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MicroRNA-21 protects from mesangial cell proliferation induced by diabetic nephropathy in db/db mice

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ABSTRACT

Diabetic nephropathy (DN) is a major diabetic complication. But the initiating molecular events triggering DN are unknown. Recent researches have addressed the role of microRNAs in diabetes and its complications. In this study, we looked for microRNAs expression during early DN, and showed microRNA-21 (miR-21) expression was downregulated in response to early DN in vitro and in vivo. Over-expression of miR-21 inhibited proliferation of mesangial cells and decreased the 24-h urine albumin excretion rate in diabetic db/db mice. Moreover, we identified PTEN as a target of miR-21. We also found PI3 K and p-Akt increased in miR-21 treated mesangial cells and db/db mice. Overall, these studies for the first time provide evidence for the potential role of miR-21 in early DN.
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1. Introduction

Diabetes is the leading cause of end-stage renal failure, and much of the morbidity and mortality of diabetes can be attributed to nephropathy. Diabetic nephropathy (DN) is characterized early in its course by glomerular hypertrophy and, importantly, mesangial hypertrophy, which is associated with the eventual glomerulosclerosis [1]. Despite the great progresses that have been made in recent decades, the mechanism involved in mesangial hypertrophy is not fully understood.

MicroRNAs (miRNAs) are a group of endogenous, small non-coding RNAs that can modulate protein expression [2]. Recently, it has been reported that miRNAs play a role in diabetes mellitus and its complications [3]. The pancreatic islet-specific miRNA, miR-375, plays a negative role in insulin secretion, and it is also a key determinant of blood homeostasis [4]. The over-expression of miR-29 aggravates insulin resistance [5]. MiR-133 promotes diabetic cardiopathy by inhibiting kalium ion channel of ERG gene, indicating the participation of miRNAs in diabetes mellitus and its complications [6]. However, the role of miRNAs in mediating mesangial hypertrophy of DN has never been studied.

In this study, we investigated the potential role of miRNA in mesangial hypertrophy during early DN in vitro and in vivo. Through a screen with miRNA array, we identified miR-21 as a regulator of early DN. First, over-expression of miR-21 inhibited proliferation of mesangial cells in high glucose condition. In addition, the 24-h urine albumin excretion rate (UAE) of diabetic db/db mice was decreased after being treated with plasmids containing miR-21 (30 mg/kg/d) for 4 weeks. Consistent with its function in vitro and in vivo, miR-21 regulates the expression of the DN-related genes, including PTEN [7], which has a role in organ size [8,9]. This has been well established. Taken together, our results strongly suggest that there is an important regulatory role of miR-21 in DN mesangial cell proliferation, implying that it might be a novel potential target for intervention and prevention of DN.

2. Materials and methods

2.1. Animals

C57BL/6J^{Lepr} background db/db, db/m mice from SLACCAS (Shanghai, China) were used and the guidelines on humane use and care of laboratory animals for biomedical research published by NIH (No. 85-23, revised 1996) were strictly complied for all

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animal experiments. Nine 8-week-old male db/db mice and nine db/m controls were killed for microarray and cell culture studies when significant elevation of UAE was detected in db/db mice. Glomeruli were taken from mice in both groups and were sieved from renal cortical tissue, as described previously [10]. A further 48 male mice at 4 weeks of age were randomized into 4 groups: control group (12 normal untreated db/m mice), miR-21-treated db/db group (12 mice), empty plasmid pGenesil-1-treated db/db group (12 mice), or untreated db/db group (12 mice). Mice were injected intraperitoneally with plasmids (30 mg/kg/d of miR-21 or 30 mg/kg/d of pGenesil-1) until UAE was significantly higher in the untreated db/db mice than the db/m normal control mice. Whole blood was obtained from the retro-orbital sinus of fed mice, and glucose and UAE were measured using an automatic biochemistry analyzer (Hitachi Ltd., Tokyo, Japan). After mice were killed, cortical sections of both kidneys from each mouse were pooled. A slice of renal cortical tissue from each mouse was also immediately frozen in liquid nitrogen for later use.

2.2. Cell culture, cloning and expression of miR-21

Primary mouse mesangial cells isolated and cultured as described previously [7,10], cultured in RPMI 1640 medium with 5 mmol/l glucose and 10% fetal bovine serum (FBS). Mmu-miR-21 gene sequences were selected from the miRBase at http://microrna.sanger.ac.uk/sequences/. Mir-21 (mmu-miR-21, MIMAT0000530) was chemically synthesized (Takara Shuzo, Shiga, Japan), and contained a SacI restriction enzyme cut site: mmu-miR-21-A 5′-CACCTAGCTTATCA GACTGATGTTGATTTTTTG-3′; mmu-miR-21-B 5′-AGCTCAAAAAAT CAACATCAGTCTGATAAGCTA-3′. The DNA segments were cloned into pGenesil-1 expression vectors (Wuhan Genesil Biotechnology Co. Ltd., Wuhan, China) and confirmed by DNA sequencing. The expression of miR-21 was induced by transfection of the plasmid into the cells using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's instructions. Cells were maintained in serum-free medium with 5 mmol/l glucose for 24 h, which was followed by incubation with fresh serum-free medium with 25 mmol/l glucose (high glucose) for 48 h. Control cells were incubated with 5 mmol/l glucose plus 20 mmol/l mannitol (low glucose) for 48 h. Mesangial cells in this study were divided into four groups: cells in low glucose as the normal control group, a miR-21-transfected high glucose group, empty plasmid pGenesil-1-transfected high glucose group, and untransfected high glucose group.

2.3. Isolation of total RNA

RNA was extracted by Trizol reagent (Invitrogen) as standard method.

2.4. MiRNA microarray

Small RNA separating, quality control, labeling, hybridization and scanning were performed by LC Sciences (Houston, TX, USA) using miRMouse_10.0_071620 miRNA array chip, based on Sanger miRBase Release 10.0. The array was repeated twice. Preliminary statistical analysis was performed by LC sciences on mean value normalized by Locally-weighted Regression (LOWESS) method on the background-subtracted data. Then, Student's test was performed to identify the different miRNA expression. MiRNAs with $P < 0.01$ was considered as having significant difference between DN and control.

2.5. Real time reverse transcription (RT)-PCR

MiR-21 expression was measured by real time RT-PCR (details shown in Supplementary data).

2.6. Growth inhibition test

MTT Cell Proliferation Assay Kit (Invitrogen) was employed to analyze cell proliferation (details shown in Supplementary data).

2.7. Glomerular morphological observation

Sieved renal glomeruli taken from mice were observed and measured under the microscope with a micrometer. The average diameter of 100 glomeruli in a group was counted in three different visual fields.

2.8. Bioinformatics analysis

Three programs, PicTar, miRanda, and TargetScan [11–13], were used to predict the targets of miR-21.

2.9. Western blot analysis

PTEN, PI3K p85α and phospho-Akt (Ser473) proteins were measured by Western blot using mouse anti-PTEN (Santa Cruz, CA), mouse anti-PI3K (Santa Cruz, CA) and rabbit anti- phospho-Akt (Cell Signaling Technology, Beverly, MA), respectively (details shown in Supplementary data).

2.10. Immunocytochemistry and immunohistochemistry

PTEN protein of mesangial cells was analyzed by immunocytochemistry, and PTEN protein of renal tissues was tested by immunohistochemistry using mouse anti-PTEN (Santa Cruz, CA) (details shown in Supplementary data). The average gray value of 50 sections in each group was measured by Beihang CM-2000 Biomedical Image Analysis System software (Beihang, Beijing, China).

2.11. Immunofluorescence staining

PI3K p85α and phospho-Akt (Ser473) proteins were detected by immunofluorescence using mouse anti-PI3K (Santa Cruz, CA) and rabbit anti-phospho-Akt antibody (Cell Signaling Technology), respectively (details shown in Supplementary data).

2.12. Statistical analysis

All statistical tests were performed using SPSS software (SPSS, Chicago, IL). Comparisons between groups were carried out by ANOVA or Kruskal–Wallis tests, as appropriate, according to the Gaussian or non-Gaussian distribution of the data. Changes in each group were analyzed using one-way ANOVA or non-parametric tests. P-values < 0.05 were considered statistically significant. All data are presented as means ± standard deviation (S.D.).

3. Results and discussion

3.1. Reduced expression of miR-21 in db/db DN mice

To study the potential role of miRNA in DN, we used the miRMouse_10.0_071620 miRNA array, which contained 568 probes, to scan miRNAs that were differentially expressed between db/db DN mice and controls. We used db/db mice with hyperglycemia and significant elevation of urinary albumin excretion were used as animal model of the early stage of DN because db/db mice develop kidney disease with similarities to human diabetic nephropathy [14]. Among 568 miRNAs screened, 66 miRNAs exhibited significantly different expression level between glomerular tissues of db/db DN mice and db/m controls (Supplementary Table 1). Of

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