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Effect of lncRNA GAS5 on the apoptosis of neurons via the notch1 signaling pathway in rats with cerebral infarction

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Abstract. – OBJECTIVE: To observe the effect of long non-coding ribonucleic acid (lncRNA) growth arrest specific 5 (GAS5) knockdown on the apoptosis of neurons in rats with cerebral infarction (CI), and to explore the potential mechanism of lncRNA GAS5 in the pathogenesis of CI.

MATERIALS AND METHODS: A total of 60 adult male Sprague Dawley (SD) rats aged 12-14 weeks old and weighing (267.14±6.49) g were randomly divided into three groups: Sham operation group (Sham group, n=20), CI group (n=20) and CI + lncRNA GAS5 knockdown group [CI + GAS5 small interfering RNA (siRNA) group, n=20]. The rat model of focal CI was constructed by carotid artery embolization. After the CI model was successfully induced, a certain amount of lncRNA GAS5 siRNAs was injected into the rat lateral ventricle in a stereotactic manner. At 24 h after operation, triphenyl tetrazolium chloride (TTC) method was used to detect the infarction area in brain tissues of rats in each group. At the same time, the pathological changes of neurons in the hippocampus and prefrontal cortex of rats in each group were observed via hematoxylin and eosin (H&E) staining. The expressions of B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (BAX) were detected via Western blotting. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was adopted to detect the number of apoptotic neurons in brain tissues of rats in each group. Meanwhile, the expression level of Notch intracellular domain (NICD) proteins was measured using the Western blotting technique and immunohistochemical staining.

RESULTS: Reverse transcription-polymerase chain reaction (RT-PCR) showed that the lncRNA GAS5 expression in brain tissues of rats in CI group was significantly higher than that of rats in Sham group ($p<0.05$). TTC staining results revealed that lncRNA GAS5 knockdown could remarkably reduce the CI area of rats in CI group ($p<0.05$). In addition, inhibiting lncRNA GAS5 could also significantly reduce the level of pro-apoptotic gene BAX and increase the expression level of anti-apoptotic gene Bcl-2 ($p<0.05$). In the meantime, the number of apoptotic neurons in CI + GAS5 siRNA group was also evidently decreased ($p<0.05$). Finally, it was found that lncRNA GAS5 knockdown notably inhibited the expression of NICD proteins ($p<0.05$).

CONCLUSIONS: The inhibitory effect of lncRNA GAS5 knockdown on the apoptosis of neurons in CI rats may be related to the activation of the Notch1 signaling pathway. LncRNA GAS5 may be a new target for clinical treatment of CI.

Key Words:
LncRNA GAS5, Apoptosis of neurons, Cerebral infarction, Notch1.

Introduction

Ischemic stroke is one of the main causes of disability and death in industrialized countries in recent years, with a very high death rate[1]. Lack of cerebral blood flow can cause hypoxia, inflammation, oxidative stress and glutamate excitotoxicity, and blood flow reperfusion can aggravate these symptoms. This process is called ischemia-reperfusion injury[2,3]. However, the pathogenesis of ischemic stroke is still unclear, and effective treatment methods are insufficient[4]. Current treatment strategies rely mainly on drug and/or mechanical thrombolysis. If the cerebral vessels are not unblocked in time, it will lead to ischemia and infarction of local brain tissues thus resulting in cerebral infarction (CI). CI can cause death and apoptosis of a large number of neurons in brain tissues, seriously damaging the nerve function[5]. Therefore, a better understanding of the molecular mechanism of the apoptosis of neurons may provide new ideas for CI therapy. The Notch signaling pathway is considered to be an important adaptive signaling pathway in cells and participates in many pathological processes[6]. Up to now, four types

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of Notch family receptors (Notch1-4) have been reported to be specifically recognized by Notch ligands (Jagged1/2 and Delta 1/3/4)[7]. After the Notch ligand binds to the receptor, the Notch intracellular domain (NICD) is cleaved by the secretory enzyme, nuclear translocation occurs, and transcription activates downstream target genes such as Hes1, Hey1, and Cyclin D. The Notch signaling pathway plays multiple roles in regulating cell proliferation, apoptosis, inflammation and immunity[8]. Notch1 is the main receptor of the Notch signaling pathway and has been found to participate in various anoxic or ischemia-reperfusion pathophysiological processes in the liver, intestine, myocardium and brain[9,10]. There is more and more evidence that Notch1 is also involved in CI injury[11]. However, the potential mechanism of Notch1 in ischemic stroke remains to be fully elucidated. Long non-coding ribonucleic acids (lncRNAs) are a group of non-coding RNAs with a length of more than 200 nt[12]. Due to the lack of coding ability, many lncRNA genes are regarded as simply transcribed "cloned artifacts" or "noises", so they are not valued by people[13]. In recent years, the role of lncRNAs in various human diseases (such as proliferation, migration and invasion of tumor cells as well as formation and apoptosis of blood vessels) has gradually been revealed[14]. LncRNA growth arrest specific 5 (GAS5) was isolated from 3T3 mouse fibroblasts for the first time. It was named for its increased expression level in the rapamycin-induced cell cycle arrest[15]. Hu et al[16] have shown that lncRNA GAS5 has a negative regulatory effect on cell survival, and its expression is up-regulated in hypoxia-induced neurons. However, the role of lncRMA GAS5 in CI has not been reported yet.

In this study, small interfering RNAs (siRNAs) were used to construct rat models of low-expression lncRNA GAS5, and rat models of CI were induced simultaneously using the ligation method. Besides, the effect of low-expression lncRNA GAS5 on the apoptosis of neurons in brain tissues of CI rats and the related signaling pathways were detected, so as to reveal the related mechanism of lncRNA GAS5 affecting the apoptosis of neurons.

Materials and Methods

Animal Grouping and Modeling

A total of 60 male Sprague Dawley (SD) rats aged 12-14 weeks old and weighing (267.14±6.49) g were randomly divided into three groups: Sham operation group (Sham group, n=20), CI group (n=20) and CI + lncRNA GAS5 knockdown group (CI + GAS5 siRNA group, n=20). There were no differences in basic data such as weeks of age and body weight among the three groups of rats. The specific operation process is as follows: 1) after inhaling a certain dose of ether for anesthesia, the rats were fixed on the insulation board. 2) The skin was carefully opened, and the left common carotid artery and vagus nerve were separated. 3) The left common carotid artery and the proximal end of the external carotid artery were ligated in sequence. 4) A thread was prepared to form a live knot at the distal end of the left common carotid artery. 5) The internal carotid artery was separated, and an incision was made at the proximal end of the internal carotid artery. 6) The suture was inserted from the incision and pushed forward gradually. When it was about 18 mm, the resistance could be clearly felt. At this time, it was proved that the head of the suture reached the middle cerebral artery. 7) Suturing and disinfection were carried out. After the successful establishment of the CI model, lncRNA GAS5 siRNAs were injected into the rat's brain by using a brain locator. After 24 h, scoring was performed, and materials were obtained. All operations against animals were approved by the Shenyang Animal Ethics Committee of China Medical University Animal Center.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Detection

(1) TRIzol assay (Invitrogen, Carlsbad, CA, USA) was adopted to extract the total RNA from brain tissues, and then the concentration and purity of the RNA were detected by an ultraviolet spectrophotometer. When the ratio of the absorbance at 260 to that at 280 (A_{260}/A_{280}=1.8-2.0), the RNA could be used. (2) Messenger RNAs (mRNAs) were synthesized into complementary deoxyribonucleic acids (cDNAs) through RT and stored in a refrigerator at 80°C. (3) RT-PCR system: 2.5 μL 10 × Buffer, 2.5 μL cDNAs, 0.25 μL forward primers (20 μmol/L), 0.25 μL reverse primers (20 μmol/L), 0.5 μL deoxy-ribonucleotide triphosphates (10 mmol/L), 0.5 μL Taq enzymes (2×10^6 U/L) and 19 μL double distilled water. The amplification systems of RT-PCR were the same. The primer sequences of genes to be detected are shown in Table I.

Hematoxylin and Eosin (H&E) Staining

Brain tissues obtained in each group were placed in 10% formalin overnight, dehydrated and embedded in wax blocks. Subsequently, all

Table I. Primers of genes to be detected.

Target gene		Primer sequence
GAPDH	Forward	5'-GACATGCCGCCTGGAGAAAC-3'
	Reverse	5'-AGCCCAGGATGCCCTTTAGT-3'
LncRNA GAS5	Forward	5'-TGCTGCCTTTTCTGTTCCTT-3'
	Reverse	5'-AAGGTGCTGGGTAGGGAAGT-3'

the brain tissues were cut into thin slices with a thickness of 5 μm, fixed on glass slides and dried for staining. According to the instructions, they were soaked in xylene, ethanol at gradient concentration and hematoxylin, and then sealed with resin. After the slices were dried in the air, they were observed and photographed under the optical microscope. The morphology of neurons in the hippocampus and prefrontal cortex of rats in each group were observed.

Triphenyl Tetrazolium Chloride (TTC) Staining

1) Fresh brain tissues were put into a rat brain slice grinder and frozen in a refrigerator for 30 min at -20°C for slicing. 2) The brain tissues were cut into slices with a thickness of about 2 mm and no more than 6 slices per tissue. 3) The cut slices were placed in fresh TTC solution (2%) (Oxoid, Hampshire, UK) and fully contacted the TTC solution for an incubation time of not less than 0.5 h. 4) After 0.5 h, the slices were taken out and fixed with 4% paraformaldehyde, followed by photography.

Detection of the Apoptosis of Neurons in Brain Tissues Via Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Staining

The cut brain tissues were sliced, baked in an oven at 60°C for 30 min, dewaxed with xylene (5 min × 3 times) and dehydrated with 100%, 95% and 70% ethanol, respectively, each one for 3 times. Next, the slices were incubated with protein kinase K for half an hour. After washing with phosphate-buffered saline (PBS), the TdT and luciferase-labeled dUTP were added. After reaction for 1 h at 37°C, the specific antibody labeled with horseradish peroxidase (HRP) was added for incubation again in an incubator for 1 h (37°C). Subsequently, the slices reacted at room temperature for 10 min, with diaminobenzidine (DAB) as the substrate. After the nucleus was stained with hematoxylin, the slices were photographed and counted under an optical microscope.

Western Blotting Detection

After the rats' brain tissues in each group were taken out from the refrigerator, they were placed in dry ice and cut into slices with a piece of scissors. Subsequently, the slices were fully ground in lysis buffer added, followed by ultrasonic lysis, and the lysis buffer was centrifuged to extract supernatant, which was successively split into Eppendorf (EP) tubes. The protein concentration was measured *via* bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA) and the ultraviolet spectrophotometry, and the protein volume of all samples was set constant to equal concentration. Then the total protein was subpackaged and put into a refrigerator at -80°C. Next, the target protein was subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), after which the protein in the gel was transferred onto the cellulose acetate polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), incubated in the primary antibody (diluted at 1:100-200) at 4°C overnight, and incubated in the goat anti-rabbit secondary antibody for 1 h away from light. Protein bands were scanned and quantified using an Odyssey membrane sweeper, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to correct the level of proteins to be tested.

Immunohistochemical Staining

The cut brain tissue slices were baked in an oven at 60°C for 30 min and then dewaxed with xylene (5 min × 3 times), followed by dehydration with 100%, 95% and 70% ethanol, respectively, for 3 times. The endogenous peroxidase activity was inhibited by 3% hydrogen peroxide methanol, and then the tissues were sealed with sheep serum for 1 h. Antibodies against NICD were diluted at 1:200 [phosphate-buffered saline (PBS)] and incubated at 4°C overnight, followed by washing with PBS for 4 times in a shaker. After the second antibody was added, the color was developed with diaminobenzidine. After the color development, 6 samples were randomly selected from each group, and 5 fields of view were randomly selected from each sample, followed by photography under a 400× optical microscope.

Statistical Analysis

All the data were analyzed by Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA). Measurement data were expressed as mean ± standard deviation, and the comparisons of data between two groups were conducted using the *t*-test. $p<0.05$ represented that the difference was statistically significant.

Results

Expression of lncRNA GAS5 in Brain Tissues of CI Rats and Identification of Rats with lncRNA GAS5 Knockdown

As shown in Figure 1, RT-PCR results revealed that the lncRNA GAS5 expression in brain tissues in CI group was significantly up-regulated compared with that in Sham group ($p<0.05$). After the injection of lncRNA GAS5 siRNAs into the lateral ventricles of rats for 24 h, the expression level of lncRNA GAS5 in the CI area was notably inhibited ($p<0.05$), indicating that the lncRNA GAS5 knockdown model is successfully established.

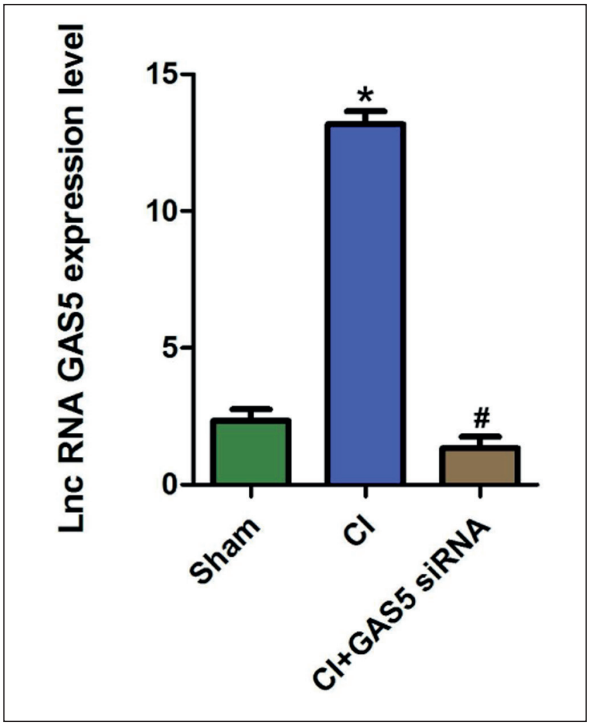

Figure 1. Expression of lncRNA GAS5 in brain tissues of rats in each group. Sham: Sham operation group, CI: CI group and CI + GAS5 siRNA group: CI + GAS5 knockdown group. *$p<0.05$ *vs.* Sham group, and #$p<0.05$ *vs.* CI group, with statistical differences.

Effect of lncRNA GAS5 Knockdown on Pathological Injury of the Hippocampus and Prefrontal Cortex in CI Rats

The pathological changes in the hippocampus and prefrontal cortex of each group of rats were examined using H&E staining. The results manifested that neurons in the hippocampus and prefrontal cortex of rats in Sham group were closely arranged with clear nuclei. However, in CI group, a large number of nuclear pyknotic cells appeared in the hippocampus and prefrontal cortex of rats. After lncRNA GAS5 was knocked down, the number of intact neurons in brain tissues of rats was significantly larger than that in CI group (Figure 2).

Comparison of the CI Area of Rats in Each Group

After TTC staining, brain tissues in the non-infarction area were red, while the infarction area became gray and white. TTC staining results (Figure 3) showed that there was no obvious infarction area in the brain tissues of rats in Sham group, but obvious CI appeared in CI group, and certain infarction area appeared in the brain tissues of rats in CI + GAS5 siRNA group, which was markedly lower than that in CI group ($p<0.05$). The above results indicate that lncRNA GAS5 knockdown can reduce the CI area caused by middle cerebral artery ischemia in rats to some extent.

TUNEL Staining Results of Neurons in the Hippocampus of Rats in Each Group

In addition, TUNEL staining (Figure 4) was applied to evaluate the apoptosis level of neurons in brain tissues of rats in each group. The TUNEL-positive rates of neurons in the hippocampus in the three groups were (1.24±0.72)%, (62.51±2.14)% and (18.55±1.42)%, respectively ($p<0.05$). The above results suggest that inhibiting lncRNA GAS5 can remarkably reduce the apoptosis of neurons in the hippocampus of CI rats.

Effect of lncRNA GAS5 Knockdown on The Expression of Apoptosis-Related Proteins in Brain Tissues of CI Rats

In order to further evaluate the effect of lncRNA GAS5 intervention on the apoptosis of neurons after the infarction of brain tissues in rats, the expression of apoptosis-related proteins in each group was detected *via* Western blotting. According to the results (Figure 5), inhibiting ln-

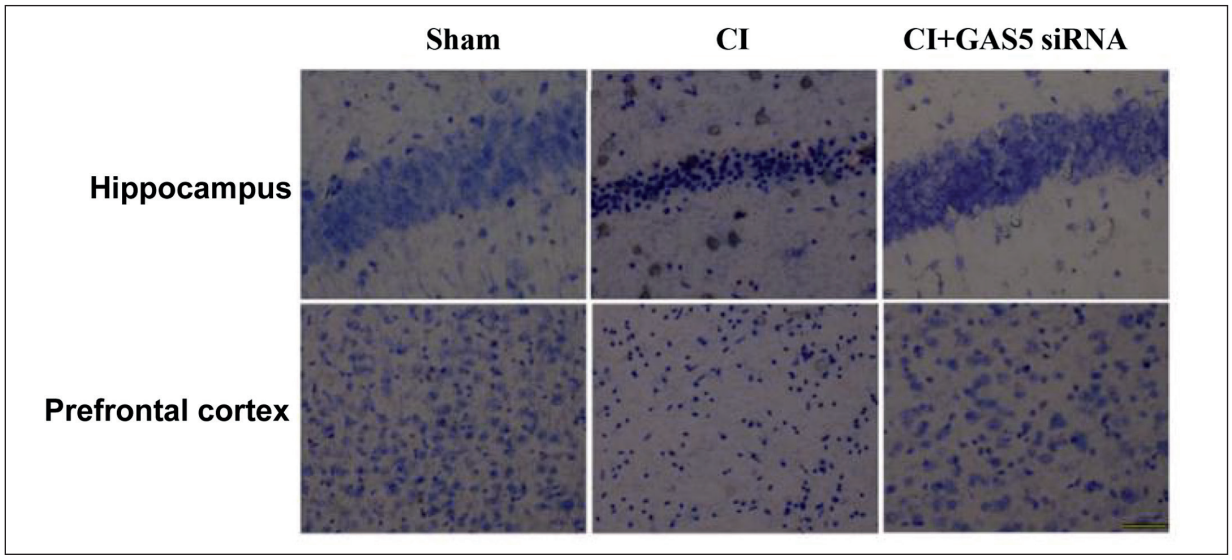

Figure 2. H&E staining of brain tissues of rats in each group. Sham: Sham operation group, CI: CI group, and CI + GAS5 siRNA group: CI + GAS5 knockdown group (magnification: 200×).

cRNA GAS5 could evidently enhance the protein expression of apoptosis-promoting gene B-cell lymphoma 2 (Bcl-2) in brain tissues of CI rats but block that of apoptosis-inhibiting gene Bcl-2-associated X protein (BAX), thus finally reducing the Bax/Bcl-2 ratio in neurons (p<0.05).

Effect of lncRNA GAS5 Knockdown on the Notch1 Signaling Pathway in Neuron Tissues

To clarify whether the inhibition of the Notch1 signaling pathway in CI can be regulated by lncRNA GAS5, Western blotting technique was adopted to quantify the NICD content in the active area of Notch1 proteins in brain tissues of rats in each group. It was found that compared with that in Sham group, the expression level of NICD in CI group was markedly inhibited (p<0.05). However, after lncRNA GAS5 siRNA intervention, the inhibitory effect on NICD was terminated (p<0.05) (Figure 6). The above results reveal that the regulation of lncRNA GAS5 on the apoptosis of neurons in CI rats is mediated by the Notch1 signaling pathway.

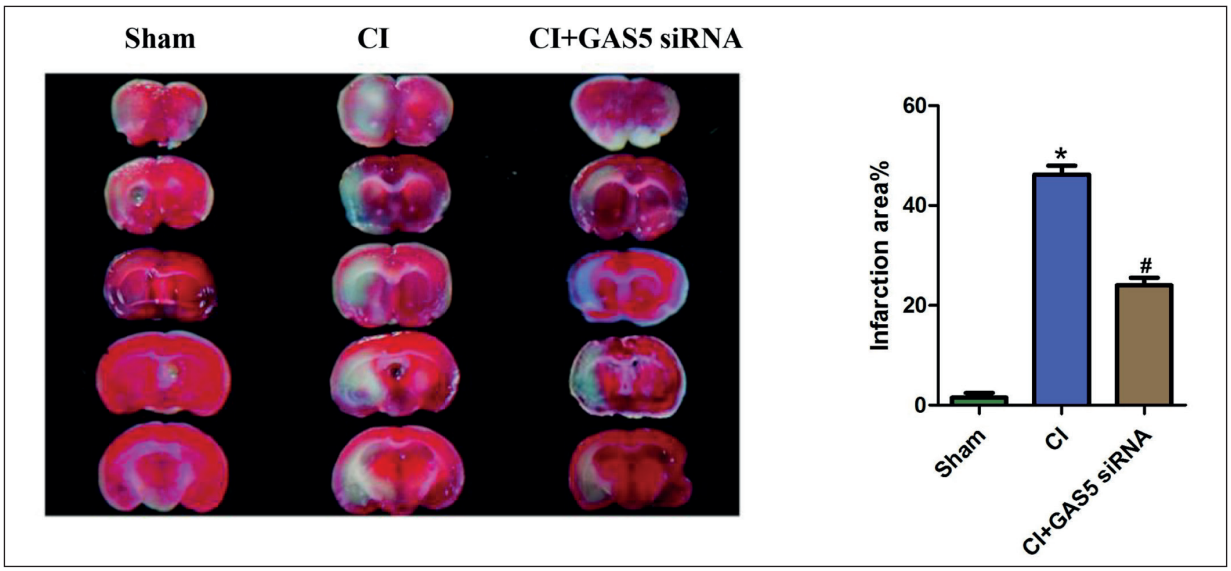

Figure 3. TTC staining of the infarction area in brain tissues of rats in each group. Sham: Sham operation group, CI: CI group and CI + GAS5 siRNA group: CI + GAS5 knockdown group. *p<0.05 *vs.* Sham group, and #p<0.05 *vs.* CI group, with statistical differences.

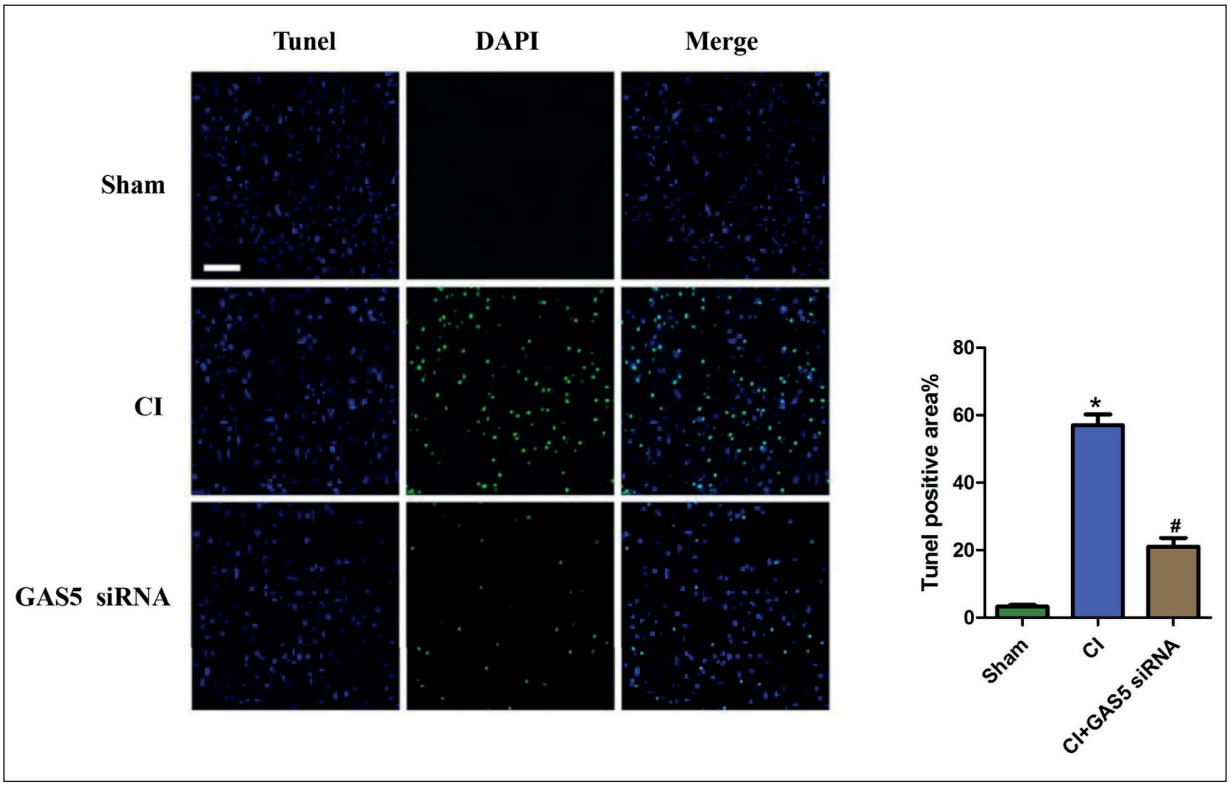

Figure 4. TUNEL staining of the infarction area in brain tissues of rats in each group (magnification: 40×). Sham: Sham operation group, CI: CI group and CI + GAS5 siRNA group: CI + GAS5 knockdown group. **p*<0.05 *vs.* Sham group, and #*p*<0.05 *vs.* CI group, displaying statistical differences.

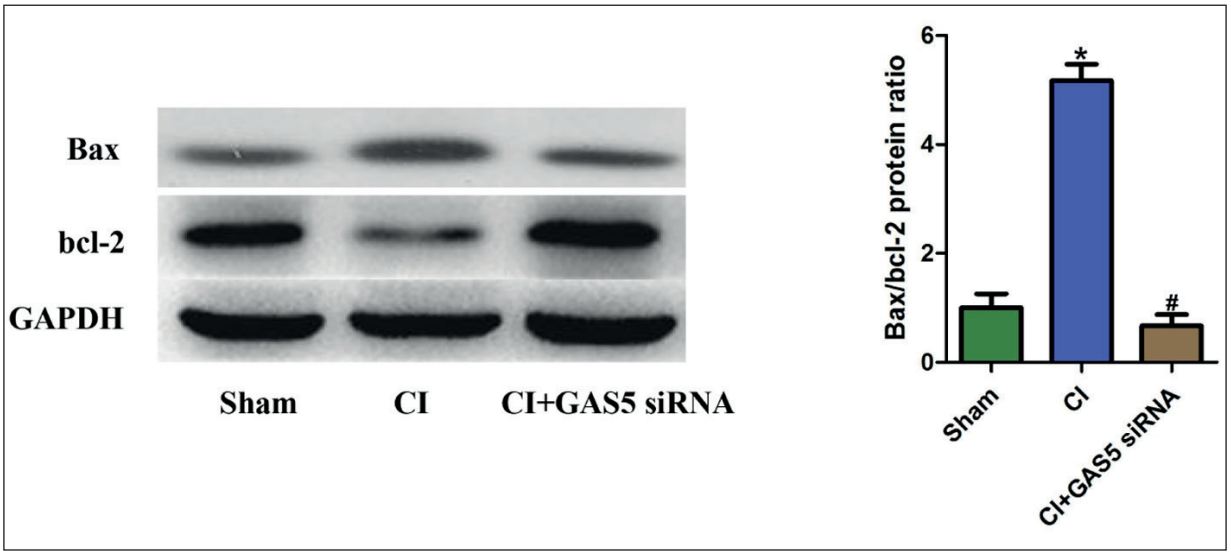

Figure 5. Western blotting results of apoptosis-related proteins in the infarction area in brain tissues of rats in each group. Sham: Sham operation group, CI: CI group and CI + GAS5 siRNA group: CI + GAS5 knockdown group. **p*<0.05 *vs.* Sham group, and #*p*<0.05 *vs.* CI group, showing statistical differences.

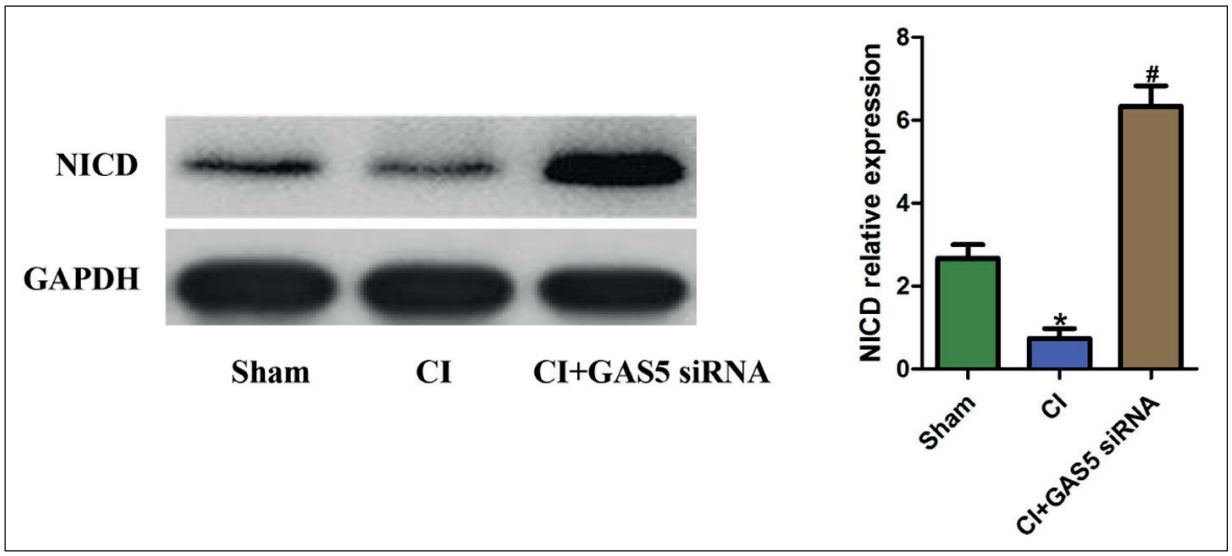

Figure 6. Western blotting results of NICD proteins in the infarction area in brain tissues of rats in each group. Sham: Sham operation group, CI: CI group and CI + GAS5 siRNA group: CI + GAS5 knockdown group. *p<0.05 *vs.* Sham group, and $^{\#}p$<0.05 *vs.* CI group, showing statistical differences.

Immunohistochemical Staining Results of Notch1 Proteins in Neuron Tissues of Rats in Each Group

Furthermore, the expression level of Notch1 in brain tissues of rats in each group was measured by immunohistochemical staining. The results (Figure 7) demonstrated that Notch1 was mainly expressed in brain tissues in the hippocampus of rats. When CI occurred in rats, the Notch1 expression was obviously inhibited, but after lncRNA GAS5 was knocked down, the Notch1 signaling pathway in the hippocampus of rats was further activated.

Discussion

In recent years, more and more studies have revealed the abnormal expression of various lncRNA in cerebrovascular diseases and play an important role in ischemic stroke, so lncRNA is expected to become a new target for the treatment of cerebrovascular diseases[17,18]. As known, the pathophysiology of lncRNA GAS5 in cerebrovascular diseases is only on the threshold. LncRNA GAS5 is a tumor inhibitor that negatively regulates cell survival and plays a role in promoting growth stagnation and apoptosis in many cell types[19]. Ln-

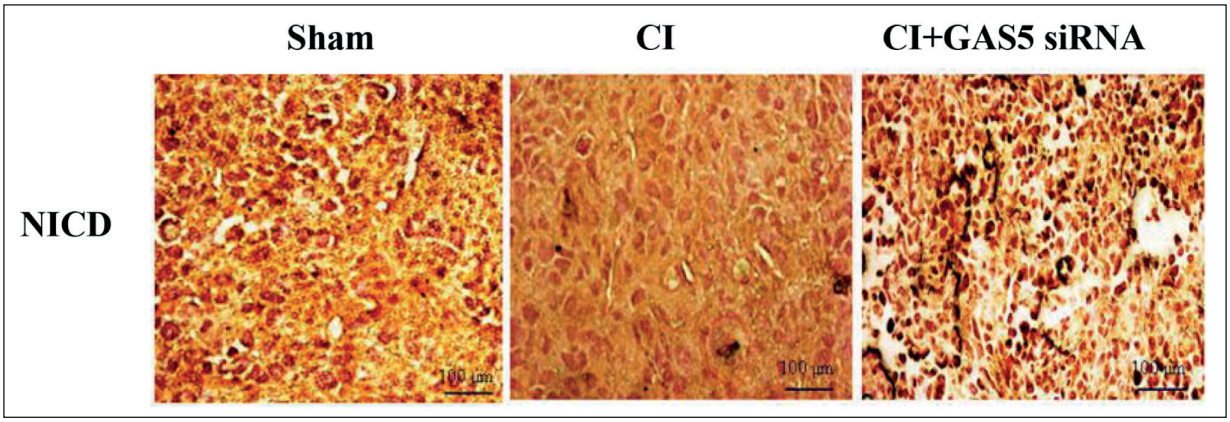

Figure 7. Immunohistochemical staining results of NICD proteins in the infarction area in brain tissues of rats in each group (magnification: 100×). Sham: Sham operation group, CI: CI group and CI + GAS5 siRNA group: CI + GAS5 knockdown group. *p<0.05 *vs.* Sham group, and $^{\#}p$<0.05 *vs.* CI group, showing statistical differences.

cRNA GAS5 down-regulates the PTEN/MMP-2 signaling pathway by targeting miR-21, and finally inhibits the activation of cardiac fibroblasts and myocardial fibrosis[20]. Besides, lncRNA GAS5 can finally suppress the proliferation of prostate cancer cells by targeting p27[21]. In this study, the new role of lncRNA GAS5 in CI was revealed. The results showed that the expression level of lncRNA GAS5 was significantly increased in neurons of CI rats, while the expression of Notch1 proteins was notably decreased. The down-regulation of lncRNA GAS5 could alleviate the injury of ischemia and hypoxia to neurons and reduce the apoptosis level of neurons. At the same time, it was found that inhibiting lncRNA GAS5 could up-regulate the expression level of Notch1 in brain tissues. Stump et al[22] have revealed that Notch1 and the Notch signaling pathway play a protective role in various organs, especially in the heart and brain, and they can protect cells from cell injury caused by hypoxia or ischemia-reperfusion. However, blocking Notch signals can reduce neuronal injury in rats with focal ischemic stroke. Furthermore, it has also been found that[22] the Notch signaling pathway is an important regulatory pathway for the occurrence and formation of vascular morphology, and it plays an important role in the development of blood vessels and diseases. The Notch signaling pathway[23] can regulate cell fate and embryo pattern and play a key role in embryogenesis and vascular development in zebrafish, mice and human mammals. Li et al[24] have shown that γ secretase inhibitor DAPT can reduce neuronal injury caused by ischemia/reperfusion in rats by inhibiting the expression of Notch1 and NF-κB. On the contrary, the present study found that the expression of Notch1 was inhibited in brain tissues in the CI area of CI rats, while the activation of Notch1 could exert a protective effect on neurons. It is speculated that the possible reason for this difference is that the models in the two studies are different. In other words, it is a CI model in this study but an ischemia-reperfusion model in the above study. Ischemia-reperfusion usually causes a secondary attack on neurons due to hypoxia/reoxygenation. Notch1, as an important regulatory protein in life activities, is located in the very upstream of the signal transduction pathway and is therefore very sensitive to various external stress attacks, which may partly explain the differential expression of Notch1 in CI and ischemia-reperfusion models. Previous studies have reported that Notch1 plays a dual role in cerebral ischemia injury. On the one hand, Notch1 can promote the proliferation of neural progenitor cells and inhibit the differentiation of neurons *in vitro* and *in vivo*, so it can stimulate the neuronal regeneration response under hypoxic conditions[10]. On the other hand, Notch1 inhibits the differentiation of neural progenitor cells into neurons *in vitro* and *in vivo* by regulating the development and activation of inflammatory cells such as T lymphocytes and microglia, thus promoting inflammation and apoptosis[25, 26]. The above findings suggest that the balance of the Notch1 signaling pathway may affect the degree of brain injury after cerebral ischemia.

Conclusions

In summary, inhibiting lncRNA GAS5 can alleviate neuronal injury and apoptosis caused by cerebral ischemia-reperfusion through *in-vivo* experiments, which may be mediated by the Notch1 signaling pathway.

Conflict of Interests

The authors declared no conflict of interest.

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