microRNA-196a attenuates ischemic brain injury in rats by directly targeting high mobility group A1

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Abstract. Dysfunction of the microRNA (miR) network has been indicated as a major regulator in neurological diseases. However, there is limited understanding regarding the functional significance of miRs in ischemic brain injury. In the present study, miR-196a expression was significantly increased in rat brains and neurons following transient middle cerebral artery occlusion (MCAO) or oxygen-glucose deprivation, respectively. In addition, repression of miR-196a significantly decreased neuron cell apoptosis and the infarct size in rats subjected to MCAO (P<0.05). Furthermore, miR-196a was indicated to directly target and inhibit high mobility group A1 expression, which indicated a potential role for miR-196a in ischemic brain injury. These findings suggested that miR-196a may be involved in regulating neuronal cell death, thus offering a novel target for the development of therapeutic agents against ischemic brain injury.

Introduction

Ischemic brain injury is caused by insufficient blood flow to the brain, is characterized by oxidative stress, hypoxia, inflammation and glutamate excitotoxicity, which leads to cell apoptosis and death (1). Ischemic stroke has been indicated as a leading cause of death and long-term disability worldwide (2). Neonatal hypoxic-ischemic brain injury or neonatal stroke is a major cause of hypoxic-ischemic encephalopathy and cerebral palsy (3). The advances in therapy for the purpose of decreasing ischemic brain injury have been limited because the pathophysiological mechanisms remain unknown. Therefore, it is of great importance to understand the pathological mechanisms of ischemia-associated cell death in order to develop effective therapies for ischemic brain injury.

MicroRNAs (miRNAs or miRs) are a family of small RNAs (~22 nucleotides) that modify the expression of genes implicated in biological processes, including tumor cell differentiation, proliferation and apoptosis (4,5). Previous studies have indicated that miRNAs are abundantly expressed in the nervous system and have been initially identified as crucial molecular mediators in the regulation of neuronal cell survival (6-8). miR-497 was the first miRNA identified to promote ischemic neuronal death by negatively regulating anti-apoptotic proteins, B-cell lymphoma-2 (Bcl-2) and Bcl-2-like protein 2 (9). miR-210, miR-29b and miR-124 have also been demonstrated to participate in the biological processes concerning the nervous system (10-12).

These findings suggest that several miRNAs may be potential candidates for possible biomarkers or therapeutic targets in stroke. In the present study, the expression of miR-196a was explored during OGD in vitro and MCAO in vivo. The role of miR-196a in ischemic brain injury was investigated.

Materials and methods

Primary culture of rat cortical neurons. A total of 15 pregnant, female Sprague Dawley rats (mean weight, 245±10 g) were obtain from the SLAC Laboratory Animal Corporation (Shanghai, China). All rats housed at 20-25˚C with 60% humidity under 12 h light:dark cycles with free access to food and water throughout the experiment. After 19-20 days, 115 neonatal rats were born and cerebral cortexes were removed within 24 h by aseptic decapitation. Then cortices were placed in D-hanks solution (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and sliced into 1-mm² fragments. Subsequently, the cortical pieces were dissociated using 0.25% trypsin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 100 ng/ml DNase (Roche Diagnostics, Basel, Switzerland) at 37˚C for 35 min. The digestion was terminated with Dulbecco’s modified Eagle’s medium (DMEM; Hyclone; Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). Isolated cells were suspended in neurobasal-A medium supplemented with 2% B27, 1% glutamine (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 g/ml streptomycin, and seeded into 6-well plates at 5x10⁴ cell/well; the plates were precoated with 10 µg/ml poly-D-lysine (Sigma-Aldrich; Merck KGaA). Cells were incubated in a humidified incubator containing 5% CO₂.
and 95% O₂ at 37°C for 7 days. The present study and all animal experiments were approved by the Ethics Committee at Beilun People's Hospital of Ningbo (Ningbo, China).

**OGD model.** To construct an OGD model, glucose-free Earl's balanced salt solution (EBSS) medium (consisting of 6,800 mg/l NaCl, 400 mg/l KCl, 264 mg/l CaCl₂, 200 mg/l MgCl₂, 2,200 mg/l NaHCO₃, and 140 mg/l Na₂PO₄ mg/l; pH 7.2) was purged with N₂/CO₂ (95/5%) at 37°C for 20 min, resulting in an oxygen content of 1%. Cortical neuronal cell cultures were washed three times with glucose-free EBSS medium and incubated at 37°C for 4 h in oxygen-free N₂/CO₂ (95/5%) gas as previously described (13). For reoxygenation, cells were incubated in fresh neurobasal-A medium supplemented with 2% B-27 in an atmosphere containing 5% CO₂ at 37°C for 12 and 24 h. The control group was incubated in EBSS with 10 mM glucose at 37°C in 5% CO₂ for 12 and 24 h.

**MCAO rat model.** A total of 32 adult male Sprague Dawley rats (age, 10 weeks; mean weight, 289±10 g) were subjected to MCAO from the SLAC Laboratory Animal Corporation. All rats housed at 20-25°C with 60% humidity under 12 h light/dark cycles with free access to food and water throughout the experiment. Rats were anesthetized using 1% sodium pentobarbital (40 mg/kg; Shanghai Xitang Company, Shanghai, China). Subsequently, the right common carotid artery, external carotid artery and internal carotid artery were exposed via a midline cervical incision. A piece of 4/0 monofilament nylon suture with a heat-induced rounded tip was inserted through the right internal carotid artery to the base of the middle cerebral artery, which occluded the blood flow to the cortex and striatum. Following 90 min of MCAO, the rats were allowed to recover for 24 h, as described previously (14). Sham-operated mice underwent the internal carotid artery separation without the suture insertion. Additionally, the rats were randomly separated into four groups: Sham operation, MCAO model, MCAO model treated with antagomiR control (5’-CAGAUC UUUUGUGUGAUCAA-3’) and antagomiR-196a (5’-CCCC AACACAAUGAAAACUCCA-3’). The antagomiR control and antagomiR-196a (both 80 mg/kg) were injected into the caudal vein.

**Cell transfection.** A total of 2x10⁶ cortical neurons were plated in 6-well plates and cultured at 37°C for 24 h. miR-196 mimics, miR-196 inhibitor or high mobility group A1 (HMGA1) vectors were transfected using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at a final concentration of 30 mM. The sequences for the miRNAs were as follows: NC control (5’-CAGAUCUUUUGUGUAGUCA-3’), miR-155 mimic (5’-UAGGAUGUAAUUCAUGUUGUUGG-3’), miR-155 inhibitor (5’-UAGGAUGUAAUUCAUGUUGUUGG-3’). Neurons were harvested for terminal deoxynucleotidyl-transferase-mediated dUTP nick end labelling (TUNEL) assay, western blot analysis or luciferase activity assay, at the indicated time points.

**Infarct volume analysis.** A total of 24 h after reperfusion, the rats were re-anesthetized and sacrificed. Then, the brain tissues of each group were removed, frozen for 30 min at -20°C and sliced into four coronal sections (1 mm thick). The sections were placed in a 1% solution of 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich; Merck KGaA) for 10 min at 37°C and fixed in 4% formalin at 4°C for 24 h. The infarct region lacked staining and appeared white, whereas the normal non-infarct tissue appeared red. Images were captured of both sides of each stained coronal slice using a digital camera and infarction was measured using ImageJ Pro 6.0 digital image analysis software (National Institutes of Health, Bethesda, MD, USA). The infarct volume was expressed as a percentage: Total infarct volume of ipsilateral structure/total volume of contralateral structure x100.

**TUNEL assay.** Coronal brain tissues were fixed in 10% paraformaldehyde at 4°C for 24 h, dehydrated in ethanol (100, 95, 90, 80 and 70%; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) and embedded in paraffin. The specimens were cut into 4-µm thick slices for TUNEL and immunocytochemistry analysis. TUNEL assay was performed according to the manufacturer's instructions of the TdT-mediated dUTP nick end labeling analysis kit (Roche Diagnostics). All cell nuclei were indicated via blue staining and the positive nuclei were indicated via green staining. The number of positive nuclei was determined by manually counting the positively labeled nuclei in five randomly selected fields under a fluorescence microscope (magnification, x400). Immunochemistry. The 4-µm thick sections were deparaffinized and rehydrated with ethanol (100, 95 and 75%), and exposed to the antigen retrieval system (10 mM sodium citrate and 0.05% Tween 20; pH 6.0) at room temperature for 15 min. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 15 min at room temperature. Subsequently, the slides were rinsed in 0.01 mol/l PBS three times and subsequently incubated with the primary mouse polyclonal HMGA1 antibody (1:200; cat. no. ab226112; Abcam, Cambridge, MA, USA) for 1 h at room temperature. Following washing with PBS twice for 5 min, the slides were incubated with horseradish peroxidase-labeled goat anti-mouse antibody (1:100; cat. no. A0192; Beyotime Institute of Biotechnology, Haimen, China) at room temperature for 1 h. Samples were stained using a 3,3'-diaminobenzidine peroxidase substrate kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. Positive nuclei were indicated as brown in color. Slides were observed using a fluorescence microscope (magnification, x400) and the number of positive cells was analyzed by Image J pro 6.0.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA obtained from rats and cells was extracted using TRIzol Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. A total of 1 µg total RNA was reverse-transcribed into cDNA using a TaqMan microRNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) in a 15-µl total volume according to the manufacturer's protocol. The reactions were incubated for 5 min at 95°C, followed by 40 cycles consisting of a 15 sec interval at 95°C and 1 min interval at 60°C. Expression levels of miR-196a were measured by TaqMan MicroRNA Assay kits (Applied Biosystems;
Thermo Fisher Scientific, Inc.) in triplicate on an IQ5 real-time PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and normalized using GAPDH as the internal control. The quantification was performed by the comparative \( 2^{-\Delta\Delta C_T} \) method (15). The primer sequences (5’→3’) were: HMGAI, forward: TTACCAGTACCCACGGTA, reverse: AGG CTGGGACAAATACTGGC; GAPDH, forward: GATGGT GAAGTCTGGTGTTGA, reverse: TGAACCTGCGGTGGG TAGAG; miR-196a RT-primer: CTCACCTGGTGTCTGGA GTGGGCAATTCTAGTTAGCCCAAAAC, forward: ACA CTCCAGCTGTTAGTGTACCTGTT, reverse: TGG TGTCGTGGAGTCG.

**Western blot analysis.** Total proteins from cells were extracted with radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology) and protein concentration was tested using the BCA assay (Beijing Solarbio Science & Technology Co., Ltd.). A total of 30 μg protein were loaded and separated using 10% Tris–glycine SDS-PAGE, and transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA) using the electrophoretic transfer system (Bio-Rad Laboratories, Inc.). The membranes were blocked with 5% non-fat dry milk in Tris-HCl buffer saline (pH 7.4) containing 0.1% Tween 20 for 1 h at room temperature and incubated with primary antibodies against HMGAI and GAPDH (both: 1:1,000; cat. no. ab181602; Abcam) overnight at 4°C. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:2,500; cat. no. A0192) at room temperature for 2 h followed by band detection with a Super Signal Enhanced Chemiluminescence Kit (Pierce; Thermo Fisher Scientific, Inc.). Quantification of the bands was performed using densitometric analysis and the ImageJ Pro 6.0 analysis system (National Institutes of Health).

**Bioinformatics analysis.** To predict the target genes of miR-196a, a series of bioinformatics analyses were used. The authors predicted the target genes for miR-196a with miRBase (http://www.mirbase.org/), TargetScan (http://www.targetscan.org/), and miRDB (mirdb.org/mirDB/). The predicted targets included HMGAI.

**Luciferase assay.** Cortical neuronal cells were plated at a density of 4x10³ cells/well in 96-well plates and cultured at 37°C for 24 h. Cells were co-transfected with 40 ng recombinant wild-type (WT) or mutated (MUT) luciferase vectors [pGL3-HMGAI-3′-untranslated region (UTR)-WT or pGL3-HMGAI-3′-UTR-Mut], pRL-TK vectors and miR-196a mimics or a negative control (NC) mimics (Guangzhou Ribobio Co., Ltd., Guangzhou, China) at a final concentration of 45 nM using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Luciferase assays were performed with a dual luciferase reporter assay system (Promega Corporation, Madison, WI, USA) 48 h after transfection. *Renilla* luciferase activity was normalized to that of Firefly luciferase.

**Statistical analysis.** All data were expressed as the mean ± standard deviation of at least three separate experiments. Difference between two groups was compared by Student's t-test. Difference among multiple groups was analyzed by one-way analysis of variance followed by a post-hoc Student-Neuman-Keuls test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Expression of miR-196a and HMGAI was changed in cortical neurons subjected to OGD.** To investigate the role of miR-196a and HMGAI in ischemic brain injury, an OGD model of cortical neurons was established. RT-qPCR was performed to evaluate the gene expression levels (Fig. 1). As Fig. 1B demonstrated, OGD induced apoptosis and the difference between the control and OGD groups at 12 and 24 h was significant (P<0.05; Fig. 1A). Notably, the miR-196a expression was elevated significantly following OGD at 12 and 24 h compared with the control (P<0.05; Fig. 1C). Conversely, the mRNA and protein expression levels of HMGAI were significantly down-regulated after 12 and 24 h of OGD compared with the control (P<0.05; Fig. 1D and E).

miR-196a reduces the neuronal rate of apoptosis and infarction following ischemic brain injury in vivo. A rat MCAO model was constructed to further explore the effect of miR-196a on ischemic brain injury and its mechanism (Fig. 2). The expression of miR-196a was determined to assess the effects of antagoniR-196a. As presented in Fig. 2A, miR-196a expression in MCAO was significantly higher than the sham group (P<0.01), whereas antagoniR-196a injection significantly decreased the miR-196a expression compared with the antagoniR control group (P<0.05). The infarct volume and cortical neuron apoptosis rate was increased in the MCAO group compared with the sham and this difference was indicated as statistically significant (P<0.05; Fig. 2B-E), which indicated the successful establishment of the *in vivo* model. As expected, knockout of miR-196a by intracerebroventricular injection of antagoniR-196a resulted in a significant decrease in miR-196a expression compared with the MCAO group (P<0.05; Fig. 2A) and significantly improved the recovery of brain function as the infarct volume and the apoptosis rate were significantly decreased compared with the MCAO groups (P<0.05; Fig. 2B-E). Furthermore, TUNEL staining indicated that the proportion of apoptotic cells was decreased in miR-196a antagoniR-injected rat brains compared with the antagoniR control (Fig. 2D) and the difference was statistically significant (Fig. 2E). Considering the abnormal expression of HMGAI in cells undergoing OGD (Fig. 1E), the expression of HMGAI using immunocytochemistry in rat brains subjected to MCAO was evaluated. Consistent with the previous study, the expression of HMGAI was markedly downregulated in the MCAO group compared with that in the sham group and antagoniR-196a injection significantly enhanced the HMGAI expression to sham group (P<0.05; Fig. 2F and G, respectively).

miR-196a directly targets HMGAI and inhibits its expression. To identify the target genes of miR-196a in regulating ischemic brain injury, candidate genes were investigated using miRBase, TargetScan 5.1 and miRNA databases. HMGAI was selected as a potential candidate gene due to its abnormal expression in cortical neuronal cells subjected to OGD and in rat brains following MCAO. miR-196a mediated HMGAI
expression by directly binding to the 3'UTR of HMGA1 in cortical neuronal cells (Fig. 3A). Luciferase assay was performed to indicate whether miR-196a targeted HMGA1. Co-transfection of miR-196a mimics significantly inhibited the luciferase activity produced by the reporter construct that contained the wild-type 3'UTR segment of HMGA1 (P<0.05), whereas no significant effect was observed with the construct containing the mutated segment of HMGA1 3'UTR (Fig. 3B). RT-qPCR demonstrated that miR-196a expression was significantly increased in the miR-196a mimics group, but was reduced in miR-196a inhibitor group when compared with the OGD group (P<0.05; Fig. 3C). In addition, western blot analysis was applied to observe the protein expression levels of HMGA1. The results revealed that transfection of miR-196a mimics significantly decreased HMGA1 in OGD-induced cells, whereas miR-196a inhibitor increased HMGA1 compared with the OGD group (P<0.05; Fig. 3D).

**HMGA1 overexpression suppresses cell apoptosis in cortical neurons subjected to OGD.** To directly determine the effect of HMGA1 on OGD-induced neuronal damage, neuronal cells were transfected with pcDNA3.1/HMGA1 vector and the TUNEL assay was performed to evaluate the apoptosis of cortical neurons under different treatments (Fig. 4A). Compared with the control, HMGA1 overexpression significantly suppressed the apoptosis induced by OGD (P=0.043; Fig. 4B).

**HMGA1 reverses the induction of apoptosis by miR-196a.** In accordance with the findings in the in vivo study, miR-196a overexpression was indicated to promote the apoptosis in OGD neurons. To explore whether miR-196a targets HMGA1, neuronal cells were co-transfected with HMGA1 vector and miR-196a mimics. As expected, HMGA1 overexpression significantly reversed the induction of apoptosis by miR-196a (P<0.05; Fig 4B).

**Discussion**

miRNA binding is mediated by argonaute proteins within the RNA-induced silencing complex, which is dependent on the degree of sequence complementarity and leads to either cleavage of the target mRNA or a reduction in its translational efficiency (16). A subset of miRNAs are abundantly expressed in the human brain (17) and have critical roles in the pathophysiology of brain seizures, ischemia and trauma (18,19).

miR-196a was initially identified as an oncogene that was associated with apoptosis, invasion and proliferation (20). Increasing evidence has suggested the aberrant expression of miR-196a is a frequent event in various cancers, including head and neck squamous cell carcinomas, laryngeal cancer, pancreatic cancer and gastric cancer (21-24). In addition, miR-196a is a putative diagnostic biomarker or therapeutic target for various human diseases, including adrenomyeloneuropathy, chronic hepatitis and Huntington's Disease (25-27). To date, limited understanding exists regarding the association between miR-196a and ischemic brain injury. In the present study, miR-196a expression was significantly increased during OGD in vitro and MCAO in vivo. To determine whether miR-196a has a role in ischemic brain injury, its expression was downregulated and the effects explored. The results demonstrated that knockdown of endogenous miR-196a expression significantly reduced brain infarct size and protected against ischemic-induced brain cell apoptosis. To identify the target genes of miR-196a in regulating cell.
Figure 2. miR-196a reduced neuronal apoptosis and infarction following ischemic brain injury in vivo. (A) Rats were pretreated with a miR-196a antagonim, antagonim control and subsequently subjected to 1.5 h MCAO and 24 h reperfusion. Reverse transcription-quantitative polymerase chain reaction was used to determine the expression of miR-196a in rat brain tissues. (B) Coronal sections exhibiting the different treatments were stained with 1% 2,3,5-triphenyltetrazolium chloride and were evaluated. The infarct region lacked staining and appeared white, whereas the normal non-infarct tissue appeared red. (C) The infarct volume was determined and (D and E) TUNEL assay was performed to evaluate the apoptosis of brain tissue following MCAO (magnification, x100). Blue fluorescence indicates nuclei and green fluorescence indicates apoptotic cells. (F and G) Immunocytochemistry was used to evaluate the HMGA1 expression in brain tissue. Positive nuclei were indicated as brown in color. Data are expressed as the mean ± standard deviation (n=8). *P<0.05 and **P<0.01 vs. the sham group; #P<0.05 vs. the MCAO group. HMGA1, high mobility group A1; miR-196a, microRNA-196a; MCAO, middle cerebral artery occlusion; TUNEL, terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling.
apoptosis, miRBase and TargetScan 5.1 were used to search for candidate genes. Due to the low expression of HMGA1 in the current study, HMGA1 was selected as a potential target gene for miR-196a. However, whether miR-196a directly targets HMGA1 required further elucidation. Luciferase assay results indicated that miR-196a significantly repressed luciferase activity in the cortical neuron cells transiently transfected with wide-type HMGA1 3'UTR compared with cells transfected with mutated HMGA1 3'UTR, which suggested that HMGA1 may be a direct target of miR-196a.
In addition, western blot analysis revealed that knockdown of miR-196a significantly increased the protein expression of HMGA1. The present study indicated that HMGA1 may be a physiological target of miR-196a and regulate its expression at the transcriptional level.

HMGA1 proteins are non-histone proteins that regulate chromatin structure and gene expression during embryogenesis, tumourigenesis and immune responses (28,29). HMGA1 is considered as a key hub for several oncogenic pathways, including the Wnt/β-catenin, Notch pathways and Ras/extracellular-related kinase signaling (30,31). Previous results have indicated that HMGA1 represses p53 apoptotic activity by promoting the cytoplasmic relocalization of the p53 proapoptotic activator homeodomain interacting protein kinase 2 (32). Furthermore, the anti-apoptotic effect of HMGA1 has also been associated with the deregulation of Bcl-2 (33). In addition, ischemic brain injury has been suggested to be associated with widespread molecular and biochemical changes, inflammatory responses, oxidative stress, free radical production and neuronal apoptosis (34-36). Notably, neuronal apoptosis is the prominent cause of cell death in secondary brain damage, which greatly affects the functional outcome of ischemic injury (37). It was hypothesized that miR-196a targeted HMGA1 and regulated neuronal apoptosis following OGD and MCAO. However, the underlying mechanism requires further exploration and further studies are required to explore whether HMGA1 regulates p53 or Bcl-2.

In conclusion, the present study demonstrated that miR-196a was induced by ischemia and may contribute to the pathogenesis of ischemic brain injury by directly targeting HMGA1. Accordingly, the inhibition of miR-196a may become a potential therapeutic option for ischemia-associated brain damage. However, further studies using neuron-specific miR-196a are warranted.

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Availability of data and materials
The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors’ contributions
JH designed the study and collected the data. WS analyzed the data and drafted the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate
The present study and all animal experiments were approved by the Ethics Committee at Beilun People’s Hospital of Ningbo (Ningbo, China).

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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