

Original Article

MiR-124 inhibits neural apoptosis in ischemic stroke

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Abstract: Stroke is a common disease around the world, which causes cerebral tissue damage due to rupture or occlusion of cerebral vessels. Previous studies showed that a large number of microRNA was involved in post-stroke damage or regeneration of neural tissue, among which miR-124 was reported to play a pivotal role in neural differentiation. Our study was focused on the expression of miR-124 in ischemic stroke and the effect of miR-124 on neural apoptosis. 40 male C57BL6 mice were prepared for establishment of cerebral infarction model with suture method. Tissues of stroke lesion were extracted for examination of miR-124 expression by qRT-PCR and in situ molecular hybridization. Mice neurons were cultured *in vitro* and transfected with lentiviral expression vector of miR-124. Proliferation of neurocytes was assessed by MTT assays. Western-Blot was performed to examine the expression of Bcl-2, P53 and Caspase-3. Our results showed cerebral infarction model was successfully established as demonstrated by significant infarcted lesion. qRT-PCR and in situ molecular hybridization showed that, compared with normal tissues, tissues of stroke lesion had a higher expression of miR-124 ($P<0.01$). Overexpression of miR-124 up-regulated apoptosis-associated Bcl-2 expression ($P<0.05$), inhibited expression of activated caspase-3 ($P<0.05$), and enhanced proliferation of neurocytes ($P<0.05$). In conclusion, miR-124 was up-regulated in infarcted tissues after ischemic stroke. MiR-124 inhibited neural apoptosis and enhanced proliferation of neurocytes via apoptosis-associated pathways.

Keywords: MiR-124, ischemic stroke, neuron, proliferation, apoptosis

Introduction

Stroke is known as cerebral apoplexy, which is the leading cause of disability and death worldwide [1]. There are two main types of stroke, ischemic stroke and hemorrhagic stroke. Ischemic stroke accounts for 85% of all stroke cases and always results from cerebral circulation insufficiency due to occlusion of the brain feeding arteries, including carotid artery and vertebral artery [2]. The mechanisms of ischemic stroke are unclear. However, some studies demonstrated that both nerve regeneration and neural apoptosis in infarcted tissues play important roles in neural functional recovery in ischemic stroke patients [3, 4]. Thus, it is of great significance to further study the regulatory mechanism underlying neural apoptosis in ischemic stroke.

microRNA is a kind of non-coding RNA with a length of 21 to 25 bp. Previous studies showed

that microRNA influenced multiple cellular events via RNA interference [5]. MiR-124 is highly expressed in neural tissues with high conservation across different species [6]. MiR-124 is proved to play important roles in neural development and differentiation. MiR-124 regulates polypyrimidine tract-binding protein 1 (PTBP1), which changes alternative splicing patterns of multiple mRNA in neuron, and influences neural differentiation via targeting PTBP1 mRNA [7]. In addition, clinical trials showed that serum level of miR-124 was increased in ischemic stroke patients, suggesting miR-124 might be involved in the stroke occurrence and post-stroke nerve regeneration [8-10].

Our study aimed to explore the expression of miR-124 in ischemic stroke and the effect of miR-124 on neural apoptosis so as to unravel the role of miR-124 in the pathogenesis of ischemic stroke.

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Table 1. Primers of miR-124

Name	Sequence
miR-124-F	5'AGGCCTCTCTCTCCGTG3'
miR-124-R	5'CAGCCCCATTCTTGGC3'

Materials and methods

Animal grouping and cerebral infarction model establishment

C57BL6 mice were purchased from Experimental Animal Center of Medical School of Zhejiang University (Hangzhou, China). 40 male adult C57BL6 mice were randomly assigned into two groups with equal numbers. Cerebral infarction model was established with suture method. Detailed protocols for experimental group were as follows: Mice were fixed on the experiment table followed by shaving hair along the neck. Incise neck skin and find carotid artery. Ligate distal part of carotid artery with 5/0 sutures and proximal part with 2/0 sutures. Establish a slipknot in the middle part of carotid artery with 0# sutures, clip a small hole above the slipknot and put 1 cm line embolism into vessels. Perform separation of vascular but not put the line embolism into vessels in control group. Euthanasia was performed to harvest tissues. All mice experiments were processed according to requirements of the International Council for Laboratory Animal Science.

All procedures were approved by the Animal Ethics Committee of the First affiliated Hospital of Medical School of Zhejiang University.

qRT-PCR

Cerebrum was harvested for RNA extraction. RNeasy pure Tissue Kit (QIAGEN) was used to extract RNA. Amplification was performed with miR-124 primers (GeneBank NR_029668, **Table 1**). mirVana qRT-PCR miRNA detection kit (Ambion) was used to perform qRT-PCR. U6-RNA sequence was used as an internal control. $2^{-\Delta\Delta Ct}$ method was used to analyze the results [11].

In situ hybridization

Paraffin section of cerebrum was performed. Antisense locked nucleic acid was used to modify miR-124 oligonucleotide probe (5'-TGGCATT-CACCGCGTGCCCTTAA-3', underlined sequence was locked nucleic acid). Dewax paraffin section with routine protocols, and block non-spe-

cific binding sites after hydration. Add prehybridization solution after rinse, and incubate for 4 hours at 65°C. Add probe and incubate for 15 hours at 65°C. Block non-specific binding sites after SSC solution rinse. Add antibody and incubate for 30 minutes. Perform developing in dark place, and observe under a microscope [12].

Interpretation of the results: bluish violet particles suggested positive expression of miR-124, and expression of miR-124 is positively correlated with the degree of color [13].

Culture of primary neuron

Suckling mice were prepared for extraction of primary neurons. Put harvested brain into cold PBS, and remove ependyma, meninges and cerebral vascular tissues. Add trypsin and collect primary neurons. Primary neurons were cultured for 20 minutes at 37°C, 5% CO₂, and remained trypsinization. Then, 10% fetal bovine serum was added to terminate trypsinization. Resuspend primary neurons with pipettor. Perform cell counting after filter screening. Change medium and add 5-fluoro-deoxyuridine after 24 hours culture [10].

Cell transfection and overexpression of miR-124

Overexpression of miR-124 in primary neurons was performed with lentivirus infection. Lentiviral expression vector with both green fluorescent protein and miR-124 was constructed followed by cell transfection with routine protocols. Replace culture medium with routine medium after 24 hours transfection. Analyze cell fluorescence with fluorescence microscope after 3 days' culture. MiR-124 overexpressed primary neurons were screened in the medium with puromycin (1 mg/L).

MTT assays

MiR-124 overexpressed primary neurons were prepared for culture at different time points (20 h, 44 h, 68 h and 92 h). Remove the medium and add DMSO (150 µL). Cell proliferation was assessed according to the absorbance value (A) at 490 nm which was measured by a microplate reader. Growth curve was established with absorbance values.

Western blot

Primary neurons were cultured for 48 hours followed by extraction of total proteins for SDS-

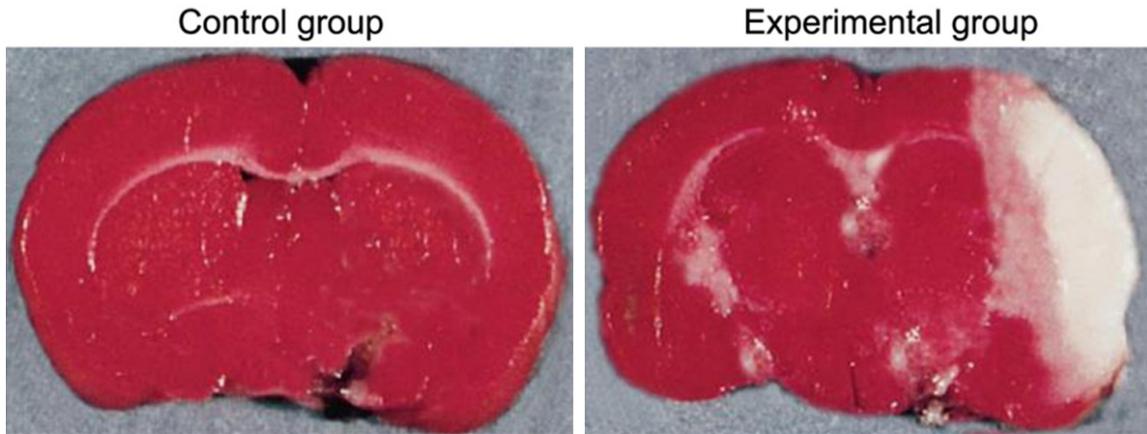


Figure 1. TTC staining to determine cerebral infarction.

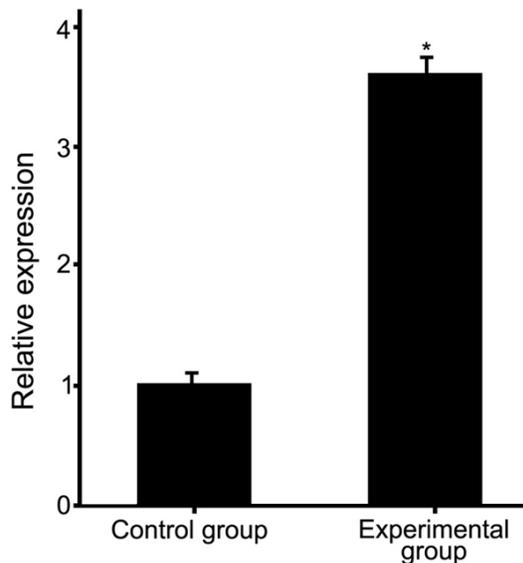


Figure 2. Analysis of the expression of miR-124. * $P < 0.01$, versus control group.

PAGE electrophoresis. Primary antibodies were rabbit-anti-mouse Bcl-2 antibody (Proteintech, 25850-1-AP), rabbit-anti-mouse P53 antibody (Proteintech, 10442-1-AP), rabbit-anti-mouse caspase-3 antibody (Proteintech, 19677-1-AP) and rabbit-anti-mouse β -actin antibody (Proteintech, 20536-1-AP). β -actin was used as an internal control. Gel imaging system was used to analyze the relative expression of specific protein bands [14].

Statistical analysis

SPSS 20.0 software was used for data processing. Measurement data were represented

as mean \pm standard deviation (SD). One-Way ANOVA was performed for analysis of statistical significance. P value < 0.05 was considered to be statistically significant.

Results

Establishment of cerebral infarction model

Cerebrum of mouse was harvested after 24 hours cerebral infarction. TTC stain was performed to determine the infarction of cerebrum [9]. As showed in **Figure 1**, cerebrum of control mice was totally stained (red), while cerebrum of experimental mice had a remarkable infarction area, which was not stained (white). All staining results suggested the successful establishment of cerebral infarction model.

Increased expression of miR-124 in infarction tissues

qRT-PCR showed that, compared with control group, experimental group had a higher expression of miR-124 in infarcted tissues (3.5 fold increase) (**Figure 2**, $P < 0.01$). Consistent with this, in situ hybridization also confirmed the increased expression of miR-124 in infarcted tissues as showed more tissues with more bluish violet particles in infarcted experimental group than that in control group (**Figure 3**).

Overexpression of miR-124 in cultured primary neurons

qRT-PCR was performed to examine the expression of miR-124 in transfected primary neu-

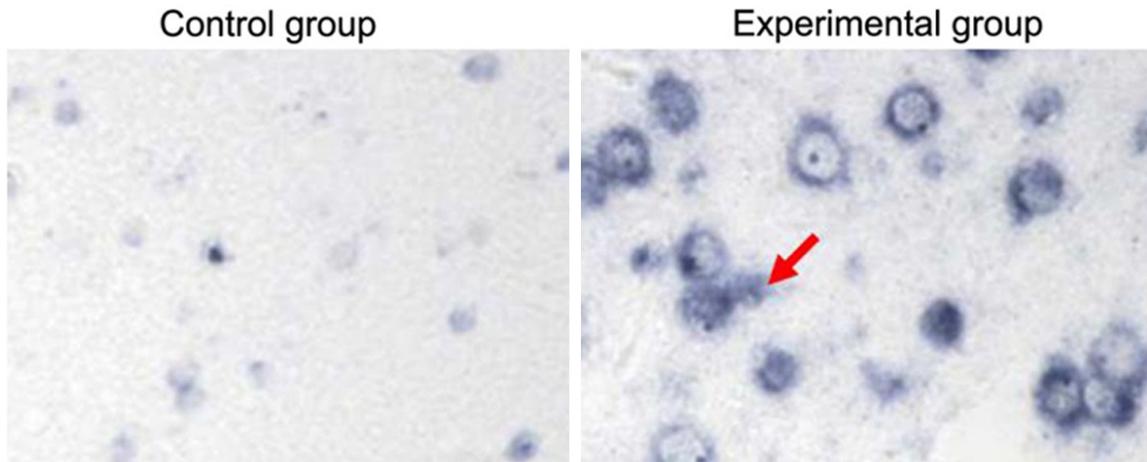


Figure 3. Image of in situ hybridization analysis of miR-124 expression. Red arrow refers to bluish violet particle, suggesting enhanced expression of miR-124.

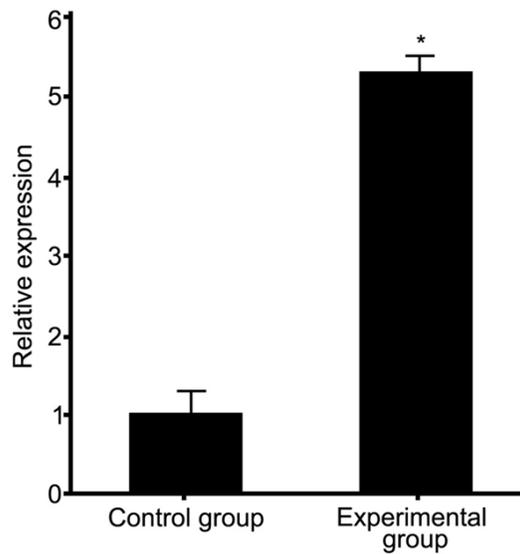


Figure 4. Analysis of the expression of miR-124 in primary neurons. * $P < 0.05$, versus control group (normal primary neurons).

rons. Compared with normal primary neurons, transfected primary neurons had a higher expression of miR-124 (**Figure 4**, $P < 0.05$), suggesting overexpression of miR-124 was successfully established in primary neurons.

MiR-124 enhanced proliferation of neurocytes

MTT assays showed that all primary neurons proliferated gradually along with time, while the cell number of miR-124 transfected primary neurons was significantly higher than that of normal primary neurons after 48 hours' culture

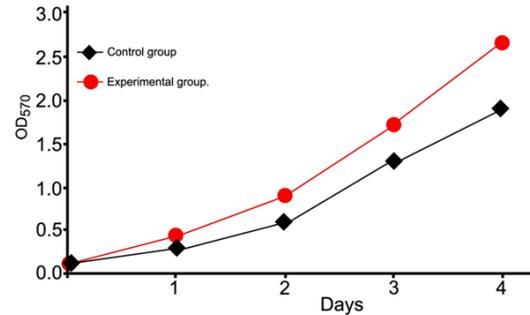


Figure 5. Analysis of cell proliferation at different time points. * $P < 0.05$, versus control group (normal primary neurons).

(**Figure 5**, $P < 0.05$), suggesting overexpression of miR-124 enhanced the proliferation of neurocytes.

MiR-124 regulated expression of apoptosis-related proteins

Western blot was performed to examine the expressions of apoptosis-related proteins, including Bcl-2, P53 and caspase-3. Overexpression of miR-124 significantly increased the expression of Bcl-2 and decreased the expression of caspase-3 (**Figure 6**, $P < 0.05$), while no difference was observed in the expression of P53.

Discussion

As a kind of small non-coding RNA, microRNA regulates cellular events and metabolism [5]. Our mice cerebral infarction models showed

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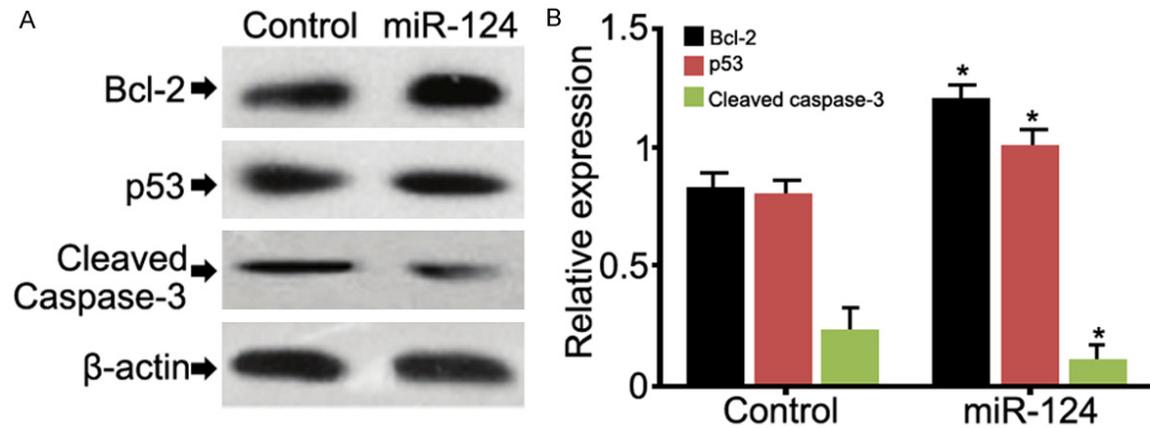


Figure 6. A. Western blot analysis of expression of Bcl-2, p53, and caspase-3. B. Quantitative analysis of the expression of Bcl-2, p53, and caspase-3. * $P < 0.05$, versus control group (normal primary neurons).

that the expression of miR-124 was up-regulated in infarcted brain tissues, suggesting miR-124 might be involved in post-stroke neuropathology progress. Further experiment of transfection demonstrated that overexpression of miR-124 increased the expression of Bcl-2 in primary neurons, decreased the expression of caspase-3 and enhanced proliferation of neurocytes.

Plenty of current studies have elucidated that microRNA indeed regulates post-stroke neuron injury or regeneration, such as miR-125, miR-145, miR-181 and miR-424 [12-15]. Dharap et al. found that miR-145 expression was increased in infarcted brain tissues, while inhibition of miR-145 expression remarkable attenuated stroke lesion [12]. On the other hand, inhibition of miR-424 expression could exacerbate stroke [15]. All findings suggested that microRNA was a potential therapeutic target for stroke treatment. Our study firstly prove that miR-124 influenced post-stroke prognosis, and overexpression of miR-124 had promising effect on the proliferation of neurocytes, which will enhance our understanding of how microRNA regulated stroke progression. However, the exact mechanism by how microRNA was regulated during the development of stroke remains unclear. There are some scholars believed that methylation of microRNA transcripts promoter region influenced the expression of microRNA [16]. In addition, recent studies indicated that proteins encoded by microRNA target genes could reversely regulate the expression of microRNA [17]. Further studies are required to perform to

investigate the molecular mechanisms underlying the changes of miR-124 expression in stroke patients.

MiR-124 is specifically expressed in nerve tissues and involved in neural differentiation [18]. Our study found that miR-124 also regulated the proliferation of neurocytes and inhibited apoptosis via mediating the expression of apoptosis-related proteins, such as Bcl-2, caspase-3. Previous study proved that Bcl-2 inhibited apoptosis through directly inhibiting activation of caspase-3 and other apoptosis-related proteins [19, 20]. Our study demonstrated that Bcl-2 expression was increased and caspase-3 expression was decreased after overexpression of miR-124, which can explain the phenomena that miR-124 promoted proliferation possibly due to inhibited apoptosis.

Inflammation and neurological damage are two main pathological processes in stroke. Recent study demonstrated that neurological damage could be attenuated when apoptosis was reduced. Thus, study on the regulatory mechanism of apoptosis is of great significance to not only prognosis of stroke patients, but also improvement of treatment and nervous system function [21]. Collectively, our study confirmed miR-124 was up-regulated in stroke patients, and overexpression of miR-124 could be a potential therapeutic approach to improve post-stroke prognosis via enhancing proliferation and reducing apoptosis.

In conclusion, miR-124 was up-regulated in infarcted tissues after ischemic stroke. Overex-

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pression of miR-124 reduced neural apoptosis and enhanced proliferation of neurocytes via increasing Bcl-2 expression and decreasing caspase-3 expression, suggesting miR-124 might be a new therapeutic target in the treatment of stroke.

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Disclosure of conflict of interest

None.

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