High glucose-induced apoptosis in human kidney cells was alleviated by miR-15b-5p mimics

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Abstract

MicroRNAs were involved in a wide range of biological processes of diabetic nephropathy (DN). It is reported that miR-15b-5p was downregulated in the patients with DN. However, the mechanisms underlying the regulatory effects of miR-15b-5p on patients with diabetes remain unclear. Thus, this study aimed to investigate the role of miR-15b-5p during high glucose (HG)-induced apoptosis in human kidney cells. QRT-PCR was used to detect the level of miR-15b-5p. CCK-8 assay, EdU staining assays and flow cytometry were used to detect cell proliferation, apoptosis respectively in vitro. In addition, western blotting was used to determine active caspase-3, cleaved PARP, p-AKT, p-mTOR, p-S6, p-JNK, p-p38 and p-ERK proteins levels. The expression of miR-15b-5p in patients with DN were dramatically decreased compared with health persons. Similarly, HG down-regulated the expression of miR-15b-5p in HK-2 cells. In contrast, miR-15b-5p mimics alleviated HG-induced apoptosis in HK-2 cells via decreasing the expressions of active caspase 3 and cleaved PARP. EdU detection further confirmed that miR-15b-5p mimics attenuated the anti-proliferation effect of HG in HK-2 cells. Furthermore, HG-induced Akt/mTOR pathway downregulation and JNK upregulation were markedly reversed by miR-15b-5p mimics in cells. The data suggested that miR-15b-5p mimics protects HK-2 cells from HG-induced apoptosis. The anti-apoptotic effects of miR-15b-5p may due to the activation of the Akt/mTOR pathway as well as inactivation of JNK. Taken together, miR-15b-5p might be a potential therapeutic target for the treatment of patients with DN.

Keywords: diabetic nephropathy, miR-15b-5p, glucose, HK-2 cells, apoptosis
Introduction

Recent report showed that about 7.7% of the world population will be attacked by diabetes by 2030 [1]. Both the incidence and prevalence of diabetes continue to rise [2]. Podocytes, glomerular endothelia and mesangial cells, might be damaged by diabetes [3]. Diabetes is known to trigger nephropathy [3]. DN is one of the most dangerous complications of diabetes [2, 4]. The vital pathologic features of DN include glomerular hypertrophy and hyperfiltration, tubulointerstitial fibrosis, increased urinary albumin secretion and low grade of renal inflammation [5-8]. Therefore, searching better therapeutic agents for the treatment of early stages of DN in diabetic patients is necessary.

MicroRNAs (miRNAs) are a kind of short RNAs (~18 to 24 nucleotides) [9], which affect multiple cellular processes and disease states via regulating gene expression by posttranscriptional and epigenetic mechanisms [10]. In addition, miRNAs regulate gene expression in cell proliferation, differentiation, apoptosis, and carcinogenesis [11]. Previous studies indicated that several miRNAs play a vital role during the process of diabetes [12, 13].

MiR-15b-5p is associated with proliferation, apoptosis and vascular tube formation [14-17]. It is reported that miR-15b-5p was decreased in the patients with DN [18]. However, the mechanisms underlying the regulatory effects of miR-15b-5p on patients with DN remain unclear. Therefore, this study aimed to investigate the role of miR-15b-5p during the process of diabetes by using in vitro cell model.

Material and methods

Cell cultures

HK-2 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA), and cultured in Dulbecco modified Eagle medium (DMEM) (Gibco, NY, USA) with 10% fetal bovine serum (FBS) (Sigma Aldrich St. Louis, MO, USA) with supplemented with 1% Penicillin-Streptomycin at 37°C in 95%humidified air and 5% CO₂. HKC-5 was a gift from Dr. Ke Wu, Zhengjiang University, China and
maintained in DMEM-F12 (1:1, Gibco, NY, USA) supplemented with 10% FBS.

In order to conduct the following assays, cells were seeded into plates and divided into four groups: (1) Control group (control); (2) high glucose group (HG; 50 mM glucose); (3) high glucose plus mimics-control (HG + mimics-ctrl); (4) high glucose plus miR-15b-5p mimics (HG + miR-15b-5p mimics).

MiR-15b-5p mimics transfection

MiR-15b-5p mimics (5'-UAGCAGCACAUCAUGGUUUACA-3') and mimics control (5'-UAGCAGCACCAAGGUCCACA-3') were provided by GenePharma (Shanghai, China). HK-2 and HKC-5 cells in the logarithmic phase were transfected with 10 nM miR-15b-5p mimics and mimics control with Lipofectamine® 2000 (Thermo Fisher Scientific, Waltham, MA, USA) for 6 h. After culture for 48 h, cells were collected for the following experiments.

Quantitative real-time-PCR (qRT-PCR)

qRT-PCR was used for measuring miR-15b-5p levels in cells. First, total RNAs were extracted from human kidney cells lysate using RNA extract kit (Tiangen Biotech Co., Ltd., Beijing, China). cDNA was synthesized with Reverse Transcriptase using an oligo dT primer (Thermo Fisher). Next, real-time PCR was performed on ABI PRISM-7700 sequence detection system (Thermo Fisher) with a total of 20 ml PCR reaction mixture using SYBR Green Real-Time PCR Master Mixes (Thermo Fisher). The forward and reverse primers for miR-15b-5p and U6 were contained from GenePharma (Shanghai, China). MiR-15b-5p: ATCCAGTGCGTGTCGTG-F; TGCTTAGCAGCACATCATG-R. U6: GCTTCGGCAGCACATATACTAAAAT-F; CGCTTCACGAATTTGCGTGTCAT-R. The expression of genes were measured using the 2^ΔΔCt as folder changes. Calculated miR-15b-5p level was normalized to level of U6.

CCK-8 assay

Cell viability was detected by using CCK-8 kit (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer’s protocols. In order to perform a cell survival assay, HK-2 and HKC-5 cells (5×10^3 cells per/well) were incubated in 96-well
plate for 48 h. After that, cell counting kit-8 solution (10 μL) was added into each well. Then the plate was cultured for another 1.5 h. The OD value was analyzed at 450 nm using an enzyme-linked immunosorbent assay reader (ELX-800, BioTek Instruments, Inc., Winooski, VT, USA).

**Flow cytometric analysis of cell apoptosis**

Harvested HK-2 and HKC-5 cells (1×10^6 cells) were stained with dual-staining Annexin V-FITC-propidium iodide (PI) (Thermo Fisher) according to the manufacturer’s protocols. The cells were analyzed on the flow-cytometer (BD, Franklin Lake, NJ, USA) after treatments. The software WinMDI 2.9 (Thermo Fisher) was used to analyze the results. Annexin V positive: early apoptosis marker; Annexin V/PI positive: late apoptosis marker.

**Western blot analysis**

Harvested cells were collected in cell lysate buffer (Sigma, St. Louis, MO, USA). The equal amount protein was quantified by BCA protein assay kit (Beyotime). The proteins were loaded on 10% SDS-PAGE and subsequent was electrophoretic transferred to polyvinylidene fluoride membrane (PVDF, Thermo Fisher). PVDF membrane were blocked by a 5% skimmed milk incubation in TBST buffer for 50 min. Then membranes were subjected to primary antibodies: anti-active caspase 3 (Abcam ab2302) (1:1000), anti-cleaved PARP1 (Abcam ab32064) (1:1000), anti-β-actin (Abcam ab8227) (1:1000), anti-p-Akt (Abcam ab8932) (1:1000), anti-p-mTOR (Abcam S2448) (1:1000), anti-p-P70S6K (Abcam ab109393) (1:1000), anti-p-JNK (Abcam ab207477) (1:1000), anti-p-p38 (Abcam ab4822) (1:1000), anti-p-ERK (Abcam ab192591) (1:1000) overnight at 4°C. Then, incubation with peroxidase-conjugated secondary antibodies HRP-labeled anti-rabbit (1: 5000, PTG, USA) for 1 h in the next day. The bands were presented by reacting with ECL (Santa Cruz Biotechnology). In order to quantify the blots, all values were normalized to those of β-actin.
EdU staining

Harvested HK-2 cells (1×10^6 cells) were cultured with EdU labeling medium (50 µM) for 2 h. EdU detection was performed according to the EdU kit specification (Thermo Fisher) described method.

Statistical analysis

Each group were performed at least three independent experiments and all data were expressed as the mean ± SD. Student’s t-test was used to compare the difference between two groups. The differences among multiple groups were made with one-way analysis of variance (ANOVA) followed by Dunnett’s test. P<0.05 or P<0.01 was considered to demonstrate a statistically significant difference (*P<0.05, ** P<0.01).

Results

MiR-15b-5p mimics increased the level of miR-15b-5p in HK-2 cells

We first explored the effects of HG on the expression of miR-15b-5p in HK-2 cells. As indicated in Fig. 1A, both 30 and 50 mM HG time-dependently inhibited the level of miR-15b-5p in HK-2 cells. In contrast, miR-15b-5p mimics time-dependently upregulated the expression of miR-15b-5p in cells (Fig. 1B). Therefore, 48 h HG incubation was chosen for use in subsequent experiments. Furthermore, HG-induced miR-15b-5p downregulation was notably reversed by miR-15b-5p mimics (Fig. 1C). These data indicated that the levels of miR-15b-5p were down-regulated by HG treatments in HK-2 cells.

HG-induced growth inhibition and apoptosis in human kidney cells were alleviated by miR-15b-5p mimics

Next, CCK-8 assay was used to determine the effect of miR-15b-5p mimics on HK-2 cell viability with or without HG. As revealed in Fig. 2A, HK-2 cell viability was markedly decreased by HG. However, HG-induced cell growth inhibition was significantly attenuated by miR-15b-5p mimics. In addition, HG could markedly induce cell apoptosis (Annexin V positive and Annexin V/PI double positive cells), which was
alleviated by miR-15b-5p mimics as well (Fig. 2B, 2C). Moreover, apoptosis-related proteins active-caspase 3 and cleaved PARP were detected by western blotting. As shown in Fig. 2D-2F, the expressions of active-caspase 3 and cleaved PARP were dramatically increased by HG. Nevertheless, the increases of these proteins was significantly inhibited following transfection with miR-15b-5p mimics, compared with the HG group (P<0.01).

We next performed EdU fluorescence assay to further reveal the effect of miR-15b-5p mimics on cell proliferation. The data indicated that HG notably decreased the EdU positive cells, while miR-15b-5p mimics markedly increased Edu positive cells in the presence of HG (P<0.01). (Fig. 2G, 2H). Additionally, the repeated CCK8 and apoptosis experiments using another human kidney cell line HKC-5 were consistent with those results using HK-2 cells (Supplementary Fig. 1A-1D). In addition, miR-15b-5p mimics had no effect on the HK-2 cell viability and apoptosis (Supplementary Fig. 2A-2E). All these data illustrated that miR-15b-5p mimics could alleviate HG-induced growth inhibition and apoptosis in human kidney cells.

**MiR-15b-5p mimics protected HK-2 from HG via activating of Akt/mTOR pathway**

Previous studies have demonstrated that Akt/mTOR signaling pathway is vital for cellular proliferation and growth [18, 19]. Whether miR-15b-5p protected HK-2 cell from HG by activating of Akt/mTOR pathway remains unclear. As revealed in Fig. 3A-3D, the expression of p-Akt, p-mTOR and p-p70S6K were significantly decreased in HG-treated cells. However, the decrease of p-Akt, p-mTOR and p-p70S6K proteins were notably reversed by following transfection with miR-15b-5p mimics in cells. These data illuminated that miR-15b-5p mimics protected HK-2 cell from HG partly via activating of Akt/mTOR pathway.

**MiR-15b-5p mimics protected HK-2 cells from HG via inhibition of JNK pathway**

Since JNK, p38 and ERK pathways play important roles in mediating cell apoptosis [20, 21], further studies were performed to explore if these pathways were involved in. The western blot revealed HG significantly upregulate p-JNK expression, not p-p38 or
p-ERK in HK-2 cells (Fig. 4A-4D). In addition, HG-induced p-JNK upregulation was completely reversed by miRNA-15b mimics (Fig. 4A, 4B). All these results revealed that miR-15b-5p mimics protected HK-2 cells from HG partly via inhibiting of JNK pathway.

Discussion

MicroRNAs have been demonstrated to be associated with multiple cytological processes of DN, including cell proliferation and apoptosis [22-24]. In this study, our results indicated that the expression of miR-15b-5p was significantly decreased following treatment with HG in HK-2 cells. Similarly, Li et al found that miR-25 was markedly decreased in patients with DN, and miR-25 was decreased by HG treatment in HK-2 cells [25]. Thus, this finding has indicated that miR-15b-5p may play a vital role during the development of DN. In addition, the results showed that HG could induce apoptosis of HK-2 cells. Consistently, Wang et al found that HG could induce apoptosis of podocyte in DN [26]. Moreover, miR-15b-5p mimics could induce cell apoptosis through regulating Rab1A in liver cancer [27]. However, in the current study miR-15b-5p mimics alleviated HG-induced apoptosis in HK-2 cells via decreasing the levels of active-caspase 3 and cleaved PARP. The discrepancy between our result and previous studies might reflect differences in the cell types. Li et al indicated that miR-25 suppressed HG-induced apoptosis in HK-2 cells as well [25]. Our results were consistent with previous study [18], demonstrating that miR-15b-5p mimics could attenuate HG-induced apoptosis in HK-2 cells.

AKT and mTOR play critical role in regulating diverse cellular functions, including cell metabolism, growth, survival and migration [28-32]. mTOR phosphorylates and activates p70S6K on Thr 389 [31]. Lee et al found that HG could inhibit mTORC1 activity [33]. Our results verified this report by revealing that mTOR signaling pathway may be associated with HG-induced apoptosis in HK-2 cells. However, these effects were alleviated in the presence of miR-15b-5p mimics, demonstrating that
miR-15b-5p mimics activated mTOR signaling pathway via increasing the level of p-Akt, p-mTOR and p-p70S6K. Therefore, we proposed miR-15b-5p may protect HK-2 cells from HG partly via activation of mTOR pathway.

MAPKs include three kinases, JNK, p38 and ERK. MAPKs signaling pathway mediate a plenty of biological processes and receive and transmit all kinds of cellular signals such as cell apoptosis [34]. Our results indicated that HG activated the expression of p-JNK, while miR-15b-5p mimics completely attenuated the upregulation of p-JNK. Zhang et al found that apoptosis induction was dependent on the activation of JNK signaling pathway [35]. Han et al found that agmatine decreased the expression of p-JNK in HG-treated Müller cells [34]. Our results were consistent with these findings. Therefore, miR-15b-5p mimics may protect HK-2 cells from HG via partly inactivation of JNK. In addition, the results indicated that neither miRNA-15b mimics nor mimics-ctrl influenced the expression of p-p38 and p-ERK in HG-treated HK-2 cells. Thus, the role of MAPKs in HG-treated HK-2 cells has not yet been well recognized and require further investigation. The novelty of this study lies in the finding that miR-15b-5p mimics play an important role in protecting HK-2 cells through activation of mTOR pathway and downregulation of JNK signaling. However, the direct target of miR-15b-5p is still under investigation and we do not reach a conclusion yet.

Conclusions

In conclusion, miR-15b-5p may protect HK-2 cells from HG via upregulation of AKT/mTOR and downregulation of JNK pathways. Therefore, miR-15b-5p might act as a potential therapeutic target for the treatment of patients with DN.

Conflicts of interest

The authors declare no conflict of interest.
Supplementary Materials

The online version of this article contains supplementary materials.
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Figure 1 MiR-15b-5p mimics upregulated the level of miR-15b-5p in HG-treated HK-2 cells. (A) HK-2 cells were incubated with 30 or 50 mM HG for 12, 24, and 48 h, and qRT-PCR was used to detect the level of miR-15b-5p. (B) After transfection with miR-15b-5p mimics, the level of miR-15b-5p in cells were detected with qRT-PCR. (C) HK-2 cells were treated with HG or/and miR-15b-5p for 48h, and qRT-PCR was used to detected the level of miR-15b-5p. **P < 0.01 vs. control, ##<0.01 vs. HG.
Figure 2 MiR-15b-5p mimics attenuated HG-induced growth inhibition and apoptosis in HK-2 cells. HK-2 cells were treated with HG or/and miR-15b-15p mimics for 48h. (A) Cell viability was measured using CCK-8 assay. (B) Apoptotic cells were observed with Annexin V/PI staining. The rate of apoptotic cells were calculated. (C) The rate of apoptotic cells were calculated. (D) Expressions of active caspase-3, cleaved PARP in HK-2 cells were analyzed by western blotting. (E) Active caspase-3 relative expression was quantified by normalizing to β-actin. (F) Cleaved PARP relative expression was quantified by normalizing to β-actin. (G) Images of the HK-2 cells stained with EdU and DAPI. (H) Relative fluorescence expressions were quantified by calculation of EdU and DAPI positive cells. **P<0.01 vs. control.
Figure 3 MiR-15b-5p mimics protected HK-2 cells from HG via activating of Akt/mTOR pathway. HK-2 cells were treated with HG or/and miR-15b-15p mimics for 48h. (A) Expressions of p-Akt, p-mTOR and p-p70S6K analyzed by western blotting in HK-2 cells. (B) P-Akt relative expression was quantified by normalizing to β-actin. (C) P-mTOR relative expression was quantified by normalizing to β-actin. (D) P-p70S6K relative expression was quantified by normalizing to β-actin. **P<0.01 vs. control, ##P<0.01 vs. HG.
Figure 4 MiR-15b-5p mimics protected HK-2 cells from HG via inhibition of JNK pathway. HK-2 cells were treated with HG or/and miR-15b-15p mimics for 48h. (A) Expressions of p-JNK, p-p38 and p-ERK analyzed by western blotting after in HK-2 cells. (B) P-JNK relative expression was quantified by normalizing to β-actin. (C) P-p38 relative expression was quantified by normalizing to β-actin. (D) P-ERK relative expression was quantified by normalizing to β-actin. **P<0.01 vs. control, ###P<0.01 vs. HG.