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LncRNA MEG3 promotes neuronal apoptosis in rats with ischemic cerebral infarction *via* the TGF-β1 pathway

Shiyu Long[1], Jingli Chen[1], Shuwei Tan[1], Rong Zhou[1], Su He[2], Jiedeng Jia[3*]

[1] Department of Neurology, Gaoping District People's Hospital of Nanchong, Nanchong, China
[2] Department of Neurology, Nanchong Second People's Hospital, Nanchong, China
[3] Dean's office, Gaoping District People's Hospital of Nanchong, Nanchong, China

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ABSTRACT

To study the influence of long non-coding ribonucleic acid maternally expressed gene 3 (lncRNA MEG3) on the neuronal apoptosis in rats with ischemic cerebral infarction, and to analyze its regulatory effect on the transforming growth factor-beta 1 (TGF-β1) pathway. A total of 36 Sprague-Dawley rats were randomly assigned into sham group, model group and low expression group. Ischemic cerebral infarction modeling was constructed in rats of the model group and low expression group. Corresponding adenoviruses were intracranially injected in rats of low expression group to knock down lncRNA MEG3 expression. At 24 h after the operation, the neurological function of rats was evaluated in each group, and the expression level of lncRNA MEG3 in cerebral tissues was determined using quantitative polymerase chain reaction (qPCR). The infarct size was measured *via* 2,3,5-triphenyltetrazolium chloride (TTC) staining. The apoptosis level of neurons in cerebral tissues was determined using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining. Besides, enzyme-linked immunosorbent assay (ELISA) was performed to determine the contents of inflammatory factors in cerebral tissues. Expression levels of apoptosis-associated proteins and vital genes in the TGF-β1 signaling pathway in rat cerebral tissues were measured using Western blotting. Compared with the sham group, rats in the model group exhibited substantial increases in the neurological score and apoptosis level of neurons ($p<0.01$). Relative levels of lncRNA MEG3, interleukin (IL)-6, tumor necrosis factor-alpha (TNF-α), Caspase-3, TGF-β1, small mothers against decapentaplegic homolog 2 (Smad2) and Smad3 ($p<0.01$) were higher in a model group than those in sham group. Notable declines in the content of IL-10 ($p<0.01$) and the ratio of B-cell lymphoma 2 (Bcl-2)/Bcl associated X protein (Bax) ($p<0.01$) were seen in the model group compared with the sham group. The abovementioned changes in the model group were partially abolished in the low expression group. LncRNA MEG3 is upregulated in the cerebral tissues of rats with ischemic cerebral infarction. It induces an inflammatory response, expands cerebral infarct size, and promotes neuronal apoptosis and impairment by activating the TGF-β1 pathway.

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Introduction

Stroke is featured by extremely high mortality and disability rates. It can be classified into hemorrhagic cerebral infarction and ischemic infarction, the latter of which accounts for 70-80% of the total cases (1,2). According to epidemiological investigation and research findings, ischemic cerebral infarction is the leading cause of death in the elderly and it is the number three killer that seriously influences the life quality and health of patients (3). Currently, surgery combined with adjuvant medications is preferred to ischemic cerebral infarction. Although angioplasty can effectively treat ischemic cerebrovascular disease through expanding infarcted blood vessels, with the advantages of exact efficacy and small traumas, the prognosis of patients is still relatively poor (4). Therefore, there is an urgent need of exploring new treatment strategies of ischemic cerebral infarction. As molecular biological techniques develop, searching for pathogenic genes has become a hotspot. Long non-coding nucleic acids (lncRNAs), a type of RNAs of exceeding 200 nucleotides in length, have no protein-encoding function (5). Studies have evidenced that lncRNAs are tissue-specific, which can regulate multiple physiological and pathological processes, such as cell apoptosis, cell differentiation and stress responses (6). Li et al. (7) found that the expression level of lncRNA maternally expressed gene 3 (MEG3) is dramatically upregulated in the myocardial tissues of patients with myocardial infarction. Transforming growth factor-beta 1 (TGF-β1), a subtype of TGF-β, is widely expressed in multiple cells and tissues. It activates the TGF-β1 pathway, thereby activating small mothers against decapentaplegic homolog 2 (Smad2) and Smad3, and participating in the regulation of inflammatory responses *in vivo* (8) So far, there have not yet been studies on the effect of lncRNA MEG3 on the TGF-β1 pathway, and their potential regulations on neuronal apoptosis induced by ischemic cerebral infarction. The present research group, therefore, aims to evaluate the influences of lncRNA MEG3 on neuronal apoptosis in rats with ischemic cerebral infarction and the involvement of the TGF-β1 pathway. Our findings provide a theoretical basis for the in-depth elucidation of the pathogenesis and

* Corresponding author. Email: jiangjd2000@126.com

treatment strategy of ischemic cerebral infarction.

Materials and Methods

Construction of Recombinant Adeno-Associated Viruses

The recombinant adeno-associated viruses were constructed by Shanghai GenePharma Co., Ltd. (Shanghai, China). The published lncRNA MEG3 sequences were obtained to establish MEG3-RNA interference (RNAi). After its biological titer was determined, transfection of MEG3-RNAi was performed to knock down the expression of lncRNA MEG3 in the cerebral tissues of rats.

Laboratory Animals and Grouping

Male Sprague-Dawley (SD) rats were adaptively fed in a specific pathogen-free animal breeding room for 7 d at a room temperature of 22±2°C and a humidity of 45% in the circadian rhythm. They had free access to water and food. Within 12 h before modeling, they were fasted for food, but not water. This study was approved by the Animal Ethics Committee of Gaoping District People's Hospital of Nanchong Animal Center.

Experimental grouping: the 36 SD rats were randomly divided into the sham group (n=12), model group (n=10) and low expression group (n=10). Artery ligation was performed in rats of both the model group and the low expression group, while those in the sham group received the same procedures except for ligation. Intracranial stereotactic positioning was adopted to transfect MEG3-RNAi into the cerebral tissues of rats in the low expression group. Rats in sham and model groups were injected with the same dose of empty viruses.

Establishment of Rat Model of Ischemic Cerebral Infarction

The rats were first anesthetized by 10% chloral hydrate (Sigma-Aldrich, St. Louis, MO, USA) and fixed in the supine position. The hair on the neck was cleared using depilatory creams, followed by iodine-based disinfection and draping of a surgical hole towel. Then, the neck skin was cut open along the midline of the neck, and the cervical muscle tissues were bluntly separated using tweezers, while the left common carotid artery, external carotid artery and internal carotid artery were also separated. Subsequently, the lower common carotid artery and external carotid artery were ligated by appropriate sutures. With the distal end of the internal carotid artery clamped by arterial clips, an incision was made at the proximal end, and a 0.3 mm thread was inserted to the optimal depth. Afterward, the internal carotid artery was ligated and blocked using the thread. The cervical muscle and epidermis were sutured. Rats were placed on a thermostatic blanket for heat preservation and moved into the breeding cages after analepsis. The rats in the sham group underwent anesthesia and blood vessel separation, without the insertion of threads.

Evaluation of Neurological Function

At 24 h after the operation, the neurological function of rats was evaluated according to Zea-Longa's score criteria in each group to assess the neurological impairment in rats. Neurological function was assessed from 0-4 points. 4 points: rats die or have a disorder of consciousness and are unable to walk; 3 points: rats walk and topple towards the contralateral side of the infarcted; 2 points: rats walk and circle around the contralateral side of the infarcted; 1 point: the anterior paws of rats on the contralateral side of the infarcted cannot stretch freely, and 0 points: rats can move normally.

Determination of LncRNA MEG3 Expression in Cerebral Tissues Using Quantitative Polymerase Chain Reaction (qPCR)

After evaluation of neurological function, the rats were immediately sacrificed. The whole brain tissues were separated, weighed, and lysed in TRIzol (Invitrogen, Carlsbad, CA, USA) at 100 mg/mL and ultrasonically homogenized until there was no tissue debris visible to the naked eye. Then the resulting homogenate was shaken 10 times, and let stand for 10 min. The total RNAs were harvested for determining their concentration and purity. Subsequently, the reverse transcription was performed at 37°C for 15 min and at 85°C for 5 min according to the instructions of the kit (Wuhan Vazyme Biotech Co., Ltd., Wuhan, China). QPCR system was prepared as follows: 2 μL of total RNAs, 1 μL of Oligo $(dT)_{20}$, 1 μL of 10 mM dNTP, 3 μL of 0.1 M DTT and diethyl pyrocarbonate (DEPC)-treated water (Beyotime, Shanghai, China) added until the total volume was 25 μL. Primers (synthesized by Invitrogen Corporation, Carlsbad, CA, USA) were amplified, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal reference. The sequences of each primer were shown in Table 1. Following PCR, the baseline and threshold were set, and the relative expression level of lncRNA MEG3 was calculated using the formula of $2^{-\Delta\Delta Ct}$ and expressed as lncRNA-MEG3/GAPDH.

Measurement of Cerebral Infarct Size in Each Group of Rats Using 2,3,5-Triphenyltetrazolium Chloride (TTC) Staining

After neurological function evaluation, the rats were immediately sacrificed for collecting the whole brain tissues. They were washed using pre-cooled normal saline, placed on a plate and cryopreserved at -20°C for 30 min. Then, brain tissues were sliced to about 2 mm-thick coronal sections, placed in 2% TTC solution (Oxoid, Hampshire, UK) and stained at 37°C in dark for 30 min, during which the sections were turned over for full staining. Upon completion of staining, the TTC staining solution on the brain tissue sections was blotted up using filter paper, and sections were fixed in 4% paraformaldehyde and stored in a refrigerator at 4°C overnight. Subsequently, the fixed sections were taken out, and photographed. The infarct

Table 1. Primer sequences.

Gene	Forward	Reverse
LncRNA MEG3	5' GCTCTACTCCGTGGAAGCAC 3'	5' CAAACCAGGAAGGAACGAG 3'
GAPDH	5' GCACCGTCAAGGCTGAGAC 3'	5' TGGTGAAGACGCCAGTGGA 3'

zone was white, while the non-infarct zone was red. The cerebral infarct size was computed based on the following formula: Infarct size (%) = (contralateral brain area − ischemic non-infarct size)/contralateral brain area ×100%.

Counting of Apoptotic Cells Via Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL) Staining

The rats were immediately sacrificed after neurological function evaluation, and the whole brain tissues were extracted, followed by TUNEL staining strictly according to the instructions of the kit (Beyotime, Shanghai, China). Then, an antifade mounting medium was added dropwise to seal the stained sections. Neuronal apoptosis in the infarcted cortex tissues was observed under a confocal laser scanning microscope, among which yellowish-green fluorescent TUNEL-positive cells were apoptotic cells.

Determination of Inflammatory Factor Content Via Enzyme-Linked Immunosorbent Assay (ELISA)

The contents of inflammatory factor interleukin (IL)-6, IL-10 and tumor necrosis factor-alpha (TNF-α) in the brain tissues of rats were determined using the ELISA kit (Wuhan Boster Biological Technology Co., Ltd., Wuhan, China) strictly in accordance with the instructions of the kit.

Detection of Relevant Protein Expression Via Western Blotting

Rat brain tissues were extracted, lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (Beijing TDY Biotech Co., Ltd., Beijing, China) supplemented with 1% phosphatase and protease inhibitors, ultrasonically homogenized for 10 min and centrifuged at 12,000 rpm at 4°C for 10 min. The supernatant was aspirated, and the total protein was obtained and quantified using the bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Subsequently, the loading system was prepared using the proteins at an equal concentration, loaded for electrophoresis, transferred onto membranes, and sealed using freshly prepared 5% skim powder for 2 h. The target bands were cut off, incubated with the corresponding primary antibodies [Caspase-3, B-cell lymphoma 2 (Bcl-2), Bcl-2 associated X protein (Bax), TGF-β1, Smad2, Smad3 and the internal reference GAPDH primary antibodies (1:1,000, CST, Danvers, MA, USA)] at 4°C overnight. After washing in Tris-Buffered Saline and Tween (TBST) for 5 min × 3 times, bands were incubated with the horse radish peroxidase (HRP)-labeled secondary antibodies (1:5,000, Shanghai Yihyson Biotechnology Co., Ltd., Shanghai, China) at room temperature for 1 h. Enhanced chemiluminescence (ECL) (Thermo Fisher Scientific, Waltham, MA, USA) was conducted for image development and exposure in a darkroom. Finally, the expression level of each protein was calculated.

Statistical Analysis

In the present study, the data were expressed as mean ± standard deviation, and processed with Statistical Product and Service Solutions (SPSS) 19.0 software (SPSS Inc., Chicago, IL, USA). Intergroup comparisons were made using analysis of variance (ANOVA), and the results conforming to the homogeneity of variance were subjected to a Bonferroni test, while those fulfilling heterogeneity of variance were analyzed using Welch's t-test. $P<0.05$ represented statistically significant differences.

Results

Neurological Score of Each Group of Rats

The neurological score of rats was recorded in each group. It is shown that the neurological score of rats in the model group was prominently higher than that in the sham group ($P<0.01$), while the rats in the low expression group had a lower neurological score than those in the model group ($P<0.01$) (Figure 1).

Expression Level of lncRNA MEG3 in all Groups of Rats

As shown in Figure 2, the model group exhibited a notably higher expression level of lncRNA MEG3 in the cerebral tissues of rats than the sham group ($P<0.01$), whereas the expression level of lncRNA MEG3 in the low expression group was remarkably lower than that in model group ($P<0.01$).

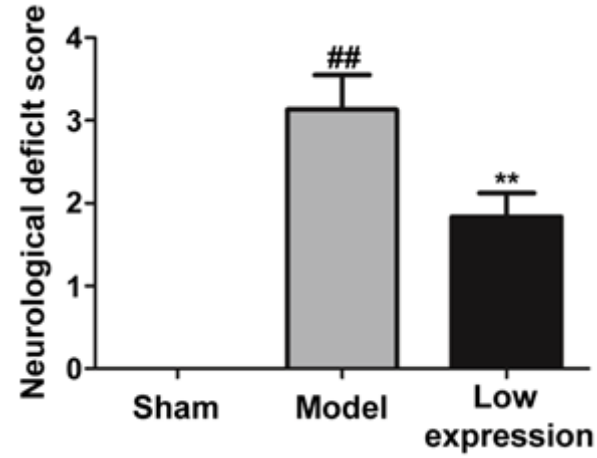

Figure 1. Neurological score of rats. The neurological score of rats in the model group is substantially higher than that in both the sham group and low expression group ($^{\#\#}P<0.01$ *vs.* sham group, $^{**}P<0.01$ *vs.* model group).

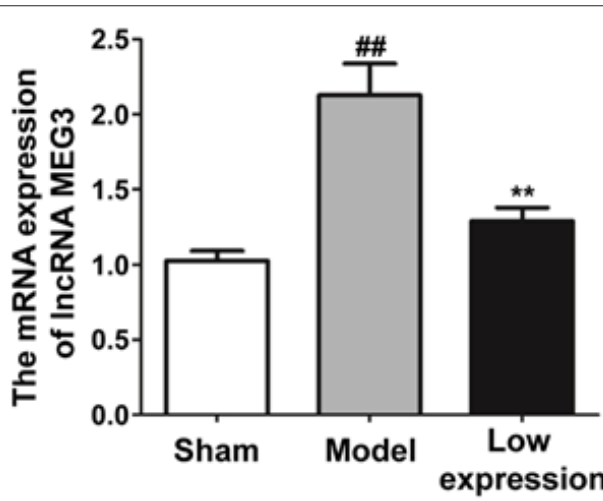

Figure 2. The expression level of lncRNA MEG3 in the cerebral tissues of rats determined *via* qPCR. The expression level of lncRNA MEG3 in the cerebral tissues of rats in the model group is considerably higher than that in both the sham group and low expression group ($^{\#\#}P<0.01$ *vs.* sham group, $^{**}P<0.01$ *vs.* model group).

Cerebral Infarct Size of Rats in Each Group

The cerebral infarct size of rats was measured *via* TTC staining in each group. It was found that the cerebral infarct size of rats in model group was markedly larger than that in sham group (P<0.01), and low expression group exhibited a prominently smaller cerebral infarct size of rats than model group (P<0.01) (Figure 3).

Neuronal Apoptosis Level in all Groups of Rats

TUNEL staining was performed to determine the apoptosis level of neurons. The results revealed that the apoptosis level of neurons in rats rose remarkably in the model group compared with that in the sham group (P<0.01), whereas the neurons of rats in the low expression group had a lower apoptosis level than those in the model group (P<0.01) (Figure 4).

Expression Levels of Inflammatory Factors in Rat Cerebral Tissues

As shown in Figure 5, compared with the sham group, the model group had substantially increased IL-6 and TNF-α contents (P<0.01, P<0.01), and notably lowered IL-10 content (P<0.01) in the cerebral tissues of rats. In the low expression group, the contents of IL-6 and TNF-α were dramatically lower (P<0.01, P<0.01), but the content of IL-10 was remarkably higher than those in model group (P<0.01).

Expression Levels of Apoptosis-Associated Proteins in Each Group of Rats

According to the Western blotting results (Figure 6), in comparison with those in the sham group, the ratio of Bcl-2/Bax declined considerably (P<0.01), while the Caspase-3 level rose substantially (P<0.01) in the cerebral tissues of rats in model group. Besides, the low expression group had a notably higher ratio of Bcl-2/Bax (P<0.01), but prominently lower Caspase-3 level (P<0.01) in the cerebral tissues of rats than the model group (P<0.01).

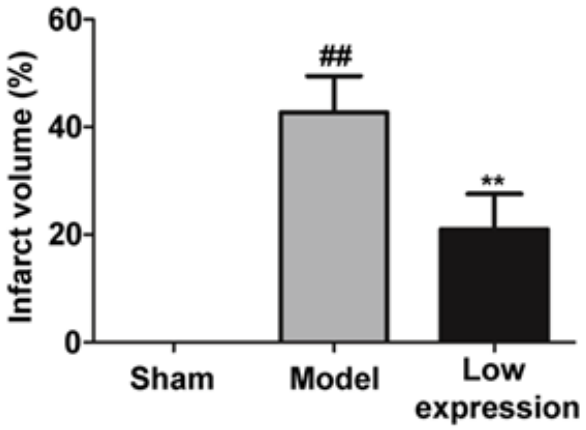

Figure 3. Cerebral infarct size of rats in each group measured *via* TTC staining. (A) Images of cerebral tissue sections. (B) Statistical graph of infarct size. The model group shows a considerably larger cerebral infarct size of rats than both the sham group and low expression group ($^{\#\#}P$<0.01 *vs.* sham group, $^{**}P$<0.01 *vs.* model group).

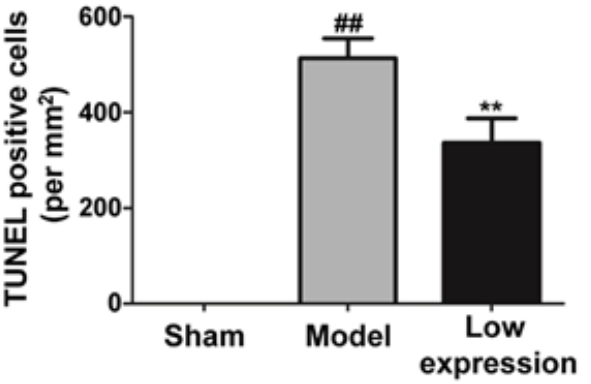

Figure 4. Neuronal apoptosis level in each group of rats determined *via* TUNEL staining. (A) Micrograph (magnification: 400×). (B) Statistical graph (scale bar =50 μm). The neurons of rats in the model group have a substantially higher apoptosis level than those in both the sham group and low expression group ($^{\#\#}P$<0.01 *vs.* sham group, $^{**}P$<0.01 *vs.* model group).

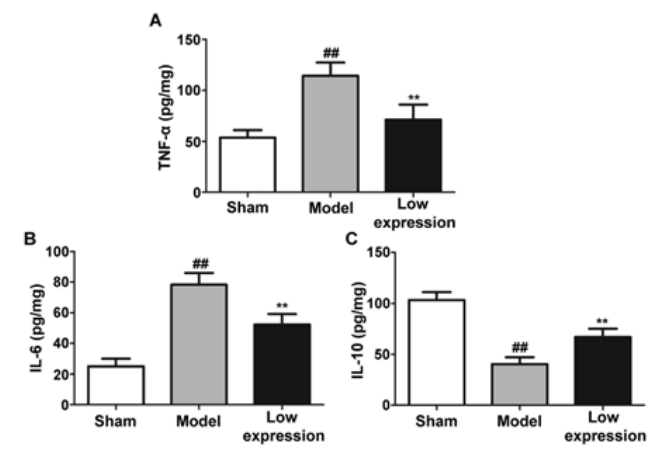

Figure 5. Content of inflammatory factors in the cerebral tissues of rats in each group. (A) TNF-α content, (B) IL-6 content, (C) IL-10 content. The contents of TNF-α and IL-6 in the cerebral tissues of rats in the model group were markedly higher, whereas the IL-10 content is prominently lower than those in the sham group and low expression group ($^{\#\#}P$<0.01 *vs.* sham group, $^{**}P$<0.01 *vs.* model group).

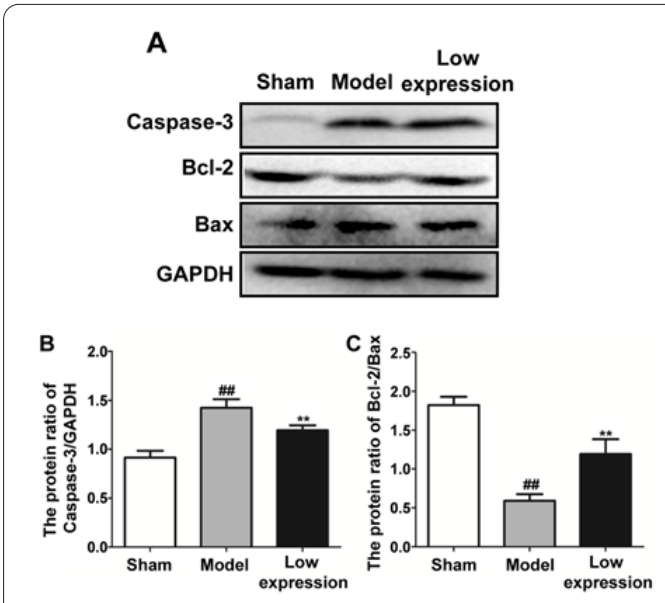

Figure 6. Expression levels of apoptosis-associated proteins in the cerebral tissues of rats in each group. (A) Protein bands. (B) Caspase-3 expression level. (C) The ratio of Bcl-2/Bax. The model group has a considerably higher expression level of Caspase-3 in the cerebral tissues of rats and a markedly lower ratio of Bcl-2/Bax than sham group and low expression group ($^{\#\#}P$<0.01 *vs.* sham group, $^{**}P$<0.01 *vs.* model group).

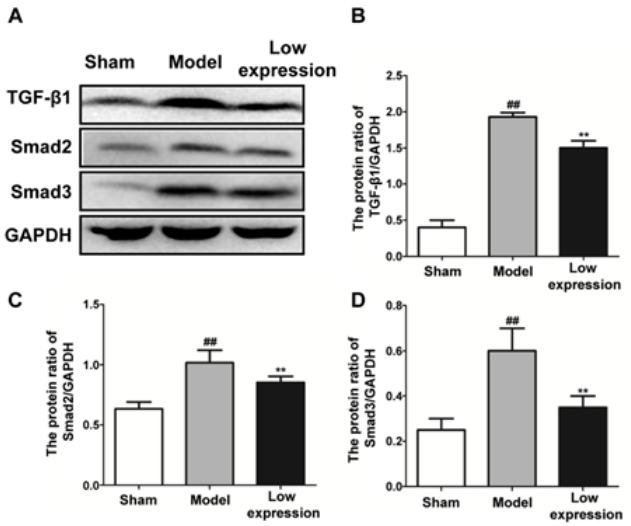

Figure 7. Expression levels of TGF-β1 signaling pathway-associated proteins in the cerebral tissues of rats in each group. (A) Protein bands, (B) Statistical graph of TGF-β1 expression level, (C) Statistical graph of Smad2 expression level, (D) Statistical graph of Smad3 expression level. The expression levels of TGF-β1, Smad2 and Smad3 in the cerebral tissues of rats in the model group are markedly higher than those in both the sham group and the low expression group ($^{\#\#}P<0.01$ *vs.* sham group, $^{**}P<0.01$ *vs.* model group).

Expression Levels of TGF-β1 Signaling Pathway-Associated Proteins in Cerebral Tissues of Rats

Compared with those in the sham group, the expression levels of TGF-β1, Smad2 and Smad3 were dramatically elevated in the cerebral tissues of rats in the model group ($P<0.01$), whereas their expression levels in the low expression group were remarkably lower than those in model group ($P<0.01$) (Figure 7).

Discussion

Ischemic stroke, an acute cerebrovascular disease, is characterized by high morbidity and mortality rates. Its main clinical manifestations include sudden faintness, loss of balance coordination ability, hemiplegia, distortion of commissure, unconsciousness and unsmooth speech (9). Cerebral tissue damage caused by cerebral embolism-induced ischemia-hypoxia is the fundamental reason for ischemic stroke (10). In the present study, the model of ischemic cerebral infarction was established in rats. It was found that the expression level of lncRNA MEG3 rose substantially in the cerebral tissues of rats with ischemic cerebral infarction. Sheng et al. (11) discovered that highly expressed lncRNA MEG3 can significantly worsen optic nerve damage. Wu et al. (12) found that the expression level of lncRNA MEG3 is also notably raised in the myocardial tissues of patients with myocardial infarction and positively correlated with the apoptosis level of myocardial cells. The above results well indicated that the high expression of lncRNA MEG3 can greatly raise the apoptosis level of normal cells. This study further discovered that the knockdown of lncRNA MEG3 in the cerebral tissues markedly protected the infarct size and neuronal apoptosis in rats with ischemic cerebral infarction.

According to the findings in the study of Nazarinia et al. (13), ischemia-reperfusion injury can cause neuronal apoptosis in cerebral tissues by mitochondria mediating neuronal apoptosis. Cerebral tissue ischemia can change mitochondrial transmembrane potential to increase mitochondrial membrane permeability, thus inducing neuronal apoptosis (14,15). Anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax have been confirmed to affect the mitochondrial transmembrane potential. They are abnormally expressed in most mitochondrial pathway-mediated apoptosis (16,17). Besides, rats with ischemic cerebral infarction had a considerably raised neuronal apoptosis level, a markedly increased expression level of Caspase-3 and a substantially lowered ratio of Bcl-2/Bax in cerebral tissues. Meanwhile, the decline in the expression of lncRNA MEG3 in cerebral tissues prominently reduced neuronal apoptosis and Caspase-3 expression in cerebral tissues, but elevated the ratio of Bcl-2/Bax. It is suggested that ischemia cerebral infarction-induced neuronal apoptosis may be associated with the mitochondria-mediated apoptosis pathways. Smad2 and Smad3 are the intracellular signaling proteins mediating the intracellular signal transduction of TGF-β (18,19). The results of the present study showed that the expression levels of TGF-β1, Smad2 and Smad3 were notably raised, and the TGF-β1 signaling pathway was activated in the cerebral tissues of rats with ischemic cerebral infarction. The triggered release of inflammatory factors IL-6 and TNF-α in the cerebral tissues further aggravated the inflammatory responses and neuronal apoptosis. Moreover, the knockdown of lncRNA MEG3 in cerebral tissues inactivated the TGF-β1 signaling pathway and downregulated TGF-β1, Smad2 and Smad3, thereby effectively suppressing the release of the pro-inflammatory factors, promoting the release of anti-inflammatory factors and improving neurological function.

Conclusions

In conclusion, we first confirmed that lncRNA MEG3 is upregulated in the cerebral tissues of rats with ischemic cerebral infarction. It activates the TGF-β1 signaling pathway, thus protecting ischemia cerebral infarction-induced inflammatory response, neuronal apoptosis and neurological damage.

Conflict of Interest

The authors declare that they have no conflict of interest.

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