Original Article

LincRNA-p21 sponges miR-18b to promote the progression of diabetic nephropathy

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Abstract: Little is known about the role of lincRNA-p21 in the development of diabetic nephropathy. The aim of the present study was to investigate the level of lincRNA-p21 in diabetic nephropathy, and explore its underlying mechanism. The current study revealed that down-regulation of lincRNA-p21 could alleviate pathological changes of diabetic nephropathy in mice. LincRNA-p21 expression was significantly up-regulated in MMCs under high glucose condition in vitro. Besides, lincRNA-p21 promoted the proliferation of MMCs, which was reversed by miR-18b targeted with 3'-UTR. Moreover, miR-18b suppressed the expression of connective tissue growth factor (CTGF) by binding with the 3'-UTR. Furthermore, down-regulation of lincRNA-p21 expression alleviated extracellular matrix under high glucose, which could be reversed by miR-18b inhibitor significantly. In short, our study suggests lincRNA-p21 plays as an important role in progression of diabetic nephropathy in an animal model through interaction with miR-18b, providing a novel insight for the pathogenesis and an underlying therapeutic target for diabetic nephropathy.

Keywords: Diabetic nephropathy, mouse mesangial cells, lincRNA-p21, miR-18b

Introduction

Diabetes mellitus, commonly referred to as diabetes, is a group of metabolic disorders characterized by hyperglycemia due to insulin-secreting or insulin-action disorders [1]. If left untreated, diabetes can increase the risk of long-term complications [2]. The primary complications of diabetes due to damage in small blood vessels can lead to systemic organs, especially in the eyes [3], kidneys [4], and nervous [5] system, and dysfunction and failure.

Diabetic nephropathy (DN) is one of the severe complications of diabetes and always leads to end-stage kidney diseases. The key pathologic features of DN include a thickening of the basement membrane, a widening of the slit membranes of the podocytes, an increase in the number of mesangial cells, and an accumulation in extracellular matrix [6]. Evidences suggest that proliferation of mesangial cells is critical in the initiation and progression of DN [7]. However, the molecular mechanisms underlying the development of DN have not been fully understood.

As a member of non-coding RNAs, long non-coding RNA (lncRNA) is defined as greater than 200 nucleotides in length and plays an important role in diverse cellular processes, such as regulation of gene expression, posttranscriptional processing and tumorigenesis [8, 9]. Long intergenic non-coding RNA p21 (lincRNA-p21) was first reported as a direct transcriptional target of p53, it mainly regulates the cell cycle and apoptosis [10]. LincRNA-p21 has been previously found to play an important role in liver fibrosis and pulmonary fibrosis [11, 12]. Nevertheless, the role of lincRNA-p21 in DN remains elusive.

MicroRNAs are a class of highly conservative non-coding small RNAs (about 19-23 nucleotides) that exhibit its biological functions by binding to the 3' untranslated region (3'-UTR) of target gene [13]. Accumulating evidences suggest miRNAs are important regulators in pathology of diabetes and its relevant renal injury. For example, miR-192 could lead to renal fibrosis proteinuria by activating TGF-β signaling pathway and up-regulation of miR-21 induces the overactivation of Akt signaling pathway followed...
by renal fibrosis and hypertrophy [14, 15]. Whether miR-18b acts on the development of DN is still not very clear.

Thus, the aim of the present study was to investigate the levels of lincRNA-p21 in the progression of DN in vitro and in a db/db mouse model of diabetes, and explore its underlying mechanism.

Materials and methods

Animals

The C57BL/6 db/db and control db/m mice at the age of 8 weeks old weighing 20-22 g were purchased from the laboratory animal center of Xinjiang Medical University. All mice were maintained in the same room under conventional conditions with a regular 12 h light/dark cycle with the temperature controlled at 20-22°C. Mice were given standard chow and autoclaved water ad libitum. Mouse models of diabetes were constructed by intraperitoneal injections of streptozotocin (STZ, 100 mg/kg) for three consecutive days [16]. Mice were considered DN when tail vein blood glucose levels ≥300 mg/dl and urine albumin creatine ratio (ACR) ≥300 µg/mg. Db/db and db/m mice were randomized into groups with 10 mice in each: (1) db/m group, (2) db/db group, (3) si-control-treated db/db group, (4) si-lincRNA-p21-treated db/db group. Renal cortical tissues were dissected from kidney as previously described [17]. The fresh kidney tissue samples were fixed by neutral formalin.

Biochemical determination

Tail vein blood glucose levels were measured by Glucose LiquiColor Test (Stanbio Laboratory, Boerne, TX, USA) every 4 weeks. Urinary samples (24 hours) were collected by metabolic cage every 4 weeks. BUN, creatinine and urine albumin were determined by competitive ELISA according to the manufacturer’s instruction.

Histological examination

Renal cortical tissues were fixed in 4% paraformaldehyde for 48 h, dehydrated through a graded series of ethanol, embedded in paraffin wax, and cut into 4 µm sections. The tissues were then stained with Hematoxylin-eosin (H&E) and Periodic acide Schiff (PAS) to observe glomerular morphological changes. Four random fields were chosen by light microscope.

Cell culture and transfection

293T cells were cultured in DMEM with 5 mmol/l glucose and 10% fetal bovine serum at 37°C in a humidified, 5% CO₂ atmosphere. Mouse mesangial cells (MMCs) cultured in DMEM were treated with 25 and 5 mmol/l glucose respectively to mimic diabetic pathological and normal physiological environments as previously described [18]. For transfection, cells were seeded into plates and transfected with lincRNA-p21 mimic, lincRNA-p21 inhibitor, miR-18b mimic or miR-18b inhibitor (Gene Pharm, Shanghai, China) mixed with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, MA, USA) according to the manufacturer’s protocols.

Real time quantitative PCR analysis

The total RNA was extracted from cells using Trizol reagent (Invitrogen, Shanghai, China) according to the manufacturer’s instructions. Complementary DNA (cDNA) was synthesized using a reverse transcription kit (Takara Biotechnology, Dalian, China). Relative levels of gene expression was expressed relative to GAPDH and calculated using the 2⁻ΔΔCt method.

Cell proliferation assay

Cells (3×10⁵) were cultured in 96-well plates and incubated for 24 h and stained with 0.5 mg/ml MTT for 4 h. Supernatant was discarded and 200 µl of dimethylsulfoxide (DMSO) was added to dissolve precipitates. Samples were measured at 490 nm using an ELISA reader.

Luciferase activity assay

The 3’-UTR of lincRNA-p21 and CTGF containing miR-18b binding sites was amplified and cloned into the pGL3-basic luciferase vector (Promega, USA), respectively. Similarly, the mutant 3’-UTR of lincRNA-p21 and CTGF was cloned into the same vector. Transfected cells were detected using the Dual-Luciferase Reporter Assay System (Promega) 48 h later.

Western blotting analysis

The cultured cells were washed with ice-cold PBS and lysed in RIPA buffer supplemented with protease inhibitor mixture. After electrophoresis, the protein samples were incubated with the primary antibody (anti-CTGF, anti-Collagen I, anti-Collagen IV and anti-fibronectin, 1: 1000 dilution). Samples were incubated with secondary antibodies conjugated by HRP. Bands were quantified using ImageJ software.
Figure 1. The role of lincRNA-p21 in renal function and pathological changes of DN in db/db mice. The level of clinical biochemical indexes were up-regulated remarkably in db/db mice, which was significantly improved by si-lincRNA-p21, including blood glucose, BUN, creatinine and urine albumin (A-D; *P<0.05, #P<0.05). HE and PAS staining showed the degree of pathological changes was lightened when db/db mice received si-lincRNA-p21 treatment (E).
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Results

The role of lincRNA-p21 in renal function and pathological changes of DN in db/db mice

We first detected the change of blood glucose. The data of the present study demonstrated

the level of blood glucose was up-regulated remarkably in db/db mice, which was significantly improved by si-lincRNA-p21 (Figure 1A, *P<0.05). Results of qRT-PCR manifested that lincRNA-p21 level was significantly elevated in MMCs transduced with lincRNA-p21 mimic compared with lincRNA-NC (B, *P<0.05). A MMT assay revealed that the growth rate of MMCs in high glucose conditions was increased, which could be further elevated by lincRNA-p21 over-expression (D, *P<0.05). LincRNA-p21 silencing was established via siRNA transfection in cells in vitro (C, *P<0.05). MTT assay showed that lincRNA-p21 silencing inhibited the proliferation of MMCs in high glucose conditions significantly (E, *P<0.05).

LincRNA-p21 promoted the proliferation of MMCs in vitro, which was reversed by LincRNA-p21 silencing. A showed that lincRNA-p21 mRNA expression was significantly up-regulated in MMCs under high glucose condition over time (A, *P<0.05). Results of qRT-PCR manifested that lincRNA-p21 level was significantly elevated in MMCs transduced with lincRNA-p21 mimic compared with lincRNA-NC (B, *P<0.05). A MMT assay revealed that the growth rate of MMCs in high glucose conditions was increased, which could be further elevated by lincRNA-p21 over-expression (D, *P<0.05). LincRNA-p21 silencing was established via siRNA transfection in cells in vitro (C, *P<0.05). MTT assay showed that lincRNA-p21 silencing inhibited the proliferation of MMCs in high glucose conditions significantly (E, *P<0.05).

Statistical analysis

Results are expressed as the mean ± standard error. The Student’s t-test and ANOVA were performed among different groups. All calculations were performed using SPSS 17.0 software (IBM Software, Chicago, IL, USA) and GraphPad (version 6.0, USA). A value of P<0.05 indicated a statistically significant difference.

Results

The role of lincRNA-p21 in renal function and pathological changes of DN in db/db mice

We first detected the change of blood glucose. The data of the present study demonstrated
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**Figure 3.** LincRNA-p21 acts as a molecular sponge for miR-18b. The 3'-UTR binding sites can be seen in (A). Luciferase reporter assay confirmed the binding with the decreasing fluorescence within miR-18b mimic and lincRNA-p21 wild type (B). MiR-18b expression levels were significantly up-regulated after cells were transfected with si-lincRNA-p21 (C, *P*<0.05). (D) showed that miR-18b mRNA expression was significantly down-regulated in MMCs under high glucose condition over time (*P*<0.05). MTT assay revealed that miR-18b inhibitor could rescue the suppression by si-lincRNA-p21 in the proliferation of MMCs under high glucose for 72 h (*P*<0.05).

LincRNA-p21 promoted the proliferation of MMCs in vitro, which was reversed by LincRNA-p21 silencing

To investigate the effect of lincRNA-p21 on the development of DN, the expression of lincRNA-p21 in MMCs was analyzed by RT-qPCR. The MMCs were induced by high glucose (25 mM) for 12, 24, 48, and 72 hours. Figure 2A showed that lincRNA-p21 mRNA expression was significantly up-regulated in MMCs under high glucose condition implying the elevated level of lincRNA-p21 mRNA induced by high glucose was time-dependent. Moreover, the lincRNA-p21-overexpression in MMCs was firstly established by retrovirus infection. Results of qRT-PCR manifested that lincRNA-p21 level was significantly elevated in MMCs transduced with lincRNA-p21 mimic compared with lincRNA-NC.
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To explore the role of lincRNA-p21 in cell proliferation, a MMT assay was performed to assess the viability of MMCs. Results revealed that the growth rate of MMCs in high glucose conditions was increased, which could be further elevated by lincRNA-p21 over-expression. To further verify the role of lincRNA-p21 in the development of DN, we stably established lincRNA-p21 silencing via siRNA transfection in cells in vitro. MTT assay showed that lincRNA-p21 silencing inhibited the proliferation of MMCs in high glucose conditions significantly.

LincRNA-p21 acts as a molecular sponge for miR-18b

Increasing evidence had illustrated that lincRNAs function as sponges to bind specific miRNAs. Bioinformatics analysis was used to predict the candidate targets of miRNAs binding with lincRNA-p21. Results found 3’UTR of LincRNA-p21 was highly conserved to bind with miR-18b. The 3’-UTR binding sites can be seen in Figure 3A. Luciferase reporter assay confirmed the binding with the decreasing fluorescence within miR-18b mimic and lincRNA-p21 wild type (Figure 3B). Moreover, after cells were transfected with si-lincRNA-p21, miR-18b expression levels were significantly up-regulated (Figure 3C). Figure 3D showed that miR-18b mRNA expression was significantly down-regulated in MMCs under high glucose condition over time. Furthermore, MTT assay revealed that miR-18b inhibitor could rescue the suppression by si-lincRNA-p21 in the proliferation of MMCs under high glucose for 72 h (Figure 3E).

MiR-18b directly targets with 3’-UTR of CTGF

We examined computationally predicted targets of miR-18b using bioinformatics analysis. Results revealed 3’UTR of CTGF was highly conserved to bind with miR-18b. The 3’-UTR binding sites can be seen in Figure 4A. Luciferase reporter assay showed transfection of miR-18b could significantly restrict the relative luciferase activity in cells (B, *P<0.05). Overexpression of miR-18b obviously inhibited CTGF expression in cells at the mRNA and protein levels. (C, D; *P<0.05).

Figure 4. MiR-18b directly targets with 3’-UTR of CTGF. The 3’-UTR binding sites can be seen in (A). Luciferase reporter assay showed transfection of miR-18b could significantly restrict the relative luciferase activity in cells (B, *P<0.05). Overexpression of miR-18b obviously inhibited CTGF expression in cells at the mRNA and protein levels. (C, D; *P<0.05).
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in MMCs, the expression of Collagen I, Collagen IV and fibronectin were detected to predict the accumulation of ECM under high glucose condition by RT-PCR and western blot. When cells were transfected with si-lincRNA-p21, the expression of these indexes was down-regulated remarkably, which was reversed by miR-18b inhibitor significantly. (*P<0.05).

**Discussion**

As one of the most important long-term complications of diabetes, DN is the major cause of end-stage renal disease [22]. Progression of diabetic nephropathy (DN) is characterized by the gradual scarring of the renal glomerulus and fibrosis of the tubulointerstitial region [23]. The function of lincRNA-p21 has been widely investigated in various diseases. Yu, F. et al. found lincRNA-p21 inhibits the progression of
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To our knowledge, this is the first study investigating the impact of lincRNA-p21 on the development of DN. The main clinical features of DN are persistent albuminuria and progressively declined glomerular filtration rate. The results demonstrated the clinical indexes reflecting renal function was up-regulated remarkably in db/db mice, which was significantly improved by si-lincRNA-p21. These data suggested lincRNA-p21 could promote the progression of DN.

Given that mesangial expansion is the primary driver of glomerulosclerosis [23], we next observed the role of lincRNA-p21 in MMCs under high glucose condition. The result illustrated lincRNA-p21 mRNA expression was significantly up-regulated in MMCs under high glucose condition. Besides, lincRNA-p21 could promote the proliferation of MMCs in vitro, which was reversed by lincRNA-p21 silencing significantly. The study implied lincRNA-p21 act as a positive regulatory role in the growth of MMCs in vitro.

LncRNAs function as sponges for common miRNAs and abolished the endogenous suppressive effect of these miRNAs on their targeted transcripts [25]. In this study, we found that miR-18b was reduced by lincRNA-p21. Luciferase activity assays revealed that lincRNA-p21 is a target of miR-18b. Further study revealed that miR-18b inhibitor could rescue the suppression by si-lincRNA-p21 in the proliferation of MMCs under high glucose. These data showed lincRNA-p21 exerted the pro-fibrogenic role by targeting miR-18b.

CTGF is a 349-amino acid cysteine-rich polypeptide belonging to the CCN family [26]. Under the diabetic milieu, CTGF expression is found to be markedly upregulated in mesangial cells [27]. Bioinformatics analysis revealed 3'-UTR of CTGF was highly conserved to bind with miR-18b. Over-expression of miR-18b obviously inhibited CTGF expression in cells at the mRNA and the protein levels, suggesting miR-18b suppresses the progression of DN by targeting CTGF gene at 3'-UTR. When cells were transfected with si-lincRNA-p21, the expression of Collagen I, Collagen IV and fibronectin was down-regulated remarkably, which was reversed by miR-18b inhibitor significantly. These data suggests that lincRNA-p21 could promote extracellular matrix accumulation of MMCs in vitro.

In short, the present study characterizes the role of lincRNA-p21 in the pathogenesis of DN through interaction with miR-18b, and provides a novel therapeutic target for the treatment of DN.

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Disclosure of conflict of interest

None.

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