Int J Clin Exp Med 2017;10(1):965-971
www.ijcem.com /ISSN:1940-5901/IJCEM0031233

Original Article

MicroRNA-18a targets ATXN1 to alleviate injury induced by permanent middle cerebral artery occlusion in mice

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Received April 26, 2016; Accepted August 6, 2016; Epub January 15, 2017; Published January 30, 2017

Abstract: Background: Ischemic stroke injury is caused by cerebral hypoperfusion, characterized by oxidative stress, hypoxia. Nowadays, miRs and their roles in ischemic stroke have attracted much attention. Our purpose was to investigate the role of miR-18a in cerebral ischemic disease. Methods: qRT-PCR was used to analyze the expression level of miR-18a after oxygen-glucose deprivation in PC12. ATXN1 expression was detected by Western blot and qRT-PCR. MiR-18a mimics was transferred into PC12 cells to up-regulated miR-18a. The target of miR-18a was predicted by bioinformatic analysis and confirmed by luciferase reporter assay. Agomir-18a was intracerebroventricular injected into mice after 1 h of middle cerebral artery occlusion (MCAO). Brain infarct volume, neurological deficit score and the LDH level in ischemia brain was assessed in mice subjected to 24 h of MCAO. Results: The expression level of miR-18a in PC12 was remarkably down-regulated after subjected to hypoxia, while ATXN1 mRNA level and protein expression under hypoxic conditions were significant higher compared with normoxia. Luciferase reporter analysis indicated that ATXN1 was a direct target of miR-18a. Furthermore, dramatically increase in the protein and mRNA of ATXN1 when PC12 was transfected with miR-18a mimics. Moreover, post-injected with miR-18a agomir, the brain infarct volume was greatly decreased after 24 h MCAO, the same change were found in neurological deficit score and the LDH level in ischemia brain. Conclusion: miR-18a alleviated injury induced by permanent MCAO in mice by targeting ATXN1. Post-treatment with miR-18a agomir may be an effective new approach for stroke therapy.

Keywords: Oxygen-glucose deprivation, middle cerebral artery occlusion, miR-18a, ATXN1

Introduction

Ischemic stroke injury is caused by cerebral hypoperfusion, characterized by oxidative stress, hypoxia, inflammation, and glutamate excitotoxicity, finally leading to cell death [1]. Although several random clinical trials have supported that there are opportunities to prevent and treat acute stroke, the disability and mortality leading by ischemic stroke remains at a high level. Therefore it is still necessary to further dissect the mechanisms and explore effective therapies for acute ischemic stroke.

MicroRNAs (miRs) are a class of small, non-coding RNAs that regulate diverse many biological processes, including differentiation, proliferation, development, migration, and apoptosis [2-4]. Nowadays, miRs and their roles in ischemic stroke have attracted much attention. Some of them, including miR-122, miR-let7e and miR-145 have been reported to involve in the progression of acute stage ischemic stroke and modulate the biological properties of cells [5-7]. As a consequence, the identification and the respective targets of miRNAs may provide novel molecular insight and new therapeutic strategies to treat cardiovascular and cerebrovascular diseases [8, 9].

Recently, miR-18a has been reported to increase the permeability of the blood-tumor barrier via Kruppel-like factor 4 [10]. In vitro study revealed thatmiR-18a can decrease choroidal endothelial cell proliferation [11]. In addition, miR-18a was also demonstrated to affect the rate of cell apoptosis and the cell invasion ability in gastric carcinoma cells subjected to hypoxic conditions [12]. These studies supported a role for miR-18a as a regulator for many biological processes. However, the role of miR-18a in cerebral ischemic disease remains un-

known. The aim of the present study was to investigate the role of miR-18a in middle cerebral artery occlusion (MCAO) mice and provide novel molecular of miR-18a in PC12 cells subjected to hypoxic conditions.

Methods

Model of oxygen-glucose deprivation (OGD)

PC12 were exposed to DMEM without serum or glucosein a humidified atmosphere containing 95% N_2 and 5% CO_2 for many hours. Cells cultured in DMEM (Gibco, CA, USA) containing 10% FBS (Gibco, NY, USA) under normoxic condition were used as control.

Model of middle cerebral artery occlusion (MCAO)

Permanent coagulation of the middle cerebral artery was performed as previously described [13]. Briefly, C57BL/6J mice were anesthetized with 0.4% pentobarbital sodium (45 μg/kg), then make a 1 cm skin incision between the ear and eye using little operation scissors. After identifying the MCA below the transparent skull, skull was thined out with the drill right above the MCA branch until it has a thin and translucent texture. Coagulated the artery with the electrocoagulation forceps proximal, and then relocated the temporal muscle to its position, covering the burr hole.

Transfection of miR-18a

In vivo, C57BL/6J mice were anesthetized with 0.4% pentobarbital sodium (45 μg/kg). A 26-gauge brain infusion cannula was placed stereotaxically into the left lateral ventricle (bregma: -0.58 mm; dorsoventral: 2.1 mm; lateral: 1.2 mm) as previously described [14]. miR-18a agomir (5 nmol/kg in 1 μl) or negative control (RiboBio Company, Guangzhou, China) was infused over 5 min.

In vitro, PC12 cells were cultured to 80% confluence and transfected with 50 nM miR-18a mimic or negative control (RiboBio Company, Guangzhou, China) according to the manufacturer's protocol.

RNA extraction and quantitative real-time PCR

Total RNA, including miRNA, was extracted from the cell using TRIzol reagent (Invitrogen, NY, USA). RNA was reverse-transcripted into cDNA using a reverse transcriptase kit (Takara, Dalian, China). And then cDNA was analyzed using the ABI 7500 Fast Realtime PCR system (Applied Biosystems, Life Technologies, USA). The relative of miR-18a expression level was calculated by the comparative CT method $2^{-\Delta\Delta}$Ct using U6 as an internal reference. And the relative of ATXN1 expression level was calculated by the comparative CT method $2^{-\Delta\Delta}$Ct using GAPDH as an internal reference. The primers for miR-18a, U6 and ATXN1 were purchased from RiBoBio.

Luciferase assay

The wide-type (WT) or mutant (MT) 3'UTR of ATXN1 was cloned into the luciferase gene system (Shanghai GeneChem Co., Ltd, Shanghai, China). The PC12 were cotransfected with the vectors carrying WT 3'UTR or MT 3'UTR and miR-18mimic or negative control. After 48 h transfection, cells were harvested and analyzed by the Dual-Luciferase Reporter Assay System (Promega Corporation, Fitchburg, USA).

Western blot analysis

Protein was quantitated according to the BCA method using the BCA protein assay kit (Beyotime Biotechnology, Haimen, China). A total of 30 μg of samples were run on 10% SDS-PAGE. Then, the proteins were transferred to NC filter membranes. The membranes were blocked overnight in 5% BSA in PBS. For immunoblotting, the membranes were incubated at 4°C overnight with the ATXN1 antibody (1:1000; Cell Signaling Technology, NY, USA) or GAPDH antibody (1:5000; Cell Signaling Technology, China), followed by the incubation 30 min with the IRDye 800CW conjugated secondary antibody (1:10000; Li-Cor Bioscience, USA). The infrared fluorescence image was obtained using the Odyssey infrared imaging system (Li-Cor Bioscience, USA).

Evaluation of neurological deficit score

Neurological deficit score was evaluated previously described [15]. Neurological deficits of each mouse were evaluated at 23 h after MCAO by the Zea-Longa method: 0 = normal spontaneous movement; 1 = failure to extend the contralateral forelimb; 2 = circling to affected side; 3 = partial paralysis on affected side; 4 = no spontaneous motor activity.

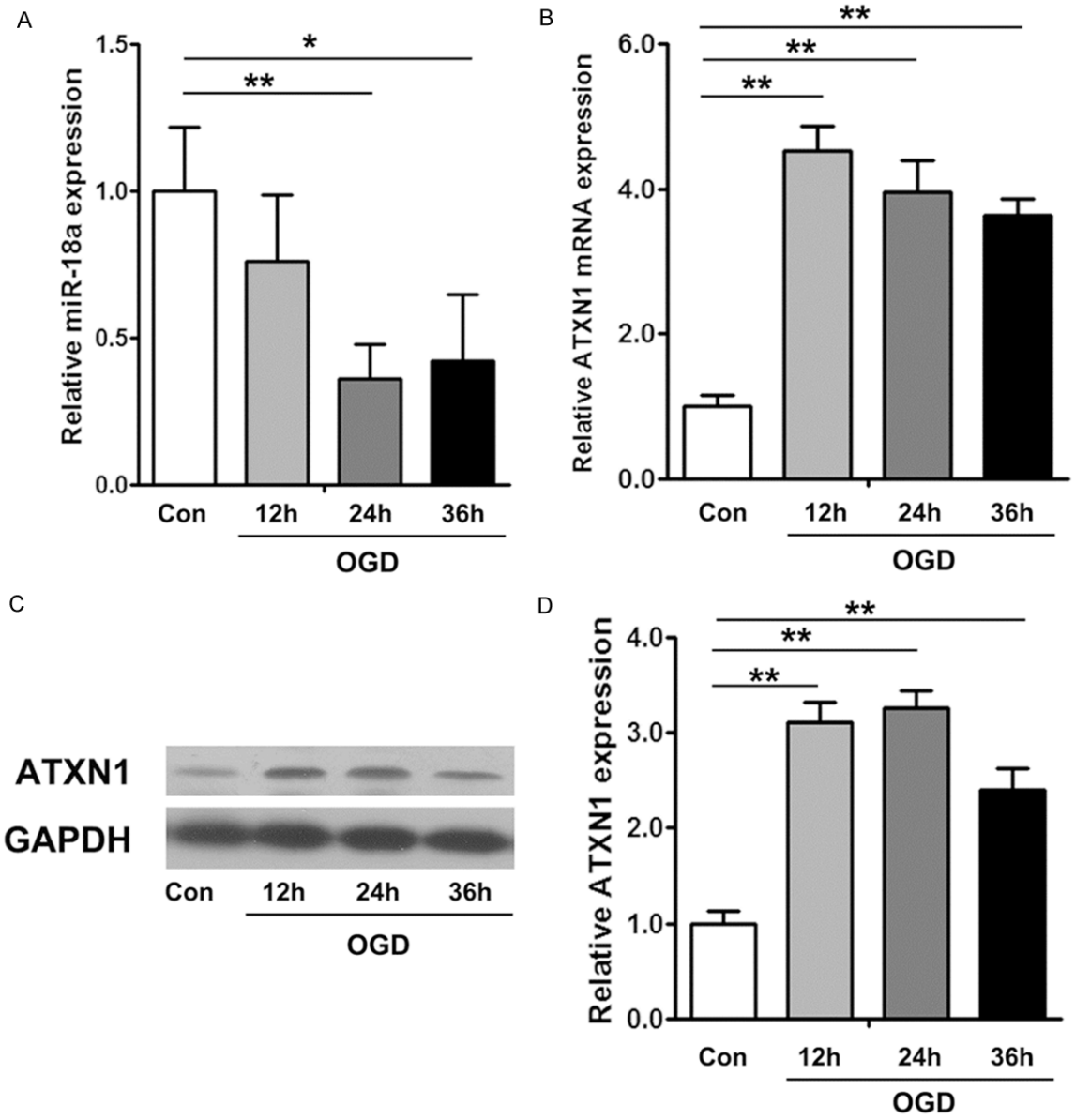

Figure 1. *Hypoxia down-regulates the expression of miR-18a and up-regulates the expression of ATXN1.* A. The miR-18a expression levels in PC12 cell after OGD; B. The mRNA expression levels of ATXN1 in PC12 cell after OGD; C and D. The protein expression levels of ATXN1 in PC12 cell after OGD. *P<0.05. **P<0.01. OGD, oxygen-glucose deprivation.

Measurement of infarct volume

Mice brains were removed after 24 h MCAO and sliced into six 2-mm thick coronal sections on a brain matrix. The slices were stained with 2% TTC (2,3,5-triphenyltetrazolium chloride) (Sangon Biotech, Shanghai, China) for 15 min at 37°C. The percent of infarct volume was calculated using a derived formula in which infarct.

Measurement of LDH

The ischemic hemisphere was homogenized inlysis buffer and the lysis was centrifuged at 12000 g and 4°C for 15 min, the supernatant was used to assess thelevel of LDH in ischemic brain by theassay kits (Nanjing Jianchen, Nanjing, China).

Statistical analysis

All data reported were mean ± sd. Statistical analysis was performed using the SPSS 17.0 software. Data were analyzed using Student's t-test, and all the tests performed were two-tail. A *p*-value of <0.05 was considered statistically significant.

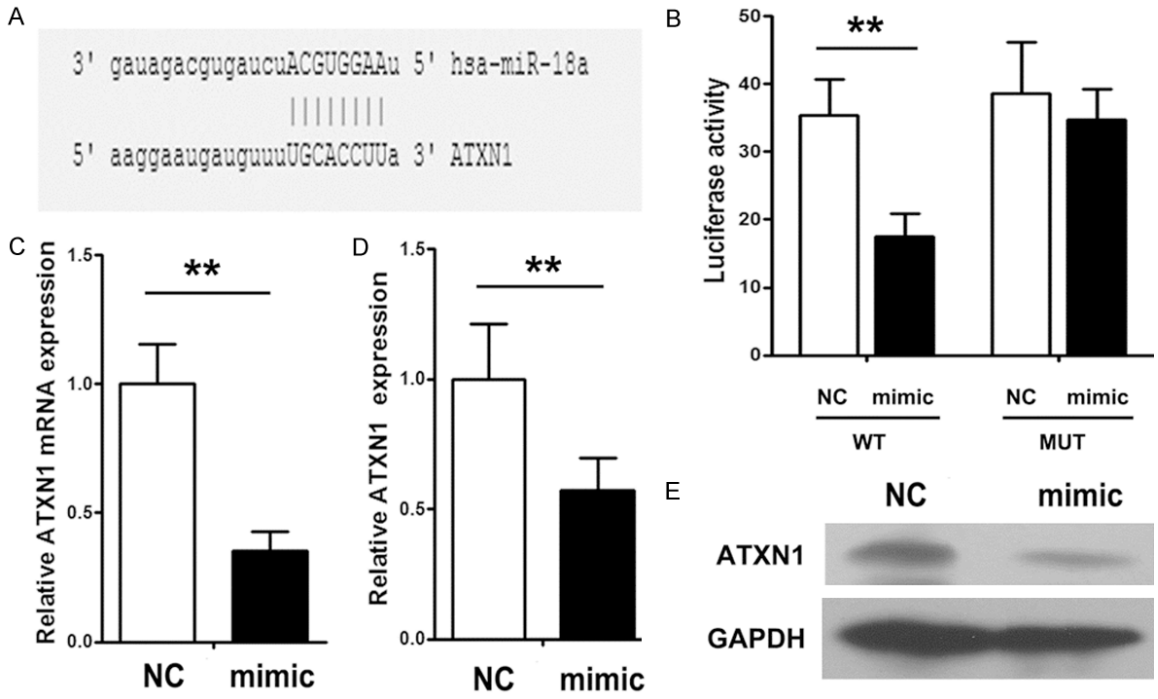

Figure 2. *ATXN1 is a direct target of miR-18a in PC12*. A. Predicted binding site of ATXN1 and miR-18a. B. Luciferase reporter assay in PC12 cell co-transfected with miR-18a mimics or NC and wide-type (WT) or mutant-type (MT). C. The mRNA expression levels of ATXN1 in PC12 cell after transfected with the miR-18a mimics or NC. D, E. The protein expression levels of ATXN1 in PC12 cell after transfected with the miR-18a mimics or NC. **P<0.01. NC, negative control.

Results

Hypoxia down-regulates the expression of miR-18a and up-regulates the expression of ATXN1

To assess the influence of hypoxia on neuronal cells, the expression level of miR-18a in PC-12 cell lines was detected. As shown in **Figure 1A**, compared with the expression under normoxic conditions, the expression level of miR-18a in PC12 was remarkably down-regulated after subjected to hypoxia. Furthermore, as shown in **Figure 1B**, ATXN1 mRNA under hypoxic conditions was significant higher compared with normoxia. Additionally, as western blot analysis shown in **Figure 1C** and **1D**, protein level of ATXN1 after hypoxia in PC12 was also significant higher compared with control.

ATXN1 is a direct target of miR-18a in PC12

To further investigate the relationship between ATXN1 and miR-18a, we searched for the specific binding site of miR-18a and ATXN1 using biological software (http://www.microrna.org/microrna/get), and the predicted binding site of miR-18a and ATXN1 is shown in **Figure 2A**. To verify the prediction, we constructed theluciferase reporter vector containing wild-type or mutant 3'UTR of ATXN1. As shown in **Figure 2B**, miR-18a distinctly induced the luciferase activity of vector carrying wild-type 3'UTR of ATXN1 compared to that carrying mutant 3'UTR of ATXN1. Next, we assayed the effect of miR-18a on the expression of ATXN1, we found that dramatically increase in the protein and mRNA expression of ATXN1 when PC12 was transfected with miR-18a mimics 48 h as shown in **Figure 2C-E**.

Overexpression of miR-18a alleviates the brain injury induced by MCAO

To detect the functional roles of miR-18a in ischemic stroke, we up-regulate the miR-18a level in brain by intracerebroventricular injection of miR-18a agomir 1 h after MCAO. It was shown that miR-18a overexpression was able to alleviate infarct volume induced by MCAO (**Figure 3A**, **3B**). Similarly, neurological deficit score after MCAO was ameliorated in miR-18a agomir injection animals (**Figure 3C**). Further-

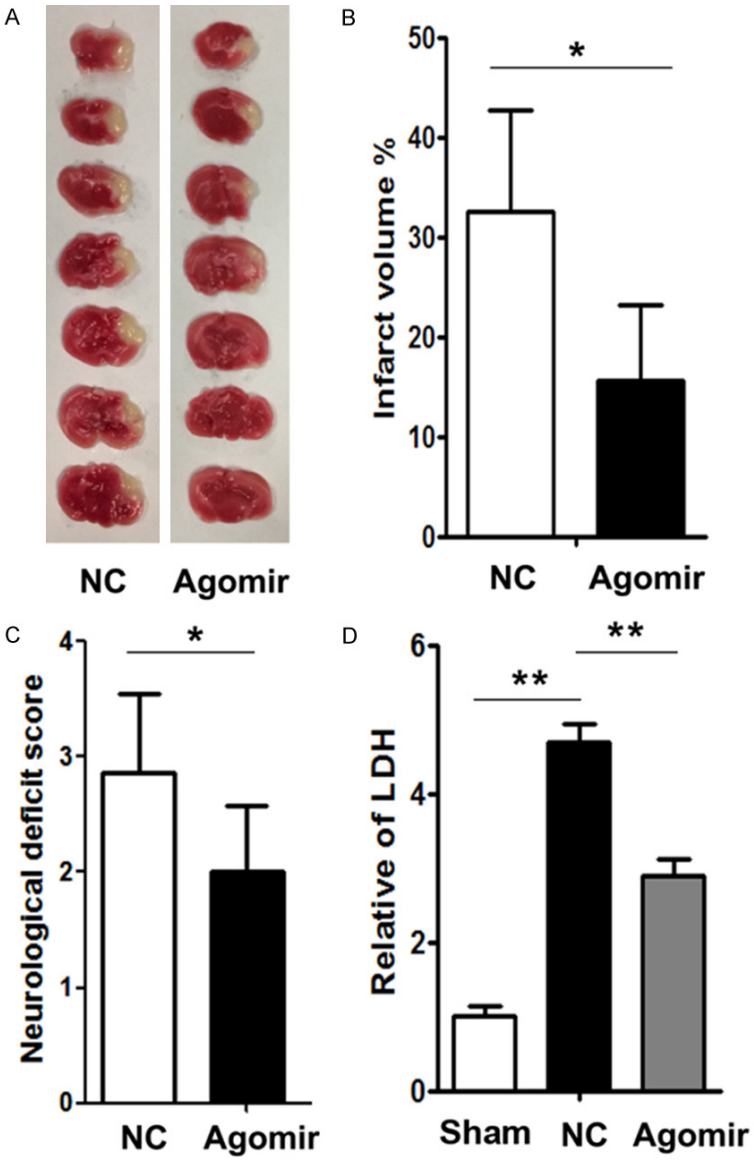

Figure 3. *Overexpression of miR-18a alleviates the brain injury induced by MCAO*. A. Representative TTC-stained coronal sections of mice after 24 hours MCAO; B. Bar graph shows total infarct volume; C. Neurological deficit score after MCAO; D. LDH level of ischemic brain. *P<0.05, **P<0.01; MCAO, middle cerebral artery occlusion; agomir, agomir-18a; n = 7 per group.

more, the LDH level was significantly increased in ischemic brain and decreased after miR-18a agomir injection in the model of MCAO (**Figure 3D**).

Discussion

MiRNAs are a class of sophisticated gene expression regulators that inhibit translation and/or degrade target mRNAs by recognizing them through base pairing with short regions near 3'-UTRs [16]. Previous studies have revealed that microRNAs involved in the regulation of cerebral ischemic injury and may lead to novel strategies for therapeutic interventions [17]. Down-regulation of miRNA-30a alleviates cerebral ischemic injury through enhancing beclin 1-mediated autophagy [18]. Moreover miR-207/352 regulate lysosomal-associated membrane proteins and enzymes following ischemic stroke [19]. MicroRNA-18a, another reported brain-specific miRNA, has been showed significant upregulation in frontal lobe and hippocampus in the duloxetine treatment group relative to depression mice [20]. MiR-18a can also regulate invasive meningiomas via hypoxia-inducible factor-1α [21]. Furthermore suppression of miR-18a expression promotes apoptosis of human trophoblast cells [22]. These evidences suggested that miR-18a plays a critical role in the pathophysiology of brain seizures. However, the identification and specific contribution of miR-18a in cerebral ischemic injury is still unresolved. In this manuscript we indicated that the expression of miR-18a was remarkably down-regulated under hypoxic conditions in PC12 cells. This data suggested that miR-18a should play a critical role in the regulation of the cellular response to hypoxia. In addition, the results demonstrated that miR-18a overexpression was able to alleviate infarct volume induced by MCAO. Similarly, neurological deficit score after MCAO was ameliorated in miR-18a agomir injection animals. These data was the first to report that miR-18a is the positive-regulator of the cellular response to hypoxic stress.

The ATXN1 gene provides instructions for making a protein called ataxin-1. This protein is found throughout the body. More and more evi-

dences have shown that ATXN1 involved in the pathophysiology of cerebellar seizures [23, 24]. In mouse model of polyglutamine expansion diseases, silencing of ATXN1 mRNA provides therapeutic benefit [25]. In this study, the target of miR-18a was predicted by biological software and luciferase reporter assay was performed to confirm the potential target of miR-18a. We found that miR-18a could directly bind with the 3'-UTR of ATXN1 mRNA and negatively regulate ATXN1 gene expression. Furthermore, the protein expression level of ATXN1 in PC12 cell was also significance decreased after transfected with the miR-18a mimics. Taken together, these evidence sported that miR-18a contributes to the cerebral ischemic injury by targeting ATXN1. And miR-18a might become a contribution factor of intervention therapy for cerebral ischemic stroke.

In conclusion, our results demonstrate that miR-18a alleviated injury induced by permanent MCAO in mice by targeting ATXN1. Post-treatment with miR-18a agomir may be an effective new approach for stroke therapy.

Acknowledgements

This study was supported by the grants from health and family planning commission of Hubei province of China (WJ2015MA007, 2015-060101010047).

Disclosure of conflict of interest

None.

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