-7a-5p was significantly downregulated in renal mesangial cells in vitro. Furthermore, transfection with let-7a-5p mimics induced significant decreases in the proliferation and promoted the apoptosis of renal mesangial cells cultured under high-glucose conditions; transfection with let-7a-5p inhibitors exhibited the opposite effects. These results conclusively indicated a pathological role of let-7a-5p in mesangial cell hypertrophy.

miRNAs exert their functions through the silencing of target genes. Using the online computational prediction tools TargetScan and miRanda, HMGA2 was predicted as a target gene of let-7a-5p in the present study, and Mayr et al (26) demonstrated that let-7a directly targets HMGA2. HMGA2 has been recognized as a non-histone chromosomal protein, and it binds to DNA and regulates the transcription process of its target genes (26). HMGA2 has been suggested to be involved in a number of biological processes, including cell proliferation, differentiation, embryonic development and carcinogenesis (26). It has been demonstrated previously in a numerous studies that HMGA2 is upregulated in renal diseases (27-29). However, whether HMGA2 may regulate the proliferation and apoptosis of renal mesangial cells, which leads to the development of DN, remains unclear. As it has been previously described that DN was induced by the over-proliferation of mesangial cells (30), it was hypothesized that downregulation of let-7a-5p may lead to the aberrant expression of HMGA2, which may serve critical roles in the pathogenesis.

Therefore, to explore the association between let-7a-5p and HMGA2 in DN, a series of in vitro and in vivo experiments were performed in the present study. It was observed that the expression levels of HMGA2 were markedly increased in the kidney of DN mice, and in high-glucose-treated mesangial cells. In addition, transfection with let-7a-5p mimics of high-glucose-treated mesangial cells induced a significant decrease in the expression of HMGA2; transfection with let-7a-5p inhibitors exhibited the opposite results. Finally, the results of the dual luciferase reporter assay suggested that HMGA2 is a direct target of let-7a-5p, which was consistent with the observation of Lee and Dutta (31). Taken together, these results indicated that let-7a-5p may be involved in the pathogenesis of DN by targeting HMGA2 expression.

Considerable evidence indicates that the PI3K/AKT signaling pathway is involved in the control of various cellular processes, including that of DN. For example, notoginsenoside R1 may ameliorate podocyte and renal injuries in rats with DN by activating the PI3K/AKT signaling pathway (32). Jiangtang decotion may decrease inflammation associated with DN through the regulation of the PI3K/AKT signaling pathway (33). The association between HMGA2 and the PI3K/AKT signaling pathway has been discussed previously: Tan et al (34) observed that HMGA2 may induce cell proliferation in acute myeloid leukemia cells via regulating the PI3K/AKT/mechanistic target of rapamycin signaling pathway; Cui et al (35) revealed that miR-337 may regulate
Figure 3. Let-7a-5p may affect the proliferation and apoptosis of renal mesangial cell under high-glucose conditions in vitro. (A) Effect of let-7a-5p mimics and inhibitors on the expression of let-7a-5p in renal mesangial cells. (B) Effect of let-7a-5p mimics and inhibitors on the proliferation of renal mesangial cell under high-glucose conditions in vitro measured by the Cell Counting Kit-8 method. (C) Effect of let-7a-5p mimics and inhibitors on the apoptosis of renal mesangial cell under high-glucose conditions in vitro measured by flow cytometry. (D) Quantification of the flow cytometry results. "P<0.01 vs. control. NC, negative control; OD, optical density; PI, propidium iodide; FITC, fluorescein isothiocyanate.
the PI3K/AKT signaling pathway to inhibit hepatocellular carcinoma progression via targeting HMGA2. The present study explored whether the PI3K/AKT signaling pathway was involved in the let-7a-5p/HMGA2-induced anti-DN effects within renal mesangial cells, and it was observed that the overexpression of let-7a-5p promoted the expression of PI3K and p-AKT in high-glucose-treated mesangial cells. Taken together, the results of the present study not only revealed the important role of the let-7a-5p/HMGA2/PI3K/AKT signaling pathway in the development of DN, but also suggested the potential role of let-7a-5p as a potential therapeutic target for the treatment of DN.

In conclusion, the data from the present study demonstrated that let-7a-5p was downregulated in DN and that let-7a-5p may participate in the pathogenesis of DN by targeting the HMGA2/PI3K/AKT signaling pathway. The results present
let-7a-5p as a potential diagnostic marker and therapeutic targets for the diagnosis and treatment of DN.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TW performed experiments and wrote the article. HZ contributed to the design of the study and revised the manuscript. SY designed and performed the experiments and analyzed the data. XF contributed to the design of the study and revised the manuscript. All the authors have seen and approved the manuscript.

Ethics approval and consent to participate

The present animal study was approved by the Ethical Committee of Taixing City Second People’s Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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