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Influence of lncRNA ANRIL on neuronal apoptosis in rats with cerebral infarction by regulating the NF-κB signaling pathway

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Abstract. – OBJECTIVE: The aim of this study was to evaluate the influence of long non-coding ribonucleic acid (lncRNA) antisense non-coding RNA in the INK4 locus (ANRIL) on neuronal apoptosis in rats with cerebral infarction (CI), and to further explore the underlying mechanism of lncRNA ANRIL in the occurrence and development of CI.

MATERIALS AND METHODS: A total of 60 adult male Wistar rats were randomly divided into three groups using a random number table, including sham group (n=20), CI group (n=20) and CI + lncRNA ANRIL knockdown group [CI + lncRNA ANRIL small-interfering RNA (siRNA) group, n=20]. Focal CI was constructed by suture occlusion. After successful modeling, lncRNA ANRIL siRNA was stereotactically injected into the lateral ventricle of the rats. 24 h after operation, the neurological function of the rats in each group was evaluated by the modified neurological severity score (mNSS). Meanwhile, the infarction area of brain tissues was evaluated using the triphenyl tetrazolium chloride (TTC) method. The protein expression levels of apoptosis-related genes, including B-cell lymphoma-2 (Bcl-2) and Bcl-2 associated X protein (Bax), were detected via Western blotting. Subsequently, immunofluorescence staining was performed to detect the expression and location of Caspase-3 in brain tissues. Moreover, the apoptosis level of rats in each group was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. Furthermore, the expressions of nuclear factor-κB (NF-κB) signaling pathway-related proteins were detected via Western blotting.

RESULTS: Polymerase Chain Reaction (PCR) results revealed that the expression level of lncRNA ANRIL in the CI group was significantly increased when compared with that of the sham group ($p<0.05$). The results of mNSS and TTC staining manifested that knockdown of lncRNA ANRIL could significantly reduce CI-induced neurological deficits and CI area ($p<0.05$). At the same time, knockdown of lncRNA ANRIL markedly decreased the level of Bax, whereas increased the expression of Bcl-2 ($p<0.05$). Besides, the number of apoptotic cells in the CI + lncRNA ANRIL siRNA group was remarkably decreased ($p<0.05$). In addition, lncRNA ANRIL down-regulation remarkably inhibited the phosphorylation of p65 ($p<0.05$).

CONCLUSIONS: The inhibitory effect of lncRNA ANRIL knockdown on neuronal apoptosis in CI rats may be probably related to its inhibition of the NF-κB signaling pathway. Furthermore, lncRNA ANRIL inhibitor is expected to become a targeted drug in the clinical treatment of CI.

Key Words:
LncRNA ANRIL, Apoptosis, Cerebral infarction (CI), NF-κB.

Introduction

Cerebral infarction (CI), also known as ischemic stroke, usually refers to ischemia, hypoxia and necrosis of brain tissues, neurological disorders and even deficits caused by abnormal blood supply in brain[1,2]. Despite the rapid development of medical technology, CI is still the leading cause of death and disability worldwide[3]. Rapid thrombolysis and brain protection are two important steps to prevent CI injury. However, the good prognosis of patients still cannot be ensured even if vascular remodeling is successful[4]. Therefore, clarifying the pathogenesis of CI is of important significance in early diagnosis and precise treatment of CI.

Inflammation plays an important role in the ischemic pathological injury. Nuclear factor-κB (NF-κB), as a classical nuclear transcription factor, has shown great significance in inflammation and cell survival[5,6]. Previous studies have

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demonstrated that NF-κB inhibitor IKK-NBD can significantly improve ischemia-induced neurological deficits. Meanwhile, it markedly reduces the messenger ribonucleic acid (mRNA) expression level of interleukin-1β (IL-1β) in the corpus striatum. Moreover, IKK-NBD can also inhibit ischemia-induced blood-brain barrier damage[7]. The above findings indicate that the inhibition of NF-κB may be a potential therapeutic target for CI.

Long non-coding RNAs (lncRNAs) are a class of non-protein-coding function RNAs with more than 200 nt in length[8]. These lncRNA transcripts were once considered as simple transcriptional "noise" or "clonal artifacts". Therefore, they were ignored by the research previously. In recent years, with the completion of the Human Genome Project and the rapid development of molecular-genetic techniques, the roles of lncRNAs in human diseases have attracted more attention[9,10]. Studies have indicated that lncRNAs regulate the proliferation, angiogenesis, apoptosis, migration and invasion of tumor cells. For example, knockout of lncRNA MEG3 can inhibit hypoxia/reoxygenation-induced apoptosis of brain microvascular endothelial cells in rats. The possible underlying mechanism may be related to its regulation of p53/NOX4 signal axis[11]. Besides, lncRNA SNHG14 promotes the activation of microglia in CI rats by regulating miR-145-5p/PLA2G4A axis[12]. However, the exact role of lncRNA antisense non-coding RNA in the INK4 locus (ANRIL) in CI and its mechanism have not been elucidated yet.

In the present work, the mRNA expression of lncRNA ANRIL in brain tissues in the infarction region of CI rats was detected. LncRNA ANRIL low-expression model was successfully constructed by transfection of lncRNA ANRIL small-interfering RNA (siRNA). Meanwhile, the influence of lncRNA ANRIL knockdown on neuronal apoptosis in brain tissues was detected. Finally, the expression of related pathways was detected to explore the possible mechanism of lncRNA ANRIL.

Materials and Methods

Animal Grouping and Modeling

A total of 60 male Wistar rats aged 12-14 weeks and weighing (281.42±5.62) g were divided into three groups using a random number table, including sham group (n=20), CI group (n=20) and CI + lncRNA ANRIL knockdown group (CI + lncRNA ANRIL siRNA group, n=20). No statistically significant differences were observed in basic data among the three groups, including week age and body weight. The operation process was as follows: 1) the rats were first anesthetized and fixed, 2) the left common carotid artery and vagus nerve were isolated, 3) the proximal ends of the left common carotid artery and external carotid artery were ligated, 4) a slip knot was made at the distal end of the left common carotid artery, 5) the internal carotid artery was isolated, 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 about 18 mm until there was significant resistance, proving that the head of the suture had reached the middle cerebral artery, and 7) the incision was sutured and disinfected. After successful establishment of the CI model, lncRNA ANRIL siRNA was injected into the cerebral ventricle using stereotaxic apparatus. 24 h later, the samples were collected and scored. All operations for rats were approved by the Animal Ethics Committee of China Medical University Animal Center.

Triphenyltetrazolium Chloride (TTC) Staining

The specific procedure for triphenyl tetrazolium chloride (TTC) staining was as follows: 1) Fresh brain tissues were placed in a grinding cutter and frozen in a refrigerator at -20°C for 30 min. 2) The brain tissues were sliced into about 2 mm-thick sections (not more than 6 sections for each tissue). 3) The sections were fully placed in fresh TTC solution (2%; Oxoid, Hampshire, UK) for incubation for no less than 0.5 h. 4) 0.5 h later, the sections were taken, fixed with 4% paraformaldehyde and photographed.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL) Staining of Brain Tissues

Brain tissue sections were baked in an oven at 60°C for 30 min. Then, they were deparaffinized with xylene (5 min × 3 times), followed by dehydration with 100% ethanol, 95% ethanol and 70% ethanol 3 times, respectively. Subsequently, the tissue sections were incubated with protein kinase K for 0.5 h, washed with Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA), and

reacted with terminal deoxynucleotidyl transferase and Luciferase-labeled dUTP at 37°C for 1 h. After that, the sections were incubated with horseradish peroxidase-labeled specific antibody at 37°C for 1 h. After 10 min of reaction at room temperature with diaminobenzidine (DAB) as a substrate (Solarbio, Beijing, China), the nucleus was stained with hematoxylin. Finally, the sections were photographed and counted under a light microscope.

Detection of LncRNA ANRIL Expression in CI Region Via Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from CI tissues according to the instructions of TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). The concentration and purity of extracted RNA were detected using an ultraviolet spectrophotometer. RNAs with absorbance $(A)_{260}/A_{280}$ of 1.8-2.0 could be used. Subsequently, extracted total mRNA was synthesized into complementary deoxyribonucleic acid (cDNA) through RT and stored in a refrigerator at -80°C. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) system included: 2.5 μL 10 × Buffer, 2 μL cDNA, 0.25 μL forward primer (20 μmol/L), 0.25 μL reverse primer (20 μmol/L), 0.5 μL dNTPs (10 mmol/L), 0.5 μL Taq enzyme (2×10^6 U/L) and 19 μL ddH_2O. Amplification systems for RT-PCR were the same as above. Primer sequences used in this study were as follows: LncRNA ANRIL, F: 5'-GGGATACCCTCCTTACTC-3', R: 5'-ACCGGTATGTGTCCGAACGGA-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'.

Nissl Staining

The hippocampus of rats in each group was soaked to the bottom of the sucrose solution (30%) and sliced into frozen sections (30 μm). Subsequently, the brain tissue sections were placed on a glass slide pretreated with gelatin and air dried. After that, the sections of each group were stained with crystal violet at 37°C for 0.5 h. Then, they were dehydrated with ethanol in gradient concentration, treated with xylene and sealed. Finally, the sections were observed and photographed under a light microscope.

Immunohistochemical Staining

Brain tissue sections were first baked in an oven at 60°C for 30 min. Subsequently, the sections were deparaffinized with xylene (5 min × 3 times) and dehydrated with 100% ethanol, 95% ethanol and 70% ethanol three times, respectively. Then, the endogenous peroxidase activity was inhibited using 3% hydrogen peroxide methanol. After sealing with goat serum for 1 h, the sections were incubated with the anti-Caspase-3 antibody (diluted at 1:200 with PBS) at 4°C overnight. On the next day, the sections were washed with PBS four times on a shaker, and incubated with secondary antibody. The color was developed using diaminobenzidine. 6 samples were randomly selected in each group and 5 fields of view were randomly selected for each sample. Finally, the sections were photographed under a light microscope (400×).

Western Blotting

Brain tissues in each group were fully ground in lysis buffer, followed by ultrasonic lysis. After centrifugation, the supernatant was collected and sub-packaged into Eppendorf tubes (EP; Eppendorf, Hamburg, Germany). The concentration of extracted protein was detected using the bicinchoninic acid (BCA) method (Pierce, Waltham, MA, USA) by ultraviolet spectrophotometry. All the protein samples should be quantified to the same concentration, sub-packaged and placed in a refrigerator at -80°C for use. Then, the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After incubation with primary antibodies at 4°C overnight, the membranes were incubated again with goat anti-rabbit secondary antibody in the dark for 1 h. Protein bands were scanned and quantified using the Odyssey scanner. Finally, the expression level of proteins was detected. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) was used for all statistical analysis. Measurement data were expressed as mean ± standard deviation. The *t*-test was used to compare the difference between the two groups. $p<0.05$ was considered statistically significant.

Results

Expression of LncRNA ANRIL in Brain Tissues of Each Group

As shown in Figure 1, RT-PCR results revealed that the expression of lncRNA ANRIL in brain tissues of the CI group was significantly up-regulated when compared with that of the sham group ($p<0.05$). 24 h after injection of lncRNA ANRIL siRNA into lateral ventricle, the expression of lncRNA ANRIL in the CI region was markedly inhibited ($p<0.05$). This indicated that the lncRNA ANRIL knockdown model was successfully induced.

Postoperative Neurological Score of Rats in Each Group

At 24 h after operation, the symptoms of neurological deficits, including reduced activity, depression, inability to walk straight and lean or rotation to one side, occurred in all groups except the sham group. Therefore, the neurological function of rats in each group was scored by mNSS scale. As shown in Table I, mNSS in the CI group was significantly higher than that of the sham group ($p<0.05$). However, it was ly reduced in CI rats 24 h after injection of lncRNA ANRIL siRNA ($p<0.05$). The above findings suggested that lncRNA ANRIL siRNA could improve the neurological dysfunction induced by CI.

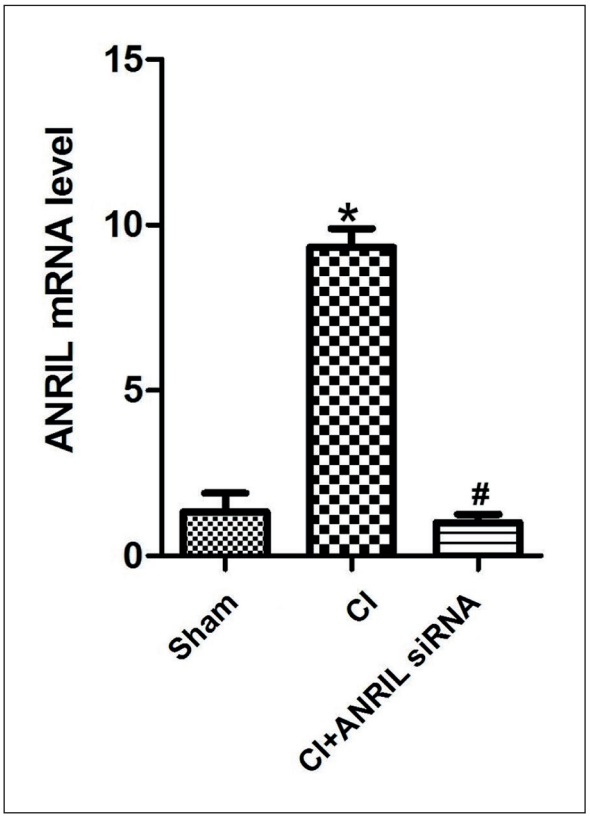

Figure 1. Expression level of lncRNA ANRIL in CI region of three different groups. Sham: Sham operation group, CI: CI group, CI+ANRIL siRNA: lncRNA ANRIL knockdown group. $p<0.05$ *vs.* *Sham group and *vs.* #CI group.

Influence of LncRNA ANRIL Knockdown on CI Area

After TTC staining, the brain tissues in the CI region displayed grey-white color. However, those in the non-CI region showed a red color. TTC staining results manifested that no significant infarction region was found in brain tissues of the sham group. However, significant CI occurred in the CI group. Meanwhile, there was also certain infarction region in brain tissues of the CI + lncRNA ANRIL siRNA group. However, the scope of the infarction region in the CI + lncRNA ANRIL siRNA group was markedly smaller than that of the CI group ($p<0.05$; Figure 2). These results indicated that lncRNA ANRIL knockdown could alleviate CI caused by middle cerebral artery ischemia to a certain degree.

Influence of LncRNA ANRIL Knockdown on Hippocampus Morphology in CI Rats

Nissl staining manifested that gliocyte proliferation and loss of Nissl's body were observed

Table I. Neurological score of rats in each group after CI (No=20).

Group	Score ($\bar{x} \pm s$)	*F*	*p*
Sham	0	194.6	0.00
CI	16.52 ± 1.66		
CI+lncRNA ANRIL siRNA	7.56 ± 0.62		

Postoperative neurological score of rats in each group, Sham: Sham operation group, CI: CI group, CI+ANRIL siRNA: lncRNA ANRIL knockdown group. $p<0.05$ *vs.* *Sham group and *vs.* #CI group.

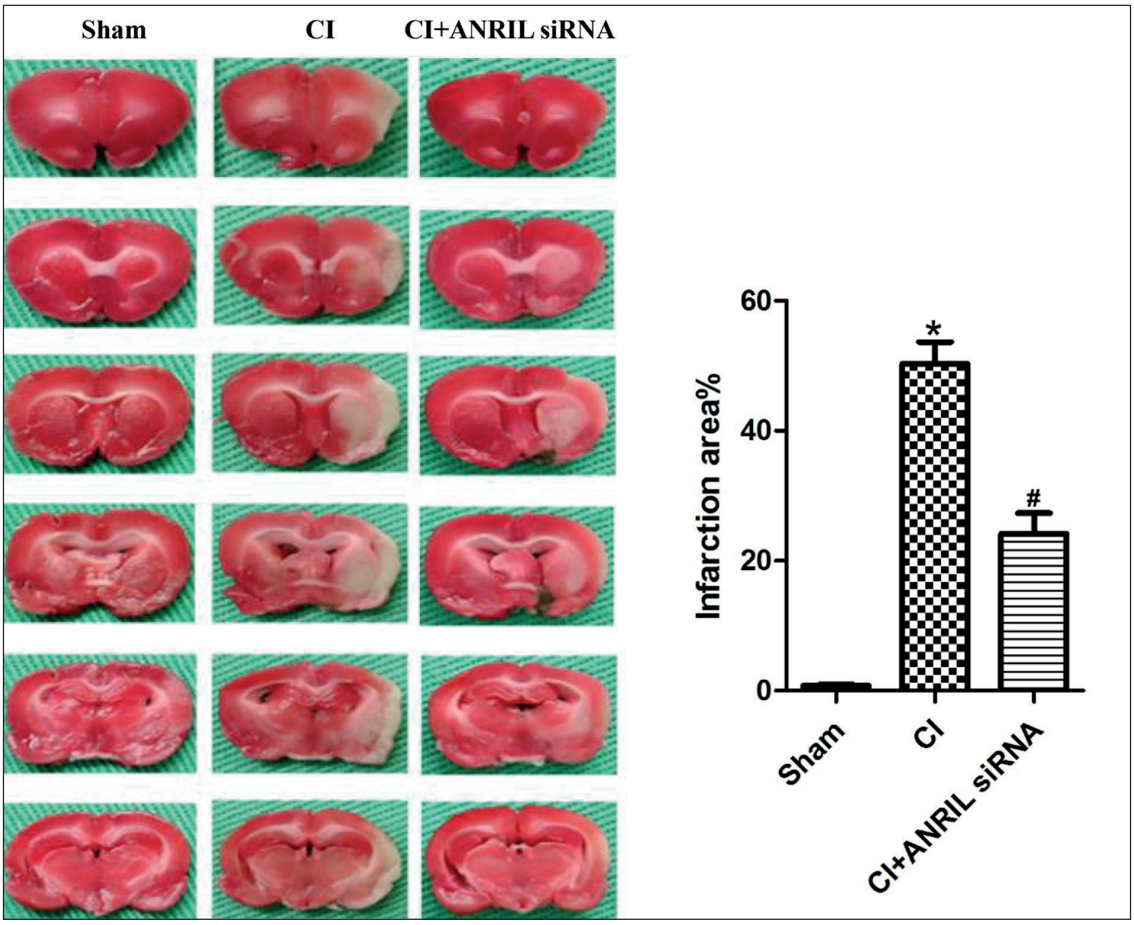

Figure 2. CI area in different groups. Sham: Sham operation group, CI: CI group, CI+ANRIL siRNA: lncRNA ANRIL knockdown group. $p<0.05$ *vs.* *Sham group and *vs.* #CI group.

in the hippocampus of the CI group. However, the number of gliocyte was markedly reduced, whereas that of Nissl's body was remarkably increased after lncRNA ANRIL knockdown ($p<0.05$; Figure 3).

TUNEL Staining of Neurons in Hippocampus in Rats of Each Group

According to the results of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL; Beyotime, Shanghai, China) staining

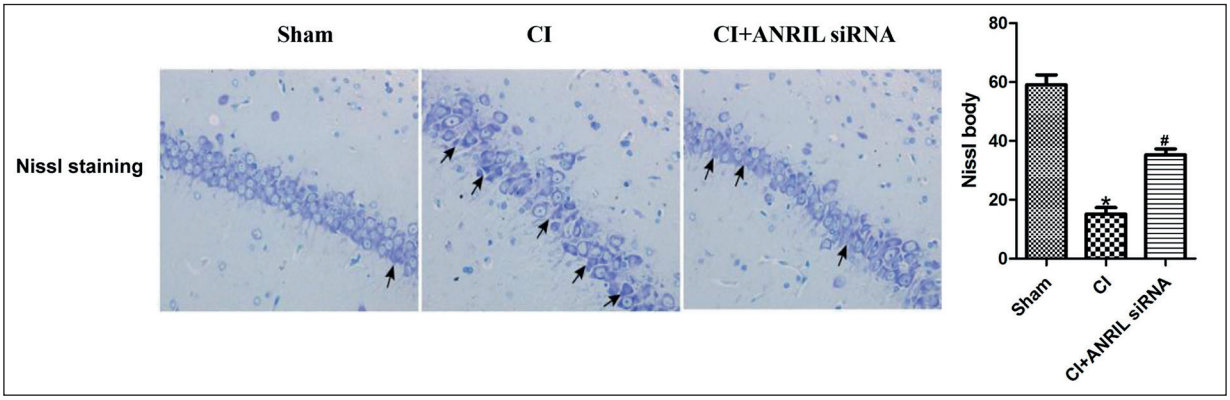

Figure 3. Influence of lncRNA ANRIL knockdown on hippocampus morphology in CI rats (magnification: 40×). Sham: Sham operation group, CI: CI group, CI+ANRIL siRNA: lncRNA ANRIL knockdown group. $p<0.05$ *vs.* *Sham group and *vs.* #CI group.

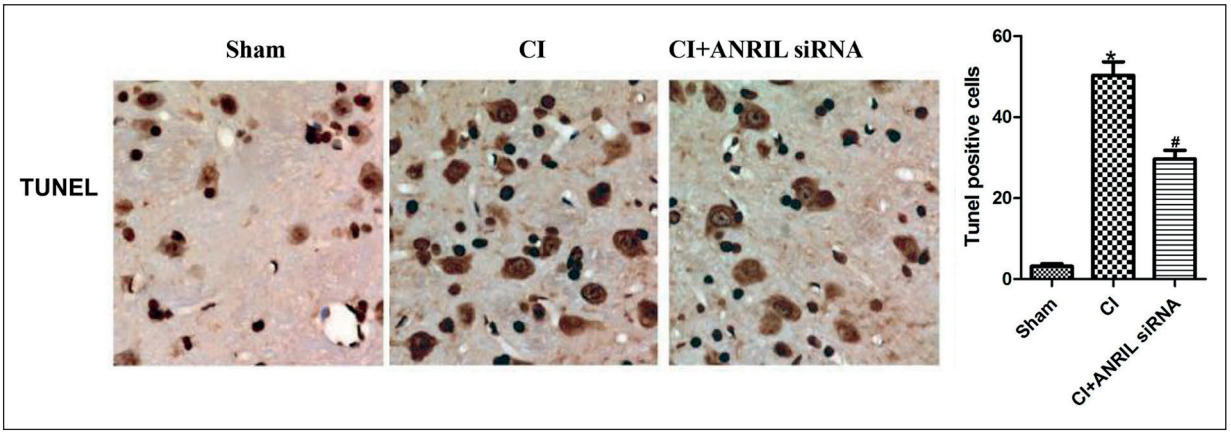

Figure 4. Influence of lncRNA ANRIL knockdown on neuronal apoptosis in hippocampus of CI rats (magnification: 40×). Sham: Sham operation group, CI: CI group, CI+ANRIL siRNA: lncRNA ANRIL knockdown group. $p<0.05$ *vs.* *Sham group and *vs.* #CI group.

(Figure 4), TUNEL-positive rate of neurons in the hippocampus of three groups was (4.49±1.23)%, (59.51±3.66)% and (26.67±2.96)%, respectively ($p<0.05$). This demonstrated that inhibiting lncRNA ANRIL could remarkably reduce neuronal apoptosis in the hippocampus of CI rats.

Expression of Apoptosis-Related Proteins in CI Tissues in Rats of Each Group

To further evaluate the influence of lncRNA ANRIL intervention on neuronal apoptosis after CI, the expression levels of apoptosis-related proteins in each group were detected *via* Western blotting. The results showed that the inhibition of lncRNA ANRIL could significantly down-regulate the expression of B-cell lymphoma-2 (Bcl-2) associated X protein (Bax) (a pro-apoptotic gene), whereas enhancing the expression of Bcl-2 (an anti-apoptotic gene) in brain tissues of CI rats ($p<0.05$; Figure 5).

Immunohistochemical Staining of Caspase-3 in CI Tissues in Rats of Each Group

The expression and location of Caspase-3 in brain tissues of each group were detected *via* immunohistochemical staining. As shown in Figure 6, lncRNA ANRIL knockdown could remarkably inhibit Caspase-3 cleavage, thereby blocking the process of neuronal apoptosis.

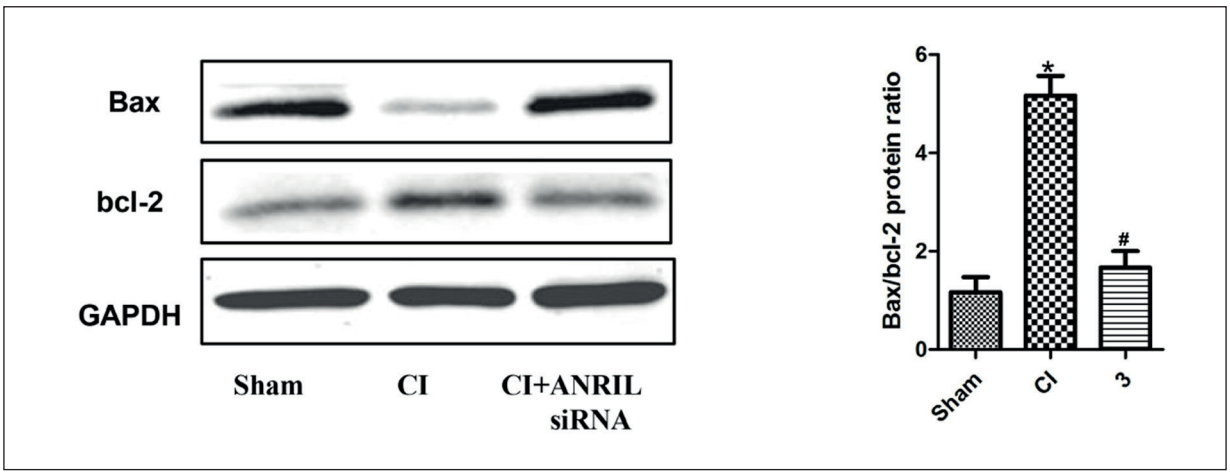

Figure 5. Influence of lncRNA ANRIL knockdown on the expression of apoptosis-related proteins in hippocampus of CI rats. Sham: Sham operation group, CI: CI group, CI+ANRIL siRNA: lncRNA ANRIL knockdown group. $p<0.05$ *vs.* *Sham group and *vs.* #CI group.

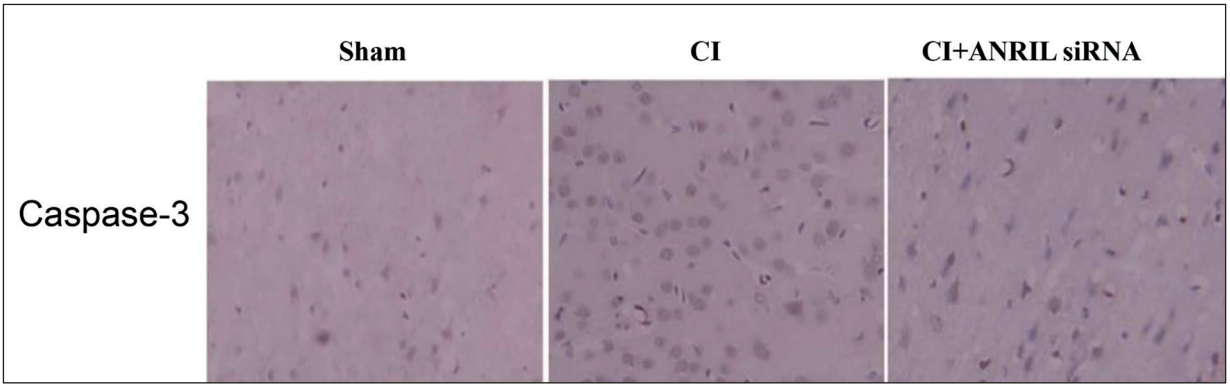

Figure 6. Immunohistochemical staining of Caspase-3 in CI tissues of each group (magnification: 40×)**.** Sham: Sham operation group, CI: CI group, CI+ANRIL siRNA: lncRNA ANRIL knockdown group.

Regulatory Effect of LncRNA ANRIL Knockdown on NF-κB Signaling Pathway

Whether the activation of the NF-κB signaling pathway in CI can be regulated by lncRNA ANRIL remains unclear. Therefore, the protein levels of phosphorylated p65 (P-p65) and total p65 (T-p65) in CI tissues of each group were quantified *via* Western blotting. The results demonstrated that the expression level of P-p65 in the CI group was significantly increased when compared with that of the sham group ($p<0.05$). However, the activation of P-p65 was reversed after lncRNA ANRIL siRNA intervention ($p<0.05$; Figure 7). The above findings further revealed that the regulatory effect of lncRNA ANRIL on neuronal apoptosis in CI rats was mediated by the NF-κB signaling pathway.

Discussion

Currently, CI is one of the most common cerebrovascular diseases in the world[13]. It is reported that the morbidity and mortality rates of CI have greatly increased year by year in European and American countries, especially in the United States. Previous studies have demonstrated that the occurrence and development of CI are closely related to genetic changes[14]. With the development of modern molecular biology and bioinformatics, increasingly more genes, RNAs and proteins have been confirmed to be involved in the occurrence and development of CI[15]. Current studies have indicated that neuronal apoptosis plays a crucial role in the cerebral ischemia-reperfusion injury. Meanwhile, the apoptosis level can directly determine the severity of the disease and prognosis

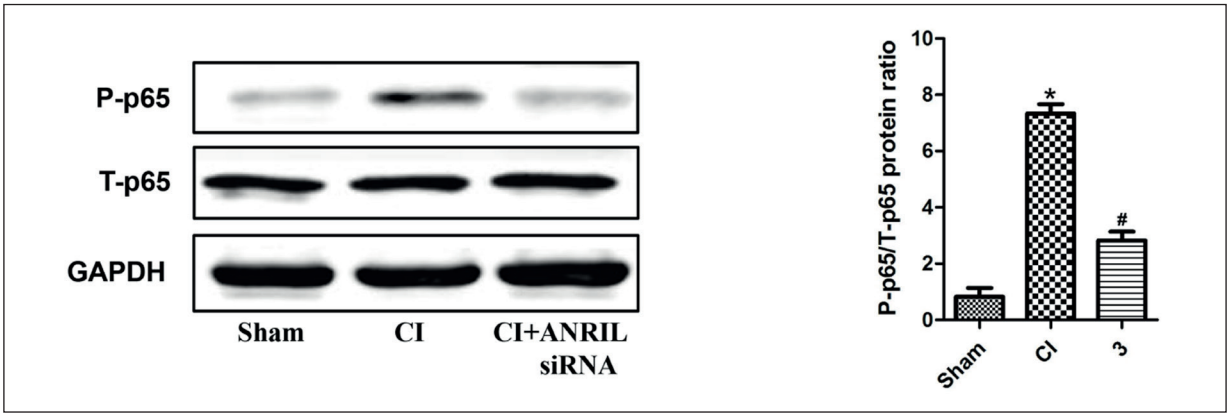

Figure 7. Influence of lncRNA ANRIL knockdown on the expression of NF-κB signaling pathway in hippocampus of CI rats. Sham: Sham operation group, CI: CI group, CI+ANRIL siRNA: lncRNA ANRIL knockdown group. $p<0.05$ *vs.* *Sham group and *vs.* #CI group.

of patients[16]. In the present work, SPF Wistar rats were used as research objects. The suture was inserted through the carotid artery to occlude the middle cerebral artery, which was conducive to simulate the pathogenic process of CI. At the same time, lncRNA ANRIL siRNA was injected into the lateral ventricle, and the rat model of lncRNA ANRIL knockdown was successfully established. This revealed the important therapeutic effect of lncRNA ANRIL siRNA on CI.

Apoptosis, also known as programmed death, refers to programmed cell death controlled by genes in order to maintain homeostasis under physiological or pathological conditions[16]. Studies have shown that various apoptosis-inducing signals are activated during CI. This may induce neuronal edema, apoptosis or necrosis in infarction regions. In particular, neurons die mainly in the form of necrosis within the first 6 h after CI. Subsequent, they die in the form of apoptosis[17]. In the present work, the samples were collected at 24 h after CI to further investigate the influence of lncRNA ANRIL on neuronal apoptosis. During apoptosis, cell fate is directly determined by the preferential expressions of Bax and Bcl-2. When the pro-apoptotic gene Bax is dominated, programmed cell death occurs. On the contrary, when the expression of anti-apoptotic gene Bcl-2 is increased, apoptosis will be inhibited[18]. We found that lncRNA ANRIL siRNA transfection markedly up-regulated the expression of anti-apoptosis gene Bcl-2, whereas inhibited the level of the pro-apoptosis gene Bax, ultimately inhibiting CI induced by ischemia and hypoxia.

LncRNA ANRIL is an important member of the lncRNA family. The roles of lncRNA ANRIL in human diseases have been increasingly revealed, including embryonic development, angiogenesis and cell proliferation[19]. Current researches have found that the expression of lncRNA ANRIL is significantly up-regulated in nasopharyngeal cancer tissues. This can facilitate the occurrence and development of nasopharyngeal cancer by promoting cell proliferation, reprogramming cellular glucose metabolism and inducing side-population stem cells[20]. In liver cancer, the expression of lncRNA ANRIL is remarkably up-regulated. Meanwhile, inhibiting lncRNA ANRIL can significantly suppress the proliferation, metastasis and invasion of liver cancer cells. The possible underlying mechanism may be related to the endogenous competitive adsorption of miR-122-5p by lncRNA ANRIL[21]. Moreover, up-regulation of lncRNA ANRIL promotes the proliferation and migration of prostate cancer cells by activating let-7a/TGF-β1/Smad signaling pathway[22]. The above results all indicate that lncRNA ANRIL may serve as a cancer-promoting gene. In the rat model of diabetes mellitus complicated with CI, overexpression of lncRNA ANRIL promotes the expression of vascular endothelial growth factor *via* activating the NF-κB signaling pathway, thereby promoting angiogenesis[23]. In the present work, the lncRNA ANRIL knockdown model was successfully established. The rat model of CI was constructed using the suture-occluded method. Subsequently, the role of lncRNA ANRIL in the occurrence and development of CI in rats, as well as its underlying mechanism, was detected. Our results revealed that rats with lncRNA ANRIL knockout could significantly resist ischemic CI. Meanwhile, the neurological score and milder pathological injury of rats with lncRNA ANRIL knockout were markedly higher than wild-type rats. The number of apoptotic neurons was remarkably smaller in rats with lncRNA ANRIL knockout. Moreover, the expression level of pro-apoptotic proteins was significantly lower. Furthermore, it was found that the neuroprotective function of lncRNA ANRIL might be related to its inhibition of the NF-κB signaling pathway. However, there were still some limitations in this study: (1) cell experiments were not performed for verification, and (2) no direct target of lncRNA ANRIL was found.

Conclusions

This study proved, for the first time, that lncRNA ANRIL knockdown could improve CI-induced neurological deficits and reduce the number of apoptotic neurons in rats. The possible underlying mechanism might be related to the inhibition of lncRNA ANRIL siRNA on the NF-κB signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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