MicroRNA-132 modifies angiogenesis in patients with ischemic cerebrovascular disease by suppressing the NF-κB and VEGF pathway

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Abstract. In the present study, the expression of microRNA (miR)-132 and the mechanism by which it modifies angiogenesis in patients with ischemic cerebrovascular disease (ICD) was investigated. RNA isolation and reverse transcription-quantitative polymerase chain reaction were used to measure miR-132 expression in patients with ICD. Inflammatory factors were measured using ELISA kits and western blotting measured B-cell lymphoma-2 (Bcl-2)-associated X/Bcl-2 ratio (Bax/Bcl-2 ratio), nuclear factor (NF)-κB p65, matrix metalloproteinase-9 (MMP-9), vascular cell adhesion molecule-1 (VCAM-1) and protein expression of inducible nitric oxide synthase (iNOS), and vascular endothelial growth factor (VEGF) protein expression. miR-132 expression in patients with ICD was lower compared with healthy volunteers. PC12 cells were used to create an oxygen glucose deprivation (OGD) model. miR-132 overexpression in an in vitro model was able to reduce tumor necrosis factor-a, interleukin (IL)-1β, IL-6, IL-8, cyclooxygenase-2, caspase-3 and caspase-9 levels, suppress Bax/Bcl-2 ratio, NF-KB p65, MMP-9, VCAM-1, iNOS, VEGF protein expression. The results suggested that miR-132 may modify angiogenesis in patients with ICD by suppressing the NF-KB pathway and promoting the VEGF pathway, and may develop into a therapy for ICD in future research.

Introduction

Ischemic cerebrovascular disease (ICD) refers to a group of clinical syndromes arising from cerebral circulatory dysfunction of various causes, and brain damage and neurological dysfunction of patients caused by cerebral ischemia, hypoxia and necrosis at the associated blood vessel zone (1). The incidence rate of ICD among cerebrovascular diseases is ~70% in

China (2). It has a serious effect on the health and life of patients, is a burden to the family and society, and inflicts substantial social and economic costs (3). According to the data of one survey, it possesses the highest disability and mortality rate in China (4). The incidence rate is rising at an annual growth rate of 8.7%, reflecting China's aging population (1).

ICD originates from a blockage in the local cerebral blood supply following a vascular occlusion, leading to necrosis due to brain tissue ischemia and hypoxia, and the formation of an ischemic penumbra around the necrotic zone (5). A previous study demonstrated that if the blood supply of the ischemic penumbra is recovered in a timely manner at the early stage of cerebral ischemia, the moribund cerebral ischemia tissue may be saved and the damage to the nerve cells of the ischemic region reversed (6). In recent years, in-depth studies on ICD have been performed. By observing numerous clinical records, it may be noted that different levels of increases in microvascular density are observed in the cerebral ischemia tissue of patients with cerebral ischemic injury (7). A study on angiogenesis have demonstrated that the scope and degree of capillary proliferation at the ischemic region are directly associated with reperfusion of the ischemic region, and affect the recovery of neural functions and improve the prognosis of patients (7). These studies are important for the investigation of angiogenic factors, and for the treatment and recovery of patients with ICD (8).

Vascular endothelial growth factor (VEGF) is the specific mitogen that acts directly on vascular endothelial cells. It is the most powerful angiogenic growth factor (9) and promotes angiogenesis via the VEGF receptor (VEGFR). A study demonstrated that the combination of VEGF and VEGFR promotes reclamation of blood vessels at ischemic penumbra, is conducive to the establishment of collateral circulation and serves an important function in the recovery of cellular function following cerebral ischemia (10). At present, a study investigating the involvement of the VEGF/VEGFR system in the promotion of ICD micro-angiogenesis has been performed (11).

The mechanism of ICD involves inflammatory reactions, changes in gene expression, activation of free radicals, protease activation, mitochondrial dysfunction and disorder in the Ca²⁺ balance (12). With the development of molecular biology, inflammatory reactions have received increased attention. Studies have reported that inflammatory reactions and cell

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apoptosis are important causes in the aggravation of cerebral ischemia-reperfusion injury. Inflammatory reactions inhibit neuron reclamation and functional rehabilitation following cerebral apoplexy (12,13). At the early stage of cerebral ischemia-reperfusion, the blood-brain barrier is damaged, leading to an increase in vascular endothelium permeability, and the exudation of albumin and other macromolecular proteins into the blood, which subsequently cause local edema (14).

Nuclear factor (NF)- κ B promotes adherence, the transmembrane migration of neutrophil granulocytes, leukocyte infiltration, the release of cytokines and chemokines, and leads to the activation of a wide range of brain cells and leukocyte infiltration, which cause further damage to the blood-brain barrier. It also promotes encephaledema aggravation, the apoptosis of neurons, glial and vascular endothelium cells, and serves an important role in reperfusion injury (15,16). ICD may lead to an inflammation cascade reaction and further aggravate cerebral tissue damage (17).

Human source microRNA (miR)-132 is located in chromosome 17pl3.3 and is rich in brain tissues, with a particularly high expression in the hippocampus (18). miR-132 expression is downregulated in a number of diseases associated with the nervous system or in animal models, including Alzheimer's disease, anencephaly, Huntington's disease, schizophrenia and bipolar affective disorder, and Parkinson's disease (19,20). In addition, miR-132 affects the expression of synapse-associated proteins, including NF- κ B and VEGF (21,22). The present study investigated the role of miR-132 in modifying angiogenesis in patients with ICD and the underlying mechanism.

Materials and methods

Ethical statement. Study protocols were approved by the Institution Review Board of Beijing Luhe Hospital, Capital Medical University (Beijing, China). Written informed consent was obtained from all participants. Patients with ICD (n=6; 3 male and 3 female; 47.7±12.78 years of age) and healthy volunteers (n=6; 3 male and 3 female; 45.23±8.12 years of age) were recruited from the Department of Neurology, Beijing Luhe Hospital, Capital Medical University between March 2016 and April 2016. Between 8:00 and 9:00 a.m., cerebrospinal fluid (CSF) of all patients and volunteers was collected at L3/L4 or L4/L5 interspace by lumbar puncture.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from CSF samples (0.5 ml) was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. An iScript cDNA Synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to transcribe to cDNA. A StepOnePlus[™] Real-Time PCR System and SYBR Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) were used for qPCR according to the manufacturer's protocols. PCR amplification conditions were as follows: 95°C for 30 sec, followed by 40 cycles at 95°C for 15 sec, 60°C for 30 sec, 72°C for 30 sec and 4°C for 1 min. The following primers were used: U6, CTCGCTTCGGCAGCACA (forward) and AAC GCTTCACGAATTTGCGT (reverse); miR-132, GCCCGT AACAGTCTACAGCCAT (forward) and GCAGGGTCCGAG GTATTC (reverse). The expression of miR-132 was quantified using $2^{-\Delta\Delta Cq}$ method (n=3) (23).

Cell culture and transfection. PC12 cells were purchased from the Cell Bank of the Shanghai Institute of Cell Biology at the Chinese Academy of Sciences (Shanghai, China) and were cultured in Dulbecco's modified Eagle medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (Hyclone; GE Healthcare Life Sciences) at 37°C and 5% CO2. miR-132 mimics (5'-CCG CCCCCGCGUCUCCAGGGCAACCGUGGCUUUCGAUUG UUACUGUGGGAACUGGAGGUAACAGUCUACAGCCAU GGUCGCCCCGCAGCACGCCCACGCGC-3') and negative mimics (5'-CCGCCCCCGCCCC-3') were purchased from Beijing Zoman Biotechnology Co., Ltd. (Beijing, China). A total of 100 nM miR-132 mimics and negative mimics were used to transfect into PC12 cells using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.). Following transfection for 48 h, Lipofectamine was replaced with Earle's balanced salt solution (Hyclone; GE Healthcare Life Sciences) and placed in an incubator (ThermoForma 3111; Thermo Scientific, Inc.) containing 5% O₂ and 95% N₂ at 37°C for 6 h to create an oxygen glucose deprivation (OGD) model.

ELISA. Total protein from PC12 cells was extracted using radioimmunoprecipitation assay (RIPA) buffer (BioTeke Corporation, Beijing, China) and quantified using a BCA protein assay kit (BioTeke Corporation). Total protein (~10 μ g per well) was measured to determine the levels of tumor necrosis factor (TNF)- α (cat no. PT516; Beyotime Institute of Biotechnology, Haimen, China), interleukin (IL)-1 β (cat no. PI303; Beyotime Institute of Biotechnology), IL-6 (cat no. PI328; Beyotime Institute of Biotechnology), IL-8 (cat no. H008; Nanjing Jiancheng Bioengineering Institute, Nanjing, China), caspase-3 (cat no. C1116; Beyotime Institute of Biotechnology) and capase-9 (cat no. C1158; Beyotime Institute of Biotechnology) using ELISA kits (RayBiotech, Inc., Norcross, GA, USA).

Western blotting. Total protein from PC12 cells was extracted using RIPA buffer and quantified using a BCA protein assay kit. Total protein (~50 μ g per well) was loaded on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to a polyvinylidene fluoride membrane (Thermo Fisher Scientific, Inc.). Membranes were initially blocked with 5% skimmed milk powder at 37°C for 1 h in 0.1% TBS-Tween-20 and incubated with cyclooxygenase-2 (Cox-2; cat no. sc-7951; 1:500), Bcl-2 (cat no. sc-783; 1:500), Bcl-2-associated X (Bax; cat no. sc-6236; 1:500), NF-кB p65 (cat no. sc-372; 1:500), matrix metalloproteinase (MMP-9; cat no. sc-10737; 1:500), vascular cell adhesion molecule-1 (VCAM-1; cat no. sc-8304; 1:500), inducible nitric oxide synthase (iNOS; cat no. sc-649; 1:1,000), VEGF (cat no. sc-13083; 1:500) and GAPDH (cat no. sc-25778; 1:1,000) primary antibodies, which were all purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) at 4°C overnight. Membranes were washed with PBS three times and probed with anti-rabbit horseradish peroxidase-conjugated secondary antibodies (cat no. sc-2004; 1:5,000; Santa Cruz Biotechnology, Inc.) for 1 h at 37°C, developed with a Luminol chemiluminescence detection kit (cat no. 12015218001; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and analyzed using sodium Image_Lab version 3.0 (Bio-Rad Laboratories, Inc.).

Statistical analysis. Data are presented as the mean \pm standard error of the mean using SPSS software version 20.0 (IBM Corp., Armonk, NY, USA). Statistical significance was determined by one-way analysis of variance with Dunnett's post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-132 expression in patients with ICD. To investigate whether miR-132 may be neuroprotective, the expression of miR-132 in patients with ICD was determined. As demonstrated in Fig. 1, miR-132 expression in patients with ICD was lower compared with the normal group.

Effects of miR-132 overexpression on inflammation. To evaluate the effect of miR-132 expression on ICD-induced inflammation *in vitro*, OGD-injured PC12 cells were established. As demonstrated in Fig. 2, ELISA results demonstrated that there were significant increases in the levels of TNF- α , IL-1 β , IL-6 and IL-8 in OGD-injured PC12 cells. Furthermore, it was observed that miR-132 overexpression significantly reduced OGD-induced TNF- α , IL-1 β , IL-6 and IL-8 levels in OGD-injured PC12 cells (Fig. 2).

Effects of miR-132 overexpression on caspase-3 and caspase-9 levels. The effects of the expression of miR-132 on apoptosis were determined by measuring caspase-3 and caspase-9 levels. As demonstrated in Fig. 3, ELISA results demonstrated that caspase-3 and caspase-9 levels were significantly increased in OGD-injured PC12 cells. Overexpression of miR-132 significantly inhibited OGD-induced increases in caspase-3 and caspase-9 levels in OGD-injured PC12 cells.

Effects of miR-132 overexpression on Cox-2 and Bax/Bcl-2 expression. To evaluate the effect of miR-132 expression on Cox-2 and Bax/Bcl-2 expression protein expression *in vitro*, Cox-2 and Bax/Bcl-2 protein expression was measured by western blotting. As demonstrated in Fig. 4, there was a significant increase of Cox-2 and Bax/Bcl-2 protein expression in OGD-injured PC12 cells compared with the control group. However, overexpression of miR-132 significantly suppressed Cox-2 and Bax/Bcl-2 protein expression in OGD-injured PC12 cells (Fig. 4).

Effects of miR-132 overexpression on MMP-9 and NF- κ B protein expression. Western blotting was performed to analyze MMP-9 and NF- κ B protein expression in OGD-injured PC12 cells. As expected, MMP-9 and NF- κ B protein expression were significantly increased in OGD-injured PC12 cells compared with control cells (Fig. 5). However, overexpression of miR-132 significantly suppressed MMP-9 and NF- κ B protein expression in OGD-injured PC12 cells (Fig. 5).

Effects of miR-132 overexpression on VCAM-1, iNOS and VEGF protein expression levels. VCAM-1, iNOS and VEGF protein expression in OGD-injured PC12 cells were also



Figure 1. miR-132 expression in patients with ICD and healthy volunteers. miR, microRNA; ICD, patients with ischemic cerebrovascular disease; normal, normal volunteer group.

determined by western blotting. In OGD-injured PC12 cells overexpressing miR-132, VCAM-1 and iNOS protein expression were significantly induced compared with non-OGD cells, and VEGF protein expression was significantly reduced (Fig. 6). However, overexpression of miR-132 significantly suppressed VCAM-1 and iNOS protein expression, and induced VEGF protein expression in OGD-injured PC12 cells (Fig. 6).

Discussion

In the 21st century, stroke has become the third highest cause of mortality in the majority of developed countries, and a major cause of mortality in China (24). It affects productivity and is a heavy burden for the family of patients and for society. The fibrinolytic method has been considered as an effective therapeutic method for ICD at present (25). However, <50% of patients are treated with this method. Circumstances have not improved in spite of research efforts and numerous clinical trials aiming to reduce the rates of disability and mortality (3). Thus far, pharmaceutical research has focused on ion channel (Ca²⁺ and Na⁺) blockers, oxygen free radical scavengers, and excitability and toxicity neurotransmitter blockers (primarily glutamate and glycine receptors) (26). However, the majority of the clinical trials involving such targets have failed due to low efficacy, the number of side effects or study difficulties (26).

Inflammatory reactions encompass the transfer of liquid and cells from blood vessels to tissue, and protection and repair of tissue. (14). It is caused by the interaction of different cells at injured tissues, including neurons, astrocytes, myofibroblasts, smooth muscle cells, endothelial cells and hemocytes (27). The most typical infarction center is an excitatory neurotransmitter released by the damaged neurons, successively followed by Ca²⁺ overload, lipid peroxidation, karyoclasis and neuron death (27). Ischemia and reperfusion have been reported to induce inflammatory reactions (28). Inflammatory reactions are characterized by the expression of local inflammatory cytokines, including TNF- α and IL-1 β , the release of chemotactic factors and upregulation of cell adhesion molecules (29). The results of the present study revealed that miR-132 overexpression significantly reduced OGD-induced TNF-α, IL-1β, IL-6 and IL-8 levels in OGD-injured PC12 cells. Kim et al (30)



Figure 2. Effects of miR-132 overexpression on inflammation. miR-132 overexpression exhibited effects on (A) TNF- α , (B) IL-1 β , (C) IL-6 and (D) IL-8 levels in PC12 cells. **P<0.01 vs. miR-132 mimics-only group and ##P<0.01 vs. OGD + miR-132 mimics group. miR, microRNA; TNF, tumor necrosis factor; IL, interleukin; OGD, oxygen glucose deprivation; miR-132 mimics, miR-132 overexpression group; miR-scramble, miR-scramble control group; OGD + miR-132 mimics, OGD + miR-132 overexpression group; miR-scramble control group; OGD + miR-132 mimics, OGD + miR-132 mimics, OGD + miR-scramble, OGD + miR-scramble control group.

Figure 3. Effects of miR-132 overexpression on caspase-3 and caspase-9 levels. miR-132 overexpression exhibited effects on (A) caspase-3 and (B) caspase-9 levels. **P<0.01 vs. miR-132 mimics-only group and ##P<0.01 vs. OGD + miR-132 mimics group. miR, microRNA; OGD, oxygen glucose deprivation; miR-132 mimics, miR-132 overexpression group; miR-scramble, miR-scramble control group; OGD + miR-132 mimics, OGD + miR-132 overexpression group; OGD + miR-scramble, OGD + miR-scramble control group.

Figure 4. Effects of miR-132 overexpression on Cox-2 and Bax/Bcl-2 expression. (A) Effects of miR-132 overexpression on Cox-2 and Bax/Bcl-2 protein expression were determined by western blotting. Densitometric analysis was performed to quantify protein expression of (B) Cox-2 and (C) Bax/Bcl-2 ratio. **P<0.01 vs. miR-132 mimics-only group and $^{\#}P$ <0.01 vs. OGD + miR-132 mimics group. miR, microRNA; Cox-2, cyclooxygenase-2; Bax, Bcl-2-associated X; OGD, oxygen glucose deprivation; miR-132 mimics, miR-132 overexpression group; miR-scramble, miR-scramble, miR-scramble control group; OGD + miR-132 mimics, OGD + miR-132 overexpression group; OGD + miR-132 mimics, OGD + miR-scramble control group.

Figure 5. Effects of miR-132 overexpression on MMP-9 and NF- κ B p65 protein expression. (A) Effects of miR-132 overexpression on MMP-9 and NF- κ B protein expression were determined by western blotting. Densitometric analysis was performed to quantify protein expression of (B) MMP-9 and (C) NF- κ B p65. **P<0.01 vs. miR-132 mimics-only group and ##P<0.01 vs. OGD + miR-132 mimics group. miR, microRNA; MMP, matrix metalloproteinase; NF- κ B, nuclear factor- κ B; OGD, oxygen glucose deprivation; miR-132 mimics, miR-132 overexpression group; miR-scramble, miR-scramble control group; OGD + miR-132 mimics, OGD + miR-132 overexpression group; OGD + miR-scramble, OGD + miR-scramble control group.

Figure 6. Effects of miR-132 overexpression on iNOS, VEGF and VCAM-1 protein expression levels. (A) Effects of miR-132 overexpression on iNOS, VEGF and VCAM-1 protein expression were determined by western blotting. Densitometric analysis was performed to quantify protein expression of (B) VCAM-1, (C) VEGF and (D) iNOS. **P<0.01 vs. miR-132 mimics-only group and #*P<0.01 vs. OGD + miR-132 mimics group. miR, microRNA; iNOS, inducible nitric oxide synthase; VEGF, vascular endothelial growth factor; VCAM, vascular cell adhesion molecule; OGD, oxygen glucose deprivation; miR-132 mimics, miR-132 overexpression group; miR-scramble, miR-scramble control group; OGD + miR-132 mimics, OGD + miR-132 overexpression group; OGD + miR-scramble, OGD + miR-scramble control group.

previously demonstrated that miR-132 is a critical mediator in the pathogenesis of inflammatory bowel disease by negatively regulating Forkhead box O3a to enhance the expression of inflammatory cytokines. The proliferation and migration of endothelial cells induced by VEGF promotes the formation of new blood vessels (11). Increased vasopermeability serves an important regulatory role in angiogenesis. In the case of cerebral ischemia and hypoxia, VEGF was reported to increase the vasopermeability and promote the exosmosis of plasma fibrinogen, vasoconstriction factors and blood coagulation factors (31). Macromolecular substances in the blood, such as fibrinogen, enter the extracellular matrix and form fibrin glue. Extracellular protein fiber, as a temporary substrate, allows and supports the introversion growth of new blood vessels and stromal cells, and is conducive to angiopoiesis (32). The increase in permeability destroys the connective structure of endothelial cells, leading to an imbalance in the dynamic equilibrium of the blood-brain barrier with low permeability. This, in turn, may cause microvascular structural damage to the diseased tissue and plasma extravasation, which aggravates encephaledema following ischemia damage (33). The results of the present study demonstrated that miR-132 overexpression suppressed caspase-3 and caspase-9 levels, reduced the Bax/Bcl-2 ratio and increased VEGF protein expression in OGD-injured PC12 cells. Mulik et al (34) reported that miR-132 inhibited angiogenic Ras activity in corneal CD31-enriched cells via VEGF and VEGF-A.

The promotion of Cox-2 is part of the development of neuronal apoptosis in brain ischemia and neurodegenerative diseases (35). NF- κ B exists in almost all types of cells. In the nervous system, it is extensively distributed in nerve cells, astrocytes and microglial cells. In addition, vascular endothelial cells include an NF- κ B locus that serves important roles in the pathological process of cerebral ischemia-reperfusion (16). Following ICD, NF-KB is one of the transcription factors that regulate inflammatory gene expression. NF-kB is involved in body defense functions and inflammatory response-associated gene expression. It is an important transcription factor that binds to the promoters of inflammatory genes, including TNF-α, Cox-2, IL-1β, IL-6, IL-8, VCAM-1 and iNOS (29). NF- κ B is bound to inhibiting factors in its inactive state (36). Under the effects of a stimulating factor, NF-kB in neuronal cells, endothelial cells, neurogliocyte and inflammatory cells around the blood vessel is activated (37). NF-κB subsequently participates in inflammation and immune reaction, and serves a vital role in the pathological process of ischemia-reperfusion injury (38). Li et al (21) indicated that miR-132 enhanced the transition from inflammation to proliferation during wound healing by suppressing the NF- κ B pathway. The present study hypothesized that miR-132 may affect Cox-2 expression in ICD, and the results demonstrated that miR-132 overexpression suppressed Cox-2, iNOS, NF-κB and VCAM-1 protein expression in OGD-injured PC12 cells.

In conclusion, the present study demonstrated that miR-132 may adjust angiogenesis in patients with ICD by suppressing the NF- κ B pathway and promoting the VEGF pathway. These findings may provide novel evidence to support the use of miR-132 therapies for ICD.

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