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Research article

Upregulation of miR-215 exerts neuroprotection effects against ischemic injury via negative regulation of Act1/IL-17RA signaling

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ABSTRACT

Objective: This study investigated the role of miR-215 and nuclear factor-κB activator (Act)1 and their mechanisms of action in ischemic stroke.

Methods: Cell viability was examined with the 3-(4,5-dimethythiazol-. 2-yl)-2,5-diphenyl tetrazolium bromide assay; cell apoptosis was detected by flow cytometry; and mRNA and protein expression was assessed by quantitative real-time PCR and western blotting, respectively. A mouse model of middle cerebral artery occlusion (MCAO) was treated with or without miR-215 mimic to verify the in vitro results. The relationship between miR-215 and interleukin (IL)-17 was evaluated in human peripheral blood from 29 patients.

Results: Act1 was upregulated whereas miR-215 was downregulated in ischemic stroke. Overexpression of miR-215 by transfection of a mimic repressed Act1 protein levels in vitro and in vivo, although the luciferase assay showed that miR-215 did not directly bind to the 3′ untranslated region of Act1. MiR-215 overexpression inhibited cell apoptosis and autophagy. Increasing miR-215 levels reduced ischemic infarction and improved neurological deficit, while loss of miR-215 phenocopied the effects of IL-17.

Conclusion: Upregulation of miR-215 exerts neuroprotection against ischemic injury by negatively regulating Act1/IL-17 receptor A signaling. These findings provide potential therapeutic targets for the treatment of ischemic stroke.

1. Introduction

Stroke is a primary cause of disability and mortality worldwide [1], and is often treated by intravenous or intra-arterial thrombolysis [2]. Temporary cessation of blood flow to the brain and subsequent reperfusion can lead to irreversible tissue injury and cell death [3].

Nuclear factor (NF)-κB activator (Act)1 activates both the c-Jun N-terminal kinase (JNK) and canonical NF-κB pathways [4,5]. It also binds to several interleukin (IL)-17 family member receptors via homotypic interactions between their respective similar expression to fibroblast growth factor and IL-17 receptor (SEFIR) domains. However, Act1 was recently reported to function as an E3 ubiquitin ligase in IL-17A-mediated JNK and NF-κB-activation [6]. Whether this is related to the autophagy pathway and cerebral ischemia-reperfusion remains unclear.

Micro (mi) RNAs are small, non-coding single-stranded RNA molecules that negatively regulate target gene expression and thereby modulate various biological functions; they also influence the stability and translational efficiency of mRNAs. MiRNAs play important roles in many diseases including cancer as well as heart and brain diseases, and may regulate factors involved in ischemia reperfusion injury [7]. MiRNA (miR)-215 was first discovered as a positive regulator of p53 but has since been shown to have oncogenic effects in various tumors, including human gastric cancer and glioma [8–10]. The expression and function of miR-215 in human glioma tissues have been explored recently indicating that miR-215 may play a vital role in central nervous system. In addtion, miR-215 affects neuron migration and proliferation in disease of aganglionosis [11]. However, there have been no studies of the role of miR-215 in ischemic injury. We previously reported that Act1 may play an important role in IL-17 signaling and hypothesized

Abbreviations: Act1, nuclear factor activator 1; CCA, common carotid artery; ECA, external carotid artery; FITC, fluorescein isothiocyanate; HEK293T, human embryonic kidney 293T; ICA, internal carotid artery; IL-17RA, interleukin-17 receptor A; JNK, c-JuneN-terminal kinase; MAP, mitogen-activated protein; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; MEKK1, mitogen-activated protein kinase kinases 1; N2a cell, the mouse neuroblastoma N2a cell; NF-kB, nuclear factor kappa B; OGD/R, oxygen glucose deprivation/reperfusion; pBcl-2, phosphorylated B-cell leukemia/lymphoma 2; PI, propidium Iodide; SEFIR, similar expression to fibroblast growth factor and IL-17 receptor; TIR, toll-Interleukin-1R; TRAF, tumor necrosis factor receptor-associated factor; TTC, triphenyl tetrazolium chloride; UTR, untranslated region

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that it may involved in cerebral ischemic injury, and a bioinformatics analysis revealed that Act1 is a potential target of miR-215.

In this study, we investigated the role of miR-215 and Act1 in ischemic stroke and their mechanisms of action using oxygen glucose deprivation/reperfusion (OGD/R) in vitro and middle cerebral artery occlusion (MCAO) in vivo models. Our results indicate that miR-215, Act1, and IL-17 can serve as therapeutic targets in the treatment of ischemic stroke.

2. Materials and methods

Details can be found in the Supplementary Data.

2.1. Protocol

In vitro experiments were first performed to identify the relationship between miR-215 and Act1 and their role in ischemic stroke with cell viability and apoptosis assays and by evaluating mRNA and protein expression. We then validated these results through in vivo experiments using mice subjected to MCAO model with or without miR-215 mimic treatment. Finally, peripheral blood samples from 29 patients were analyzed to determine the relationship between miR-215 and IL-17 levels.

2.2. Cell lines and culture conditions

N2a mouse neuroblastoma cells (Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China) and human embryonic kidney (HEK) 293T cells were grown in Dulbecco's Modified Eagle's medium containing 10% fetal bovine serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2.

2.3. OGD/R model

The OGD/R model was established using N2a cells; the details can be found in the Supplementary Data. For miRNA experiments, N2a cells were pretreated with miR-215 mimic for 24 h.

2.4. Animals and surgery

The study protocol was approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Harbin Medical University. C57BL/6 mice were housed in individual cages on a 12:12-h light/dark cycle at 22 °C ± 2 °C with free access to food and water. The middle cerebral artery occlusion (MCAO) model of ischemic stroke was established as follows. Mice were deeply anesthetized by intraperitoneal injection of sodium pentobarbital (40 mg/kg). Blunt dissection was performed under a stereomicroscope to expose the right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA). The proximal end of the ipsilateral CCA was ligated, and an arterial clamp was applied to the CCA. This was followed by a small incision to the ECA artery between the permanent and temporary sutures and insertion of a Doccol suture (0.21 mm in diameter) into the ICA about 10 mm beyond the carotid bifurcation, which occluded the origin of the MCA. After 1 h, the suture was removed and mice were allowed to recover for 12 h, 24 h, 48 h, 3 days, and 5 days (Ischemia and reperfusion were confirmed in **Supplementary Fig. S1**). Sham animals underwent the same procedure except for insertion of an intraluminal filament. The surgeries were carried out by the same technician. Rectal temperature was monitored during surgery and body temperature was maintained at 36 °C (± 1 °C) using a warming lamp.

2.5. MiRNA treatment for MCAO model

For miRNA treatment, 100 μM miR-215 mimic (3 μl; Genechem, Shanghai, China) was injected into the right cerebral ventricle of mice. The mimic or negative control mimic was considered as being effective only when cerebral blood flow dropped to below the baseline during injection. All mice were evaluated for neurologic deficits 24 h after reperfusion, and the brain was removed for later analysis.

2.6. Patients and blood samples

Blood samples were obtained from 29 patients with ischemic stroke 3 days after intravenous thrombolysis at the Department of Neurology, the First Affiliated Hospital of Harbin Medical University (Harbin, China). Informed consent was obtained in writing from patients and the protocol was approval by the ethics committee of the First Affiliated Hospital of Harbin Medical University (no. 201677).

2.7. Cell transfection

Act1 cDNA lacking the 3′ untranslated region (UTR) was inserted into the pcDNA3.1(+) vector (Invitrogen) to generate pcDNA3.1(+)-Act1. N2a cells were seeded in 96- or 6-well plates; when the cells reached 70% confluence, they were transfected with miR-215 mimic (100 nM) or pcDNA3.1(+)-Act1 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.8. Determination of inhibition ratio

The 3-(4,5-dimethythiazol-. 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was carried out in order to assess inhibition of cell viability. N2a cells were pretreated with miR-215 mimic or left untreated and then subjected to OGD for 1 h followed by reoxygenation for 12, 24, 48, 72, or 96 h. The inhibition ratio was calculated using the following formula:

Inhibition of cell viability = (1 − average absorbance value of the experimental group/average absorbance value of the control group) × 100%.

2.9. Detection of apoptosis

Apoptotic cells were detected by flow cytometry, as detailed in the Supplementary Data.

2.10. Quantitative real-time (qRT-)PCR

Total RNA was extracted from the cortical peri-infarct area of the ipsilateral hemisphere or cultured N2a cells and reverse transcribed into cDNA using the Revert Aid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Beijing, China) according to the manufacturer's instructions. PCR was carried out with the Plexor One-Step qRT-PCR system (Promega, Madison, WI, USA) on a CFX96 Detection System (Bio-Rad, Hercules, CA, USA) using the primers listed in **Table S1**.

2.11. Luciferase reporter assay

The 3′ UTR of the *Act1* gene (Act1-3′ UTR) including miR-215 binding sites as well as mutant *Act1* 3′ UTR (Act1-3′ mUTR) were amplified by PCR and cloned into the pmirGLO vector (Promega). The inserts were confirmed by sequencing. Primers used to amplify wild-type (WT) and mutant (MUT) Act1 3′ UTR constructs listed in **Table S2**.

2.12. Western blot analysis

Total protein was extracted from the mouse brain or N2a cells and analyzed by western blotting using primary antibodies against Act1,