European Review for Medical and Pharmacological Sciences 2019; 23: 10976-10981

Effects of lncRNA gm4419 on rats with hypertensive cerebral atherosclerosis through NF-κB pathway

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Abstract. – OBJECTIVE: To explore the effects of long non-coding ribonucleic acid (lncRNA) Gm4419 on rats with hypertensive cerebral atherosclerosis through the nuclear factor-kappa B (NF-κB) pathway.

MATERIALS AND METHODS: Healthy male rats were selected and randomly divided into control group, model group (hypertensive cerebral atherosclerosis model), and lncRNA group (hypertensive cerebral atherosclerosis model + lncRNA injection). Neurological deficit scoring criteria, flow cytometry, Western blotting, and staining method were adopted to measure the differences in the neurological function score, NF-κB activity, and chemerin level of rats in the three groups.

RESULTS: The neurological scores revealed that the neurological function of rats was not damaged in control group, while it was severely damaged in model group. However, the neurological function of rats was more severely damaged in lncRNA group than that in control group and model group, while the neurological function deficits were slighter in model group. In terms of NF-κB expression activity in mononuclear cells, the serum activity of NF-κB in control group appeared the lowest among the three groups and was significantly higher in lncRNA group than in model group. The serum chemerin level was evidently increased in model group compared with control group, while it was significantly decreased in lncRNA group compared with model group and control group. Moreover, the levels of NF-κB and chemerin were most evidently influenced in lncRNA group.

CONCLUSIONS: Activating the NF-κB signal, lncRNA Gm4419 promotes the expression of chemerin signal, accelerates the apoptosis of nerve cells, and motivates the deterioration of hypertensive cerebral arteriosclerosis.

Key Words:
NF-κB pathway, Atherosclerosis, LncRNA Gm4419.

Introduction

Atherosclerosis is a kind of ischemic and inflammatory cerebrovascular disease arising from the aggregation of macrophages and foam cells in blood vessels[1,2]. Formation of fatty streaks and plaque rupture are two important processes in thrombogenesis. Liu et al[3] realized that inflammation plays a critical role throughout the process from the occurrence to the development of atherosclerosis, thus promoting its steady processing. In recent decades, the morbidity rate of atherosclerosis has risen significantly. Moreover, hypertension is a primary factor that causes atherosclerosis and can also lead to a series of cardiovascular and cerebrovascular diseases which seriously threaten the health of human beings. Therefore, it is crucial to find out the factors that affect atherosclerosis. Nuclear factor-kappa B (NF-κB) is a dimer protein formed by RelA (p65), RelB, NF-κB1 (p50 and its precursor) or NF-κB2 (p52 and its precursor)[4]. The Rel/NF-κB family has been involved in regulating the transcription of multiple target genes[5] and the expression of cytokines in coronary arteriosclerosis. The immune and inflammatory responses mediated by NF-κB result in endothelial cell damage and other injuries which are manifested as atherosclerosis, cerebral infarction, etc. The previous research has revealed that the activation of NF-κB can induce the apoptosis of brain cells and finally result in atherosclerosis and other diseases. Recently, long non-coding ribonucleic acids (lncRNAs) have been gradually showing vital transcriptional regulation and play important roles in cell growth and apoptosis. It has been found that lncRNAs are closely related to the development of tumor diseases, atherosclerosis, cerebral hemorrhage, and some other diseases. Moreover, the abnormally ex-

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pressed lncRNAs have been reported in endothelial dysfunction, proliferation of smooth muscle cells, formation of foam cells, lipid mechanism, and other processes, thereby regulating the occurrence and development of atherosclerosis. However, there are few researches about the role of lncRNA Gm4419 in the development of atherosclerosis. In the present work, rat models of hypertensive cerebral atherosclerosis were established to explore the potential role of lncRNA Gm4419 and NF-κB in hypertensive cerebral atherosclerosis.

Materials and Methods

Experimental Animals and Grouping

A total of 72 male rats weighing 100-120 g and aged 3-4 months old were fed with the basal diet for 8 weeks. All the rats were randomly divided into 3 groups: control group, model group (hypertensive cerebral atherosclerosis model), and lncRNA group (hypertensive cerebral atherosclerosis model + lncRNA injection, once a day for four consecutive weeks) with 24 rats in each group. This investigation was approved by the Animal Ethics Committee of Zhejiang University School of the Medicine Animal Center.

Main Reagents and Instruments

Flow cytometer (Beckman Coulter, Miami, FL, USA), enzyme-linked immunosorbent assay (ELISA; Wuhan USCN Business Co., Ltd., Wuhan, China), kit [Siemens Medical Diagnostic Products (Shanghai) Co., Ltd, Shanghai, China], and ultracentrifuge (Thermo Fisher Scientific, Waltham, MA, USA).

Longa Score Scale6

According to Longa Score Scale: Grade 0: no evident neurological symptoms; Grade 1: inability to stretch the left forelimb; Grade 2: rotate to the left side; Grade 3: fall to the left side when walking; Grade 4: the ability to walk spontaneously with disturbance of consciousness. Grade 1 means no evident signs of neurological deficits, grade 1-2 indicates mild neurological deficits, and grade 3-4 demonstrates severe neurological deficits. The accumulative grade 1 and above suggested the successful modeling.

Modeling of Hypertensive Cerebral Atherosclerosis

After the rats in model group and lncRNA group were narcotized, a median incision was made on the neck, while the skin and connective tissue were loosened. The extracranial pterygopalatine artery was isolated under the microscope. After the *oscula* were cut off, the artery was inserted into the internal carotid artery at a depth of (20±0.5) mm.

Flow Cytometry

To collect the brain cells, the brain tissues of the rats were cut into pieces (1 mm) in a clean container by a blade and then transferred into a 1.5 mL Eppendorf (EP) tube. The tissue homogenate was suspended with 1.5 mL of tissue digestive juice containing 200 U/mL type II and 1 U/mL neutral protease at 37°C for 20 min. The digested tissue fluid was filtered through a screen (pore diameter of 75 μm) and collected into 15 mL of Hank's balanced salt solution containing 2% fetal bovine serum (FBS; Hyclone, South Logan, UT, USA) to terminate the enzyme digestion reaction. The suspension was centrifuged at 1600 rpm/min (centrifugal radius of 5 cm) for 10 min. After the supernatant was discarded, the pellet was suspended with 1 × phosphate buffer solution and single cell suspension of brain tissues was collected for further use.

Detection of NF-κB and Chemerin Protein Expression in Rats Via the Western Blot

A total of 100 μg trypsin was added into a 6-well plate and the digestion was terminated with 2 mL of culture medium. The cell extract mixture was then transferred into the EP tubes, mixed with the trypsin extract at the ratio of 1:100 and then frozen in a refrigerator for 8 min. The E solution was obtained after the complete fission of the cells. Brain cells of the rats were completely lysed in an EP tube with 1.5 mL of trypsin extract at the ratio of 1:100 to get F solution. The working solution was prepared by mixing E and F solution at the volume ratio of 80:1 and placed in an incubator at 37°C for 25 min. Finally, the concentration of protein was calculated after cooling.

The bicinchoninic acid (BCA) method was performed to quantitate the protein concentration. Protein samples were electrophoresed on polyacrylamide gels and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk, the membranes were incubated with primary antibody (Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. The membrane was incubated with the secondary an-

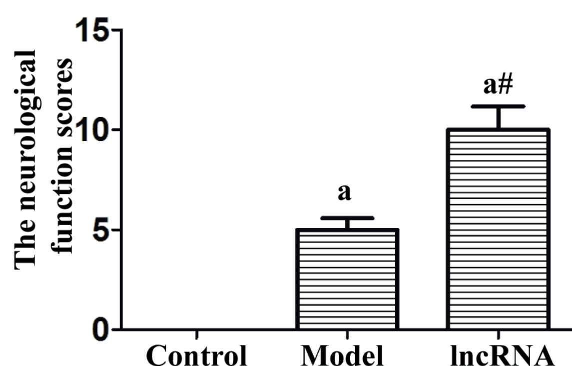

Figure 1. Neurological deficit score. Note: 1. ^{a}p<0.05 *vs*. control group, $^{\#}p$<0.05 *vs*. model group.

tibody after rinsing with Tris-Buffered Saline and Tween (TBST). Chemiluminescence was used to expose the protein bands on the membrane.

Staining Treatment

Pathological changes in each layer structure of brains cells and capillaries were observed with hematoxylin-eosin (HE) staining (Boster, Wuhan, China). HE staining images of all groups were collected under an object glass (400×). Six images were selected from each group using the random number table. The brain cells were determined using the Image-Pro Plus 6.0 (Media Cybernetics, Silver Springs, MD, USA) software.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 (SPSS, Chicago, IL, USA) software was adopted for the analysis of all data. The *t*-test was employed for the comparison of neurological function score, NF-κB level, and chemerin level. Correlation analysis was used to explore the relation between NF-κB and chemerin among control group, model group, and lncRNA group. The measurement data were presented as the mean ± standard deviation of each group. Data were compared among groups using the one-way analysis of variance (ANOVA). p<0.05 suggested that the difference was statistically significant.

Results

Longa scores of rats in different groups

The neurological function was not impaired in control group, while rats in lncRNA group showed severely damaged neurological function compared with model group and control group. Besides, the neurological function in model group was less injured than that in lncRNA group (Figure 1). Compared with that in model group, the neurological function of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) cells was not damaged in control group, while it was more severe in model group than in control group. Compared with that in model group, the neurological function score of TUNEL cells in lncRNA group was significantly increased and the difference was statistically significant (p<0.05) (Figure 2).

Serum NF-κB level in rats of the three groups

Among the three groups, control group exhibited the lowest activity of serum NF-κB and lncRNA group showed the highest NF-κB activity, following model group (Figures 3 and 4).

Chemerin level in the peripheral blood of rats

The activity of chemerin in rats was evidently decreased in control group comparing to that in model group and lncRNA group, while it was sig-

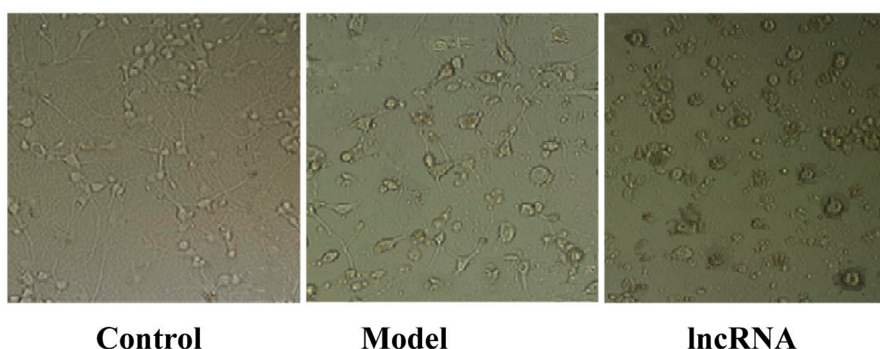

Figure 2. Expression of cells with neurological deficit (magnification × 400).

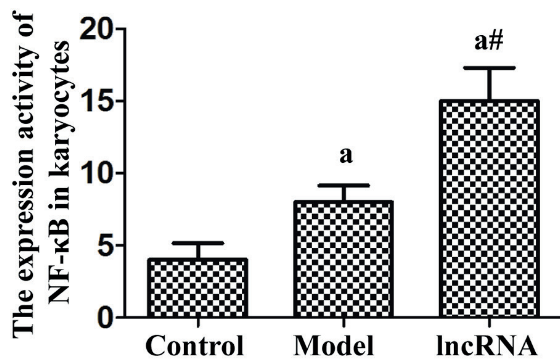

Figure 3. The expression activity of NF-κB in karyocytes. Note: 1. $^{a}p<0.05$ *vs*. control group, $^{\#}p<0.05$ *vs*. model group.

nificantly increased in lncRNA group compared with that in model group (Figure 5).

Expressions of Chemerin and NF-κB in the peripheral blood of rats in different groups

The expression levels of NF-κB and Chemerin were the highest in lncRNA group, followed by model group ($p<0.05$). The expression of NF-κB was positively correlated with chemerin (Figure 6).

Discussion

With the improvement of people's living conditions, the disease spectrum is gradually changing. However, chronic diseases, such as arterial diseases, have been on the rise year by year. Atherosclerosis is a primary manifestation of vascular diseases and also the common pathological basis for coronary heart diseases and cerebrovascular disease, which severely endangers the health of mankind[7,8]. Atherosclerosis is featured by deposition of intravascular lipids in the intima of the large- and medium-sized arteries[9]. Oxidized low-density lipoprotein, which was considered as the most important factor contributing to atherosclerosis, can turn into foam cells, and produce a large amount of proinflammatory factors[10,11], leading to narrowing and blockage of cerebral vessels. The affection mechanism of atherosclerosis includes endothelial injury response[12], blood flow shear stress[13], and many other theories. Therefore, it is urgent to explore a new therapeutic target, thus improving the development of atherosclerosis.

The results of this work indicated that the neurological function of rats in control group was not damaged, while that in lncRNA group was severely damaged, and the injury in model group was moderate. Increasing evidence has demonstrated that lncRNAs showed great potential for diagnosing cardiovascular diseases[14] and are also closely correlated with the growth process of atherosclerosis. LncRNAs can increase the proliferation of vascular smooth muscles which is an important cause of the formation atherosclerotic plaques. Wu et al[15] has revealed that the expression level of lncRNA p21 in atherosclerotic plaques of mice was significantly decreased compared with that in wild-type mice, while the proliferation of plaques in the blood caused platelet aggregation and led to a remarkable rise in the neurological function score of mice. Therefore, it can be determined that the formation of atherosclerosis can increase the neurological function score, which is similar to the results of the present study.

The expression of NF-κB was significantly increased in model group compared with that in control group and it was markedly higher in lncRNA group than in model group ($p<0.05$).

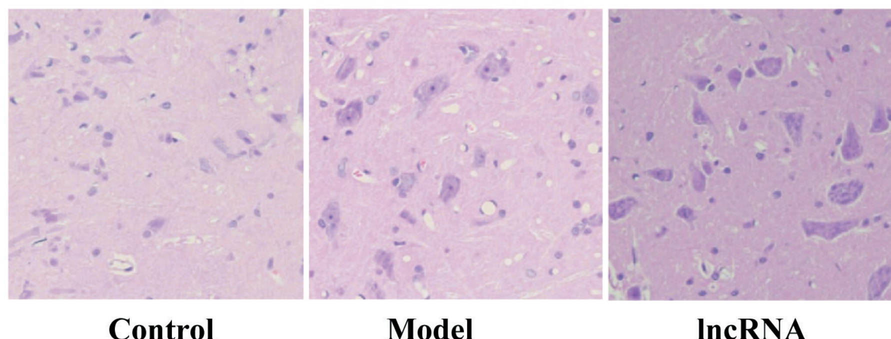

Figure 4. Expression of NF-κB in cells (magnification × 400).

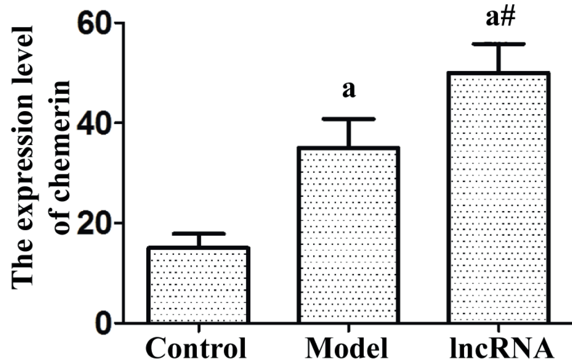

Figure 5. Expression level of chemerin in peripheral blood. Note: 1. $^a p<0.05$ *vs*. control group, $^\# p<0.05$ *vs*. model group.

Furthermore, a large number of specific inflammatory factors related to the occurrence of atherosclerosis have been detected, including toll-like receptor 4 (TLR4). It is reported that TLR4 had proinflammatory effects on endothelial cells of atherosclerotic plaques and could protect inflammatory factors[16]. Consistent with that, the present report demonstrated that the activation of NF-κB increased the spreading of atherosclerotic plaques.

The expression level of chemerin in rats was increased in model group and lncRNA group compared with that in control group, and that in lncRNA group was the highest, followed by model group. It has been proved that the activity of chemerin was evidently higher in patients with atherosclerosis and hypertension than in normal people, and its activity is positively correlated with the course of atherosclerosis and hypertension[17]. Chemerin is an intermediate target of risk factors for cardiovascular diseases like atherosclerosis and hypertension. Bobbert et al[18] considered that chemerin influenced the systolic and diastolic functions, as well as the structure of cerebral vessels, promoted the insulin resistance mechanism by motivating inflammatory reaction, and thus participated in the occurrence and development of hypertension. Here, we also observed a significant increase of chemerin. The activity of NF-κB and chemerin was most evidently expressed in lncRNA group. According to Takahashi et al[19], TLR4 is an important nuclear transcription factor of NF-κB. In an atherosclerosis rats' model, chemerin activated the intracellular NF-κB pathway by regulating the expression of TLR4 on the surface of fibroblasts and the production of a large number of inflammatory factors. Our data indicated that NF-κB and chemerin might be competitive in adjusting the expression of TLR4, while further research is warranted for exploring the specific mechanisms. Chemerin is significantly up-regulated after activation of NF-κB and positively related to the expression of NF-κB, suggesting that NF-κB signal might be involved in chemerin-medicated inflammatory reaction.

Conclusions

LncRNA Gm4419 promoted the expression of chemerin signal by activating NF-κB signal, accelerated the apoptosis of nerve cells, and had a negative effect on hypertensive cerebral arteriosclerosis.

Conflict of Interests

The Authors declare that they have no conflict of interests.

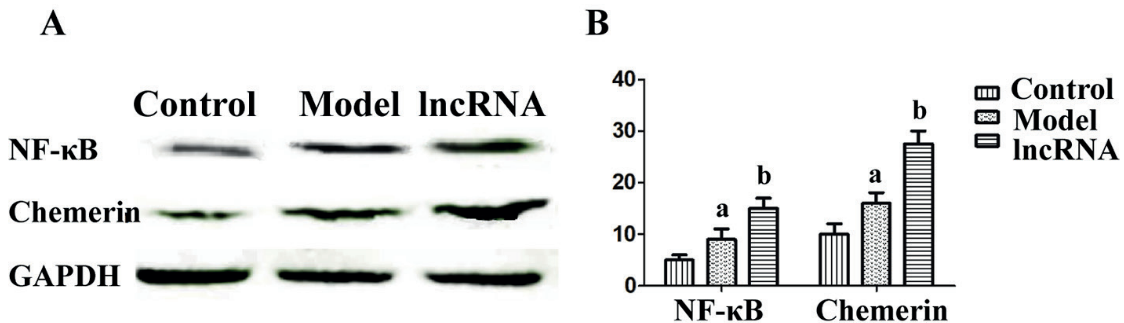

Figure 6. Expressions of Chemerin and NF-κB in the peripheral blood detected by Western blot.

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