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Original Article

MiRNA-214 ameliorates neuronal apoptosis in an experimental rat stroke model by targeting Bax

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Abstract: Accumulating evidences suggest that most neurons undergo apoptosis upon the exposure to the blood flow reduction in ischemic stroke. MicroRNAs are a class of endogenous small non-coding RNAs, which have been associated with many diseases, including ischemic stroke. In this study, we aimed to investigate the effects of miR-214 overexpression on neurological outcomes, infarct volume and neuronal death in middle cerebral artery occlusion (MCAO) mice, and to explore the underlying mechanisms. Our results showed that miR-214 expression was significantly decreased in the brain of MCAO mice. Infusion with miR-214 agomir improved the neurological outcomes and reduced the infarct volume. Moreover, neuronal apoptosis in MCAO mice was markedly inhibited after upregulation of miR-214. Furthermore, the luciferase receptor assay identified the binding of miR-214 to the 3'-untranslated region (UTR) of Bax mRNA. Overexpression of miR-214 with transfection of miR-214 mimics reduced Bax protein expression, whereas inhibition of miR-214 increased Bax expression in vitro. Additionally, in the brain of MCAO mice, upregulation of miR-214 resulted in a decrease in Bax expression, with an increase in Bcl-2 expression, concomitantly with inhibition of cytochrome c release from mitochondria to cytosol and inactivation of caspase-3 and -9. Taken together, our data demonstrate that miR-214 inhibits neuronal apoptosis by negatively regulating Bax, and consequently attenuates ischemic injury in MCAO mice. These findings may provide a potential therapeutic target for stroke treatment.

Keywords: Ischemic stroke, neuronal apoptosis, miR-214, Bax, mitochondria

Introduction

Worldwide, stroke is the major cause of neurological long-term disability and the second leading causes of death, with an incidence of about 17 million per year [1]. In China, it is estimated that 1 million people die from stroke-related disability per year, of which 2.5 million suffer from stroke [2]. Currently, although many clinical stroke trials and tremendous progresses have been made, the only effective therapeutic strategy to date is thrombolysis by using intravenous recombinant tissue plasminogen activator [3]. Moreover, the risk of intracranial bleeding may be higher than the benefits beyond the 4.5-h window [4]. Previous studies have demonstrated that stroke triggers multiple signaling pathway and subsequently results in neuronal acute necrosis, necroptosis and apoptosis in the ischemic brain [5, 6]. Thus, to understand the underlying cellular and molecular mechanisms of neuronal death will benefit for the stroke treatments.

Cerebral ischemia triggers a cascade of events including oxidative stress, inflammation, and glutamate excitotoxicity, resulting in necrotic and apoptotic death [7, 8]. Over the past decades, multiple studies have indicated that neuron in the ischemic penumbra may mostly undergo apoptosis which could be salvaged by treatment for some time after the onset of stroke [8, 9]. Therefore, targeting apoptosis-related genes may a useful strategy for attenuating neuronal death in ischemic stroke.

MicroRNAs (miRNAs) are endogenous small (containing about 22 nucleotides) non-coding regulatory RNAs, with high conservation between species [10]. At posttranscriptional level, miRNAs bind with their targets at 3'-untranslated region (UTR) region, leading to translational

repression [11]. Recently, it has been reported that many miRNAs are involved in the development of neurological diseases, including Parkinson disease, Alzheimer disease, multiple sclerosis, and stroke [12-15]. In addition, it is worthy to note that more than 20% of the miRNAs expressions are altered in ischemic stroke [15, 16], suggesting that miRNAs are promising mediators in pathogenic and pathological of ischemic stroke. In this study, we found that miR-214 expression was significantly decreased in the brain of middle cerebral artery occluded (MCAO) mice, indicating miR-214 may play an important role in ischemic stroke. This led us to elucidate the effect of miR-214 on neuronal apoptosis in the mouse focal ischemic stroke in vivo, and to explore the underlying mechanisms. Our results may provide a possible novel therapeutic target for the treatment of ischemic stroke.

Materials and methods

MiR-214 agomir, mismatched agomir (MA), miR-214-mimics, miRNA mimics negative control (NC-i), miR-214-inhibitor and miRNA inhibitor negative control (NC-i) were obtained from Ribobio (Guangzhou, China). Dulbecco's modified Eagle's (DMEM)/F-12 medium, fetal calf serum (FCS), penicillin, streptomycin, Trizol Reagent and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA, USA). All chemicals and reagents unless otherwise indicated were purchased from Sigma (Louis, MO, USA).

Experimental design

Male C57/BL6 mice (8-10 weeks, 18-22 g) were purchased from Slaccas Lab Animal Ltd (Shanghai, China) and maintained in a 12 h light/dark cycle with access to food and water at room temperature. All animal protocols were approved by the Institutional Animal Ethics Committee. A total of 150 mice were subjected to sham or MCAO surgery, 11 were excluded from analysis, 8 animals died in the surgery, and 3 had no evidence of neurological deficit acutely. Mice were divided into the following groups: (1) sham group (n=50), (2) MCAO group (n=48), (3) MA-treated MCAO group (n=19) and (4) miR-214 agomir-treated MCAO group (n=22).

MCAO-induced transient focal cerebral ischemia

The transient focal cerebral ischemia was conducted by MCAO surgery as described previously [6, 17]. Briefly, after being anesthetized with pentobarbital sodium (0.06 g/kg i.p.), a blunt-tipped 5-0 surgical nylon filament coated with silicon hardener was inserted to the right carotid through the external carotid stump and advanced to a point approximately 12 mm to the carotid bifurcation. Blood flow was recorded before and after inserting the filament. Only those mice were contained in the study which had a decrease in blood flow by at least 80% of the baseline. After 60 min of MCAO, the filament was carefully removed to restore reperfusion. The sham mice were received an identical procedure without the filament insertion. At 24 h after reperfusion, mice were deeply anesthetized, and brains were isolated and subjected to TTC staining, immunofluorescence and western blotting.

Intracerebroventricular infusion of miR-214 agomir

The intracerebroventricular infusion of miR-214 agomir and its negative control (mismatched agomir, MA) were carried out 2 days before MCAO as previously described [17]. Briefly, mice were anesthetized with pentobarbital sodium and fixed to a stereotaxic apparatus. A brain infusion cannula (Bregma: -2.2 mm, Dorsoventral: 3 mm, Lateral: 1 mm) was stereotaxically implanted into the left lateral ventricle of the brain, which was connected with a micro-pump (Model 1003D, Alzet, Cupertino, CA, USA) and placed in a subcutaneous pocket on the neck of the mouse. Continuous intracerebroventricular infusion of miR-214 agomir and its negative control (5 pmol/μL) were delivered to the brain at a rate of 1 μL/h.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from brain tissue and blood sample by using the Trizol Reagent and then reverse transcription was performed using the miRcute miRNA first-strand cDNA synthesis kit (TIANGEN, Beijing, China). qRT-PCR reactions were conducted with the miRcute miRNA qPCR kit (TIANGEN) using an ABI 7500 real-time PCR system (Applied Biosystems, Forster City, CA, USA) at 95°C for 10 min, followed by 40 amplification cycles of 95°C for 10 s and 60°C for 1 min. The expression of miR-214 was normalized using U6 as an internal control. Predesigned primers for mouse miR-214 and U6 were from Applied Biosystems.

Behavioral testing

Motor function was assessed with the string test as previously described [18, 19]. All mice were received training in string test for 7 days before MCAO surgery, and the mice that achieving criteria were not used for further study. The mouse was placed on the string (Diameter: 2 mm; Length: 50 cm) between the supports at a height of 40 cm. After performance of tests, scores were calculated as follows: 0, falls off; 1, hangs onto the string with two forepaws; 2, hangs onto the string with two forepaws and attempts to climb onto the string; 3, hangs onto the string with two forepaws and one hind paw; 4, hangs onto the string with two forepaws and both hind paws; 5, escape. Animals not able to achieve to at least 4 score were exclude from the MCAO surgery. String test was performed 3 times after 24 h of MCAO and by blinded observer.

Cerebral infarction volume determination

Cerebral infarction volume was determined with 2,3,5-Triphenyltetrazolium chloride (TTC) staining. At 24 h after MCAO surgery, the mouse brains were dissected coronally and cut into 2-mm sections. Afterward, brain sections were stained with 2% TTC for 30 min at 37°C, and then fixed in 4% paraformaldehyde at 4°C overnight. The red and white brain regions represented as the normal and infarct area, respectively. The relative infarct volume was expressed as a percentage of the infarct volume compared to the contralateral hemisphere volume and was assessed by computerized image analysis software (Image-Pro-Plus, Media Cybernetics, Rockville, MD, USA).

Immunofluorescence

To assess neuronal injury, we performed Fluoro-Jade C staining, which is a specific marker for identification irreversibly degenerating neurons [20]. The brain tissue sections were deparaffinized and rehydrated, and then were bathed in a solution of 0.001% Fluoro-Jade C (Millipore, Billerica, MA, USA) dissolved in 0.1% acetic acid for 10 min. After 3 times 1-min washes with distilled water, the air-dried sections were cover-slipped and imaged with a confocal microscope (FV1000, Olympus, Tokyo, Japan).

Apoptosis was analyzed by in situ staining of DNA ends with TUNEL according to the manufacturer's instructions (Cell Death Detection Kit, Roche, Germany). The sections were deparaffinized and rehydrated, and incubated with TUNEL Reaction Mixture for 60 min at 37°C. TUNEL-positive cells were observed using a confocal microscope (FV1000).

Cell culture and miRNA transfection

HEK293T cells were purchased from the Cell Bank of Chinese Academy of Medical Science (Shanghai, China) and maintained in DMEM/F-12 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 U/ml streptomycin in a humidified incubator with 5% CO_2 at 37°C. For overexpression or knockdown of miR-214, the cells were transfected with miR-214 mimics, miR-214 inhibitor, or their negative control for 48 h by using Lipofectamine 2000 according to the manufacturer's instructions.

Luciferase reporter assay

The luciferase experiment was performed as previously described [6]. Briefly, the human Bax 3'-UTR luciferase reporter construct (WT Bax) was amplified by PCR from the Bax mRNA (NM_004324) 3'-UTR sequence of the PGL3 construct (Ambio Inc., Austin, TX, USA). We also generated a mutant 3'-UTR of the Bax gene (Mut Bax) with substitution of 5 bp from seed region of miR-214. HEK293T cells were cultured in 96-well plates at 0.6×10^4 cells per well. After 24-h culture, the cells were co-transfected with luciferase reporter and miR-214 mimics or its negative control using Lipofectamine 2000. Cells were collected and assayed using a luciferase assay kit 48 h after transfection (Promega, Madison, WI, USA).

Western blotting analysis

Brain tissues were dissected and lysed with radioimmuno precipitation assay buffer (RIPA buffer, Beyotime, Jiangsu, China) containing protease and phosphatase inhibitors (Merck, Darmstadt, Germany). For preparation of mitochondrial fractions, mitochondrial and cytoplasmic proteins were isolated using the Mitochondria Isolation Kit (Thermo Fisher Scientific Inc., Rockford, IL, USA) according to manufacturer's instructions. The protein content was determined by Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Equal amount of total pro-

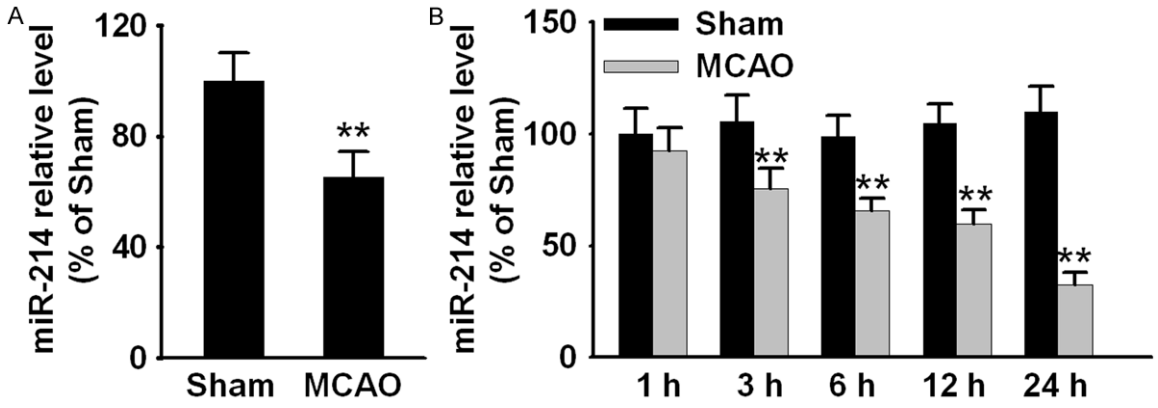

Figure 1. miR-214 expression is decreased in MCAO mice. A. The serum samples were harvested at 24 h after MCAO surgery and the expression of miR-214 was detected by qRT-PCR. **P<0.01 vs. sham, n=10. B. The total RNA was isolated from brain tissues of the sham or MCAO mice at indicated time, and then the expression of miR-214 was determined. **P<0.01 vs. corresponding sham mice, n=8.

tein were separated by 8% SDS-PAGE and then transferred to PDVF membranes (Roche, Germany). The membranes were blocked with 5% skim milk for 1 h and then incubated with the following primary antibodies at 4°C overnight: Bax, Bcl-2, cytochrome c, cleaved caspase-3, cleaved caspase-9 (1:1000, Cell Signaling Technology, Beverly, MA, USA), Cox-IV, β-actin (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing with 3 times each at 10 min intervals, the membranes were probed with secondary antibodies including peroxidase-conjugated anti-mouse or rabbit antibody (1:1000, Beyotime) for 1 h at room temperature. The signals were visualized with enhanced chemiluminescence (Amersham Pharmacia, Piscataway, NJ, USA).

Statistical analysis

All data were presented as mean ± SEM and analyzed by SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA). Comparisons were performed by one-way analysis of variance (ANOVA) or the unpaired two-tailed Student's t test. *P* value less than 0.05 was considered to be statistically significant.

Results

Decreased expression of miR-214 in MCAO mice

An animal model of MCAO was established in mice for 24 h. The expression of miR-214 was determined in the serum samples from Sham and MCAO mice. The results showed that miR-214 expression was significantly decreased in the serum of MCAO mice (**Figure 1A**). Consistently, miR-214 expression in the brains of MCAO mice was gradually decreased. At 1, 3, 6, 12 and 24 h, the expression of miR-214 was reduced to 92.5%, 78.4%, 62.1%, 54.4%, and 27.2% of corresponding sham mice (**Figure 1B**).

MiR-214 reduces neurological deficit and infarct volume in MCAO mice

To investigate the potential role of miR-214 in ischemic injury induced by MCAO, we determined whether restoring miR-214 expression using stereotaxic injection of miR-214 agomir for 2 days before MCAO surgery. The expression of miR-214 in brain tissue was examined by qRT-PCR. The results showed that miR-214 expression was significantly higher than those in the control or mismatched agomir (MA) infusion group (**Figure 2A**). Behavioral assessment showed that cerebral ischemia remarkably decreased the string test score from 4.3 in control group to 2.2 in MCAO group. However, overexpression of miR-214 significantly increased neurological scores compared to MCAO group (**Figure 2B**). Furthermore, the infarct volume of the brain sections was determined with TTC staining after MCAO surgery. TTC generates red color in normal brain tissue whereas infarcted area was shown in white. As shown in **Figure 2C**, infusion with miR-214 agomir obviously decreased the infarct volume in MCAO mice. Quantitative analysis revealed that the percentage of the infarct volume was reduced from

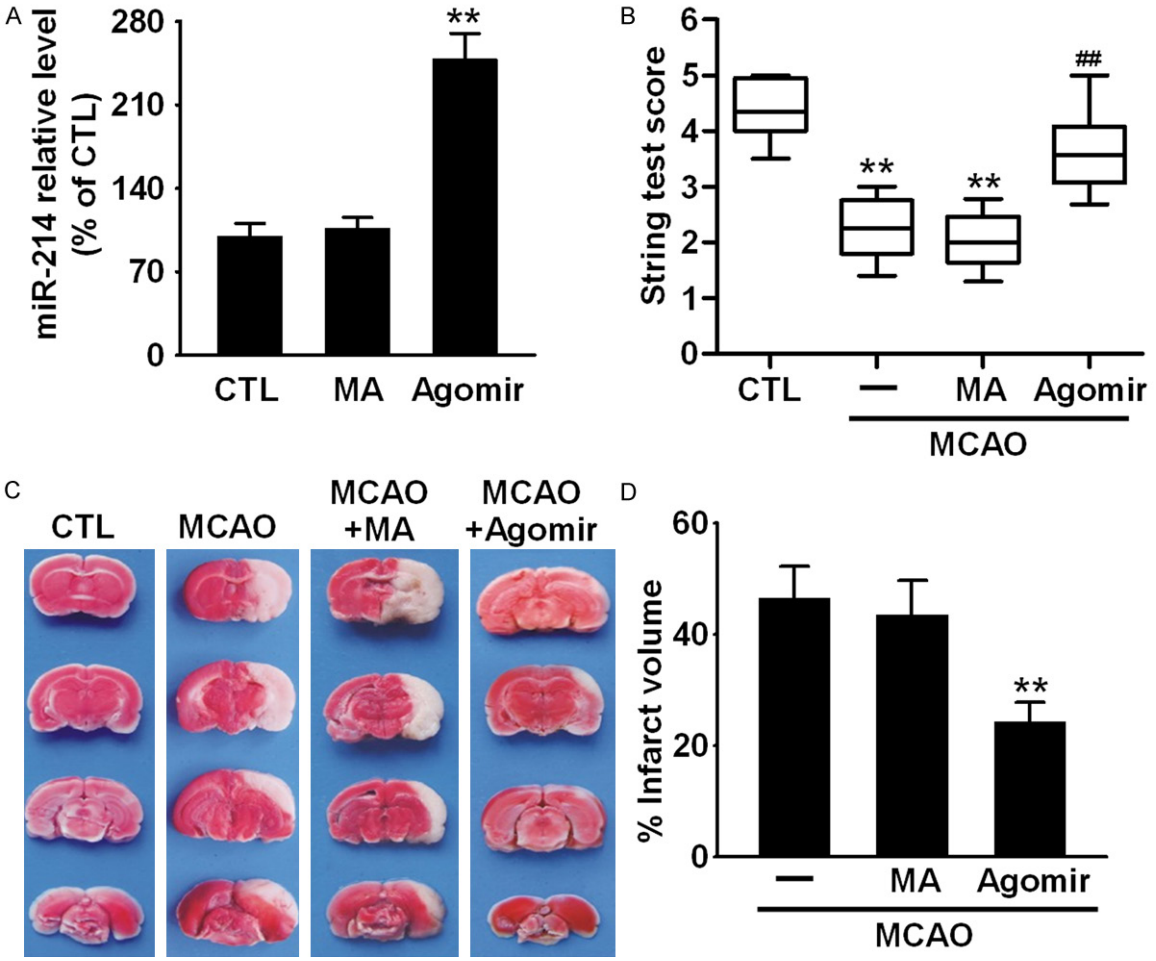

Figure 2. Upregulation of miR-214 improves neurologic functional outcomes. A. MiR-214 agomir or mismatched agomir (MA) was delivered to brain, and the transfection efficiency was determined by qRT-PCR. **P<0.01 vs. control, n=8. B. Quantitative analysis of the scores of the string test of MCAO mice infused with MA or miR-214 agomir. **P<0.01 vs. control; ##P<0.01 vs. MCAO, n=10. C. Representative images of TTC-stained brain sections showing the infarct area 24 h after MCAO. D. Quantitative analysis of the total infarct volume. **P<0.01 vs. control; ##P<0.01 vs. MCAO, n=8.

46.3% in the MCAO group to 23.5% in the miR-214 agomir group (**Figure 2D**). These results indicate that forced miR-214 expression could partially improve neurologic functional outcomes in MCAO mice.

Overexpression of miR-214 inhibits neuronal apoptosis in MCAO mice

To determine whether the neuroprotection of miR-214 was mediated by anti-apoptotic effects, the neuronal survival was examined by using FJ staining. We found that FJ-positive neurons were rarely seen in the sham group, whereas MCAO surgery significantly increased the number of FJ-positive neurons. Infusion of miR-214 agomir dramatically reduced the number of FJ-positive neurons (**Figure 3A** and **3B**). Moreover, as shown in **Figure 3C**, MCAO surgery resulted in strong positive green fluorescent nuclei, as evidenced by the increased intensity of TUNEL fluorescence. However, the DNA damage caused by MCAO surgery was markedly alleviated by miR-214 overexpression (**Figure 3C** and **3D**).

Bax is a direct target of miR-214

Analysis of the database TargetScan (www.Targetscan.org) showed that Bax was the potential target of miR-214. A 7-bp fragment of the 3'-UTR of Bax gene is complementary to the miR-214 seed sequence (**Figure 4A**). To confirm the direct targeting of Bax by miR-214, we gen-

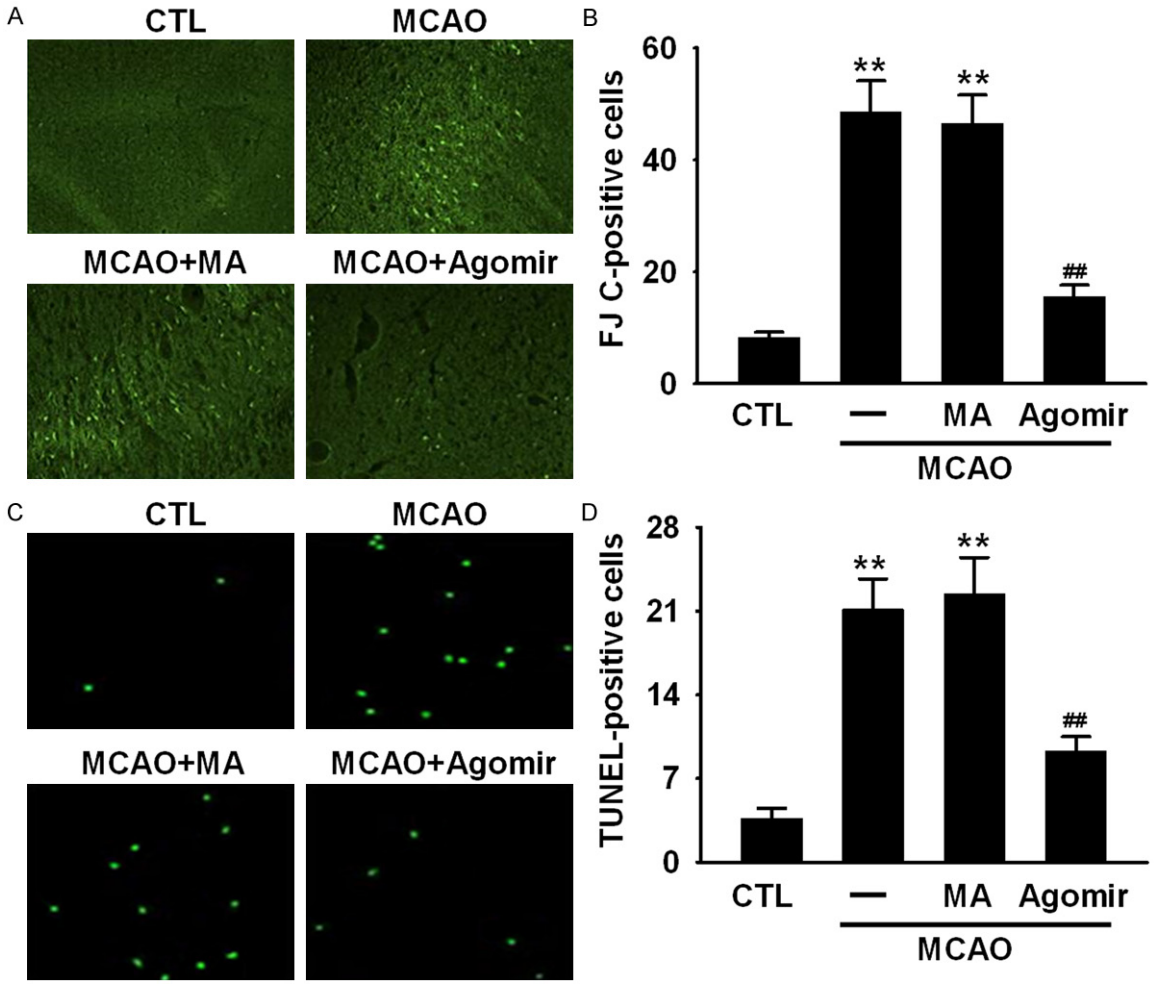

Figure 3. Overexpression of miR-214 reduces neuronal death in MCAO mice. A. The brain sections were stained with Fluoro-Jade C (FJ C) and the representative images were shown. B. Quantitative analysis of FJ C-positive area. C. Representative images of TUNEL staining in the brain tissues. D. Quantitative analysis of TUNEL-positive cells. **P<0.01 vs. control; ##P<0.01 vs. MCAO, n=6.

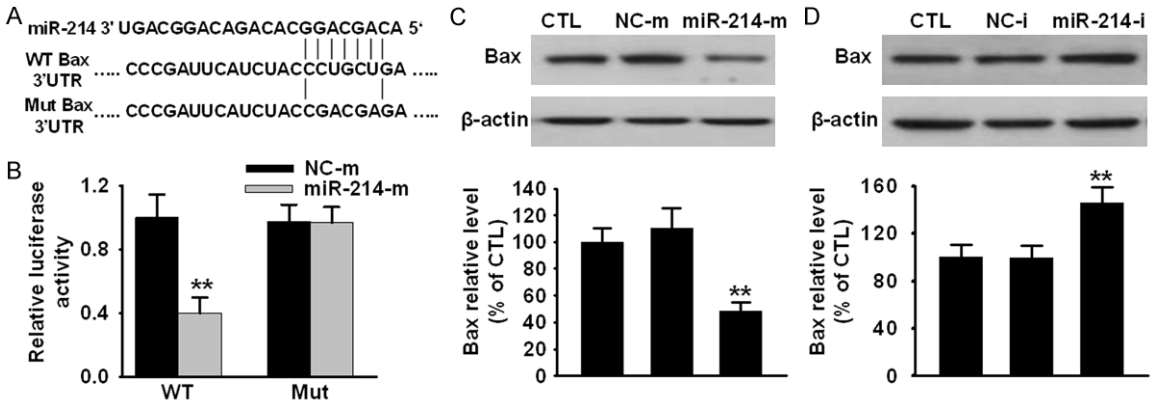

Figure 4. Bax is a direct target of miR-214. A. The diagram of specific complement sequences in the miR-214 seed region and the 3'-UTR of Bax. B. Luciferase reporter assay was performed to examine the luciferase activity of the reporter vectors. **P<0.01 vs. mimics negative control (NC-m), n=6. C. Upregulation of miR-214 by miR-214 mimics in HEK293T cells and the cell lysis was harvested for western blotting to analyze the protein expression of Bax. D. Inhibition of miR-214 by miR-214 inhibitor in HEK293T cells and the cell lysis was for western blotting to analyze the protein expression of Bax. **P<0.01 vs. control, n=6.

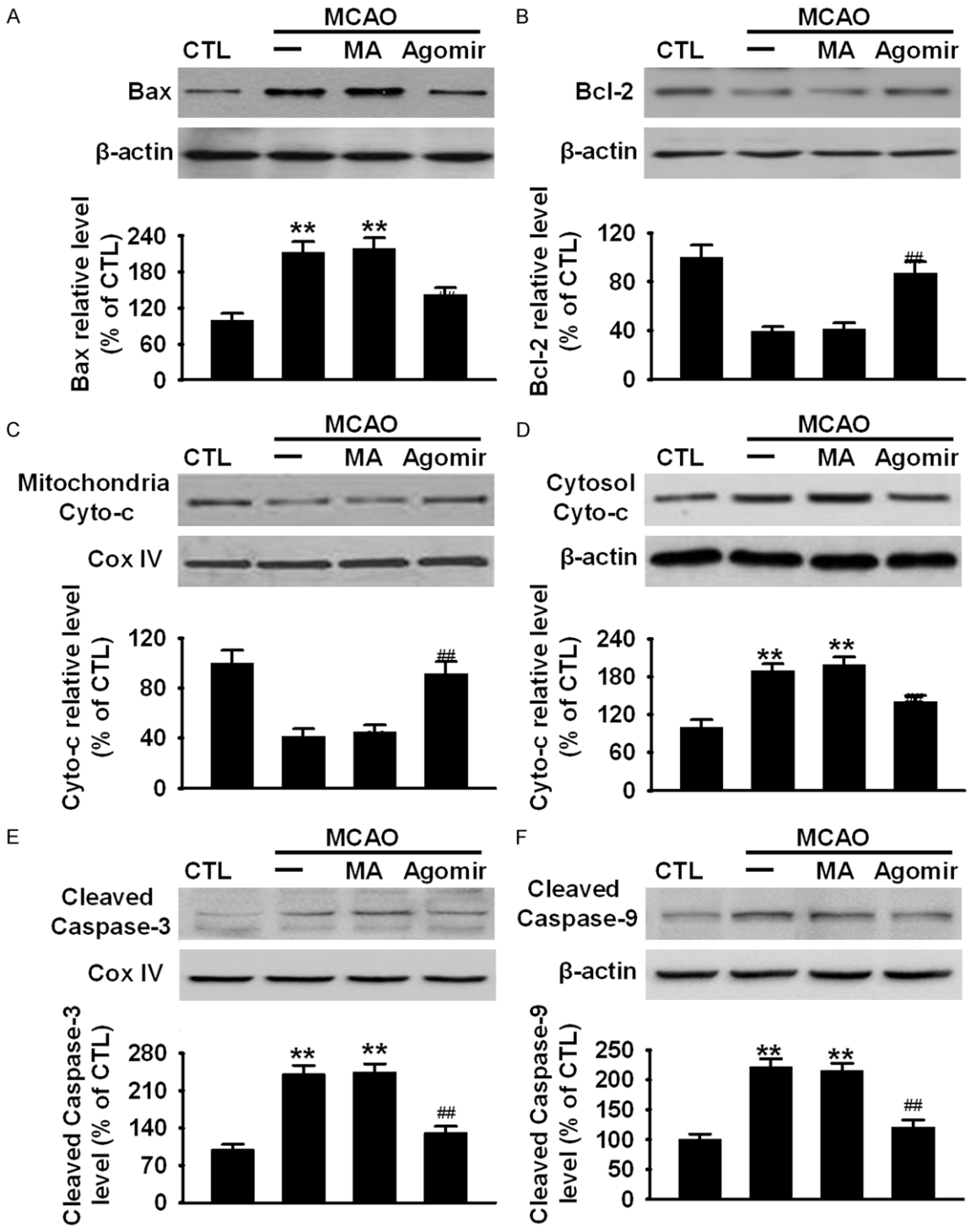

Figure 5. MiR-214 protects against neuronal apoptosis via inhibition of mitochondria-dependent pathway. (A, B) The protein expressions of Bax (A) and Bcl-2 (B) in the brain tissues were analyzed by western blotting. (C, D) Western blotting analysis of cytochrome c protein expression in the mitochondrial (C) and cytosol (D). Cox IV was used as a loading control of mitochondrial protein. (E, F) Protein expressions of cleaved caspase-3 (E) and cleaved caspase-9 (F) were measured by western blotting. **P<0.01 vs. control; ##P<0.01 vs. MCAO, n=5.

erated a firefly luciferase reporter plasmid containing the 3'-UTR of Bax, or a fragment whose target site was mutated, and then transfected these plasmids into HEK293T cells with miR-

214 mimics or negative control. The luciferase assay demonstrated that the relative luciferase activity was reduced in the WT reporter-transfected HEK293T cells that were co-transfected with miR-214 mimics as compared with those cells co-transfected with negative control, whereas this inhibitory effect was completely abolished in the cells transfected with mutated 3'-UTR of Bax (**Figure 4B**). Furthermore, overexpression of miR-214 by transfection with miR-214 mimics dramatically decreased Bax protein expression, whereas downregulation of miR-214 was associated with increased Bax protein expression (**Figure 4C** and **4D**). The above data strongly suggest that miR-214 negatively regulates Bax expression by directly targeting the 3'-UTR of Bax.

MiR-214 attenuates neuronal apoptosis via inhibition of mitochondria-dependent pathway

Bax is an important member of Bcl-2 family, and the ratio of Bcl-2/Bax seems to be the determinants of cell survival and death [21]. Thus, we investigated the effects of miR-214 on Bax and Bcl-2 expression in brain tissue of MCAO mice. Western blotting showed that MCAO surgery remarkably increased Bax expression whereas reduced Bcl-2 expression. However, infusion of miR-214 agomir obviously inhibited the above effects (**Figure 5A** and **5B**). Imbalance of Bax and Bcl-2 could result in mitochondrial dysfunction and consequently cytochrome c release from mitochondria to cytosol [22]. We next determined the release of cytochrome c. The results showed that the release of cytochrome c from mitochondria to cytosol was significantly increased in MCAO mice as compared with sham mice. As expected, upregulation of miR-214 markedly inhibited cytochrome c release (**Figure 5C** and **5D**). Cytochrome c released from mitochondrial will cleave and activate downstream apoptosis-associated proteins, caspase-3 and caspase-9, which in turn initiates the mitochondria-dependent apoptosis [20]. MCAO surgery resulted in a marked increase in caspase-3 and caspase-9 cleavage. However, the activation of caspase-3 and caspase-9 was almost abolished after miR-214 agomir infusion (**Figure 5E** and **5F**). These findings indicate that inactivation of mitochondrial pathway may underlie partially the inhibitory effects of miR-214 on neuronal apoptosis.

Discussion

In the present study, we showed that miR-214 was decreased in brain of MCAO mice. More importantly, we found that miR-214 could protect neuron against apoptosis through negatively regulating pro-apoptotic protein Bax.

It has been reported that there are two types of stroke: ischemic and hemorrhagic [20]. Nevertheless, approximately more than 80% of stroke is ischemic, resulting from vascular occlusion and consequently insufficient blood flow [23]. In ischemic stroke, the most prominent pathological change is the neuronal death, which leads to neuronal loss and irreversible damage within minutes [24]. The peripheral region of the ischemic core area, also known as the "ischemic penumbra", represents the region in which damaged neurons can be potentially recovered if effective therapy was applied [25]. Since our data showed that miR-214 was dramatically decreased in blood samples and brain tissues of MCAO mice, we delivered miR-214 agomir to increase miR-214 level in brain and to investigate the role of miR-214 in ischemia stroke. As expected, the impaired neurological function could be reversed by miR-214 in vivo. Consistently, we observed a decreased cerebral infarction volume in MCAO mice-infused miR-214 agomir. Collectively, these results indicate that miR-214 can protect neuron against ischemic injury and provide experimental evidence for the efficient use of miR-214.

Increasing evidences suggest that apoptosis plays a critical role in mediating neuronal death after cerebral ischemia [8, 9]. Unlike necrosis, apoptosis is a highly regulated process of programmed cell death so that they may be reversed by potential treatment [22]. Here, our results showed that infusion of miR-214 agomir significantly reduced neuronal death in MCAO mice, as evidenced by FJ C staining. Moreover, we also found decreased apoptotic cells in brain tissues of MCAO mice-infused miR-214 agomir, suggesting that inhibition of apoptosis underlies, at least partially, the reduced neuronal death in miR-214 agomir-infused MCAO mice.

There are two major apoptotic pathways: the extrinsic cell death receptor-mediated pathway and the intrinsic mitochondria pathway [26].

The extrinsic apoptotic pathway is triggered by activation of death receptor including FasR, APO-1 and CD95 [27]. The pro-apoptotic protein Bax belongs to the Bcl-2 family and is the most important activator of mitochondria pathway [28]. Bax translocation to the mitochondria increases the mitochondrial membrane permeability, leading to cytochrome c release and caspase pathway activation [21]. In this study, we identified that Bax as a novel target of miR-214. The luciferase reporter gene assay and western blotting showed that miR-214 targeted the 3'-UTR region of Bax to decrease the protein level of the target gene. On the contrary, inhibition of miRNA-214 significantly increased Bax protein expression. Moreover, infusion of miR-214 agomir resulted in a decrease in Bax expression, with an increase in Bcl-2 expression, concomitantly with inhibition of cytochrome c release from mitochondria to cytosol and inactivation of caspase-3 and -9. These data suggest that miR-214 inhibits MCAO-induced neuronal apoptosis mainly via inactivation of intrinsic mitochondria pathway. However, the possibility whether the extrinsic death receptor-mediated apoptosis is also involved would be further explored in the future.

In summary, the present study demonstrated that miR-214 expression was downregulated in the brain of MCAO mice. Infusion of miR-214 agomir not only improved neurological outcomes, but also inhibited neuronal apoptosis in MCAO mice. Furthermore, we identified that miR-214 specifically bind with the 3'-UTR of Bax and repressed its translation. Overexpression of miR-214 in ischemic brain resulted in inhibition of mitochondria pathway-dependent apoptosis. These findings revealed an important biological role of miR-214 in ischemic stroke, suggesting that forced miR-214 expression may be a promising approach for the treatment of stroke.

Disclosure of conflict of interest

None.

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