miRNA-33 expression and its mechanism in patients and model rats with type 2 diabetic nephropathy

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Abstract: Purpose: The microRNA-33 (miRNA-33) expression and the underlying mechanisms were investigated in the serum of patients with type 2 diabetic nephropathy (DN) and in the serum and nephridial tissues of DN model rats. Methods: A total of 60 patients were included in our study, 30 had type 2 DN and 30 were early diabetic patients without DN. Quantitative polymerase chain reaction (qPCR) was used to detect the miRNA-33 expression in the serum of these patients. Meanwhile, model rats with type 2 DN were induced by administering high-glucose and high-fat diets and a low dose of streptozotocin. The miRNA-33 expression levels in the serum and nephridial tissues of the model rats were detected through qPCR, and the sirtuin-6 (SIRT6) and nuclear factor-kappa B (NF-κB) protein levels in their nephridial tissues were determined through Western blot. The glomerular mesangial cells (GMCs) were transfected with miRNA-33 inhibitor and subjected to high-glucose stimulation to determine the expression levels of SIRT6 and NF-κB through Western blot, and the expression level of the transforming growth factor-β (TGF-β) in the culture supernatant through enzyme-linked immunosorbent assay (ELISA). Results: The DN patients exhibited the highest miRNA-33 level (P < 0.05), followed by diabetic patients with no DN and then by healthy individuals. When compared with the control group, the DN model rats had increased levels of miRNA-33 expression in their serums and nephridial tissues. In addition, their SIRT6 expression was down-regulated and NF-κB expression was up-regulated (P < 0.05). The GMCs with high-glucose stimulation in vitro showed increased miRNA-33 mRNA levels, and the inhibition of miRNA-33 in the GMCs increased the SIRT6 expression level but reduced the NF-κB expression level after high-glucose stimulation. The TGF-β secretion in the culture supernatant also decreased (P < 0.05). Conclusion: The miRNA-33 levels increased in the serums of the DN patients as well as in the serums and nephridial tissues of the DN model rats. These increases enhanced the NF-κB/TGF-β expression levels by targeting SIRT6, thus exacerbating the renal lesion.

Keywords: MicroRNA-33 (miRNA-33), diabetic nephropathy (DN), TGF-β

Introduction

Diabetic nephropathy (DN) is a severe complication of diabetes and has been the second cause of end-stage renal disease (ESRD) in China. The pathogenesis of this disease is extremely complex and includes various factors, such as glycolipid metabolism disorders, hemodynamic changes, oxidative stress, inflammatory cytokine production, advanced glycation end-product formation, genetic factors, kinin system and autophagy [1-3]. High glucose level facilitates DN development and can induce the activation and expression of inflammation- and fibrosis-related genes in inherent renal cells [4, 5]. Transforming growth factor-β (TGF-β), a major cytokine in the pathogenic cause of DN, is an important biomarker and therapeutic target for the development of DN [6, 7]. As patients with DN have poor quality of life, the pathogenesis of DN must be elucidated to determine methods for the prevention and treatment of this disease.

microRNAs (miRNAs) are noncoding single-stranded RNAs composed of 20-25 bp of nucleotides. They mediate the silencing of target genes through base pairing with the 3’ untranslated regions (3’UTRs) of mRNAs. Gene silencing results in mRNA degradation or repression. Emerging studies have shown that variation in miRNA expression levels plays an important regulatory role in the development and progression of DN through various mechanisms.
miRNA-33a/b gene is located at the intron regions of the sterol-regulatory binding protein genes [8]. It contains the transcription of ethylene-responsive element binding protein over the same period and can target multiple mRNAs (ABCA1, CROT, CPT1A, HADHB, AMPK, SIRT6 and G6PC) and thus regulate the intracellular cholesterol output, lipid oxidation and insulin signalling pathways [9]. Some studies have shown that miRNA-33 plays a negative role in metabolic syndrome, and its overexpression is correlated with high cholesterol and atherosclerosis [10, 11]. However, it remains unclear whether miRNA-33 participates in DN development.

SIRT6 is a member of the SIRT family and is mainly expressed in the nucleus, with both deacetylase and ADP-ribosyltransferase activities. Accumulating evidence revealed that SIRT6 regulates various biological functions including metabolism, ageing and stress resistance [12]. Thus, SIRT6 is a potential target of miRNA-33 because 3'UTR in SIRT6 contains the seed sequences that combine with miRNA-33 [8]. Some reports showed that SIRT6 negatively regulates NF-κB expression through the deacetylation of the NF-κB promoter and histone acetylation and thus modulates the initiation of inflammation [13, 14].

Thus, our study aims to compare and analyse the miRNA-33 expression levels in DN patients with those in healthy individual, to analyse the effects of miRNA-33 on DN pathogenesis by performing an in vitro experiment using model rats and regulating the SIRT6 protein and to further confirm the effects of miRNA-33 on DN via the SIRT6 protein.

Materials and methods

Clinical samples

All blood samples were collected from diabetic patients who received treatment from September 2013 to February 2015 in the First Affiliated Hospital of Xinxiang Medical University, China. A total of 60 patients were included in our study, and among them, 30 had type 2 DN and 30 were early diabetic patients without DN. Thirty healthy checkups in our hospital were selected as control (see Table 1). This study was conducted in accordance with the declaration of Helsinki and with the approval from the Ethics Committee of the First Affiliated Hospital of Xinxiang Medical University. Written informed consents were obtained from all participants.

Table 1. General data in each group of patients

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Male</th>
<th>Female</th>
<th>Average age (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal group</td>
<td>30</td>
<td>15</td>
<td>15</td>
<td>56.8 ± 6.2</td>
</tr>
<tr>
<td>Diabetes without DN group</td>
<td>30</td>
<td>16</td>
<td>14</td>
<td>50.7 ± 6.8</td>
</tr>
<tr>
<td>DN group</td>
<td>30</td>
<td>18</td>
<td>12</td>
<td>64.2 ± 5.8</td>
</tr>
</tbody>
</table>

Establishment of rat model with type 2 DN

Thirty Sprague Dawley (SD) rats with an average weight of 150 ± 5 g were purchased from Shanghai Lab, Animal Research Centre. The SD rats were divided into model group (20) and normal group (10) after a 1-week adoptive feeding. The model group was fed with high-glucose and high-fat diets (10% sucrose, 10% lard and 5% cholesterol) for 10 weeks after the intraperitoneal injection of streptozotocin (35 mg/kg) into each rat. Meanwhile, each rat in the normal group was administered with equal volume of citrate buffer through intraperitoneal injection after a 10-week normal diet. Two weeks after the start of the diet programmes, the fasting blood glucose (the rats underwent fasting 12 h before the test) was monitored using a Roche blood glucose metre. A glucose concentration of > 16.7 mmol/L was considered a successful model.

Total RNA extraction, reverse transcription reaction and qPCR

Approximately 5 mL of peripheral blood samples was collected from each of the participants in the early morning and incubated at 4°C overnight. The participant fasted before the collection of their blood samples. After incubation, the samples were centrifuged at 3000 × g for 10 min, and the supernatant was separated and stored at -80°C until use.

After the rats were anesthetised with chloral hydrate, their bilateral kidneys were removed. The renal cortices were collected after the renal capsules and perirenal connective tissues were stripped (the kidney medullas were removed as far as possible) and quickly stored.
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Table 2. Comparison of the expression levels of miRNA-33 mRNA in each group of patients

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>miRNA-33 mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal group</td>
<td>30</td>
<td>1.02 ± 0.05</td>
</tr>
<tr>
<td>Diabetes without DN group</td>
<td>30</td>
<td>1.58 ± 0.009*</td>
</tr>
<tr>
<td>DN group</td>
<td>30</td>
<td>1.77 ± 0.05*</td>
</tr>
<tr>
<td>F, P</td>
<td></td>
<td>1072.35, 0.000</td>
</tr>
</tbody>
</table>

Note: * Compared with normal controls, t=34.042, 44.210, P=0.000, 0.000; * compared with diabetic patient without DN, t=10.163, p=0.000.

Figure 1. Expression levels of miRNA-33 mRNA in the serums of the three groups. The expression levels of miRNA-33 mRNA was calculated as describled in the Methods. *P < 0.05 vs. Normal group; **P < 0.05 vs. Diabetes without DN group.

GMC culture, cell transfection and high-glucose stimulation

The GMC cell line (HBZY-1) (Centre for Type Culture Collection, Wuhan, China) was cultured in Dulbecco’s Modified Eagle’s Medium containing 15% foetal bovine serum (containing 5.6 mM of glucose). The GMCs were divided into normal group (5.6 mM of glucose), control group (5.6 mM of glucose and 24.4 mM of mannitol) and high-glucose group (30 mm glucose) and then cultured for 48 h. The cells were then collected to detect the effects of high glucose on the changes in miRNA-33 levels. To investigate the role of miRNA-33 on DN pathogenesis, the transfections of both miRNA-33 inhibitors (GenePharma Co., Ltd, Shanghai, China) (inhibitor group) and interference-free RNAs (control group) were performed using a GenEscort TMIII (WiseGen Company, Nanjing, China) after the 48 h culture at high-glucose conditions. Subsequently, qPCR was used to verify the inhibitory effects on miRNA-33 expression. The cells were then harvested to detect the changes in the SIRT6 and NF-κB proteins, whereas the supernatant was collected to detect the TGF-β levels.

Western blot

The nephridial tissues and GMCs in the rats were lysed in RIPA lysis buffer (Beyotime Company, Shanghai, China), and the protein concentration was determined using a BCA kit (Pierce, Rockford, IL, USA). 5 × SDS loading buffer were mixed and boiled at 100°C for 5 min. The resulting mixture was then stored at -20°C until use. The mixture was subjected to SDS-PAGE electrophoresis and then grafted on polyvinylidene fluoride (PVDF) mem-
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was incubated for 1 h with the corresponding horseradish peroxidase-labelled secondary antibody (dilution with 1:1000) (SunShine Biotechnology, Nanjing, China) at room temperature. Finally, electrochemiluminiscence (ECL) imaging system (Tanon 5200; Tanon, Shanghai, China) was used for detection. The ECL luminescent liquid was purchased from Thermo Co., USA, whereas the β-actin, which was selected as an internal reference, was provided by Nanjing SunShine Biotechnology Co., Ltd, Nanjing, China.

Enzyme-linked immunosorbent assay (ELISA)

The rat GMCs were transfected with phosphate-buffered saline (PBS) (normal group), control miRNA (control group) and miRNA inhibitors (inhibitor group) separately. After high-glucose stimulation, the supernatant was collected and stored at -80°C. After miRNA-33 inhibitors were added, the concentration of TGF-β secreted by the GMCs under high-glucose conditions was quantified using an ELISA kit (Biolegend, San Diego, CA, USA) according to the manufacturer’s instructions.

Statistical analysis

All data were performed in SPSS version 17.0 software (SPSS Inc, Chicago, IL, USA), and statistic graphs were constructed in GraphPad Prism 5.0 software (GraphPad Software Inc, San Diego, CA, USA). The observation data, as measurement data, were expressed as mean ± standard deviation (X ± S) through normality tests. The differences among the data were compared and analysed using t-test between two groups, whereas data in multiple groups were compared using single factor analysis of variance. P < 0.05 was considered statistically significant.

Results

Expression levels of miRNA-33 mRNA in the serums of DN patients and diabetic patients without DN

The expression levels of miRNA-33 mRNA in the serums of normal individuals, DN patients and diabetic patients without DN are shown in Table 2. The results from single factor analysis of variance showed that the fold changes among the three groups were significant (P <
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The expression levels of miRNA-33 in type 2 diabetic nephropathy (DN) model rats were significantly higher than those of the normal rats ($P < 0.05$). These results corroborated the key role of miRNA-33 in DN development.

Protein levels of SIRT6 and NF-κB in the nephridial tissues of DN model rats

The SIRT6 level in the nephridial tissues of the DN rats significantly decreased in contrast to the changes in their miRNA-33 level. Meanwhile, their NF-κB protein expression level increased in contrast to changes in their SIRT6 level (Figure 3). miRNA-33 was suggested to up-regulate the expression of NF-κB by targeting SIRT6 during DN development and thus facilitates kidney inflammation, which results in increased kidney damage [8, 13].

MiRNA-33 mRNA expression in the three GMC groups

The expression levels of miRNA-33 mRNA in the normal, control and high-glucose groups are shown in Figure 4. An integrated analysis showed statistical significance ($P < 0.05$). Meanwhile, the result of pairwise analysis showed that the miRNA-33 mRNA level was significantly up-regulated in the GMCs of the high-glucose group compared with those of the normal and control groups, and the differences were statistically significant ($P < 0.05$). This result was consistent with the results of the in vivo experiment. The miRNA-33 expression level was increased in the renal GMCs under high-glucose stimulation, and this condition can damage the kidney.

Effects of miRNA-33 inhibitors on the SIRT6, NF-κB and TGF-β levels in rat GMCs

After transfecting with PBS (normal group), control miRNA (control group) and miRNA-33 inhibitor (inhibitor group), the rat GMCs were cultured at high-glucose conditions for 48 h. The level of miRNA-33 in the inhibitor group significantly decreased compared with that of the control group (Figure 5). This result indicated that the inhibitory effect was satisfactory, and the interference-free RNA in the control group did not inhibit miRNA-33 expression. As shown in Figure 6, compared with the normal and control groups, the inhibitor group demonstrated an increase in SIRT6 protein level, whereas its NF-κB expression decreased (Figure 6) through the inhibition of miRNA-33 in...
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Discussion

DN is one of the most common microvascular lesions in diabetic patients and the leading cause of ESRD in some European and American countries. In China, the proportion of DN that leads to ESRD is also increasing yearly and has become one of the main causes of death in diabetic patients. Pathological changes in DN are characterised by excessive accumulation of the extracellular matrix (ECM), thickening of the glomerular and tubular basement membranes, progressive loss of foot processes, GMC proliferation, increased amount of ECM secretion and tubulo-interstitial fibrosis, which ultimately results in the generation of proteinuria and renal failure. These complications are due to the changes in the expression levels of several pathogenesis-related genes and proteins or the regulation disorders of the signal cascade reactions [15]. In this regard, exploring the pathogenesis of DN may provide a theoretical basis for the prevention and treatment of the disease.

As the key link of DN, high glucose levels can cause glucose metabolic disorders and change the renal haemodynamics, which is the basis of kidney diseases. Meanwhile, high blood glucose can damage cells, such as GMCs and podocytes [16, 17]. As one of the most important cells of glomerulus, GMCs can proliferate and ECM accumulation increases at high-glucose condition, which is the most important characteristic of DN. In the above process, hyperglycemia and elevated TGF-β as an initial factor of DN may cause glomerulosclerosis by activating the ERK1/2, PI3K, AMAP and kinase signalling pathway, which promote cell proliferation and collagen expression [18].

In the recent years, research has focused on microRNAs (miRNAs) because of their critical role in the regulation of post-transcriptional levels of protein-coding genes that may serve as key pathogenic factors in diseases [19]. Several miRNAs participate in the pathogenesis of DN (miRNA-377, miRNA-195 and miRNA-215) [20], whereas others exhibit renal protective effects (let-7b, miRNA-93 and miRNA-25). Therefore, targeting miRNAs involved in DN may be a good prospect for DN treatment.

In this study, we found that the expression levels of miRNA-33 in the sera of patients with diabetes increased, and these levels were
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exceeded by those in DN patients. The miRNA-33 levels in the serums and nephridial tissues of DN model rats also significantly increased, whereas the protein expression levels of SIRT6 in the nephridial tissues, as the possible target gene of miRNA-33, decreased. The NF-κB levels, negatively regulated by SIRT6, increased [14]. These results suggested that miRNA up-regulated NF-κB expression by targeting SIRT6, resulting in inflammation. To verify these results, we cultured rat GMCs in vitro and subjected them to a high-glucose treatment. The result showed that the miRNA-33 level was significantly up-regulated. However, after the miRNA-33 expression was inhibited, the SIRT6 level was elevated and the NF-κB level declined. All these findings confirmed the crucial role of SIRT6 diabetes progression.

In conclusion, miRNA-33 and SIRT6 are involved in the pathogenesis of diabetes and DN and can provide potential targets for DN prevention and treatment.

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Disclosure of conflict of interest

None.

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