Downregulation of microRNA-185 expression in diabetic patients increases the expression of NOS2 and results in vascular injury

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Abstract. The aim of the present study was to investigate the regulatory effect and mechanism of microRNA (miR)-185 in diabetic angiopathy. The expression of miR-185 and nitric oxide synthase 2 (NOS2) in the blood from diabetic patients was examined by reverse transcription-quantitative PCR and enzyme-linked immunosorbent assay. After establishment of diabetic rats, the expression of miR-185 and NOS2 in vascular tissues and blood was also measured. Then, miR-185 was overexpressed in HMEC-1 cells and the expression of NOS2 was determined. Dual-luciferase reporter assay was used to identify the direct interaction between miR-185 and NOS2 mRNA. The expression of NOS2 was upregulated and the expression of miR-185 was downregulated in the blood from patients with diabetes. Vascular tissues and blood of diabetic rats showed similar trends compared with that of human. HMEC-1 cells with overexpression of miR-185 had decreased expression of NOS2. Dual-luciferase reporter assay demonstrated the direct binding between miR-185 and NOS2. The present study demonstrates that upregulation of NOS2 in diabetic patients is associated with the downregulation of miR-185, which participates in the progression of diabetes possibly through regulating NOS2 expression.

Introduction

Diabetes mellitus (DM) is a disease characterized by hyperglycemia induced by absolute or relative insufficiency of insulin (1). The total number of diabetic patients in China ranks the first in the world, and 95% of diabetic patients have type 2 DM (T2DM) (2). If blood glucose levels cannot be effectively controlled after the diagnosis of diabetes, there will be extensive microvascular and macrovascular lesions during the course of the disease (3-5).

The long-term existence of various pathogenic factors in T2DM can damage the vascular endothelium, lead to abnormal secretion function, and interrupt the normal proliferation and apoptosis balance of smooth muscle cells (6). In the disease, atherosclerosis, decreased compliance with various vasoactive substances, abnormal diastolic and systolic functions, and finally stenosis, obstruction, and complications may occur (6). At present, it is believed that the molecular mechanism of vascular lesions in T2DM is very complex, being closely associated with gene susceptibility, oxidative stress, advanced glycation end products, aldose reductase and inflammation (7).

Nitric oxide synthase 2 (NOS2) is a key enzyme for endogenous NO synthesis in the human body. An appropriate amount of NO can relax blood vessels, maintain local blood flow, and play a protective role in body tissues (8). However, with the prolongation of ischemia, NOS2 will induce a large amount of NO, which produces more toxic superoxide radicals that damage vascular tissues (9). At present, the regulation of NOS2 is a hot spot in research on vascular lesions, and regulations by microRNA (miR) have attracted increasing attention. During the preliminary bioinformatics prediction, it was discovered that miR-185 is closely associated with NOS2 and may be an upstream miRNA regulating NOS2. It is also confirmed that miR-185 plays an important role in the circulatory system. For example, miR-185 is reported to reverse myocardial hypertrophy through a variety of signaling pathways (10). However, the regulatory association between miR-185 and NOS2 has not been fully clarified in diabetic angiopathy.

In the present study, the aim was to investigate the association between NOS2 and miR-185, and the expression level of NOS2 was examined in the blood and vascular tissues from diabetic patients and rat models.

Materials and methods

Subjects. A total of 33 patients with T2DM, including 17 males and 16 females, participated in the present study between December 2017 and July 2019. The ages of the patients ranged from 38 to 60 years, and the mean age was 50.3 years. In addition, 33 age-matched healthy subjects (age match by power analysis), including 17 males and 16 females, were included in the control group (age range, 37-62 years; mean age, 51.7 years).

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Blood was drawn from all patients on the day of diagnosis and healthy subjects on the day of physical examination, and stored in tubes containing EDTA. After centrifugation at 1,200 x g at 4˚C for 10 min, plasma was separated and stored at -20˚C. All procedures performed in the current study were approved by the Ethics Committee of Lishui People's Hospital. Written informed consent was obtained from all patients or their families.

Animals. A total of 40 male SD rats [certificate no. SCXK(Yu)2018-0011; Tengxin] weighing between 150 and 200 g were used for animal experiments. Before conducting the experiments, all rats had adaptive feeding for one week and were free to eat and drink.

The rats were firstly fasted overnight. Under anesthesia by intraperitoneal injection of chloral hydrate (300 mg/kg), alloxan was injected via the tail vein at a dose of 50 mg/kg body weight. After 48 h, the diabetic rat model was established. The number of rats in the diabetic group was 20 and that in control group was also 20.

Blood was collected from the caudal vein of all rats, and stored in the presence of EDTA. Following centrifugation at 1,200 x g at 4˚C for 10 min, plasma was separated and stored at -20˚C. After the model construction was completed, and blood was drawn from the animals, the animals were euthanized by decapitation after anesthesia by intraperitoneal injection of chloral hydrate (300 mg/kg). After sacrifice, aortic vascular tissues were collected from the rats and stored in liquid nitrogen. All animal experiments were conducted according to the ethical guidelines of Lishui People's Hospital.

Extraction of RNA and reverse transcription-quantitative PCR (RT-qPCR). Tissues (100 mg) were ground into powder in liquid nitrogen and lysed with 1 ml TRIzol® (cat. no. R0016; Beyotime Institute of Biotechnology), and liquid samples (100 μl) were directly lysed with 1 ml TRIzol. Total RNA was extracted using an RNA extraction kit (R0016; Beyotime Institute of Biotechnology). The integrity of RNA bands was detected by gel electrophoresis. The ratio of absorbance at 260 nm over absorbance at 280 was measured by spectrophotometer to detect the purity of RNA. Total RNA (1 μg) was reverse-transcribed into template cDNA using the TIANScript II RT kit (Tiangen Biotech Co., Ltd.) according to the manufacturer's protocol, which was stored at -20˚C.

For RT-qPCR, the primer sequences were as follows: human U6 (upstream), 5'-GCTTCGGCAGCACATATACTAAAAT-3'; human U6 (universal downstream), 5'-CGCTTCACG AATTTGCGTGTCAT-3'; human miR-185 (upstream), 5'-TGGAGAGAAAGGCAGTTCCTGA-3'; human miR-185 (universal downstream), 5'-CGCTTCACGA ATTTGCGTGTCAT-3'; human NOS2 (upstream), 5'-GGT AGAGGCCTGGAAAACCC-3'; human NOS2 (downstream), 5'-AGCTCATCCCCTTCTCCCAT-3'; human β-actin (upstream), 5'-CTCCATCCTGGCCTCGCTGT-3'; and human β-actin (downstream), 5'-GCTGTCACCTTCACCGTTCC-3'. According to the manufacturer's instructions (2xSYBR Green qPCR Master Mix; EZBioscience), the qPCR reaction system (20 μl) contained RT-qPCR-Mix (10 μl), upstream primer (0.5 μl), downstream primer (0.5 μl), cDNA (2 μl) and ddH_2O (7 μl). The reaction protocol of RTq-PCR was: Initial denaturation, 95˚C and 30 sec; denaturation, 95˚C and 5 sec; annealing, 60˚C and 20 sec. A total of 39 cycles were performed. The results were analyzed by the $2^{-\Delta\Delta Cq}$ method (11). The ratio of NOS2/β-actin was calculated.

The primer sequences for rat NOS2 were 5'-ATCCCGAAACGCTACACTT-3' (upstream) and 5'-GGTCTGGCGAAGAACAATC-3' (downstream). The primer sequences for rat β-actin were 5'-CGTGCGTGACATTAAAGAG-3' (upstream) and 5'-CTGGAAGGTGGACAGTGAG-3' (downstream). The primer sequences for rat U6 were 5'- CTCGCTTCGGCAGCACA-3' (upstream) and 5'-AACGCTTCACGAATTTGCGT-3' (downstream). The primer sequences for rat miR-185 were 5'-ACACTCCAGCTGGGTGGAGAGAAAGGCAGT-3' (upstream) and 5'-TGGTGTCGTGGAGTCG-3' (downstream). Real-time PCR reaction system (20 μl) for rat was the same with that for human described above. The reaction protocol of RT-qPCR for rat: initial denaturation, 95˚C and 10 min; denaturation, 95˚C and 45 sec; annealing, 52˚C and 45 sec; elongation, 72˚C and 45 sec. A total of 35 cycles were performed. The results were analyzed by $2^{-\Delta\Delta Cq}$ method (11). The ratio of NOS2/β-actin was calculated.

Enzyme-linked immunosorbent assay (ELISA). The concentrations of human NOS2 (cat. no. ab253217; Abcam) and rat NOS2 (cat. no. SBJ-R0012; SenBeiJia Biological Technology) were measured by ELISA according to the protocols provided by the kit manufacturers.

Western blotting. After extraction of proteins using RIPA buffer (Beyotime Institute of Biotechnology), BCA protein concentration detection kit (cat. no. P0009; Beyotime Institute of Biotechnology) was used to measure the concentrations of target proteins. The protein samples were mixed with SDS-PAGE loading buffer before boiling for 5 min. Then, 20 μg protein samples were loaded for 10% SDS-PAGE. On ice bath, the samples were transferred onto PVDF membrane at 100 V for 2 h, and then blocked with 5% skimmed milk at room temperature for 1 h. Then, primary antibodies of NOS2 (cat. no. ab3523; 1:800; rabbit anti-rat, polyclonal; Abcam) and internal reference β-actin (cat. no. ab129348; 1:5,000; rabbit anti-rat, Abcam) were used to incubate the membrane at 4˚C overnight. Afterwards, secondary antibody (cat. no. ab6721; 1:3,000; goat anti-rabbit; Abcam) was used to incubate the membrane at room temperature for 1 h. The membrane was then soaked in electrochemiluminescence liquid (cat. no. ab65623; Abcam), and imaged in gel imaging system. Then, Image Lab (version 3.0; Bio-Rad Laboratories, Inc.) was used to analyze the protein bands, and the relative expression of target protein was expressed as the ratio of target protein greyscale against β-actin greyscale.

Bioinformatics prediction. Bioinformatics prediction is the basis and main clue for miRNA function research. The miRwalk 3.0 target gene prediction software (http://mirwalk.umm.uni-heidelberg.de) was used to predict genes that might regulate NOS2, following the instructions published on the website.

Cells and transfection. HMEC-1 and 293T cells were purchased from The Cell Bank of Type Culture Collection of

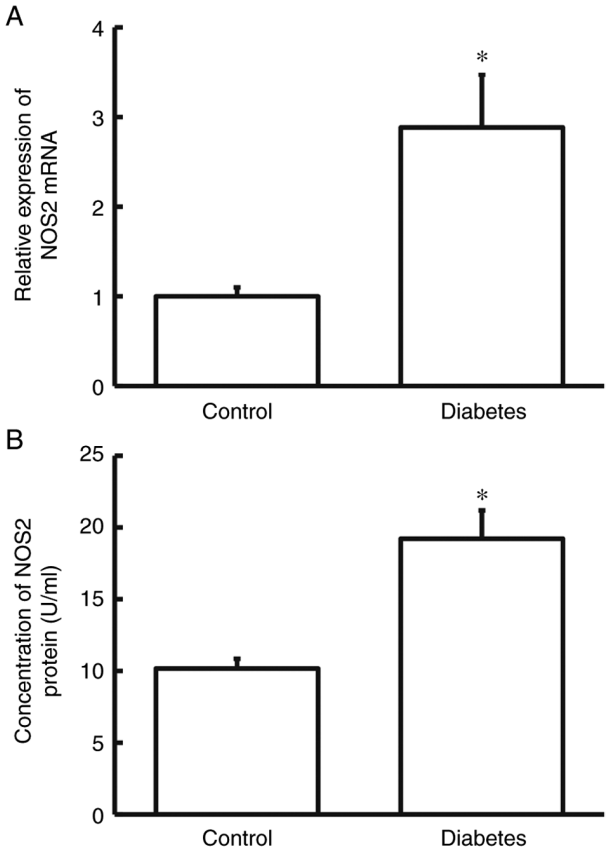

Figure 1. Levels of NOS2 in the blood from patients with diabetes compared with healthy subjects. (A) Relative expression of NOS2 mRNA in the blood determined by reverse transcription-quantitative PCR. (B) Content of NOS2 protein in the blood determined by enzyme-linked immunosorbent assay. *P<0.05 vs. diabetes group. N=33. NOS2, nitric oxide synthase 2.

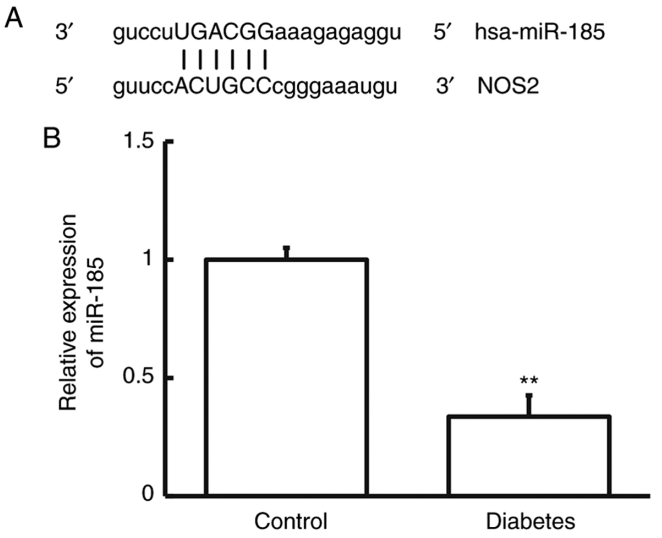

Figure 2. Level of miR-185 in the blood. (A) Possible binding sites between miR-185 and NOS2 gene, as predicted by bioinformatics. (B) Relative expression of miR-185 in blood from patients with diabetes in contrast to control group. **P<0.01 vs. control group. N=33. miR, microRNA; NOS2, nitric oxide synthase 2.

The Chinese Academy of Sciences, and cultured at 37˚C and 5% CO_2.

Before transfection, 1 ml medium containing $1x10^5$ cells was added to 6-well plates, which were incubated at 37˚C and 5% CO_2. When the cells reached 40-60% confluence, Lipofectamine® 3000 was used to transfect the cells following the manufacturer's protocols (cat. no. L3000001; Thermo Fisher Scientific, Inc.). Subsequent experiments were performed 48 h after transfection.

Dual-luciferase reporter assay. Wild-type (WT) and mutant seed regions of miR-185 in the 3'-untranslated region (UTR) of NOS2 gene were chemically synthesized *in vitro*, and Spe-1 and *Hin*dIII restriction sites were attached to the two ends. Then, the two types of DNA fragments were cloned into pMIR-REPORT luciferase reporter plasmids (Ambion; Thermo Fisher Scientific,Inc.), using the mutant 3'-UTR seeding region as control. Using Lipofectamine 3000 (Thermo Fisher Scientific, Inc.), plasmids (0.8 μg) with WT or mutant 3'-UTR DNA sequences were co-transfected with human or rat agomiR-185 (100 nM; Sangon Biotech, Co. Ltd.) into 293T cells. For control, 293T cells were transfected with agomiR-negative control (agomiR-NC). After being cultured for 24 h, the cells were lysed using dual-luciferase reporter assay kit (E1980; Promega Corporation) according to the manufacturer's manual. The luminescence intensity was measured using GloMax 20/20 luminometer (Promega Corporation). Using renilla luminescence activity as an internal reference, the luminescence values of each group of cells were measured.

Statistics. Data are expressed as mean ± SD. SPSS v.18.0 software (SPSS, Inc.) was used for statistical analysis. One-way ANOVA was performed for group comparison, with LSD post hoc test. Comparison between two groups was carried out using unpaired Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

NOS2 level in patients with diabetes is elevated. Firstly, RT-qPCR and ELISA were used to measure the levels of NOS2 in the blood. Compared with the control group, NOS2 mRNA and protein levels in the blood from diabetes group were notably higher compared with those in the control group (P<0.05 for both; Fig. 1A and B). The result suggests that NOS2 level in patients with diabetes is elevated.

Decreased miR-185 expression and elevated NOS2 expression in the blood of patients with T2DM meet the regulatory mode between miRNA and mRNA. Using bioinformatics, miR-185 was predicted to be an upstream regulatory gene for NOS2 (Fig. 2A). Using RT-qPCR, the level of miR-185 in the blood from patients with T2DM was determined. The data showed that the expression of miR-185 in patients with T2DM was notably decreased compared with that in the control group (P<0.05; Fig. 2B). The result indicates that decreased miR-185 expression and elevated NOS2 expression in the blood of patients with T2DM meet the regulatory mode between miRNA and mRNA.

NOS2 levels in vascular tissues and blood from rats with diabetes are elevated compared with normal rats. After

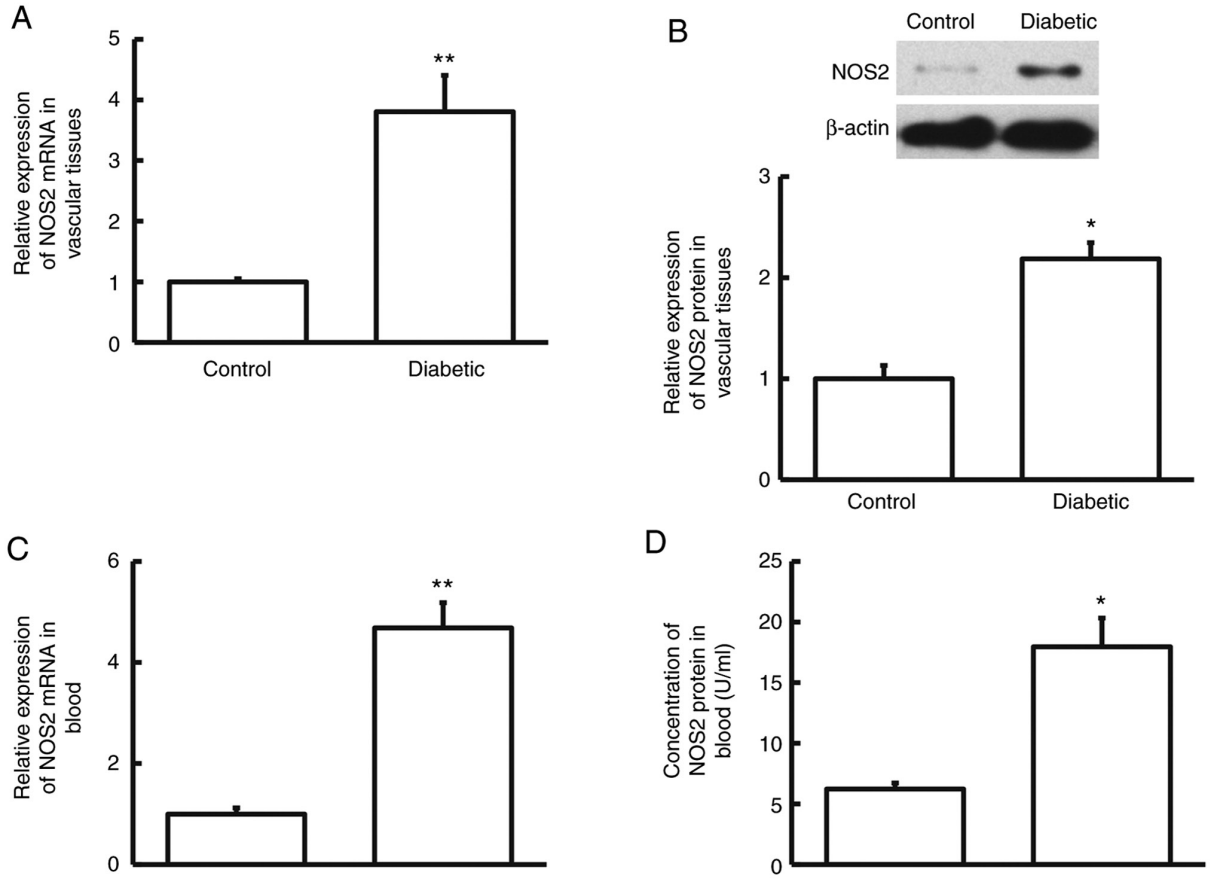

Figure 3. mRNA and protein expression of NOS2 in vascular tissues and blood from diabetic rats. (A) mRNA and (B) protein levels of NOS2 in vascular tissues from normal rats and diabetic rats, as determined by RT-qPCR and western blotting, respectively. (C) mRNA and (D) protein levels of NOS2 in the blood from normal rats and diabetic rats, as determined by RT-qPCR and enzyme-linked immunosorbent assay, respectively. *P<0.05 and **P<0.01 vs. control group. N=20. NOS2, nitric oxide synthase 2; RT-qPCR, reverse transcription-quantitative PCR.

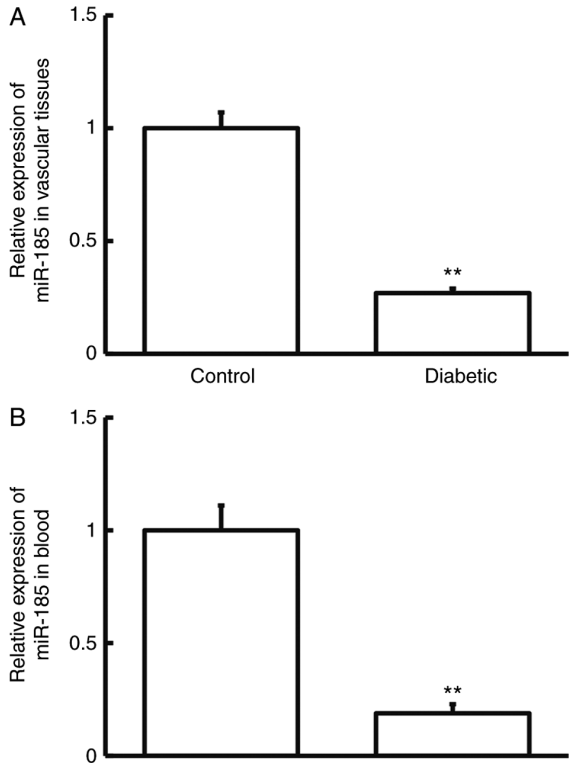

Figure 4. Expression of miR-185 in (A) vascular tissues and (B) blood from diabetic rats. **P<0.01 vs. control group. N=20. miR, microRNA.

Table I. Biochemical indexes of diabetic rats.

Groups	N	HbA1c, %	Glucose, mmol/l	Insulin, μU/l
Control	20	6.17±1.05	5.28±0.16	17.55±4.81
Diabetic	20	10.21±3.56[a]	22.18±5.83[b]	6.13±1.66[a]

[a]P<0.05 and [b]P<0.01 vs. control group.

construction of diabetic rats, the diabetes-associated biochemical indices were examined in the rats. The data showed that the levels of HbA1c and glucose in the diabetic group were notably elevated compared with the control group (P<0.05), while the concentration of insulin in the diabetic group was notably decreased compared with the control group (P<0.05; Table I). Then, mRNA and protein levels of NOS2 in vascular tissues and blood were determined. RT-qPCR and western blotting showed that the expression level of NOS2 mRNA and protein in vascular tissues from diabetic group was significantly higher compared with that from the control group (P<0.05; Fig. 3A and B). Similarly, RT-qPCR and ELISA showed that NOS2 mRNA and protein levels in the blood from diabetic group were obviously higher compared

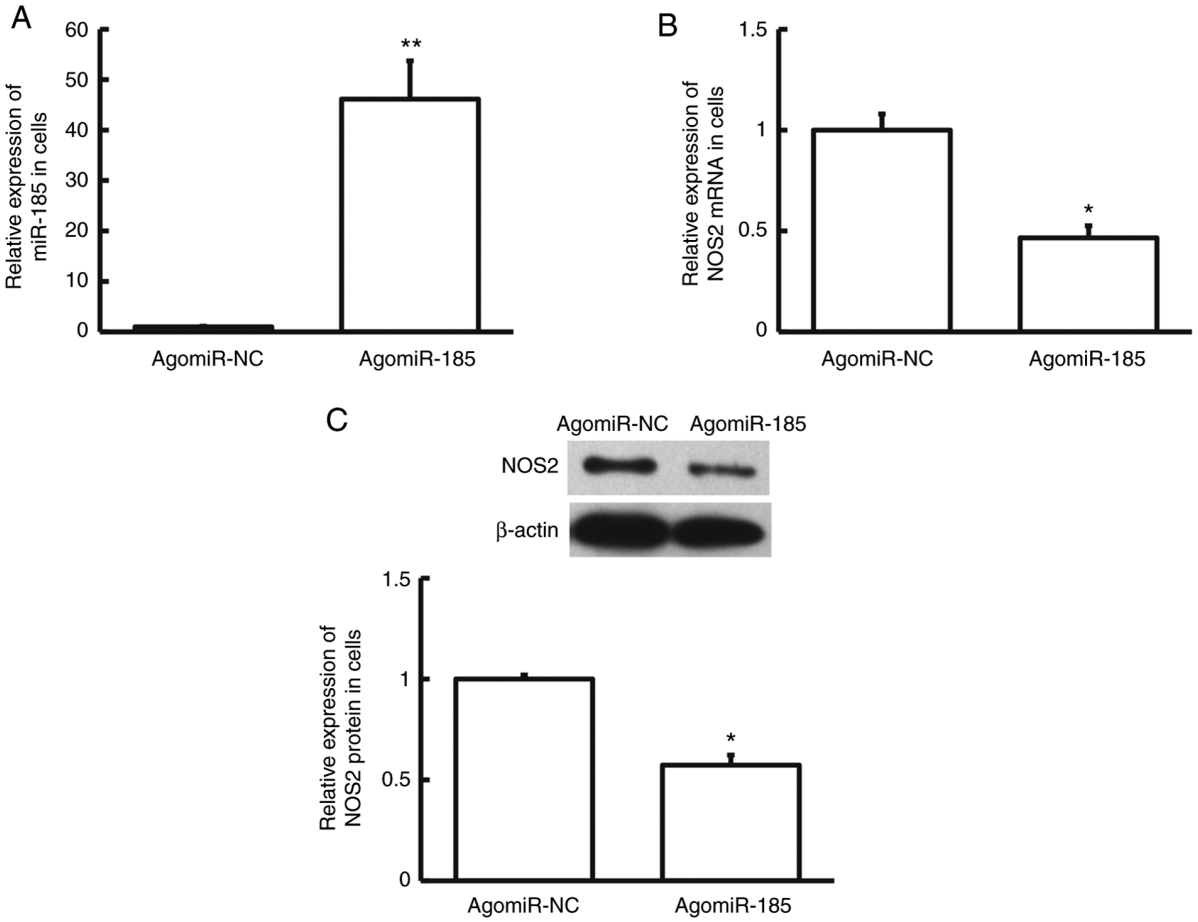

Figure 5. Effect of miR-185 expression on the expression of NOS2. (A) Expression of miR-185 in HMEC-1 cells after transfection with agomiR-NC and agomiR-185. (B) Expression of NOS2 mRNA in HMEC-1 cells after transfection with agomiR-NC and agomiR-185. (C) Expression of NOS2 protein in HMEC-1 cells after transfection with agomiR-NC and agomiR-185. *P<0.05 and **P<0.01 vs. agomiR-NC group. N=3. miR, microRNA; NOS2, nitric oxide synthase 2; NC, negative control.

with those from the control group (P<0.05; Fig. 3C and D). The results demonstrate that NOS2 levels in vascular tissues and blood from rats with diabetes are elevated compared with normal rats.

Rats with diabetes have downregulated levels of miR-185 in vascular tissues and blood compared with normal rats. Moreover, the expression of miR-185 was detected in rats. RT-qPCR showed that the levels of miR-185 in vascular tissues and blood from diabetic group were markedly decreased compared with those from the control group (P<0.01; (Fig. 4A and B). The results suggest that rats with diabetes have down-regulated levels of miR-185 in vascular tissues and blood compared with normal rats.

Overexpression of miR-185 downregulates the expression of NOS2 in vitro. In cell experiments, HMEC-1 cells were transfected with agomiR-NC or agomiR-185. RT-qPCR showed that the expression of miR-185 in agomiR-185 group was markedly elevated compared with that in the agomiR-NC group (P<0.01; Fig. 5A). Furthermore, NOS2 mRNA and protein levels in agomiR-185 group were obviously decreased compared with those in the agomiR-NC group (P<0.05; Fig. 5B and C). These results elucidate that overexpression of miR-185 downregulates the expression of NOS2 *in vitro.*

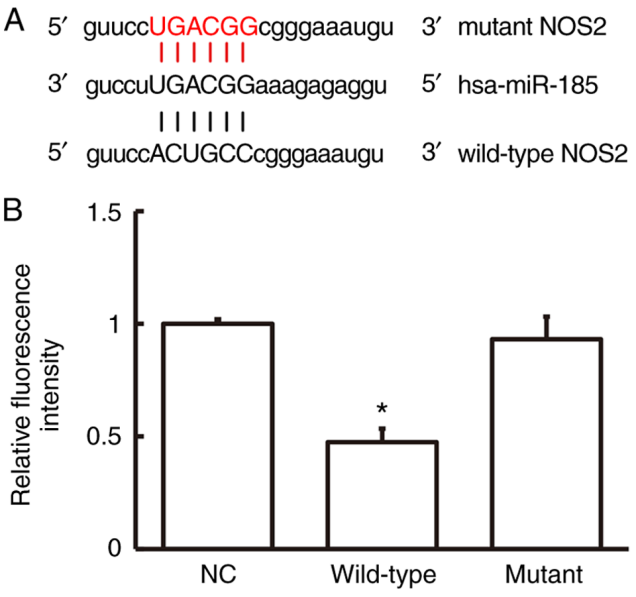

Figure 6. Direct interaction between miR-185 and NOS2 mRNA. (A) Design of mutation sequence in NOS2 seed region. (B) Fluorescence intensity of 293T cells co-transfected with miR-185 and its wild-type or mutant seed regions in the 3'-untranslated region of NOS2 gene, as examined by dual-luciferase reporter assay. One-way ANOVA was performed, followed by Least Significant Difference test. *P<0.05 vs. NC group. N=3. miR, microRNA; NOS2, nitric oxide synthase 2; NC, negative control.

miR-185 directly binds with the 3'-UTR of NOS2 gene to regulate NOS2 expression. Dual-luciferase reporter showed that the fluorescence intensity of 293T cells co-transfected with wild-type agomiR-185 and pMIR-REPORT was markedly decreased compared with that of negative control group ($P<0.05$). By contrast, the fluorescence intensity of 293T cells co-transfected with mutant agomiR-185 and pMIR-REPORT was not different from that of negative control group ($P>0.05$; Fig. 6A and B). The result indicates that miR-185 directly binds with the 3'-UTR of NOS2 gene to regulate NOS2 expression.

Discussion

Hyperglycemia leads to chronic damage and dysfunction of various tissues and organs, especially eyes, kidneys, heart, blood vessels and nerves (12). However, the pathogenesis of diabetes is not yet fully understood. The incidence rate of vascular diseases in diabetic patients is 2-4 times higher than that of non-diabetic patients (13,14). The pathological basis of diabetic angiopathy is atherosclerosis and irregular thickening, fibrosis and calcification of media and adventitia of arteries caused by the proliferation of vascular smooth muscle cells and fibroblasts (14).

Angiogenesis is a complexed, dynamic and coordinated process involving a variety of cytokines and cell components, in which the state of vascular endothelial cells plays a leading role (15). The central process of angiogenesis is the proliferation, migration, differentiation and lumen formation of vascular endothelial cells (15). Inhibition of apoptosis and promotion of survival for endothelial cells are the basic mechanisms of angiogenesis. Exogenous inducers can accelerate the establishment of collateral circulation, and improve the symptoms of myocardial ischemia and hypoxia.

Furchgott *et al* (16) discovered that under the action of acetylcholine, a substance was produced in blood vessels to relax the smooth muscle of the vessels and named it endothelium-derived relaxing factor, which was later proven to be No. NO plays a dual role in cerebral ischemia-reperfusion injury, and can be synthesized by NOS2. NOS2 is not expressed under physiological state, but can be induced by LPS, IFN-γ, TNF-α and IL-1β (17). NOS2 has important clinical significance in the occurrence and development of several diseases. It decreases the survival rate by promoting tumor metastasis in patients with triple-negative breast cancer (18). In patients with nasopharyngeal carcinoma, IL-6 and NOS2 are highly expressed and involved in the regulation of MMP9- and MMP2-dependent metastasis (19). High circulating nitrite levels may be a prognostic marker for survival (19). In addition, the expression of NOS2 is increased in hypertensive rats (20). In the present study, elevated NOS2 levels were observed in the blood from patients with T2DM and the vascular tissues and blood from T2DM rats. The aforementioned data indicated that NOS2 plays important regulatory roles in T2DM.

miRNAs, ~18-22 base pairs in length, are a class of endogenous non-coding single-stranded RNA molecules, which negatively regulate gene expression at posttranscriptional level by binding to the 3'-UTR of target genes (21). miRNAs are widely involved in the pathophysiological processes of cardiovascular diseases (22,23). Mian *et al* (24) discovered that the expression of >140 miRNAs was changed in arterial vessels of patients with arteriosclerosis obliterans of lower extremity. Knockout of Dicer, an enzyme required for miRNA production, can inhibit vascular budding, endothelial cell migration and angiogenesis (25). These studies suggest that miRNAs play important roles in arterial injury and angiogenesis. According to bioinformatics prediction, it was found that miR-185 was closely associated with NOS2 and might be an upstream miRNA that regulates NOS2. It is reported that miR-185 can inhibit the proliferation of clear cell renal cell carcinoma cells and induce their apoptosis by targeting VEGFA (26). In the meantime, miR-185 can inhibit β cell dysfunction caused by diabetes mellitus through targeting SOCS3 gene (27). In breast cancer, the target of miR-185 is VEGFA, which plays an important role in angiogenesis and biological functions (28). The data in the present study demonstrated that expression of miR-185 was decreased in the blood of patients with T2DM and the vascular tissues and blood from diabetic rats. This matched the regulatory mechanism between microRNA and its target gene. Then, miR-185 was overexpressed in vascular endothelial cells, and discovered decreased expression of NOS2 in these cells. Dual-luciferase reporter assay demonstrated that miR-185 could directly bind to NOS2 mRNA. All the aforementioned results indicated that miR-185 plays a regulatory role on NOS2 in the process of diabetes. Although NO produced by eNOS is one of the key factors on vascular homeostasis, the prediction and experimental results of the present study showed that mi-R185 does not regulate eNOS (data not shown).

To summarize, the present study demonstrates that the downregulation of miR-185 expression in vascular tissues and blood of diabetic patients leads to the upregulation of NOS2 expression, which results in a series of vascular lesions and eventually vascular injury. As a regulatory factor of NOS2, miR-185 may become a new foothold in the study of diabetic angiopathy. The present study reveals some mechanisms of miRNA-mRNA regulatory network in the course of type 2 diabetes, which may play a positive role in the prevention, early diagnosis and intervention of the disease.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YZ and HW contributed to the design of the study. YZ, LG and JT performed the experiments. YZ and LG analyzed the data. YZ and HW interpreted the results and prepared the manuscript. YZ and HW confirmed the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All procedures performed in the current study were approved by the Ethics Committee of Lishui People's Hospital (approval no. IACUC-20160201-01). Written informed consent was obtained from all patients or their families. All animal experiments were conducted according to the ethical guidelines of Lishui People's Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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