Original Article
Effects of miR-223 on cerebral ischemic injury and angiogenesis in diabetic rats

Li Zhu[1,2], Xinmin Wang[2], Jianhua Wu[3], Jingyan Yang[4], Xiaohan Sun[1], Xiangyun Fan[5], Mingli Zhang[1], Shengnian Zhou[1]

[1]Department of Neurology, Qilu Hospital of Shandong University and Brain Science Research Institute, Shandong University, P. R. China; [2]Department of Neurology, Binzhou People's Hospital, Binzhou, P. R. China; [3]Department of E.N.T, Binzhou People's Hospital, Binzhou, P. R. China; [4]Department of Pathology, The Second Hospital, Shandong University, Jinan, P. R. China; [5]Cadre Health Department, Binzhou People's Hospital, Binzhou, P. R. China

Abstract: The aim of this study is to investigate the role of miR-223 in diabetes associated cerebral ischemic damage in rats. A total of 50 rats were divided into 5 groups (10 rats per group): sham group, cerebral ischemia (CI) group, diabetes with cerebral ischemia (DCI) group, DCI+miR-223 inhibitor (inhibitor) group and DCI+scramble (scramble) group. Rats were intraperitoneally injected with Streptozotocin (STZ) to induce diabetes, and cerebral ischemic models were established by inserting a monofilament suture into right-middle cerebral artery according to Longa's methods. Mir-223 inhibitor and scramble were given through lateral ventricle for 3 weeks after diabetes induction. Twenty-four hours after cerebral ischemic induction, ischemic neuronal damage in diabetic rats was evaluated by neurobehavioral function score and cerebral infarct volume. Expression of miR-223, CD31 and VEGF was determined in cortical areas by Real-time PCR and Western blot, respectively. Expression of miR-223 was significantly increased after cerebral ischemia in rats. Compared with DCI group and scramble group, miR-223 inhibitor significantly reduced ischemic neuronal damage while increased the expression of both CD31 and VEGF. Our results indicated that miR-223 had an important role in the regulation of diabetes associated cerebral ischemic damage and angiogenesis in rats. Down-regulation of miR-223 may alleviate the ischemic neuronal damage in diabetic rats.

Keywords: miR-223, cerebral ischemic injury, diabetes

Introduction

Cerebral infarction is a severe complication of diabetes and diabetes associated acute cerebral infraction, which is characterized by increased cerebral infarct areas, more severe symptom, poor prognosis, and higher possibility of advancing stroke, accounts for 20-25% of cerebral infraction [1]. It is widely recognized that diabetes could aggravate both cerebral ischemic neuronal damage and cerebral ischemic vessel damage [2], but the mechanism for this is not fully understood and no effective treatment method is reported.

MicroRNAs (miRNAs) are a class of 18-25 nucleotide (nt) non-coding RNAs that extensively expressed in eukaryote, and they predominantly serve as translational repressor by binding to complementary sequences in the 3' untranslated region (UTR) of their target mRNAs [3]. It is reported that miRNAs could participate in multiple biological activities including cell differentiation, proliferation and revascularization by repressing the translation of their target mRNAs [4]. In human, expression of miRNAs is regulated by various signaling pathway [5]. Studies have shown that miRNAs are closely correlated with ischemic cerebrovascular disease. For example, a study on miRNA expression profile in cerebral stroke patients and healthy subjects using microarray method found many upregulated and downregulated miRNAs in the acute phase of cerebral stroke [6]. miR-223 expression was found to be significantly changed in during cerebral ischemic damage and various cerebral injuries, indicating the important role of miR-223 in the regulation of cerebral ischemic damage [7]. In addition, angiogenesis has been shown to increase

blood perfusion and improve neurological function of brain tissue after cerebral ischemia, and play an important role in diabetes associated ischemic brain injury [8].

In this study, we aimed to investigate the role of miR-223 in angiogenesis after cerebral ischemic damage by downregulating the level of miR-223 in cerebral ischemic brain tissues using synthetic miR-223 inhibitor.

Material and methods

Animals

A total of 50 healthy adult Wistar rats (SPF grade with 8-12 months age and 240-280 g body weight) were supplied by Beijing Vital River Experimental Animal Co., Ltd. (Beijing, China). Water and diet were provided as libitum. Rats were divided into 5 groups with 10 rats per group. These groups were: (1) Sham group that experienced sham operation and received 10 µL of saline water through lateral ventricle; (2) Cerebral ischemia (CI) group that received equal volume of saline water through lateral ventricle at the time of cerebral ischemia; (3) Diabetes with cerebral ischemia (DCI) group that received equal volume of saline water through lateral ventricle at the time of cerebral ischemia after diabetes induction; (4) DCI+ miR-223 inhibitor (inhibitor) group that received 10 µg antagomir-223 through lateral ventricle for 3 weeks after diabetes induction; (5) DCI+scramble (scramble) group that received negative control oligonucleotide through lateral ventricle for 3 weeks after diabetes induction. MiR-223 inhibitor and scramble were purchased from Shanghai GenePharma Co., Ltd (China). All animal experiments were conducted according to the ethical guidelines of Shandong University.

Diabetes induction in animals

Streptozotocin (STZ, Sigma Chemical Co., St. Louis, MO, USA) were dissolved by 0. 1 mmol /L sodium citrate-citrate buffer solution (pH 4.2) to the concentration of 10 mg /L. Following 8 hr fasting, rats were injected intraperitoneally with a single dose of 55 mg/kg STZ. Glucose in the blood of rats was determined by Roche glucometer after 7 days. Rats with fasting glucose concentration exceeding 16.7 mmol /L were considered diabetic.

Establishment of animal cerebral ischemic model

Diabetic rats were used for establishment of cerebral ischemic models at 6 weeks after STZ injection. Cerebral ischemic models were established by inserting a monofilament suture into right-middle cerebral artery for 24 hr according to Longa's methods [9].
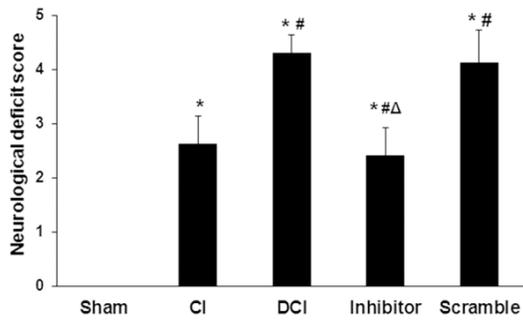
Neurobehavioral function evaluation

Twenty-four hours after cerebral ischemia, the neurobehavioral function of rats from each group was evaluated according to Zea-Longa score [9]. Rats with score 2 or scores higher than 2 were considered as successful model.
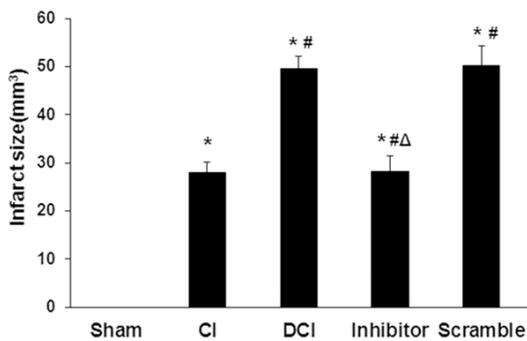
Evaluation of the cerebral infarction volume

After neurobehavioral function evaluation, rats were anesthetized with 3% Pentobarbital Sodium and sacrificed by cervical dislocation. The brain was removed rapidly and frozen for 20 min at -20°C. Then the brain was cut into coronal sections of 2 mm thickness. The sections were immersed in 2% 2, 3, 5-triphenyltetrazolium chloride (TTC) at 37°C for 30 min, followed by 4% paraformaldehyde fixation for 24 hr. The normal brain tissue showed red and infarct tissue white. Photographs were taken by digital camera and processed by Fiji Luxex-F to calculate cerebral infarct volume according to the formula $V = t (A_1 + A_2 +......A_n) - (A_1 + A_n) t/2$. A, cerebral infarct area; t, thickness of section.

RNA extraction and real-time PCR assay

Twenty-four hours after cerebral ischemia, cortical areas of the cerebral ischemia side were collected. The total RNA from each sample was extracted using miRNeasy Mini kit (Qiagen, Valencia, CA, USA) according to the manufacture's construction. RNA was reverse-transcribed into cDNA using a TaqMan® MicroRNA Reverse Transcription Kit (Life Technologies, Inc., Gaithersburg, MD, USA). Quantitative Real-time PCR of miRNAs was performed using TaqMan® MicroRNA Assays qPCR Kits (Life technology) and quantitative Real-time PCR of mRNAs was performed using TaqMan® Genotyping Master Mix (Life technology) according to the manufacture's construction. U6 and GAPDH was used as internal control for miRNA and mRNA, respectively. The reaction

Figure 1. Neurobehavioral function evaluation. Neurobehavioral function score in sham group, CI group, DCI group, DCI+miR-223 inhibitor group and DCI+scramble group was calculated by Zea-Longa method. CI, cerebral ischemia; DCI, diabetes with cerebral ischemia. Data are presented as mean ± S.D., N = 10 per group. *P < 0.05, compared with sham group; #P < 0.05, compared with CI group; ΔP < 0.05, compared with DCI group.

Figure 2. The cerebral infarction volume. The cerebral infarction volume in sham group, CI group, DCI group, DCI+miR-223 inhibitor group and DCI+scramble group was detected by TCC staining. Data are presented as mean ± S.D., N = 10 per group. *P < 0.05, compared with sham group; #P < 0.05, compared with CI group; ΔP < 0.05, compared with DCI group.

mixture was incubated for 1 cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 1 min, 60°C for 40 s, and 72°C for 30 s, and 1 cycle at 72°C for 1 min. Primers used for amplification were as follows: VEGF forward: 5'-GAAGCTCAT-CTCTCCTATGTGCTGGC-3' and VEGF reverse: 5'-CCTGGTGGACATCTTCCAGGAGTACC-3'; CD31 forward: 5'-AGGGCTCATTGCGGTGGTTGTCAT-3' and CD31 reverse: 5'-TAAGGGAGCCTTCCG-TTCTAGAGT-3'; GAPDH forward: 5'-CCATGGAG-AAGGCTGGGGCTCA-3' and GAPDH reverse: 5'-ATGGCATGGACTGTGGTCATGAG-3'. The relative expression levels were evaluated using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Rats were sacrificed by cervical dislocation. Cortical areas of the cerebral ischemia side (100 mg) were taken and were ground on ice. Pre-cooled RIPA lysis buffer was added to the brain tissue for further grinding and centrifuged at 12,000×g for 30 min at 4°C for collecting the supernatant. BCA method was used to measure the total protein. A total of 40 µg Protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then proteins were transferred onto PVDF membrane. After blocking with 5% non-fat milk, expression of CD31 and VEGF was detected with the following antibodies: rabbit anti-CD31 (1:500; ab28364), rabbit anti-VEGF (1:500; ab46154) and rabbit anti-GAPDH (1:2500; ab9485) at 4°C overnight. The primary antibodies were from Abcam (Cambridge, MA, USA). After washing, the membrane was then incubated with goat anti-rabbit HRP conjugated antibody (1:2000) at room temperature for 1 h. Finally, the membrane was developed by enhanced ECL reagent (Millipore, Billerica, MA, USA). The developed film was scanned using the Bio-Rad gel imaging systems (Bio-Rad Laboratories, Hercules, CA, USA). And the Western blot images were analyzed using image lab software (Bio-Rad Laboratories, Hercules, CA, USA). The relative absorbance ratios of CD31 to GAPDH and VEGF to GAPDH were defined as the relative values of CD31 and VEGF, respectively.

Statistical analysis

Data were expressed as mean ± S.D. SPSS 17.0 (SPSS Statistics/IBM Corp, Chicago, IL, USA) was used for data analysis. Statistical significance was determined with one-way ANOVA between groups or SNK-q between two samples. *P*-values <0.05 were considered statistically significant.

Results

Glucose concentration before establishment of cerebral ischemic model

Before establishment of cerebral ischemic models, glucose concentration in blood was detected in rats by Roche glucometer to determine the result of diabetes induction of rats. Glucose concentration of untreated rats was
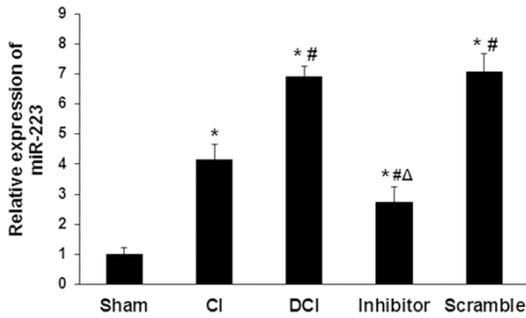
Figure 3. Expression of miR-223 in the cortical areas of the cerebral ischemia side. Real-time PCR analysis showed relative miR-223 expression in sham group, CI group, DCI group, DCI+miR-223 inhibitor group and DCI+scramble group. Data are presented as mean ± S.D., N = 10 per group. *P < 0.05, compared with sham group; #P < 0.05, compared with CI group; ΔP < 0.05, compared with DCI group.

5.81 ± 0. 28 mmol/L, which was significantly lower than that in STZ injected rats (16.91 ± 1.23 mmol/L, P < 0.05), indicating that STZ injection successfully induced diabetes in rats.

Neurobehavioral function score

To evaluate the effect of miR-223 on ischemic neuronal damage in diabetic rats, Zea-Longa score was calculated. There was no neurobehavioral dysfunction symptom in rats of the sham group, whose Zea-Longa score got 0 points. After cerebral ischemia/reperfusion injury, all animals showed neurobehavioral dysfunction. As shown in **Figure 1**, the Zea-Longa scores in CI group, DCI group, DCI+miR-223 inhibitor group and DCI+scramble group were significantly higher than that in the sham group (P < 0.05). The scores in DCI group and scramble group were significantly higher than that in CI group (P < 0.05), but no significant difference was found between these two groups. In addition, injection of miR-223 inhibitor significantly reduced Zea-Longa score (P < 0.05).

The cerebral infarction volume

Next cerebral infarction volume was detected by TTC staining. As shown in **Figure 2**, no cerebral ischemia infarction was displayed in the brain slices of the sham group, while infarction volume significantly increased in all other four groups of rats after cerebral ischemic reperfusion injury (P < 0.05). The volume of cerebral infarction in the DCI group and scramble group were significantly higher than that in the CI

group (P < 0.05), but there is no statistical difference between the DCI group and scramble group (P > 0.05). There was a significant decrease of cerebral infarction volume in rats from DCI group compared with that in rats from scramble group. These results suggested that diabetes aggravated ischemic neuronal damage and that miR-223 inhibitor had protective effect on this damage.

Expression of miR-223 in the cortical areas of the cerebral ischemia side

To find out the involvement of miR-223 in cerebral ischemic injury in diabetic rats, expression of miR-223 was analyzed in the cortical areas of the cerebral ischemia side by Real-time PCR. As shown in **Figure 3**, miR-223 relative expression in brain tissues were normalized as 1 in sham group rats, and increased significantly in all the other 4 groups of rats (P < 0.05). In the DCI group and scramble group, the expression of miR-223 was significantly higher than that in the CI group, but there was no difference between the DCI group and scramble group (P > 0.05). Injection of miR-223 inhibitor significantly decreased the expression of miR-223. These results indicated that miR-223 expression was correlated with ischemic neuronal damage in diabetic rats.

Expression of CD31 and VEGF in the cortical areas of the cerebral ischemia side

To investigate the effect of miR-223 on angiogenesis after cerebral ischemia in diabetic rats, expression of CD31 and VEGF were analyzed by Western blot in cortical areas. As shown in **Figure 4**, CD31 and VEGF were lowly expressed in sham group rats, and increased significantly in all the other 4 groups of rats (P < 0.05). Expression of these two proteins in the DCI group and scramble group was significantly higher than those in the CI group (P < 0.05), but no difference was observed between the DCI group and scramble group. Injection of miR-223 inhibitor significantly increased the expression of both CD31 and VEGF. These results indicated that miR-223 and the expression of CD31 and VEGF were negatively correlated.

Discussion

Diabetes is an important risk factor for the occurrence and development of cerebrovascu-
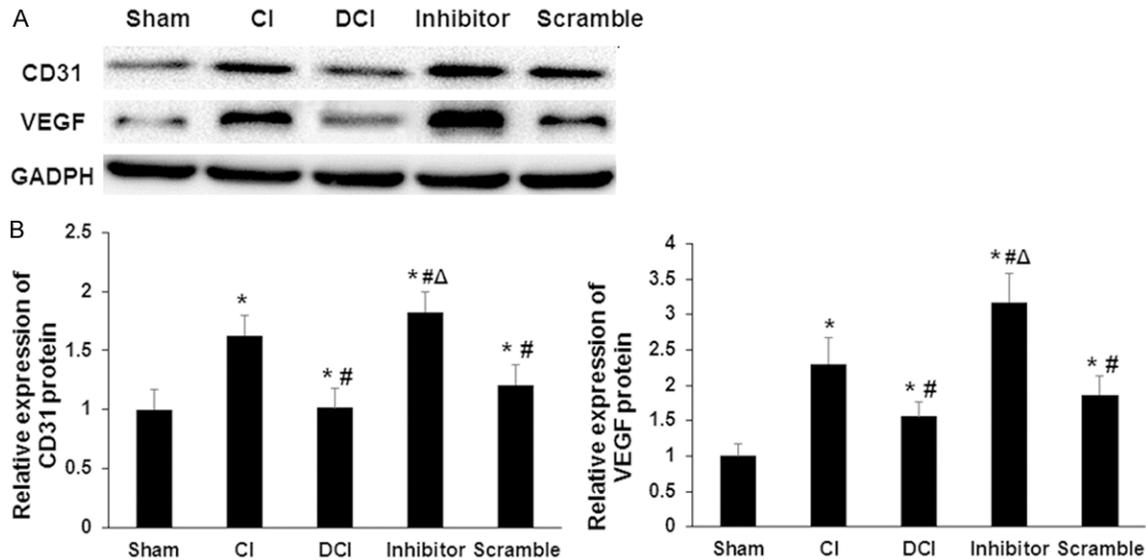
Figure 4. Expression of CD31 and VEGF in the cortical areas of the cerebral ischemia side. A. Western blot analysis showed CD31 and VEGF expression in sham group, CI group, DCI group, DCI+miR-223 inhibitor group and DCI+scramble group. B. Bar graph showed relative absorbance ratios of CD31 to GAPDH and VEGF to GAPDH. Data are presented as mean ± S.D., N = 10 per group. *P < 0.05, compared with sham group; #P < 0.05, compared with CI group; ΔP < 0.05, compared with DCI group.

lar disease. In addition to inducing ischemia, diabetes can further aggravate cerebral ischemic damage [10, 11]. In this study, we found that the neurobehavioral function score and the cerebral infarction volume of rats after DCI was significantly increased compared with that of CI group, demonstrating the aggravation of cerebral ischemic damage by diabetes. The report that angiogenesis could increase blood perfusion and improve neurological function of brain tissue after cerebral ischemia [8] provided a new way for the treatment of diabetes associated cerebral ischemia.

CD31, also known as platelet endothelial cell adhesion molecule-1 (PECAM-1), is a glycoprotein in the vascular endothelium and platelets and is a specific marker of endothelial cells [12]. It is mainly secreted by endothelial cells and differentiated monocytes in lymph nodes [12]. Expression of CD31 is closely related to microvascular density in cerebral infarct surrounding area. Microvascular density is a commonly used indicator that can effectively and intuitively reflect the number of new blood vessels and collateral circulation [13]. VEGF can increase vascular permeability of micro arteries and arterioles as well as promote vascular endothelial cell proliferation, migration and induce angiogenesis [14-16]. Expression of

VEGF and its receptor were increased in ischemic brain tissues, which was positively correlated with CD31 expression, to exert protective effect on ischemic brain tissue. Therefore, VEGF has been used as a marker to reflect the degree of angiogenesis [17].

Study showed that focal cerebral ischemia could increase VEGF expression [18], and simply increased blood glucose level could also increase VEGF expression [19]. However, we found that expression of VEGF in DCI group of rats was at a low level, indicating that no additive effect was shown by ischemia and high blood glucose level. The reason for this may be that ischemia and high blood glucose level regulated VEGF expression through different pathways. Currently it is considered that hypoxia is a potent trigger for the upregulation of VEGF by inducing the transcription and expression of related proteins [20], but increased VEGF expression by high blood glucose level is correlated with DAG-protein kinase C pathway [21]. In this study, we found that CD31 and VEGF expression was significantly decreased in the brain tissue of DCI group while neurobehavioral function score and the cerebral infarction volume of DCI group was significantly increased compared with that of CI group, further demonstrating that diabetes aggravated cerebral isch-

emia may be related to the low expression of VEGF. These results also demonstrate that angiogenesis plays an important role in blood perfusion of brain tissue after diabetes aggravated cerebral ischemia.

Involvement of miR-223 in the regulation of angiogenesis in diabetes associated cerebral ischemia has not been reported. Here we reported that expression of miR-223 in the cortical areas of the cerebral ischemia side in the DCI group was significantly increased compared with that in the CI group, indicating that miR-223 was expressed extensively and miR-223 may participate in the development of diabetes associated cerebral ischemia.

To further identify the role of miR-223 in diabetes-associated cerebral ischemia, we downregulated miR-223 expression by using miR-223 inhibitor. It was reported that inhibition of miR-145, miR-497, miR-181a, miR-1 and let-7f expression during cerebrovascular diseases had neural protective effect on cerebral ischemic damage [22]. In the present study we also found that miR-223 inhibitor could significantly decrease miR-223 expression level in the brain tissue, as well as decrease neurobehavioral function score and the cerebral infarction volume, further demonstrating the important role of miR-223 in diabetes associated cerebral ischemia. We speculated that this may due to the close relationship between the development of diabetes-associated cerebral ischemia and inflammatory responses [1].

In summary, the results showed that miR-223 in the DCI group was significantly increased while CD31 and VEGF expression was significantly decreased in the brain tissue of DCI group compared with that of CI group. Our findings indicate that miR-223 play an important role in diabetes associated cerebral ischemia.

Acknowledgements

Disclosure of conflict of interest

None.

Address correspondence to: Shengnian Zhou, Department of Neurology, Qilu Hospital of Shandong University and Brain Science Research Institute, Shandong University, 107 Wenhuaxi Road, Jinan, Shandong, 250012, P. R. China. Tel: 86-531-82168855; Fax: 86-531-82168855; E-mail: zhoushengnian678@163.com

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