

LncRNA MEG3 participates in neuronal cell injury induced by subarachnoid hemorrhage via inhibiting the Pi3k/Akt pathway

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Abstract. – OBJECTIVE: To explore the effect of LncRNA MEG3 in the subarachnoid hemorrhage (SAH) and its underlying mechanism.

PATIENTS AND METHODS: The expressions of LncRNA MEG3 in SAH patients and animal model were detected by quantitative real-time PCR (qRT-PCR). After LncRNA MEG3 was over-expressed in neurons by lentivirus, viability and apoptosis abilities were detected by cell counting kit-8 (CCK-8) assay, flow cytometry, and TUNEL assay, respectively. The apoptosis-related genes and Pi3k/Akt pathway-related proteins were further detected by a Western blot.

RESULTS: The expressions of LncRNA MEG3 in SAH patients were remarkably higher than normal controls, which were positively correlated with SAH severity. After LncRNA MEG3 over-expression, neuronal cell activity was decreased and cell apoptosis was increased. Moreover, the expressions of Bax, p53, and cleaved Caspase-3 were increased, whereas the expression of Bcl-2 and Pi3k/Akt pathway-related proteins were decreased after LncRNA MEG3 overexpression.

CONCLUSIONS: LncRNA MEG3 is up-regulated in SAH, which may promote SAH-induced neuronal cell injury via inhibition of the Pi3k/Akt pathway.

Key Words:

SAH, LncRNA MEG3, Pi3k/Akt pathway, Apoptosis.

Introduction

Subarachnoid hemorrhage (SAH) is a common clinical neurosurgical disease, accounting for about 5% of all strokes. Unfortunately, about

15% of SAH patients die before being admitted to a hospital. The 30-day mortality of SAH is as high as 30%-45%, while half of the survivors suffer from severe neurological dysfunction. The high mortality and morbidity of SAH lead to great socio-economic losses^{1,2}. Scholars have indicated that early brain injury (EBI) after SAH is the leading cause of its high mortality and morbidity. Research has also suggested that the EBI mechanisms mainly include extensive cortical depolarization, apoptosis, inflammation, oxidative stress, etc. Among them, neuronal apoptosis is one of the major mechanisms^{3,4}. However, the specific mechanism of neuronal apoptosis is not well recognized, which requires further in-depth studies.

Long non-coding RNAs (LncRNAs) are non-coding RNAs with over 200 nucleotides in length⁵. Due to the dysfunction of coding proteins, LncRNAs were initially known as “noises” in gene transcription. As genomic assays have advanced, researchers have found that LncRNAs are capable of regulating gene and protein expressions through epigenetics, transcription, and post-transcriptional levels⁶⁻⁸. Previous investigations have shown that LncRNAs participate in the development of the nervous system and the regeneration process after nervous system injury. For example, LncRNA RMST can negatively regulate the expression level of SOX2 to promote neural stem cell differentiation⁹. Additional work¹⁰ has confirmed that down-regulated

intracellular LncRNA BC089918 could prolong neuronal axons. LncRNA MEG3 is the human homologue of the mouse maternally expressed gene-trap locus 2 (Gtl2), which was initially found in 2000. LncRNA MEG3 is expressed only on maternal alleles and localized on the human chromosome 14q32.3, with 1.6 kb in length¹¹. As a tumor suppressor gene, LncRNA MEG3 is widely expressed in various normal cells, especially in the brain and pituitary gland. Studies have found that LncRNA MEG3 is involved in almost all physiological and pathological processes and exerts an inhibitory effect in several kinds of tumors. Overexpressed LncRNA MEG3 increased p53 expression, but decreased Bcl-2 expression. Moreover, overexpressed LncRNA MEG3 could induce mitochondrial apoptosis, thereby promoting the apoptosis of tumor cells^{12,13}. However, the specific effect of LncRNA MEG3 on SAH-induced neuronal cells has not been clearly elucidated.

Patients and Methods

Sample Collection

Cerebrospinal fluid samples on the 1st, 3rd, 5th, and 7th day of the SAH onset of patients with Hunt-Hess class II treated at our hospital from July 2012 to July 2017 were collected. Meanwhile, cerebrospinal fluid samples of SAH patients with Hunt-Hess grade I-II, III, and IV-V patients on the 3rd day of onset were collected as well, with 6 replicate samples in each patient. Preoperative cerebrospinal fluid samples with patients who were proposed to receive hip replacement surgery were used as controls. All samples were preserved in liquid nitrogen for the following experiments. This investigation was approved by the Ethics Committee of Lianjiang Hospital Affiliated to Guangdong Medical University/Lianjiang People's Hospital. The signed written informed consents were obtained from all participants before the study. The basic characteristics of enrolled patients were listed in Table I.

Experimental Animals and SAH Animal Model Construction

The adult male Sprague-Dawley (SD) rats weighing 280-320 g and pregnant rats were obtained from SLAC Laboratory Animal CO. LTD, Shanghai (Shanghai, China). Experimental rats were housed in an environment with 12-h cycle of light and dark. Adequate food and water

were given. The experimental procedures were approved by the Animal Ethics Committee of Guangdong Medical University.

SD rats were used for producing prechiasmatic SAH model¹⁴. Briefly, the femoral arteries of SD rats were dissected under a microscope after that chloral hydrate anesthesia was administered, autologous arterial blood samples were then collected. The prechiasmatic cistern was localized at 8 mm anterior bregma along side the midline of the skull, where 300 μ L of autologous arterial blood was injected. The rats were then assigned into the sham operation group, postoperative 3 h, 6 h, 12 h, 24 h, 72 h, 96 h, and 120 h groups, with 6 rats in each group.

After successful construction of the SAH rat model, SD rats were again injected intraperitoneally with chloral hydrate for anesthesia. The entire brain of the rat was harvested and placed in 4% formalin for fixation overnight after cardiac perfusion. Brain samples were placed at -80°C refrigerator for the following experiments.

Extraction and Culture of Rat Primary Neurons

6-well plates were coated with polylysine solution overnight. Embryonic day 14 rats were anes-

Table I. Characteristics and mark number of chosen patients.

Mark number	Age	Gender	Stage
SAH 1*	66	Female	I-II
SAH 2*	69	Female	I-II
SAH 3*	64	Male	I-II
SAH 4*	65	Female	I-II
SAH 5*	61	Male	I-II
SAH 6*	68	Male	I-II
SAH 7*	62	Female	III
SAH 8*	63	Male	III
SAH 9*	66	Male	III
SAH 10*	67	Female	III
SAH 11*	59	Female	III
SAH 12*	67	Female	III
SAH 13*	71	Male	IV-V
SAH 14*	65	Female	IV-V
SAH 15*	70	Male	IV-V
SAH 16*	66	Male	IV-V
SAH 17*	62	Female	IV-V
SAH 18*	61	Female	IV-V
Con 1	56	Female	
Con 2	57	Female	
Con 3	56	Male	
Con 4	59	Female	
Con 5	54	Male	
Con 6	57	Female	

thetized and sacrificed, followed by a collection of fetal rat brain. After digestion with 0.125% trypsin and centrifugation, the cell suspension was inoculated into the previously prepared 6-well plates. Culture medium was replaced 3 h later for discarding the non-adherent cells. Neuron culture medium supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin were slowly added and cells were maintained in a 5% CO₂ incubator at 37°C.

Construction of In Vitro SAH Model

A 100 µM hemoglobin (Hb) solution was prepared by adding 6.67 mg Hb powder into 1 mL primary neuronal complete medium. Cells were cultured in 1.7 mL of complete medium containing 300 µL of 100 µM Hb solution for 0, 3, 6, 12, 24, and 48 h, respectively¹⁵.

Cell Transfection

The cells were seeded into the 6-well plates at a dose of 4×10^5 /mL, when the cell confluence was up to 80-95%. LV-Vector or LV-MEG3 was transfected according to the instructions of Lipofectamine 2000, respectively. Transfection efficacy was verified by qRT-PCR 24 h later.

Cell Counting Kit-8 (CCK-8) Assay

Transfected cells were collected and seeded into the 96-well plates at a dose of 5×10^4 /mL. After 24 h-inoculation, 10 µL of CCK-8 solution was added into each well and incubated for 1 h in the dark. The absorbance (OD) values at the wavelength of 450 nm were obtained with a microplate reader.

RNA Extraction and qRT-PCR

The mRNAs of cells and tissues were extracted with TRIzol reagent and then were reversely transcribed to cDNAs. The reaction conditions were as follows: pre-denaturation at 94°C for 3 min, followed by denaturation at 94°C for 20 s, annealing at 55-60°C for 20 s and extension at 72°C for 30 s for a total of 40 cycles. Each sample was repeatedly performed for 3 times. Primers used in this study were as follows: lncRNA MEG3 F: CTGCCCATCTACACCTCACG; R: CTCTCCGCCGTCTGCGCTAGGGGCT.

Western Blotting

The total protein of the transfected cells was extracted. The concentration of each protein sample was determined by bicinchoninic acid (BCA) kit. Then, 50 µg of total protein was sep-

arated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% skimmed milk, followed by the incubation of specific primary antibodies overnight. The membranes were then incubated with the secondary antibody at room temperature for 1 h. Immunoreactive bands were exposed by enhanced chemiluminescence method.

Cell Cycle

Cells were fixed with ice-cold 70% ethanol, overnight. For cell cycle assay, cells were centrifuged, washed twice with phosphate buffered saline (PBS), and then incubated with 150 µL of propidium iodide (PI) in the dark for 30 min. Finally, the specific distribution of cell cycle was determined by analyzing 10000 events by flow cytometry.

Cell Apoptosis

Transfected cells were digested with EDTA-free Trypsin and washed with PBS. 500 µL of binding buffer was added. Subsequently, 5 µL of Annexin V-EGFP and 5 µL of PI were added, respectively. Cells were then incubated in the dark at room temperature for 15 min. The apoptosis rate was analyzed by flow cytometry.

TUNEL Assay

Cell slides were prepared using the TUNEL labeling kit according to the manufacturer's instructions. Following this, slides were washed with PBS and blocked with hydrogen peroxide solution. After permeabilized in Trixon-100, slides were incubated with TUNEL solution for 90 min. Negative controls were incubated with TdT labeled solution. Finally, slides were stained with 4',6-diamidino-2-phenylindole (DAPI) for the following observation of apoptotic cells.

Statistical Analysis

Statistical Product and Service Solutions (SPSS17.0, SPSS Inc., Chicago, IL, USA) statistical software was used for data analysis. Measurement data were expressed as mean $\pm$ standard deviation ($\bar{x} \pm s$). Comparison of measurement data was conducted using the *t*-test. Classification data were compared using the chi-square test. *p* < 0.05 was considered statistically significant.

Results

LncRNA MEG3 was Overexpressed in the Brain of SAH Rat Model

Higher mRNA expressions of lncRNA MEG3 in the temporal cortex of SAH rats were observed in comparison with those of the placebo operation group. 12 h after the operation, the expression of lncRNA MEG3 achieved the peak, which gradually returned to normal after 72 h (Figure 1A). For *in vitro* experiments, higher expression of lncRNA MEG3 in the SAH group was observed in comparison with that of the control group. 12 h after the SAH induction, the expression of lncRNA MEG3 peaked, which decreased gradually in a time-dependent manner (Figure 1B). Meanwhile, cell viability in the SAH group was remarkably decreased in comparison to that of the control group (Figure 1C), while cell apoptosis was remarkably increased (Figure 1D). Our results suggested that differentially expressed lncRNA MEG3 may be involved in SAH-induced neuronal damage.

LncRNA MEG3 Promoted Neuronal Apoptosis

The neuronal expression of lncRNA MEG3 was notably increased by lentiviral transfection (Figure 2A). Overexpressed lncRNA MEG3 significantly inhibited neuronal activity for 72 h (Figure 2B). Meanwhile, overexpressed lncRNA MEG3 resulted in increased neuronal apoptosis (Figure 2C). The TUNEL assay also demonstrated a similar result (Figure 2D). The above data indicated that up-regulated lncRNA MEG3 may be involved in SAH-induced neuronal damage by promoting neuronal apoptosis.

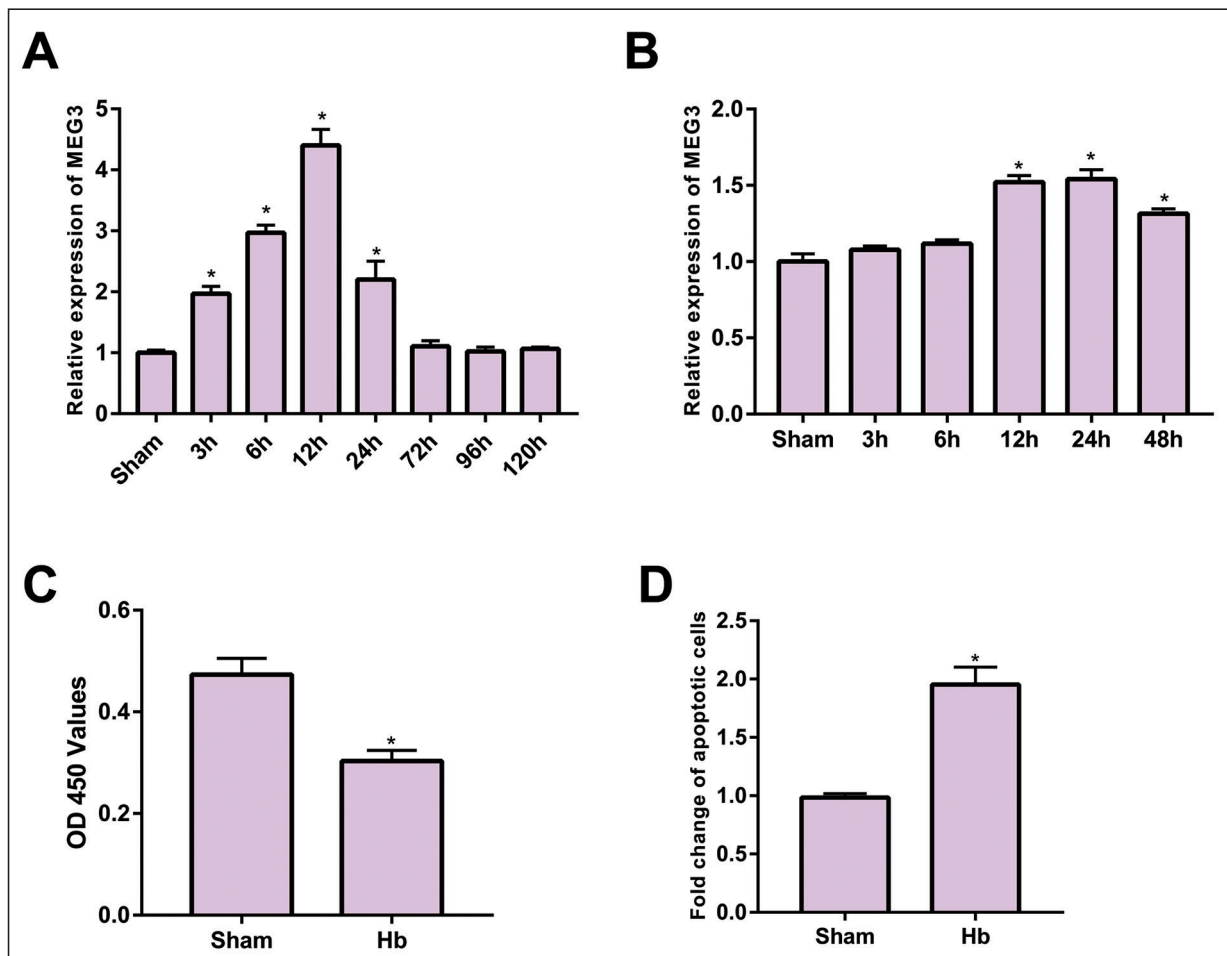


Figure 1. LncRNA MEG3 was overexpressed in the brain tissue of SAH rat model. LncRNA MEG3 expressions **A**, at different perfusion time points in SAH rat model and Hb-induced in vitro model **B**, were detected by qRT-PCR. Hb-induced neuronal cell activity **C**, and apoptosis **D**, were examined (* $p < 0.05$).

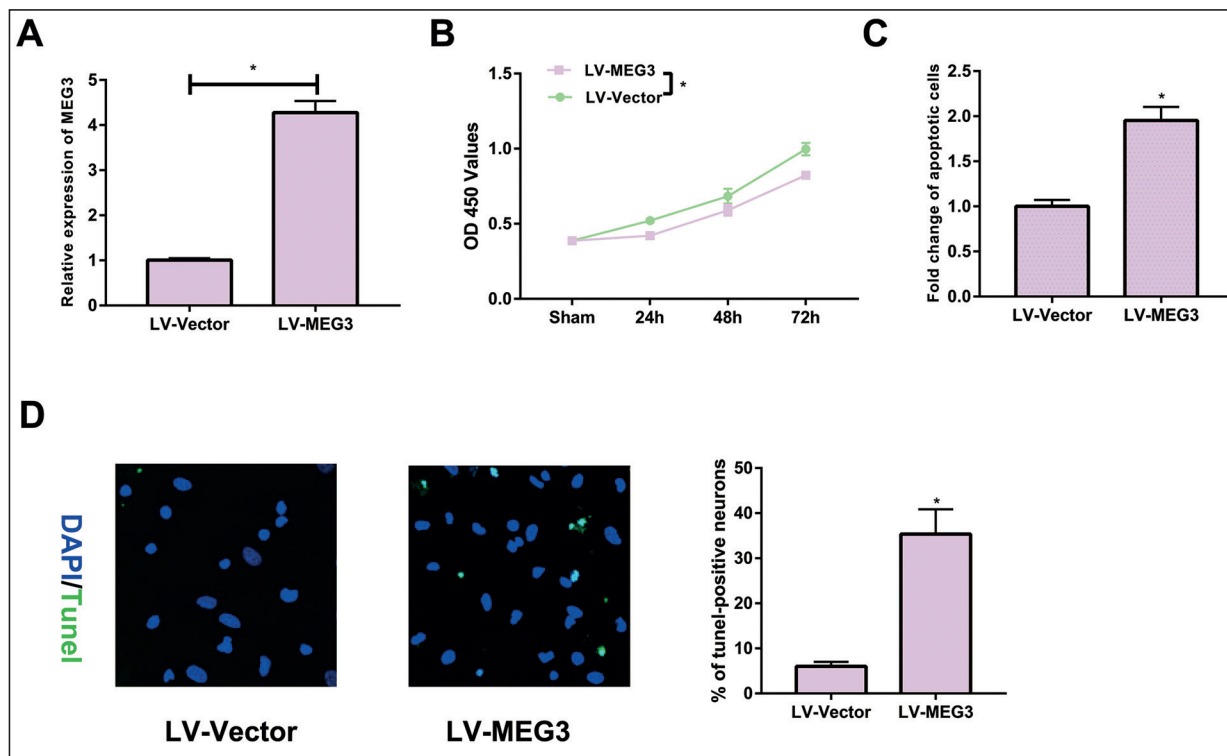


Figure 2. LncRNA MEG3 promoted neuronal apoptosis. **A**, Overexpression of lncRNA MEG3 in neuronal cells by lentivirus. **B**, The activity of MEG3-overexpressing neurons was examined by CCK-8 assay. The apoptosis of MEG3-overexpressing neurons was measured by flow cytometry **C**, and TUNEL assay **D**.

LncRNA MEG3 Suppressed the Pi3k/Akt Pathway

The expressions of cleaved Caspase-3, Bax, and p53 were remarkably elevated after the overexpression of lncRNA MEG3, whereas the expression of Bcl-2 was reduced (Figure 3A). It is well recognized that the Pi3k/Akt pathway is closely related to apoptosis¹⁶. Therefore, key proteins related to the Pi3k/Akt pathway were identified. Our findings demonstrated that the expressions of p-PI3K and p-AKT are significantly decreased after overexpression of lncRNA MEG3 (Figure 3B), suggesting that lncRNA MEG3 may promote the neuronal apoptosis by inhibiting the Pi3k/Akt pathway.

MEG3 was Positively Correlated with the SAH Severity

The mean age of SAH patients was younger than that of healthy controls (Table I). Our data revealed that the expression levels of lncRNA MEG3 in cerebrospinal fluid of SAH patients with Hunt-Hess II was remarkably higher than the healthy controls. The expression level of lncRNA MEG3 achieved the peak on the 3rd day after the operation, and then gradually decreased to the normal one (Figure 4A). In contrast with the healthy controls, the expression level of lncRNA MEG3 was increased with the elevation of Hunt-Hess grade, which achieved the peak in SAH patients with Hunt-Hess IV-V (Figure 4B), indicating that lncRNA MEG3 can be served as an indicator of disease severity.

Discussion

Subarachnoid hemorrhage (SAH) is one of the most severe cerebrovascular diseases, which mainly results from the rupture of intracranial aneurysms⁴. Secondary brain injury induced by cerebral vasospasm has been considered to be the leading cause of brain injury after SAH. However, no significant breakthrough has been made on cerebral vasospasm in the past decades. A recent multicenter trial showed that amelioration of delayed vasospasm could not improve the prognosis of SAH patients. Accumulating evidence

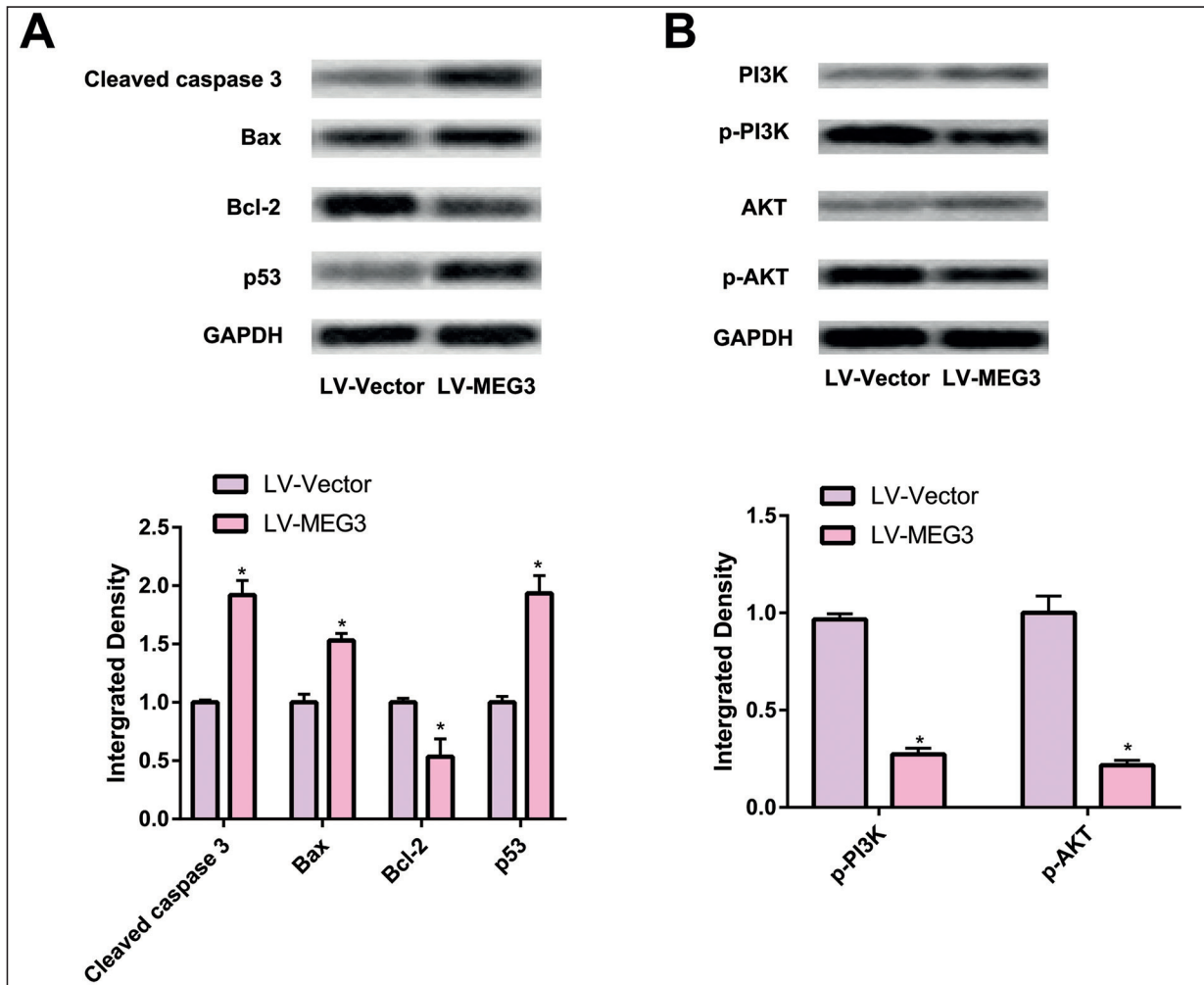


Figure 3. LncRNA MEG3 inhibited the Pi3k/Akt pathway. **A**, The expressions of cleaved Caspase-3, Bax, p53 and Bcl-2 were detected by Western blot. **B**, The expressions of PI3K, AKT, p-PI3K and p-AKT in the Pi3k/Akt pathway were detected.

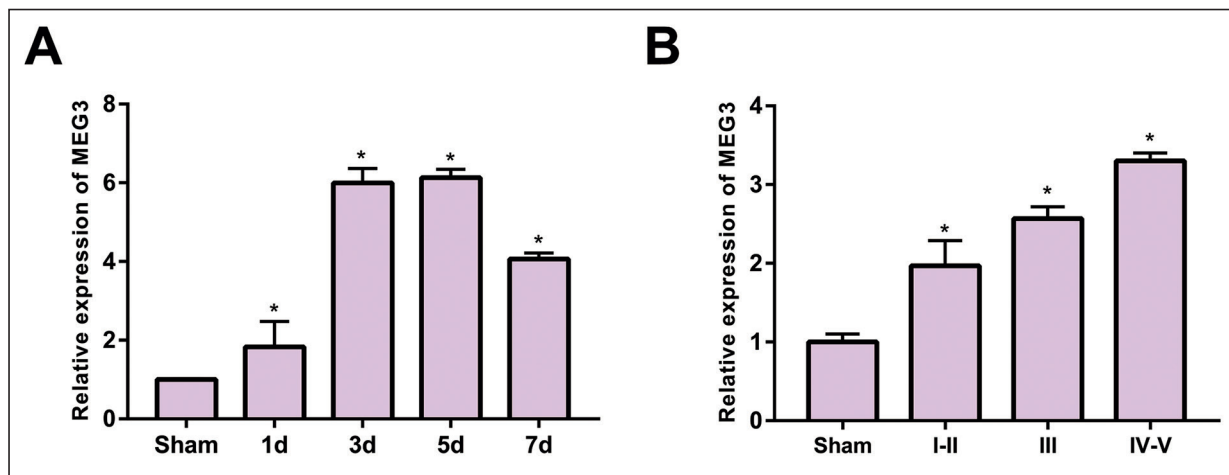


Figure 4. LncRNA MEG3 was positively correlated with the SAH severity. The expressions of lncRNA MEG3 in cerebrospinal fluid of SAH patients in different time points **A**, and different disease stages **B**, were detected by qRT-PCR ($p < 0.05$).

has indicated that other factors can also lead to brain damage after SAH. Subsequent studies have shown that EBI within 72 h after the SAH occurrence is responsible for the high mortality of SAH. The intervention of EBI can significantly improve the SAH prognosis¹⁷.

Cell death is one of the major pathological processes of EBI, including necrosis, apoptosis, and autophagy¹⁸. However, there has been controversy on the role of autophagy in brain injury after SAH. Zhao et al¹⁹ found that autophagy activation in the SAH rat model could inhibit cell death. Another research²⁰ found that autophagy activation in stroke rats is capable of promoting brain injury. As a consequence, we did not observe the autophagy in the present work. Necrosis and apoptosis are immediately induced after SAH, which have been considered as the most important forms of cell death in early stages of SAH²¹. As decreased cerebral perfusion in the early stage of SAH can be relieved in a short time²², we focused on the neuronal apoptosis in this study.

Apoptosis is a kind of programmed cell death which is initiated by various factors. Mitochondrial apoptosis has been proved to exert a crucial role in the nerve function score after SAH. The transcription factor, p53, is involved in tumorigenesis. Studies^{23,24} have shown that p53 can promote apoptosis in the early stage of SAH *via* the mitochondrial apoptotic pathway. The expression level of p53 in rat brains is significantly increased during SAH. We found that overexpressed lncRNA MEG3 can promote the expression level of p53, thereby promoting neuronal apoptosis.

B-cell lymphoma-2 (Bcl-2) family proteins are the most important ones of p53 targeting proteins, including Bcl-2, Bax, and others²⁵. Investigations have shown that Bax exerts an integral role in p53-induced apoptosis. Both Bcl-2 and Bax could regulate apoptosis *via* mitochondrial pathways. Bax promotes apoptosis by acting on mitochondria with an enhanced permeability and promoting the release of cytochrome-c and AIF. Bcl-2, however, inhibits the mitochondrial apoptosis by binding to Bax^{26,27}. In this study, we found that the overexpressed lncRNA MEG3 increases the expression of Bax, but decreases the expression of Bcl-2 *via* the Pi3k/Akt pathway.

Conclusions

We observed that up-regulated lncRNA MEG3 in SAH can promote neuronal apoptosis *via* inhi-

bition of the Pi3k/Akt pathway. Meanwhile, the expression level of lncRNA MEG3 in cerebrospinal fluid of SAH patients is positively correlated with the SAH severity.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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