Down-regulation of miR-30b reduces cardiomyocyte apoptosis by targeting Bcl-2 in diabetic cardiomyopathy

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Abstract: Diabetic cardiomyopathy (DCM) is a major complication of diabetes that contributes to the subsequent development of heart failure and increased mortality. Cardiomyocytes apoptosis is considered an important cause for the cardiomyopathy. MicroRNAs have been reported to regulate cell growth, differentiation and cell apoptosis in variety of diseases including cardiovascular disease. However, little is known about the microRNAs regulating cardiomyocytes apoptosis in DCM. We established a rat model of DCM and carried out a microarray to identify the differentially expressed microRNAs in myocardial tissue. Our findings showed that the expression of miR-30b was significantly upregulated in diabetic rats. The present study was designed to investigate the pathogenic role of miR-30b in the development of DCM. To explore the molecular mechanisms involved, we performed in vitro experiments using cultured H9c2 cells. Our results showed that miR-30b expression was associated with high glucose and increased cardiomyocytes apoptosis. Decreased expression of miR-30b by transfecting with miR-30b inhibitor was found to inhibit apoptosis in cardiomyocytes exposed to high glucose. The 3’UTR of Bcl-2 was cloned downstream of a luciferase reporter construct and co-transfected into HEK293 cells with miR-30b mimic. The results of luciferase assay indicated that Bcl-2 might be a direct target of miR-30b. The expression of Bcl-2 was downregulated in cardiomyocytes transfected with miR-30b mimic, which consequently induced cellular apoptosis. In conclusion, our study demonstrates that miR-30b is involved in the regulation of high glucose-induced apoptosis by targeting Bcl-2, which may provide a novel therapeutic strategy for the treatment of DCM.

Keywords: Down-regulation, miR-30b, apoptosis, Bcl-2, diabetic cardiomyopathy (DCM)

Introduction

Diabetes mellitus remains a worldwide health problem and is known to be associated with increased risk of cardiovascular morbidity and mortality even in the absence of hypertension and coronary atherosclerosis [1]. Diabetic cardiomyopathy (DCM) is a major diabetic complication [2].

Several studies have shown that hyperglycemia as an independent risk factor directly causes cardiac damage, leading to diabetic cardiomyopathy [3-6]. Apoptosis of cardiomyocytes is considered a significant consequence of inflammatory response and oxidative stress, which is attributable to hyperglycemia in the cardiac tissue and a key pathological change in DCM [7]. Studies have confirmed that cardiomyocytes apoptosis is the cause of contractile units lost and reparative fibrosis in DCM [8]. It is believed that cardiomyocytes apoptosis increased in hearts from streptozotocin (STZ)-induced diabetic animals [9]. Therefore, management of diabetes-associated heart disease is a major concern that has not been effectively addressed.

MicroRNAs (miRs) are involved in the pathogenesis of DM or DM-associated complications [10-12]. miR-21 contributes to myocardial impairment by regulating MAP kinase transduction pathway in cardiac fibroblasts [13]. miR-133a, miR-200c plays a role in diabetes-induced cardiomyocyte hypertrophy [14, 15]. miR-1/miR-206 regulates cardiomyocytes apoptosis in diabetic cardiomyopathy by targeting IGF1 and Hsp60 and miR-30c mediates upregulation of Cdc42 and Pak1 in Diabetic Cardiomyopathy [16, 17]. These findings sug-
gest that miRs are important mediators in DCM and might be potential therapeutic targets to prevent diabetes-related heart disease.

The underlying molecular mechanism for hyperglycemia-induced cardiomyocytes apoptosis comprises of an extensive network of signaling pathways [18] and few miRs hyperglycemia-induced cardiomyocytes apoptosis have been reported so far. Thus, far more miRs and their underlying functions in diabetes-related heart disease are required to be demonstrated.

In this study, we aimed to identify miR biomarkers in myocardial tissue from a rat model of diabetic cardiomyopathy (DCM) and to explore the potential pathogenic role of differentially expressed miRs in diabetes-induced cardiomyopathy. We showed that miR-30b is significantly increased in myocardial tissue with DCM and that increased miR-30b levels lead to increased cardiomyocytes apoptosis through downregulation of Bcl-2, a negative regulator of apoptosis.

Materials and methods

Animal model of diabetic cardiomyopathy

High-fat diet (HFD) and two low-dose streptozotocin (STZ) were used to develop the DCM rat model according to the described protocol by Zhang et al. [9]. Briefly, male Wistar rats (n = 6) (approximately 180 g of body weight) were fed with HFD (22% fat, 48% carbohydrate, and 20% protein) for four weeks, followed by two injections of STZ (i.p. 30 mg/kg body weight, in 0.1 M sodium citrate buffer and pH 4.4), a week apart. Sex and body weight matched control rats were given an equal volume of citrate buffer. DCM was confirmed by echocardiography (two-dimensional M-mode echocardiography), morphological, histopathological examination. Animal handling conformed to the guidelines for care and use of experimental animals established by the Ethical Committee of Animal Experiments.

Cell culture and induction of high-glucose state in cells

Rat cardiomyocytes cell line (H9c2) derived from the rat embryonic heart was purchased from the Chinese Academy of Science cell repository and was cultured in DMEM and supplemented with 10% fetal bovine serum. For high-glucose treatment, cells were starved in DMEM containing 0.1% FBS for 24 h and incubated in DMEM containing either 50 mM D-glucose (High-glucose group) or 5.5 mM (Normal-glucose group) for 0-72 h [19].

miRNA profiling

Total RNA was extracted from cardiac tissue of rat model of DCM using the RecoverAll Total RNA Isolation Kit (Ambion Inc, Austin, TX). RNA qualities were assessed with NanoDrop 2000 to determine the concentration and 260/280 ratio and TaqMan RT-PCR for a discrete panel of miRs. Three total RNA samples passing the quality examination from three cardiac tissues were used for miR profiling studies by Asuragen Inc (Austin, TX). One hundred nanograms of total RNA per sample was purified and hybridized to Affymetrix Gene Chip miRNA arrays (Santa Clara, CA), which contain probes of 847 rat-specific mature miRNAs. The intensity values of the microarray data were subjected to robust multi-array average background correction and quartile normalization, followed by mean summarization. A two-way ANOVA was performed to distinguish genes significantly changed between Normal and DCM group.

Real-time PCR

Total RNA was isolated from myocardial tissues and cardiomyocytes using TRizol Reagent (Invitrogen). RNA was reverse transcribed using SuperScript First Strand cDNA System (Invitrogen) according to the manufacturer’s instructions. Quantitative PCR was conducted using SYBR Green Taq Mix (Takara) on a Bio-Rad Real-Time PCR System, and β-actin was used as internal control for normalization. For miRNA real-time PCR, the miR-specific primers from the TaqMan miR assays (Applied Biosystems) were applied, and the snRNA U6 was used as an endogenous reference.

Western blotting

Protein homogenates were prepared from cardiomyocytes. Equal amounts of protein (50 μg) were separated by SDS/PAGE (10% gel), transferred onto nitrocellulose membranes and blocked by 5% non-fat milk. The membranes were incubated with primary antibodies at 4°C overnight, and then were incubated with horse radish peroxidase (HRP)-conjugated secondary
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Figure 1. Differentially expression of miRs identified by Affymetrix microRNA microarray in myocardial tissue of DCM. A. Heatmap of differentially expression of miRs. B. Real-time analysis of miR-30b in 10 myocardial tissues of Normal and DCM group. The relative amount of miRNA was normalized to U6 snRNA. Triplicate assays were performed for each RNA sample, P < 0.01 compared with Normal group.

Annexin V-FITC/PI staining

Cell apoptosis was analyzed by flow cytometry (FACS Calibur, BD) as described previously [20]. According to the manufacturer’s instructions for the Annexin V-FITC/PI Apoptosis Detection Kit (BD Biosciences), H9c2 cells treated with high glucose for 0-72 h, and cells transfected with inhibitors/NCs for 36 h, were collected. The apoptotic rate was calculated as the percentage of Annexin V-positive and PI-negative cells divided by the total number of cells in the gated region.

Luciferase reporter assay

To confirm whether Bcl-2 was a direct target of miR-30b, we performed luciferase reporter experiments in HEK-293 cells. The 3’-untranslated regions (UTR) of Bcl-2 were cloned into the downstream of luciferase gene to generate Luc-Bcl-2-Wt vector. The 3’-UTR without predicted miR-30b binding site was constructed to generate Luc-Bcl-2-Mut vector. For luciferase assay, cells were plated in 24-well culture plates, and then transfected with either wild-type or mutant construct with and without miRNA mimic or negative control. Luciferase activity was detected 48 hours after transfection using the Dual Luciferase Reporter Assay System (Promega).
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A

High-glucose Incubation

0 h

24 h

48 h

72 h

B

Normal-glucose Incubation

0 h

24 h

48 h

72 h
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**Figure 2.** Determination of cell apoptosis in cardiomyocytes in high and normal level of glucose group. A. The cell apoptosis detected by Annexin V/PI staining by flow cytometry in cardiomyocytes exposed to high level of glucose. B. The cell apoptosis detected by Annexin V/PI staining by flow cytometry in cardiomyocytes exposed to normal level of glucose. C. The columns show the qualification of apoptotic rates in the charts in the upper panel. *P* Values are significantly different from Ctrl. **P* < 0.01 Vs. 0 h group.
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miR-30b expression was upregulated in DCM

To identify the differentially expressed miRs in myocardial tissue of DCM, we established a rat model of diabetic cardiomyopathy (DCM) and compared miR expression from myocardial tissue in normal and DCM group. Using microRNA microarray, we identified 13 miRs with > 1.2-fold differences in their expression in myocardial tissue samples including baseline and repeated samples from 6 rats. Of these, miR-30b was upregulated nearly 2.5-fold in the myocardium of DCM group compared with Normal group (Figure 1A). The differential expression of miR-30b was validated by Real-time PCR including 10 myocardial tissues from rats with or without DCM respectively. Consistent with the microarray findings, miR-30b was shown to be dramatically upregulated in DCM group (Figure 1B).

High levels of glucose induced cardiomyocyte H9c2 apoptosis in vitro

To explore whether the induction of cell apoptosis in myocardium of diabetic mice is related directly to hyperglycemia, we used cardiomyocytes H9c2 to determine the effect of high levels of glucose on apoptosis in cultured cells and detected by Annexin V/PI staining by flow cytometry. As shown in Figure 2, the apoptosis rate of cardiomyocytes increased gradually with prolonged incubation in high levels of glucose (Figure 2A and 2C), while there was no changes in normal glucose group (Figure 2B and 2C).

miR-30b expression was increased in high glucose treated cardiomyocytes

High glucose treated cardiomyocytes showed significantly increased apoptotic protein (cle-
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Figure 4. MiR-30b was involved in the regulation of high glucose-induced apoptosis. A. Cardiomyocytes were transfected with miR-30b inhibitor or Scrambled sequence as a negative control and then exposed to high glucose for 48 hours. Cardiomyocytes were collected and stained with Annexin V/PI and then subjected to flow cytometry to detect apoptosis. B. The qualification of apoptotic rates in the charts in the upper panel. **P < 0.01 significantly different from normal glucose group. ##significantly different from high glucose group. C. Western Blot analysis of cleaved-Caspase-3, cleaved-PARP and Bcl-2 expression in high glucose treated cardiomyocytes (left). The histogram shows the average optical density normalized to the loading control, β-actin (right).
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DCM is an important cardiovascular complication in diabetic patients, which carries a substantial risk for the subsequent development of heart failure and increased mortality. In the present study, we established a high-fat diet (HFD) and two low-dose streptozotocin (STZ)-induced diabetic rat model to investigate the potential role of miRs in the pathogenesis of DCM. Our results showed that miR-30b expression was significantly upregulated in the myocardium of diabetic rats, which might be associated with increased cardiomyocytes apoptosis and impaired cardiac function. We then performed in vitro experiments using cultured cardiomyocytes, and our findings revealed that high glucose could induce apoptosis by regulating miR-30b/Bcl-2 pathway.

Apoptosis-related gene Bcl-2 is a direct target of miR-30b

Among the putative targets of miR-30b, we focused on Bcl-2, which belongs to Bcl-2 family that governs mitochondrial outer membrane permeabilization and functions as an anti-apoptotic gene (Figure 5A). The 3'UTR of Bcl-2 was fused to the luciferase coding region and transfected into HEK293 cells with miR-30b mimic. The luciferase assay indicated that Bcl-2 was a direct target of miR-30b (Figure 5B). The use of mutant derivatives in the miRNA recognition site confirmed the specificity of repressing activity. The expression of Bcl-2 in mRNA and protein level were downregulated in cardiomyocytes transfected with miR-30b mimic (Figure 5C and 5D), suggesting that miR-30b mimic could inhibit Bcl-2 expression in transcriptional and translation level. In addition, miR-30b was negatively correlated with the expression of Bcl-2 in a rat model of DCM.

Discussion

DCM is an important cardiovascular complication in diabetic patients, which carries a substantial risk for the subsequent development of heart failure and increased mortality. In the present study, we established a high-fat diet (HFD) and two low-dose streptozotocin (STZ)-induced diabetic rat model to investigate the potential role of miRs in the pathogenesis of DCM. Our results showed that miR-30b expression was significantly upregulated in the myocardium of diabetic rats, which might be associated with increased cardiomyocytes apoptosis and impaired cardiac function. We then performed in vitro experiments using cultured cardiomyocytes, and our findings revealed that high glucose could induce apoptosis by regulating miR-30b/Bcl-2 pathway.

MiRNAs are a class of small endogenous non-coding RNAs (20-25 nucleotide long) that regulate the expression of target genes by binding to their 3'UTR regions, leading to translational repression or mRNA degradation [22]. In this study, we found that miR-30b was upregulated in cardiomyocytes exposed to high level of glucose. Among the putative targets of miR-30b, we focused on Bcl-2, a gene functions as an anti-apoptotic gene. We cloned the 3'UTR of Bcl-2
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Bcl-2 downstream of a luciferase reporter construct and co-transfected it into HEK293 cells with miR-30b mimic. The results of luciferase assay suggested that Bcl-2 might be a direct target of miR-30b.

Apoptosis, which is characterized by cell shrinkage, plasma membrane blebbing, chromatin compaction and nuclear fragmentation, plays critical roles in the pathogenesis of DCM [12]. Hyperglycemia induced cardiomyocytes apoptosis leads to chronic diabetic complications, diabetic cardiomyopathy being the major complication [23]. In the present study, we established that miR-30b expression is upregulated in cardiomyocytes mimicking hyperglycemia in vitro as well as a rat model of DCM and decreased expression of miR-30b could attenuate high glucose-induced apoptosis in cardiomyocytes. Further research is required to explore the role of miR-30b in regulating cardiomyocytes apoptosis in vivo under hyperglycemic condition.

Bcl-2 belongs to Bcl-2 family, is localizes to the outer membrane of mitochondria, where it plays an important role in inhibiting the actions of pro-apoptotic proteins. Bcl-2 is known to regulate mitochondrial dynamics, and is involved in the regulation of mitochondrial fusion and fission. A previous study reported that Bcl-2 plays an important role in diabetic cardiomyopathy as an anti-apoptosis actor [24]. In our study, we demonstrated that miR-30b participate in cardiomyocytes cell death under diabetic condition by targeting Bcl-2, suggesting that miR-30b could act as potential therapeutic target in managing DCM. Further research is required to elucidate other pathways affecting cardiomyocytes apoptosis in patients with DCM.

In summary, these data reveals a novel function of miR-30b/Bcl-2 in inducing cardiomyocytes apoptosis under hyperglycemic condition, which may provide a new insight for understanding the pathogenic role of miR-30b in the development of DCM and can effectively contribute towards identifying a potential therapeutic target for relieving the symptoms due to cardiomyocytes apoptosis.

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Disclosure of conflict of interest

None.

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