High Glucose-Induced Apoptosis in Human Kidney Cells Was Alleviated by miR-15b-5p Mimics

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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. Quantitative real-time (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 poly(ADP-ribose) polymerase (PARP), phosphorylated (p)-AKT, p-mammalian target of rapamycin (mTOR), p-S6, p-c-Jun N terminal kinase (JNK), p-p38 and p-extracellular signal-regulated kinase (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.

Key words diabetic nephropathy; miR-15b-5p; glucose; HK-2 cell; apoptosis

INTRODUCTION

Recent report showed that about 7.7% of the world population will be attacked by diabetes by 2030. Both the incidence and prevalence of diabetes continue to rise. Podocytes, glomerular endothelia and mesangial cells, might be damaged by diabetes. Diabetic nephropathy (DN) is one of the most dangerous complications of diabetes. The vital pathologic features of DN include glomerular hypertrophy and hyperfiltration, tubulointerstitial fibrosis, increased urinary albumin secretion and low grade of renal inflammation. 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 (ca. 18 to 24 nucleotides), which affect multiple cellular processes and disease states via regulating gene expression by posttranscriptional and epigenetic mechanisms. In addition, miRNAs regulate gene expression in cell proliferation, differentiation, apoptosis, and carcinogenesis. Previous studies indicated that several miRNAs play a vital role during the process of diabetes.

MiR-15b-5p is associated with proliferation, apoptosis and vascular tube formation. It is reported that miR-15b-5p was decreased in the patients with DN. 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.

MATERIALS AND METHODS

Cell Cultures HK-2 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, U.S.A.) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, NY, U.S.A.) with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, U.S.A.) 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, U.S.A.) 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’-UAGCAGCACAUCAUGCUUACAG-3’) and mimics control (5’-UAGCAGCACAAAGGUCACGCA-3’) were provided by GenePharma (Shanghai, China). HK-2 and HKC-5 cells in the logarithmic phase were transfected with 10nM miR-15b-5p mimics and mimics control with Lipofectamine® 2000 (Thermo Fisher Scientific, Waltham, MA, U.S.A.) for 6h. After culture for 48h, cells were collected for the following experiments.

Quantitative Real-Time (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
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 was detected with qRT-PCR. (C) HK-2 cells were treated with HG or/and miR-15b-5p for 48 h, and qRT-PCR was used to detect the level of miR-15b-5p. **p < 0.01 vs. control, ***p < 0.01 vs. HG.

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 HG-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 (Figs. 2B, C). Moreover, apoptosis-related proteins active-caspase 3 and cleaved PARP were detected by Western blotting. As shown in Figs. 2D–F, 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 (\( p < 0.01 \)). (Figs. 2G, H). 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 Figs. 1A–D). In addition, miR-15b-5p mimics had no effect on the HK-2 cell viability and apoptosis (Supplementary Figs. 2A–E). 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 Figs. 3A–D, 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 path-
Fig. 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.

Fig. 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.
ways play important roles in mediating cell apoptosis, further studies were performed to explore if these pathways were involved in. The Western blot revealed HG significantly up-regulate p-JNK expression, not p-p38 or p-ERK in HK-2 cells (Figs. 4A–D). In addition, HG-induced p-JNK upregulation was completely reversed by miRNA-15b mimics (Figs. 4A, B). 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. 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. 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. Moreover, miR-15b-5p mimics could induce cell apoptosis through regulating Rab1A in liver cancer. 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. Our results were consistent with previous study, 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. mTOR phosphorylates and activates p70S6K on Thr 389. Lee et al. found that HG could inhibit mTORC1 activity. 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.

Mitogen-activated protein kinases (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. 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. Han et al. found that agmatine decreased the expression of p-JNK in HG-treated Müller cells. 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.

CONCLUSION

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.

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

REFERENCES


