

## Original Article

# Modulatory role of microRNA-124 in targeting Ku70 during post-stroke neuronal apoptosis

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**Abstract:** Cerebral ischemia, or brain stroke, is one cerebral tissues damage caused by insufficient blood supply due to rupture or blockade of cerebral vessels. Abundant microRNA (miR including miR-124 are involved in neural tissue damage and repair post-stroke. Previous study showed importance role of miR-124 in neuronal differentiation. This study aimed to investigate the regulatory role of miR-124 in targeting Ku70 during post-stroke neuronal apoptosis. Suture method was applied to establish ischemia stroke model in mice. Stroke lesion was separated and analyzed for miR-124 expression by QRT-PCR. Bioinformatic analysis was used to predict target genes of miR-124, which was confirmed by luciferase reporter gene assay. Lentiviral vector expressing miR-124 was used to transfect PC12 cell line, followed by TUNEL assay. Western blot was used to test expression of Bax and Caspase-3. QRT-PCR and *in situ* hybridization showed elevated expression level of miR-124 ( $P < 0.01$ ). Bioinformatics and luciferase reporter gene assay confirmed Ku70 as the target gene of miR-124. After lentivirus infection, miR-124 was up-regulated with elevated Bax expression ( $P < 0.05$ ), plus lower activated Caspase-3 expression and enhanced cell proliferation ( $P < 0.05$ ). MiR-124 is up-regulated in cerebral tissues of ischemia stroke model mice. Elevated miR-124 can decrease apoptosis level of neuronal apoptosis and increase proliferation ability.

**Keywords:** MicroRNA, ischemia cerebral stroke, mouse model, Ku70, cell apoptosis

## Introduction

Cerebral stroke is the major reason causing mortality and death worldwide [1]. Brain stroke can be further divided into ischemia and hemorrhage stroke. Ischemia stroke refers to a series of diseases as a result of insufficient blood supply to brain due to blockade of carotid artery or spinal artery, and consists of 85% among all stroke cases [2]. Previous studies showed that regeneration of neurons and vessels after ischemia brain stroke plus necrosis/apoptosis of neurons at stroke site were critical for recovery of patient's neural functions [3, 4]. Therefore, the investigation of modulatory mechanisms for neuron necrosis and apoptosis after ischemia brain stroke is of critical importance for recovery of patient's neural functions.

MicroRNA (miR) is one group of small non-coding RNA molecules with hairpin structures, and can participate in regulating various cellular behaviors via RNA interference mechanism [5].

MiR-124 is highly expressed in neural tissues with conserved sequences across species [6]. Previous study showed the important role of miR-124 in development and differentiation of neural cells. It can target PTBP1 mRNA to regulate its expression level. As PTBP1 can change the alternative splicing pattern of various mRNA precursors in neural cells, it can thus achieve the regulation for neural cell differentiation [7]. Ku70 protein can participate in repair process of broken double stranded DNA, and is one regulatory subunit for protein kinase [8]. Recent study has indicated the critical role of Ku70 in regulating apoptosis of neurons after brain stroke [9]. This study thus investigated the regulatory role of miR-124 in targeting Ku70 for mediating neuron apoptosis after brain stroke.

## Materials and methods

### *Animal grouping and model*

C57BL6 mice (provided by college of Clinical Medicine of Henan University of Science and

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Technology) were chosen for 20 males and 20 females (body weight 18~22 g) at 6-week age. All mice were randomly assigned into control and experiment group (N=20 each). Suture approach was adopted to establish a ischemia stroke model. In brief, mouse was fixed on the table. After fur shaving, neck skin was made a middle incision to locate carotid, on which 5/0 suture was ligated on distal site, followed by 2/0 suture on proximal site, and 0# suture in the middle ligation. An incision was made on the top of middle incision, followed by the insertion of 1 cm length clogging. In sham group, vessels were separated without clogging insertion. The whole animal procedure adopted the requirement by international committee for laboratory animals (ICLAS) [10].

Mice were used for all experiments, and all procedures were approved by the Animal Ethics Committee of the First Affiliated Hospital and college of Clinical Medicine of Henan University of Science and Technology.

### *QRT-PCR*

24 h after surgery, all mice were sacrificed and collected for the brain. Stroke lesion tissues were separated and prepared for paraffin-based sections. Total RNA was also extracted from brain tissues by RNeasy Pure Tissue Kit (QIAGEN). Using total RNA extracted from brain tissues of control mice as the control, QRT-PCR was performed to describe miRNA expression. All-in-One miRNA test kit (GeneCopoeia) was used to quantify miR-124 expression by QRT-PCR. Using U6-RNA sequence as the internal reference, relative expression of miR-124 was calculated as previously described [11].

### *Bioinformatics analysis*

TargetScan Release 5.1 ([www.targetscan.org](http://www.targetscan.org)) software was used to predict miR-124 function. Luciferase reporter gene assay was used to confirm possible targets of miR-124.

### *Luciferase reporter gene assay*

Based on prediction results, 3'UTR region of Ku70 mRNA sequence (Genebank access No. NM\_001469) was used to synthesize primer sequences (Ku70-F: 5'-AGGTG GAGTA TTCAG AAGAG GAG-3'; Ku70-R: 5'-GCAGT AAAGT AAGGA ACAGC AAAG-3'. 3'UTR sequence was amplified and was inserted downstream of luciferase gene coding region in pmirGLO vec-

tor to construct pmirGLO-Ku70 plasmid. The recombinant plasmid was used to transfect HEK293 cell lines using pmirGLO empty plasmid. Cells with successful transfection further received miR-124 mimic transfection to further enhance miR-124 expression. 48 h after transfection, fluorescent intensity was analyzed. Dual luciferase reporter gene system (Promega) was used for fluorescent intensity analysis on MicroLumaPlus LB96V spectrometry (Berthold) [12].

### *Cell transfection and miR-124 over-expression*

PC12 neural cell line (Shanghai cell bank) was cultured in DMEM medium containing 10% FBS. Lentiviral vector was applied to over-express miR-124 in neurons. Lentiviral vector with GFP and miR-124 co-expression was constructed to transfect PC12 cells. 24 h after transfection, cells were collected and cultured in fresh medium. 3 days later, fluorescent microscope was used to count the number of cells emitting fluorescence. Neurons with miR-124 over-expression were screened in culture medium containing 1 mg/L puromycin [13].

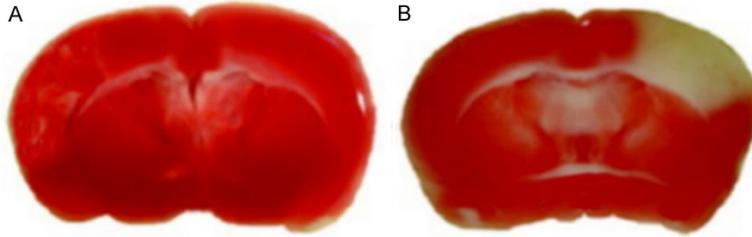
### *TUNEL assay for in situ assay of apoptosis*

Those neurons with miR-124 over-expression were inoculated into 24-well plate with coverslips, in parallel with un-transfected cells as the control group. Each experimental group consisted of six parallel samples. Coverslips were removed on the first, second, third and fourth day of culture. TUNEL assay kit (Roche) was used to quantify cell apoptosis. Under microscopic observation, brown staining of nucleus indicated cell apoptosis. Total apoptotic cell number was quantified. Apoptotic index = apoptotic cell number/total cell number [14].

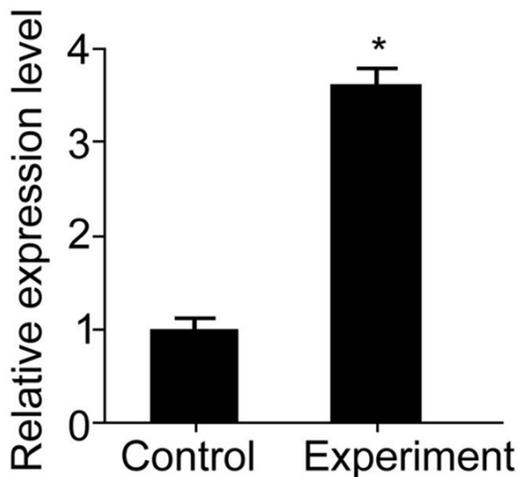
### *Western blot for expression of apoptotic related proteins*

48 h after culture, PC12 cells were collected for extracting total proteins, which were separated in SDS-PAGE and were transferred to PVDF membrane. Primary antibody used in Western blot included rabbit anti-human Bax (Proteintech), rabbit anti-human  $\beta$ -actin (Proteintech). Secondary antibody used was horseradish peroxidase (HRP) conjugated mouse anti-rabbit IgG. After blotting, freshly prepared DAB substrate was added for 10 min development. Protein bands were scanned and inputted into the computer. Integrated gray value was quanti-

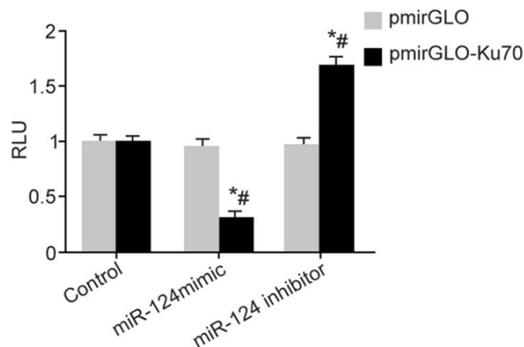
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**Figure 1.** Mouse ischemia brain stroke model generated by suture method. A. Control group; B. Experimental group.



**Figure 2.** MiR-124 expression in mouse brain tissues. \*,  $P < 0.05$  compared to control group.



**Figure 3.** Luciferase reporter gene results.

fied by gel imaging analysis system to calculate relative expression level.

### Statistical analysis

All statistical analysis was performed on SPSS20.0 software. Data were presented as mean  $\pm$  standard deviation (SD). One-way ANOVA was used to compare data among mul-

tiples groups. T test was used to compare difference between two groups. A statistical significance was defined when  $P < 0.05$ .

### Results

#### Establishment of mouse ischemia stroke model

Suture method was adopted to establish a mouse model of ischemia cerebral stroke model. 24 h after surgery, mouse brain tissues were collected. Observation found significant lesion site in model mice, indicating brain stroke lesion. Control group had complete structure of brain without significant lesion (**Figure 1**). These phenomena all suggested successful generation of mouse ischemia brain stroke model by suture method.

#### QRT-PCR for miR-124 expression

Expression profile of miR-124 was further tested by QRT-PCR in stroke lesion of ischemia mouse. Total RNA was extracted from lesion tissues for further QRT-PCR assay. As shown in **Figure 2**, we found about 3.5-fold increase of miR-124 expression level in ischemia stroke mouse brain tissues ( $P < 0.01$ ).

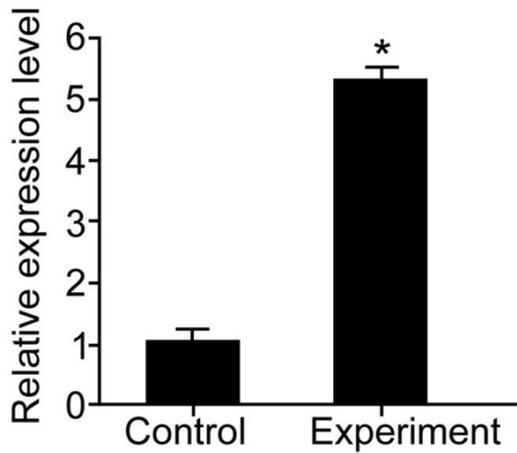
#### Functional target of miR-124 gene

TargetScan Release 5.1 software was used to predict target gene of miR-124. We found homologous sequence between miR-124 and 3'-UTR of Ku70 gene, thus predicting that Ku70 might be the target gene of miR-124. We further constructed a luciferase reporter gene system for confirmation. As shown in **Figure 3**, we found significantly lower fluorescent intensity of cells with miR-124 mimic transfection, whilst those cells transfected with miR-124 inhibitor had significantly higher fluorescent intensity ( $P < 0.05$  compared to control group). These studies indicated that 3'UTR of Ku70 gene was the targeted site of miR-124.

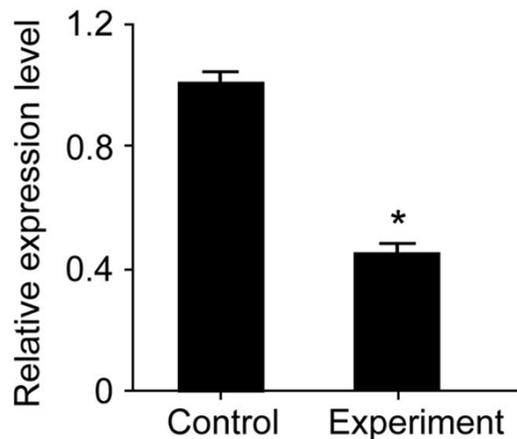
#### Cell transfection and miR-124 over-expression

Mouse embryonic neurons were separated and cultured. An miR-124 over-expression system was constructed by lentiviral vector transfection. A further screening was performed to obtain neurons with miR-124 over-expression.

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**Figure 4.** Over-expression of miR-124 in neurons. \*,  $P < 0.01$  compared to control group.



**Figure 5.** Ku70 protein expression in neurons. \*,  $P < 0.01$  compared to control group.

Total RNA was extracted from cell line for testing relative expression of miR-124 by QRT-PCR. As shown in **Figure 4**, miR-124 expression level after transfection was significantly up-regulated ( $P < 0.05$  compared to control group), suggesting successful transfection. Western blot analysis also showed significant down-regulation of Ku70 inside cells (**Figure 5**).

### Cell apoptosis level

TUNEL assay was employed to test apoptotic index. As shown in **Table 1**, the over-expression of miR-124 in neurons significantly decreased apoptotic index ( $P < 0.05$  compared to control group). With time elapsing of cell culture, apoptotic index was gradually decreased.

**Table 1.** Apoptotic index of neurons by TUNEL

Time (day)	Apoptotic index (%)	
	Control	Experiment
0	2.3±0.5	2.4±0.6
1	8.0±0.6	4.4±0.9*
2	15.6±0.9	6.2±0.8*
3	26.2±1.9	11.2±0.9*

Note: \*,  $P < 0.05$  compared to 0 d time.

### Expression of apoptosis related proteins

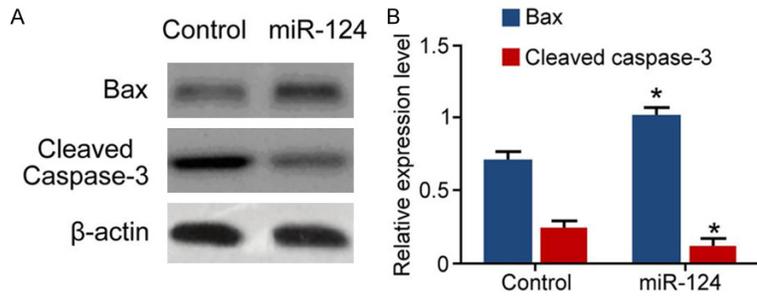
2 days after cell transfection, Western blot was used to test the expression of Bax and Caspase-3 expression level. Using  $\beta$ -actin as the control group, relative expression of three apoptotic related proteins was analyzed by gel imaging system for grey values. As shown in **Figure 6**, neurons with miR-124 over-expression had significantly elevated Bax expression and lower Caspase-3 expression ( $P < 0.05$  compared to control group).

### Discussion

MicroRNA is one type of small non-coding RNA that regulates normal cell behaviors and metabolism [5]. Previous study showed the important role of microRNA in apoptosis and proliferation of neurons. This study constructed a mouse ischemia stroke model, on which miR-124 expression was found to be up-regulated, indicating the possible involvement of miR-124 in pathological process of neural tissues after ischemia stroke. Further bioinformatics analysis and luciferase reporter gene assay predicted Ku70 to be possible target of miR-124. Further cell culture and lentiviral transfection assay successfully over-expressed miR-124 in mouse neurons. Further test of cell apoptotic index and expression of apoptosis related proteins found that over-expression of miR-124 could up-regulate Bax expression in neurons, decreased Caspase-3 activity and enhanced cell proliferation potency.

Currently large amounts of studies have revealed the participation of various microRNA molecules in neural tissue damage and repair, including miR-125, miR-145, miR-1818 and miR-424 [15-17]. Moreover, Dharap et al found significantly elevated miR-145 expression in mouse brain tissues after brain stroke, whilst inhibition of miR-145 expression could inhibit

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**Figure 6.** Western blot for the effect of miR-124 over-expression on apoptotic related protein levels. \*,  $P < 0.05$  compared to control group.

brain stroke severity [17]. All these abovementioned studies demonstrated the critical role of microRNA in progression of brain stroke disease. This study revealed up-regulation of miR-124 in mouse ischemia stroke brain tissues, further replenishing the role of microRNA in neural function recovery after brain stroke. The mechanism of microRNA expression regulation in neurons after stroke, however, is still unclear yet. Some scholars believed that modulation of microRNA expression was achieved by the alternation of promoter methylation for microRNA [18]. Other study also believed that protein products from microRNA targeted genes can regulate microRNA expression level in a negative feedback manner [19]. The mechanism of how neurons change miR-124 expression level after brain stroke still require more experimental evidences to support.

Ku70 protein is one protein kinase dependent regulatory subunit for cellular DNA repairing after injury, and plays an important role in repairing the double strand breakage. Other study showed the participation of Ku70 in neural repairmen after brain ischemia injury [9]. Bax gene belongs to BCL-2 gene family having pro-apoptotic roles. Its coding products can bind with Bcl-2 and inhibit its activity [20]. Study has shown that Ku70 could block the entry of Bax into mitochondria via specific binding. The entry of Bax into mitochondria further activates mitochondrial apoptosis pathway, further enhancing activity level of Caspase-3, thus potentiating cell apoptotic level [21].

Brain stroke is accompanied with complicated pathological processes including inflammation and neural damage. Previous study showed that prominent neuron death and apoptosis were major pathological features of brain

stroke, and causing major damage for neural functions. Therefore, the study of modulatory mechanism for neural cell apoptosis after brain stroke is of critical value for evaluating patient's prognosis, improving treatment efficacy and recovery of neural functions [22]. This study found the role of miR-124 expressional profile and its interaction with Ku70 in cell apoptotic regulation, thus providing evidences for analyzing disease acceleration of brain stroke and functional recovery in patients.

### Conclusion

MiR-124 is up-regulated in brain tissues of ischemia stroke mice. Using Ku70 as its target gene, miR-124 can modulate its expression and further regulate cell proliferation and apoptotic level.

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### Disclosure of conflict of interest

None.

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