

MicroRNA-451 relieves inflammation in cerebral ischemia-reperfusion via the Toll-like receptor 4/MyD88/NF- κ B signaling pathway

WENYAN LI¹, MINGHAO DONG², LIANGZHAO CHU², LUQIAN FENG² and XIAOCHUAN SUN¹

¹Department of Neurosurgery, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016;

²Department of Neurosurgery, The Affiliated Hospital of Guizhou Medical University, Guiyang, Guizhou 550004, P.R. China

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Abstract. The present study was designed to investigate the role of microRNA-451 (miRNA-451) on cerebral ischemia-reperfusion and to explore its possible mechanism. The expression of miRNA-451 was downregulated in rats with cerebral ischemia-reperfusion. In an *in vitro* model of cerebral ischemia-reperfusion, the downregulation of miRNA-451 increased inflammation, demonstrated by increased levels of tumor necrosis factor α , interleukin (IL)-1b, IL-6 and IL-18. However, the upregulation of miRNA-451 expression decreased inflammation in the same *in vitro* model of cerebral ischemia-reperfusion. In addition, it was found that the downregulation of miRNA-451 induced the expression of Toll-like receptor 4 (TLR4), myeloid differentiation primary response protein MyD88 (MyD88) and nuclear factor- κ B (NF- κ B)/p65. Moreover, the administration of a MyD88 inhibitor, ST 2825, reduced the expression of MyD88 and NF- κ B/p65 in the *in vitro* model of cerebral ischemia-reperfusion, inhibiting the effects of miRNA-451 upregulation on inflammation. A TLR4 inhibitor, TAK-242, was used to reduce the expression of TLR4 in the *in vitro* model of cerebral ischemia-reperfusion. TAK-242 suppressed the effects of miRNA-451 downregulation on inflammation. The present study suggested that miRNA-451 regulated cerebral ischemia-reperfusion-induced inflammation, which is mediated through the TLR4/MyD88/NF- κ B signaling pathway.

Introduction

Stroke, which can be classified into ischemic stroke and hemorrhagic stroke, is a disabling disease (1). Ischemic stroke is a condition caused by the occlusion of a blood vessel supplying the brain and represents >80% of all stroke cases worldwide (2-4). Therefore, further investigation into the pathogenesis of ischemic stroke, and the development of safe and effective intervention strategies to reduce cerebral injury induced by cerebral ischemia-reperfusion is required (5).

The brain is a sensitive organ to ischemia and hypoxia, with the potential that cerebral ischemia can lead to brain cell necrosis or apoptosis (6). Therefore, timely thrombolysis, rapid and effective reconstruction of microvascular collateral circulation, the recovery of blood reperfusion in the ischemic region and penumbra are required in cases of cerebral ischemia (7). However, reperfusion after ischemia can also lead to ischemia-reperfusion injury (IRI) (7). A number of inflammatory factors exist in the focal ischemic area at the time of cerebral ischemia-reperfusion. In addition, activation and infiltration of inflammatory cells, as well as the synthesis and secretion of adhesion molecules that initiate a cascade reaction, which enhance and promote each other, converting cerebral ischemic injury into inflammatory injury through an inflammatory signaling pathway (8). Consequently, the inflammatory response plays an important role in the mechanism of cerebral IRI (9). A number of pro-inflammatory cytokines, chemokines and white blood cell adhesion molecules are upregulated after local cerebral ischemia; the pathogenesis and pathological development of which rely on cellular signal transduction pathways (8). Some reactions can only take place after various extracellular stimulatory signals are transduced into cells (8). Typically, the Toll-like receptor (TLR) signaling pathway and the nuclear factor- κ B (NF- κ B) signaling pathway are involved in the transduction of cerebral ischemia-reperfusion inflammation (7,10).

MicroRNAs (miRNAs/miRs) are important endogenous non-coding small RNAs, which are highly conserved throughout evolution (11). miRNAs are involved in a number of biological processes, including proliferation, differentiation, apoptosis, metabolism and development (12). Previous studies have discovered that specific miRNAs play important roles in

Correspondence to: Dr Xiaochuan Sun, Department of Neurosurgery, The First Affiliated Hospital of Chongqing Medical University, 1 Youyi Road, Yuan Jiagang, Yuzhong, Chongqing 400016, P.R. China
E-mail: srntqjiuj60@126.com

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ischemic cerebrovascular diseases, including in the genesis and development of stroke, and are also involved in regulating the protection and repair mechanism after cerebral injury (13,14). Liu *et al* (15) reported that miRNA-451 protected neurons against IRI-induced cell death. Leecharoenkiat *et al* (16) showed that miRNA-451 levels may be associated with β -thalassemia/hemoglobin-E. The present study aimed to investigate the role of miRNA-451 on cerebral ischemia-reperfusion and its possible mechanism.

Materials and methods

Mouse model of cerebral ischemia-reperfusion. Male C57BL/6J mice weighing 20–21 g were purchased from Animal Experiment Center of Chongqing Medical University (Chongqing, China) and housed at 22–23°C, 55–60% humidity with a 12 h light/dark cycle and freely access to food and water. Cerebral ischemia-reperfusion was induced by middle cerebral artery occlusion (MCAo). Mice were anesthetized with 50 mg/kg pentobarbital (i.p.) and randomly assigned into two groups: Control and cerebral ischemia-reperfusion model group. In the cerebral ischemia-reperfusion model group, a midline neck incision was cut and the left common and external carotid arteries were isolated, and ligated with microvascular clips. To induce MCAo in the mice, 8–0 nylon monofilaments with silicon resin (180–190 μ m) were introduced through a small incision into the common carotid artery and advanced by 9 mm distal to the carotid bifurcation. After 1 h, reperfusion was initiated by withdrawing the monofilament. After 1 day, all mice were sacrificed for further study. The animal experiments were approved by the Ethics Committee of The First Affiliated Hospital of Chongqing.

Hematoxylin and eosin staining. The hippocampus was washed with PBS and fixed using 4% w/v paraformaldehyde in PBS for 24 h at room temperature. Tissue samples were dehydrated in a graded ethanol series, cleared in xylene, embedded in paraffin. Tissue samples were cut into 5 μ m-thick sections and stained with hematoxylin sassy for 10 min and eosin for 30 sec at room temperature. Images were captured under fluorescence microscopy (magnification, x200; Leica Microsystems GmbH).

ELISA kits. Serum samples or cell samples were collected at 2000 g for 10 min at 4°C. These samples were used to measure tumor necrosis factor- α (TNF- α , H052), interleukin (IL)-1 β (H002), IL-6 (H007) and IL-18 (H015) levels using ELISA kits (Nanjing Jiancheng Biology Engineering Institute).

Cell culture. Neuro-2a cells (Shanghai Cell Bank of Chinese Academy of Sciences) were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C with 5% CO₂. miRNA-451 (5'-AAACCGUUACCAUACUGAGU U-3'), anti-miRNA-451 and control negative mimics were transfected into cells (1x10⁵ cells) at a final concentration of 50 nmol/l using Lipofectamine[®] 2000 Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. To induce an *in vitro* model of cerebral ischemia-reperfusion, 48 h after transfection, cells were treated with 100 ng/ml lipopolysaccharide (LPS) for 6 h at 37°C.

At 24 h after transfection with anti-miRNA-451, TLR4 inhibitor TAK-242 (8 nM) was added to the cells for 24 h and they were then treated with 100 ng/ml lipopolysaccharide (LPS) for 6 h at 37°C.

At 24 h after transfection with anti-miRNA-451, MyD88 inhibitor, ST2825 (5 μ M) was added into cell for 24 h and they were then treated with 100 ng/ml lipopolysaccharide (LPS) for 6 h at 37°C.

At 24 h after transfection with anti-miRNA-451 and si-p65 (sc-422642, Santa Cruz Biotechnology, Inc.), cells were treated with 100 ng/ml lipopolysaccharide (LPS) for 6 h at 37°C.

RNA isolation, reverse transcription-quantitative (RT-q)PCR. miRNA was extracted from serum or cells samples using the miRNeasy Mini kit (Qiagen, China Co., Ltd.). RT was carried out using 50 ng miRNA using SuperScript III Reverse Transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.) at 42°C for 1 h and 82°C for 20 sec. RT-qPCR was carried out using SYBR Green I (Invitrogen; Thermo Fisher Scientific, Inc.) and a QuantStudio 6 Flex Realtime PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The sequence of the primers for RT-qPCR: TLR4: 5'-CTTCCTCTCCCC CCGTAACC-3' and 5'-GTAAGAAACCGAAGGAATCAA A-3'; MyD88: 5'-GTTCTGTTGCAACAAATTGAT-3' and 5'-CTTATCAATTTGTTGCAACGAAC-3'; p65: 5'-CTT CCAAGAAGAGCAGCGTG-3' and 5'-GCCTGGTCCCGT GAAATACA-3'; hsa-miR-451: 5'-ACACTCCAGCTGGGA AACCGTTACCATTACT-3' and 5'-CTGGTGTCGTGGAGT CGGCAA-3'; U6 forward, 5'-GCTTCGGCAGCACATATA CTAAAT-3' and reverse, 5'-CGCTTCACGAATTTGCGT GTCAT-3'. Data was analyzed using the 2^{- $\Delta\Delta$ C_q} method (17). Reaction conditions were 95°C for 15 min, denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 30 sec for a total of 40 cycles.

Microarray analysis. For microarray analysis, 500 ng mRNA was transcribed using the Low Input Quick-amp labeling kit (Agilent Technologies, Inc.) and labeled with Cy3 (Agilent Technologies, Inc.). The cDNA was hybridized onto Agilent SurePrint G3 Mouse GE Microarray Chips (Agilent Technologies, Inc.) and quantified using Feature Extraction 10.5.1.1 image analysis software (Agilent Technologies, Inc.).

Cell viability assay. Transfected cells (1x10³ cell/well) were seeded in 96-well plates and incubated with 20 μ l Cell Counting Kit-8 (CCK-8, Beyotime Institute of Biotechnology) reagent at 37°C for 2 h. The absorbance was measured at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc.).

Flow cytometry analysis of apoptosis. Transfected cells (1x10⁶ cells) were washed with PBS and stained with 5 μ l FITC-conjugated annexin-V and 5 μ l propidium iodide (BD Biosciences) for 15 min at room temperature. Apoptosis was measured using a FACS Canto instrument (BD Biosciences).

Western blot analysis. Total protein was extracted using RIPA lysis buffer (Beyotime Institute of Biotechnology) and the protein concentration was quantified using a Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology). For each sample, 50 μ g protein was resolved using SDS-PAGE

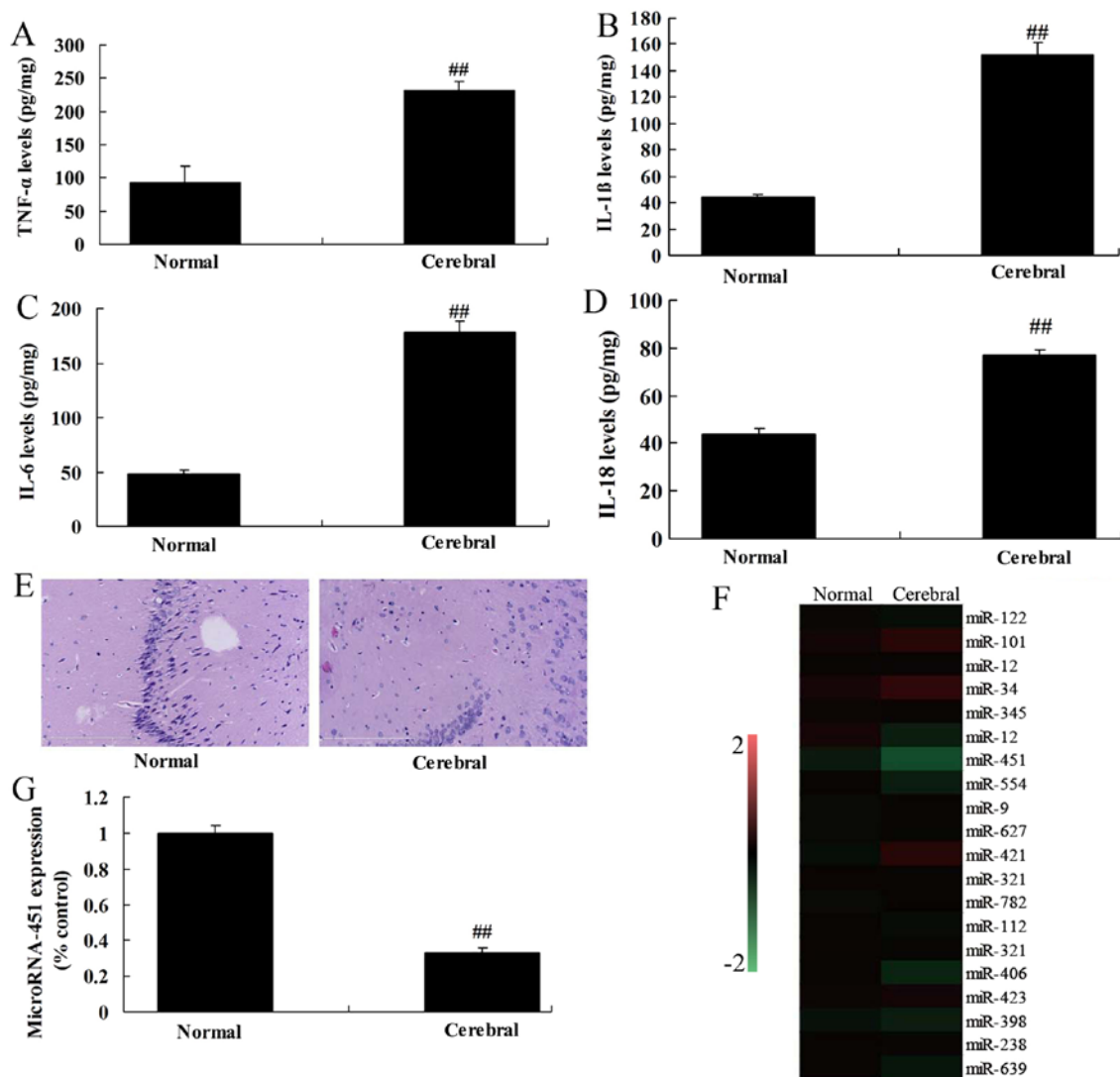


Figure 1. Expression of inflammatory factors and microRNA-451 in an *in vivo* model of cerebral ischemia-reperfusion. (A) TNF- α , (B) IL-1 β , (C) IL-6 and (D) IL-18 expression levels. (Magnification, x50.) (E) H&E staining was conducted to visualize neurons in the cerebral ischemia-reperfusion group and the control group. (Green to red: -2 to 2.) (F) A heat map was constructed to show the expression of different microRNA species in the cerebral ischemia-reperfusion group and the control group. (G) Reverse transcription-quantitative PCR was used to determine the expression level of microRNA-451 in the cerebral ischemia-reperfusion group and the control group. ^{##}P<0.01 vs. normal. Normal, control group; Cerebral, cerebral ischemia-reperfusion group; miR, microRNA; TNF- α , tumor necrosis factor- α ; IL, interleukin.

on 10-12% gels, which was then transferred onto PVDF membranes. Membranes were blocked with 5% non-fat milk in TBS-Tween-20 (TBST) for 1 h at 37°C and incubated overnight at 4°C with antibodies against TLR4 (sc-13593, 1:500; Santa Cruz Biotechnology, Inc.), MyD88 (sc-74532, 1:500; Santa Cruz Biotechnology, Inc.), NF- κ B/p65 (sc-71677, 1:500; Santa Cruz Biotechnology, Inc.) and GAPDH (sc-32233, 1:2,000; Santa Cruz Biotechnology, Inc.). The membranes were washed with TBST and incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature (sc-2004, 1:2,000; Santa Cruz Biotechnology, Inc.). Membranes were visualized using an EasyBlot ECL kit (Sangon Biotech Co., Ltd.) and quantified using ImageJ 1.49 (National Institutes of Health).

Immunofluorescence. Transfected cells (1×10^6 cells) were washed with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. Cells were permeabilized using

0.3% Triton X-100 for 15 min at room temperature and blocked with 5% BSA (Beyotime Institute of Biotechnology) in PBS for 1 h at room temperature. Cells were incubated with an antibody against TLR4 (sc-13593, 1:100; Santa Cruz Biotechnology, Inc.) at 4°C overnight. After washing with PBS, cells were incubated with Alexa 594-conjugated anti-mouse or anti-rabbit immunoglobulin G secondary antibodies (sc-362282, 1:100; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. After washing with PBS, cells were stained with DAPI (1 mg/ml) for 30 min protected from light at room temperature. Images were captured with a fluorescent microscope (x200, Leica Microsystems GmbH).

Luciferase reporter assay. Network signal path was analyzed using http://www.targetscan.org/vert_71/. The 3' UTR region of TLR4 predicted to bind to anti-miR-451 was synthesized into the pGL3 firefly luciferase reporter plasmid (Promega Corporation). Cells (1×10^6 cells) were co-transfected with

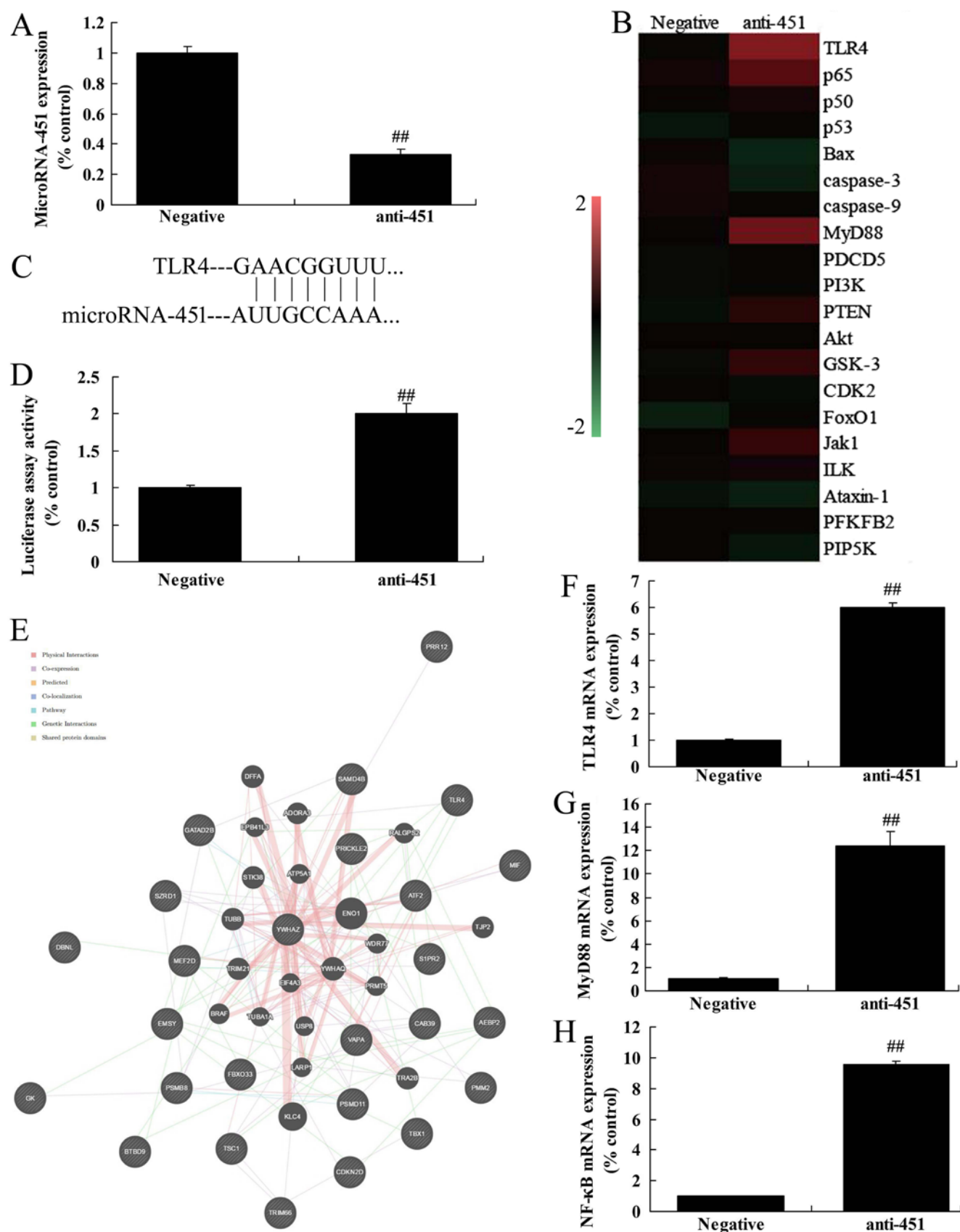


Figure 2. Downregulation of microRNA-451 affects TLR4, MyD88 and NF-κB/p65 protein expression in an *in vitro* model of cerebral ischemia-reperfusion. (A) Reverse transcription-quantitative PCR was used to analyze the expression of microRNA-451. (Green to red: -2 to 2.) (B) A heat map was constructed to show the expression of factors including TLR4, MyD88 and NF-κB/p65 following transfection with anti-451. (C) MicroRNA-451 binding sites in the 3' untranslated region of TLR4. (D) A luciferase reporter assay was used to determine the level of transcription from the TLR promoter following transfection with anti-451. (E) A network signal path revealed that TLR4 may be an important signaling pathway regulated by microRNA-451. (F) TLR4, (G) MyD88 and (H) NF-κB/p65 mRNA expression levels were determined by reverse transcription-quantitative PCR following transfection with anti-451. ##P<0.01 vs. negative. Negative, negative control; anti-451, anti-microRNA-451; TLR4, Toll-like receptor 4; MyD88, myeloid differentiation primary response protein MyD88; NF-κB/p65, nuclear factor-κB.

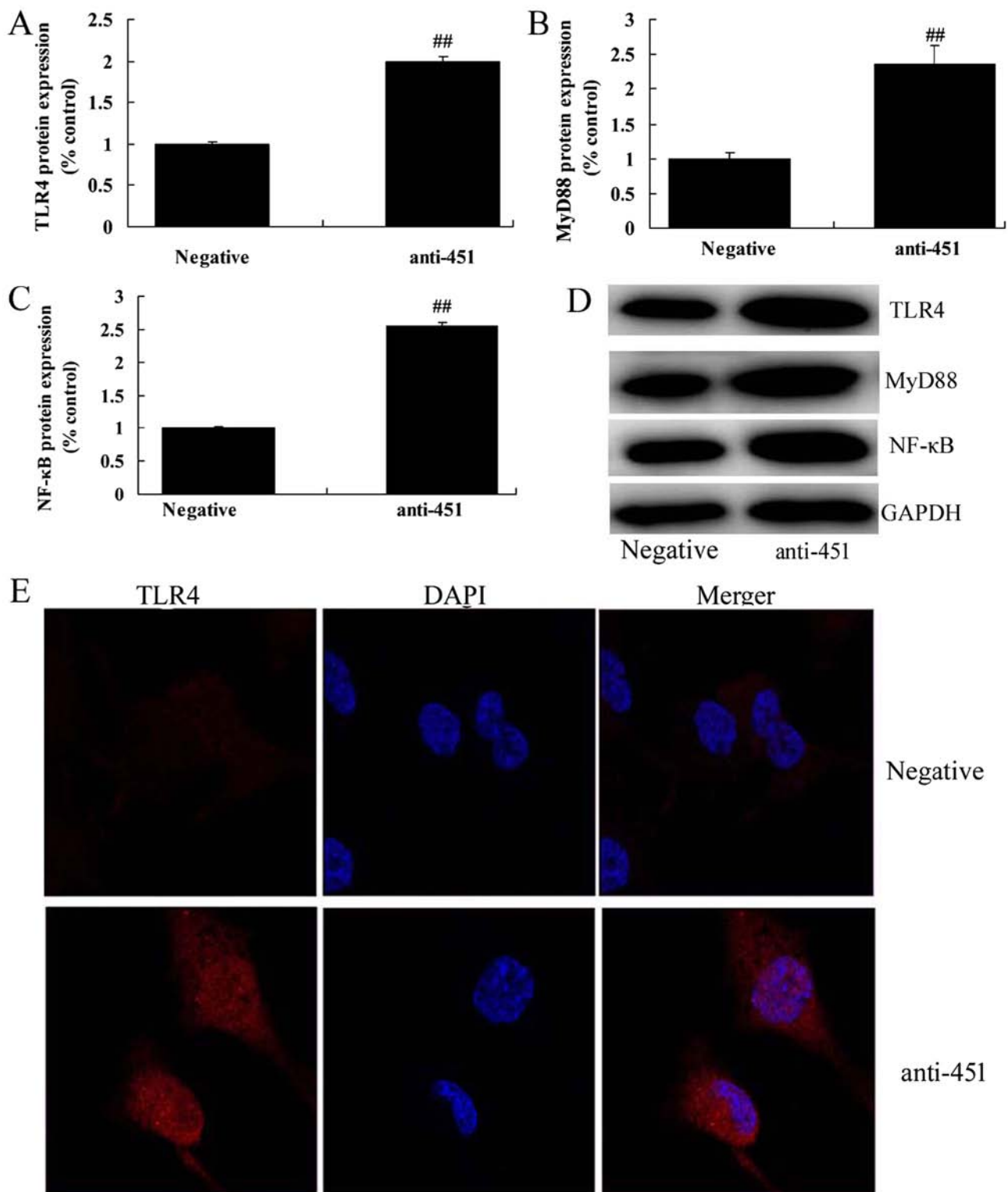


Figure 3. Downregulation of microRNA-451 affects MyD88 and NF-κB/p65 protein expression through TLR4 in cerebral ischemia-reperfusion. (A) TLR4, (B) MyD88 and (C) NF-κB/p65 protein expression was determined in an *in vitro* model of cerebral ischemia-reperfusion. (D) Western blot analysis of TLR4, MyD88 and NF-κB/p65 protein expression. (E) TLR4 protein expression was determined by immunofluorescence. Magnification, x200. ^{##}P<0.01 vs. negative. Negative, negative control; anti-451, anti-microRNA-451; TLR4, Toll-like receptor 4; MyD88, myeloid differentiation primary response protein MyD88; NF-κB/p65, nuclear factor-κB.

10 ng of anti-miRNA-451 and 10 ng of pGL3-TLR4 using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 48 h. Luciferase and Renilla signals were evaluated using the Dual Luciferase Reporter Assay kit (Promega Corporation).

Statistical analysis. All data are presented as the mean ± SEM using SPSS 21.0 (IBM Corp.), (n=3). Statistical analyses were performed using ANOVA with Tukey's post hoc test and Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

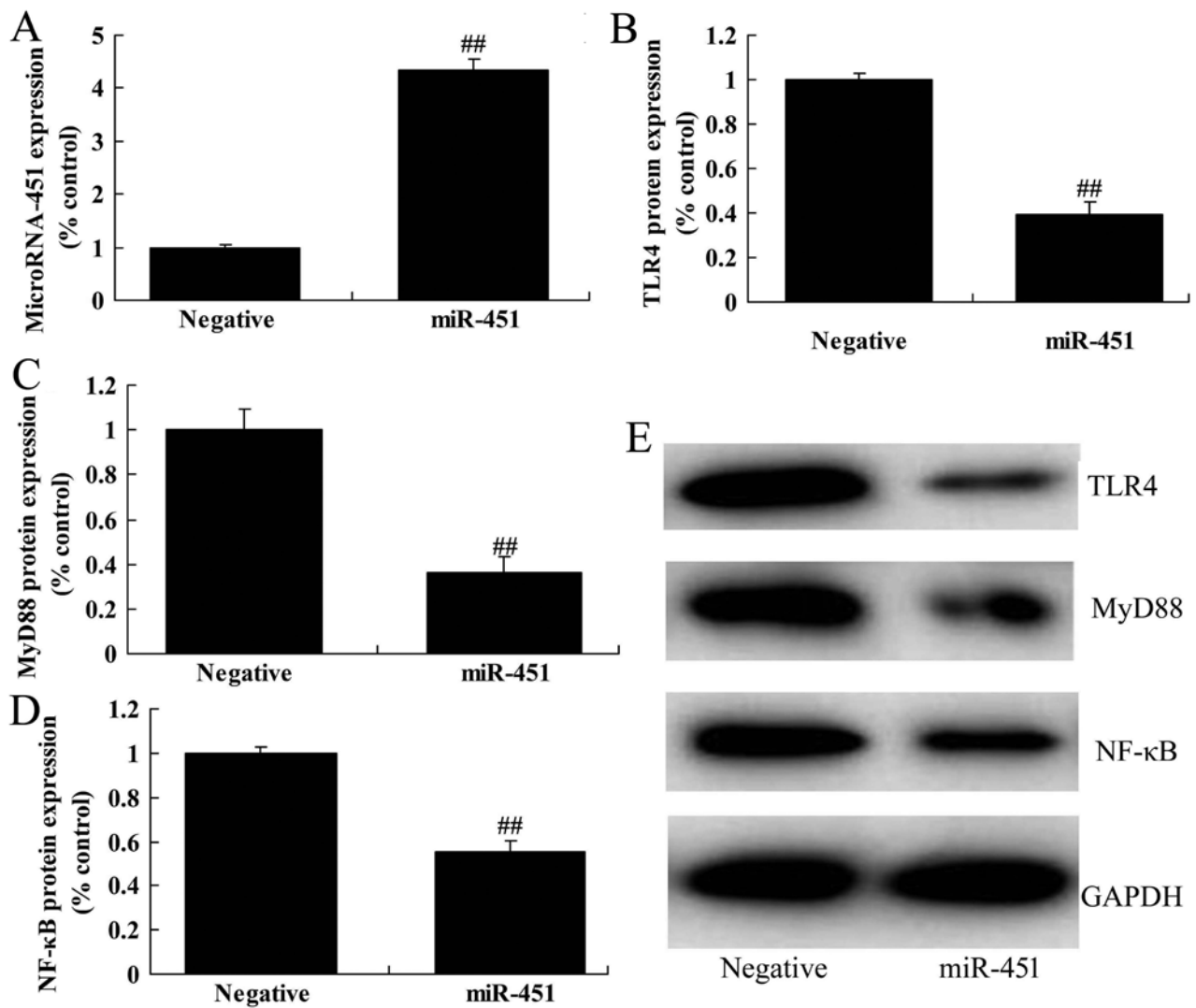


Figure 4. Overexpression of miR-451 affects MyD88 and NF-κB/p65 protein expression through TLR4 in cerebral ischemia-reperfusion. (A) Reverse transcription-quantitative PCR was used to analyze the expression miR-451. (B) TLR4, (C) MyD88 and (D) NF-κB/p65 protein expression was determined in an *in vitro* model of cerebral ischemia-reperfusion. (E) Western blot analysis of TLR4, MyD88 and NF-κB/p65 protein expression. ^{##}P<0.01 vs. negative. Negative, negative control; miR-451, miR-451; TLR4, Toll-like receptor 4; MyD88, myeloid differentiation primary response protein MyD88; NF-κB/p65, nuclear factor-κB.

Results

miRNA-451 expression in mice with cerebral ischemia-reperfusion. RT-qPCR was used to measure the level of miRNA-451 expression in rats with cerebral ischemia-reperfusion. The levels of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6 and IL-18 were increased in rats with cerebral ischemia-reperfusion compared with the control group (Fig. 1A-D). H&E staining showed that the number of nerve cells was reduced in the cerebral ischemia-reperfusion group compared with the control group (Fig. 1E). As shown in Fig. 1F and G, miRNA-451 expression was significantly lower in the cerebral ischemia-reperfusion group compared with the control group, suggesting that downregulation of miRNA-451 is a potential risk factor for cerebral ischemia-reperfusion.

Regulation of miRNA-451 affects the expression of MyD88 and NF-κB/p65 in cerebral ischemia-reperfusion by TLR4. The mechanism of miRNA-451 on inflammation of cerebral

ischemia-reperfusion was evaluated by analyzing the expression of MyD88 and NF-κB/p65. An anti-miRNA-451 mimic was used to reduce the expression of miRNA-451 *in vitro* compared with the negative control (Fig. 2A). A heat map showed that the expression of TLR4, MyD88 and NF-κB/p65 were increased *in vitro* following the down-regulation of miRNA-451 compared with the negative group (Fig. 2B). Using putative miRNA-451 binding sites in the 3' untranslated region of TLR4 and a luciferase reporter assay, it was found that the level of transcription was higher following the downregulation of miRNA-451 (Fig. 2C and D). Network signal path analysis using http://www.targetsatcan.org/vert_71/ revealed that TLR4 may be an important signaling pathway (Fig. 2E). RT-qPCR analysis showed that the downregulation of miRNA-451 induced the expression of mRNA encoding TLR4, MyD88 and NF-κB/p65 *in vitro* (Fig. 2F-H). Western blot analysis showed that the downregulation of miRNA-451 also induced the expression of TLR4, MyD88 and NF-κB/p65 at the protein level *in vitro* (Fig. 3A-D). Immunofluorescence

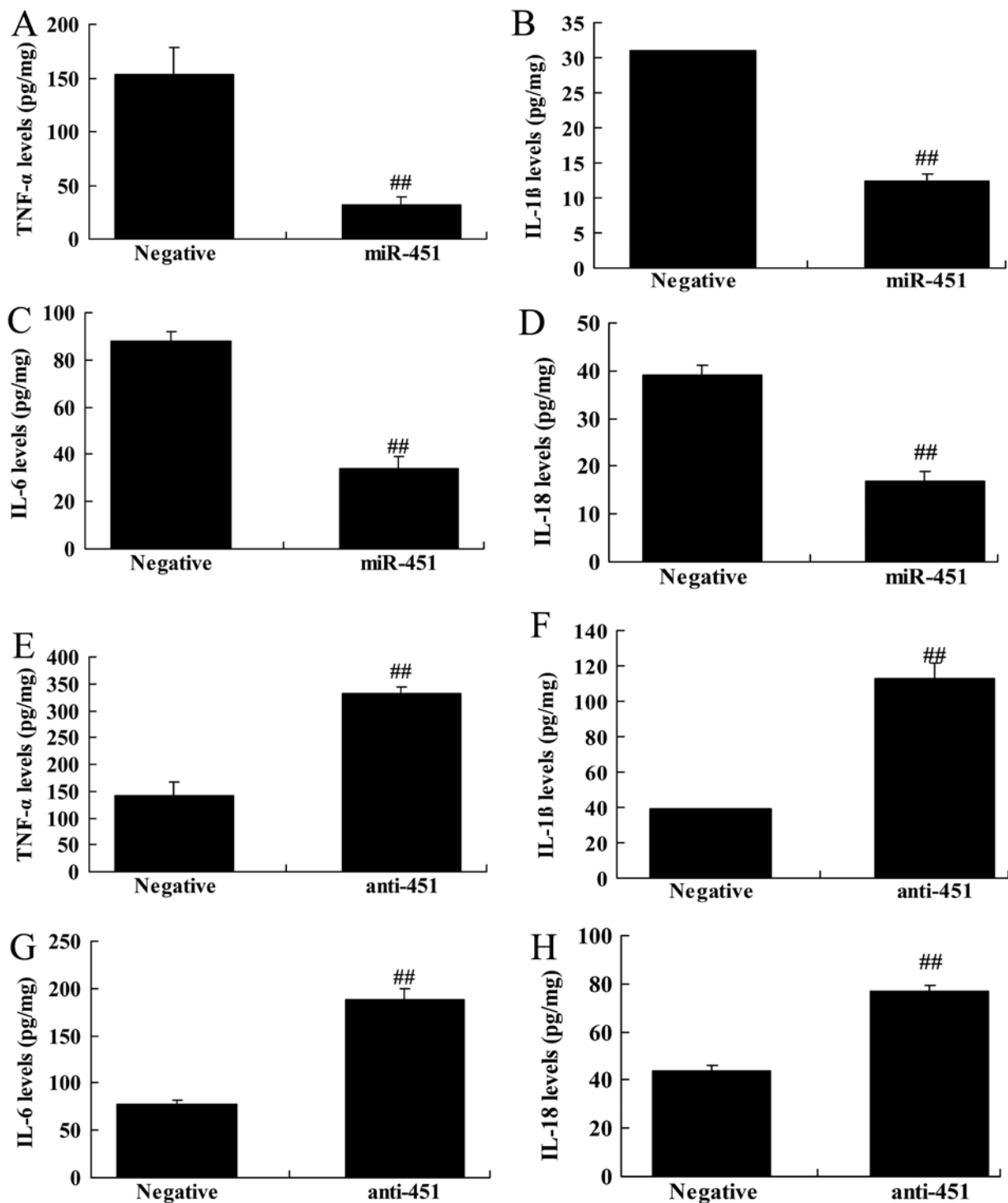


Figure 5. Regulation of miRNA-451 affects inflammation in cerebral ischemia-reperfusion. The expression levels of (A) TNF- α , (B) IL-1 β , (C) IL-6 and (D) IL-18 were determined following the overexpression of miR-451. The expression levels of (E) TNF- α , (F) IL-1 β , (G) IL-6 and (H) IL-18 were determined following the downregulation of miR-451. ##P<0.01 vs. negative. Negative, negative control; miR-451, microRNA-451; anti-451, anti-microRNA-451; TLR4, Toll-like receptor 4; MyD88, myeloid differentiation primary response protein MyD88; NF- κ B/p65, nuclear factor- κ B; TNF- α , tumor necrosis factor- α ; IL, interleukin.

also indicated that the downregulation of miRNA-451 induced the protein expression of TLR4 *in vitro* (Fig. 3E). Transfection of cells with an miRNA-451 mimic increased the expression of miRNA-451 *in vitro* compared with the negative control (Fig. 4A). The upregulation of miRNA-451 suppressed the protein expression of TLR4, MyD88 and NF- κ B/p65 *in vitro* (Fig. 4B-E). These results indicated that miRNA-451 affected

cerebral ischemia-reperfusion-induced inflammation through the TLR4/NF- κ B/p65 signaling pathway.

Regulation of miRNA-451 affects inflammation of cerebral ischemia-reperfusion. The effects of miRNA-451 on inflammation were investigated further in the *in vitro* model of cerebral ischemia-reperfusion. In comparison with the

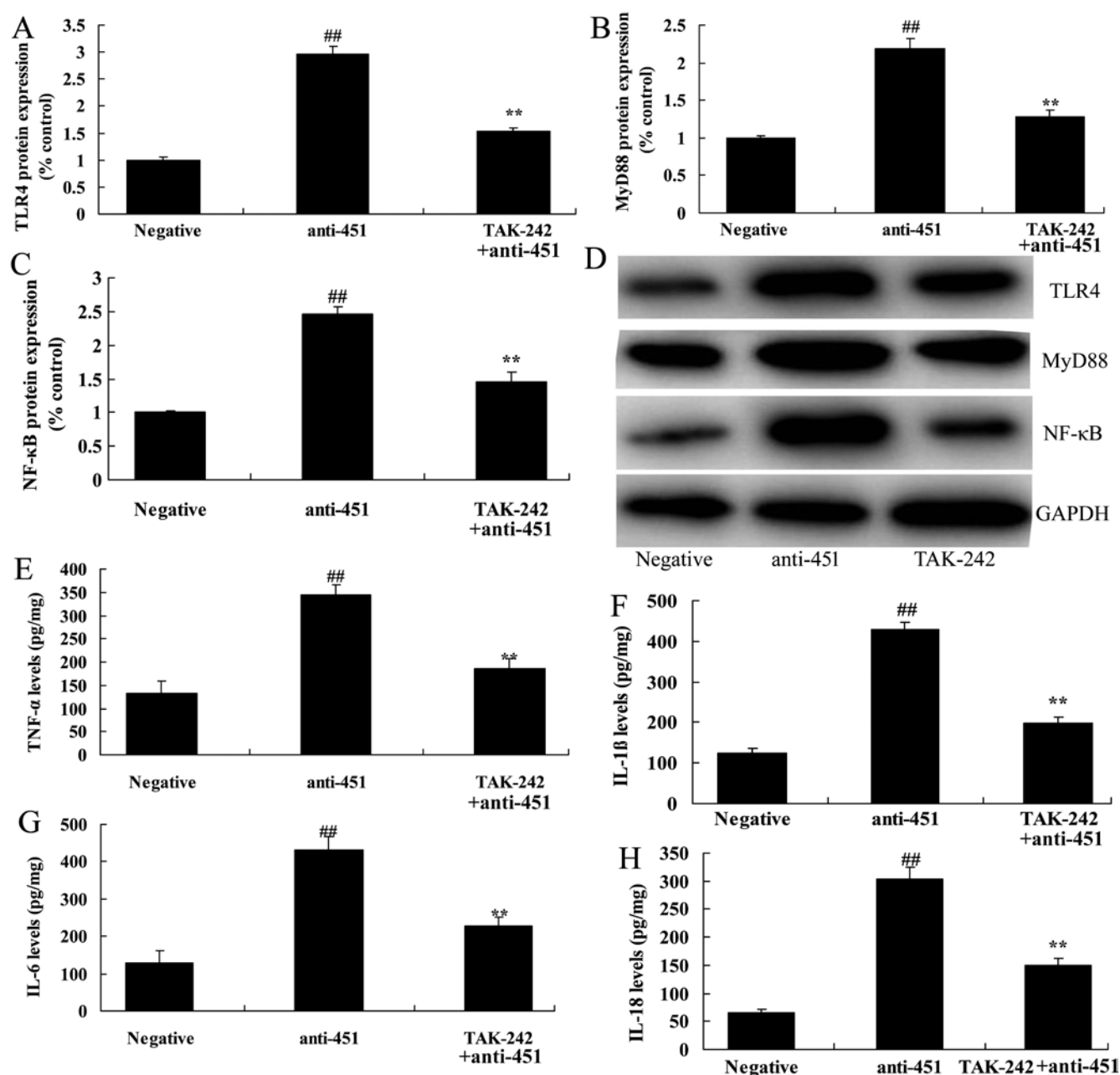


Figure 6. Inhibition of TLR4 reduces the effect of microRNA-451 on TLR4, MyD88, NF-κB/p65 protein expression in cerebral ischemia-reperfusion. The expression level of (A) TLR4, (B) MyD88 and (C) NF-κB/p65 were determined following transfection with anti-451 and exposure to the TLR4 inhibitor TAK-242. (D) Western blot analysis of TLR4, MyD88 and NF-κB/p65 protein expression. The expression levels of (E) TNF-α, (F) IL-1β, (G) IL-6 and (H) IL-18 were determined following transfection with anti-451 and exposure to TAK-242. ^{##}P<0.01 vs. negative; ^{**}P<0.01 vs. anti-451. Negative, negative control; anti-451, anti-microRNA-451; TLR4, Toll-like receptor 4; MyD88, myeloid differentiation primary response protein MyD88; NF-κB/p65, nuclear factor-κB; TNF-α, tumor necrosis factor-α; IL, interleukin.

negative control, overexpression of miRNA-451 reduced the levels of TNF-α, IL-1β, IL-6 and IL-18. By contrast, the downregulation of miRNA-451 increased the levels of TNF-α, IL-1β, IL-6 and IL-18 (Fig. 5).

Inhibition of TLR4 attenuates the effect of miRNA-451 on the expression of TLR4/MyD88/NF-κB/p65 in cerebral ischemia-reperfusion. Following the association between the level of miRNA-451 and the TLR4/MyD88/NF-κB/p65 signaling pathway in cerebral ischemia-reperfusion, the role of miRNA-451 in the expression of TLR4, MyD88 and NF-κB/p65 in cerebral ischemia-reperfusion was examined. The administration of

the TLR4 inhibitor TAK-242 reduced the expression of TLR4, MyD88 and NF-κB/p65 in cerebral ischemia-reperfusion following transfection with miRNA-451, compared with cells transfected with anti-miRNA-451 but not exposed to TAK-242 (Fig. 6A-D). The levels of inflammatory factors, including TNF-α, IL-1β, IL-6 and IL-18, were reduced in cells transfected with anti-miRNA-451 and exposed to TAK-242 compared with cells transfected with anti-miRNA-451 but not treated with TAK-242 (Fig. 6E-H).

Inhibition of MyD88 attenuates the effect of miRNA-451 on MyD88/NF-κB/p65 protein expression in cerebral

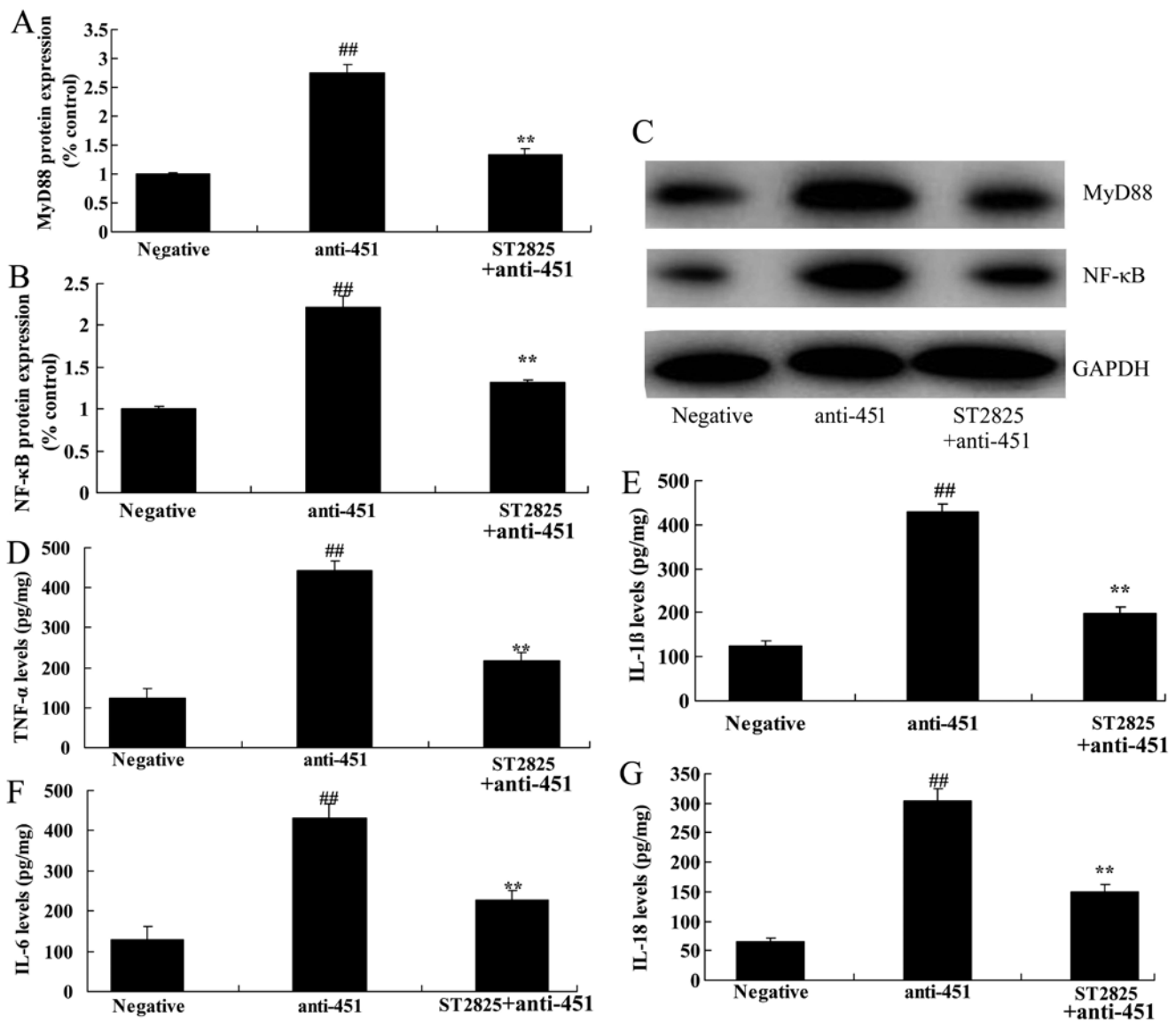


Figure 7. Inhibition of MyD88 reduces the effect of microRNA-451 on MyD88/NF-κB/p65 protein expression in cerebral ischemia-reperfusion. The expression level of (A) MyD88 and (B) NF-κB/p65 were determined following transfection with anti-451 and exposure to the MyD88 inhibitor ST2825. (C) Western blot analysis of MyD88 and NF-κB/p65 protein expression. The expression levels of (D) TNF-α, (E) IL-1β, (F) IL-6 and (G) IL-18 were determined following transfection with anti-451 and exposure to ST2825. ^{##}P<0.01 vs. negative; ^{**}P<0.01 vs. anti-451. Negative, negative control; anti-451, anti-microRNA-451; MyD88, myeloid differentiation primary response protein MyD88; NF-κB/p65, nuclear factor-κB; TNF-α, tumor necrosis factor-α; IL, interleukin.

ischemia-reperfusion. To further explore the role of MyD88 and miRNA-451 in cerebral ischemia-reperfusion, the MyD88 inhibitor, ST2825, was used. As shown in Fig. 7A-C, ST2825 suppressed the effects of anti-miRNA-451 on the expression of MyD88 and NF-κB/p65 in the *in vitro* model of cerebral ischemia-reperfusion compared with cells transfected with anti-miRNA-451 but not treated with ST2825. The inhibition of MyD88 also reduced the levels of TNF-α, IL-1β, IL-6 and IL-18 in cells transfected with anti-miRNA-451 compared with cells transfected with anti-miRNA-451 but not treated with ST2825 (Fig. 7D-G).

Inhibition of NF-κB/p65 attenuates the effect of miRNA-451 on NF-κB/p65 protein expression in cerebral ischemia-reperfusion. To further explore the role of NF-κB/p65 in and miRNA-451 in cerebral ischemia-reperfusion, small interfering (si)RNA targeting p65 (si-p65) was used. As shown in Fig. 8A

and B, si-p65 suppressed the effects of anti-miRNA-451 on the protein expression of NF-κB/p65 in the *in vitro* model of cerebral ischemia-reperfusion compared with cells transfected with anti-miRNA-451 but not with si-p65. The reduction in the expression of NF-κB/p65 also reduced the levels of TNF-α, IL-1β, IL-6 and IL-18 in cells transfected with anti-miRNA-451 compared with cells transfected with anti-miRNA-451 but not with si-p65 (Fig. 8C-F).

Discussion

Cerebrovascular accident is also referred to as a stroke, is characterized by high morbidity, high mortality, high disability and high recurrence rates (18). The number of patients with cerebrovascular disease, dominated by ischemic cerebrovascular disease, has been increasing in China (19). Survival is associated with physical disability, intellectual disability and

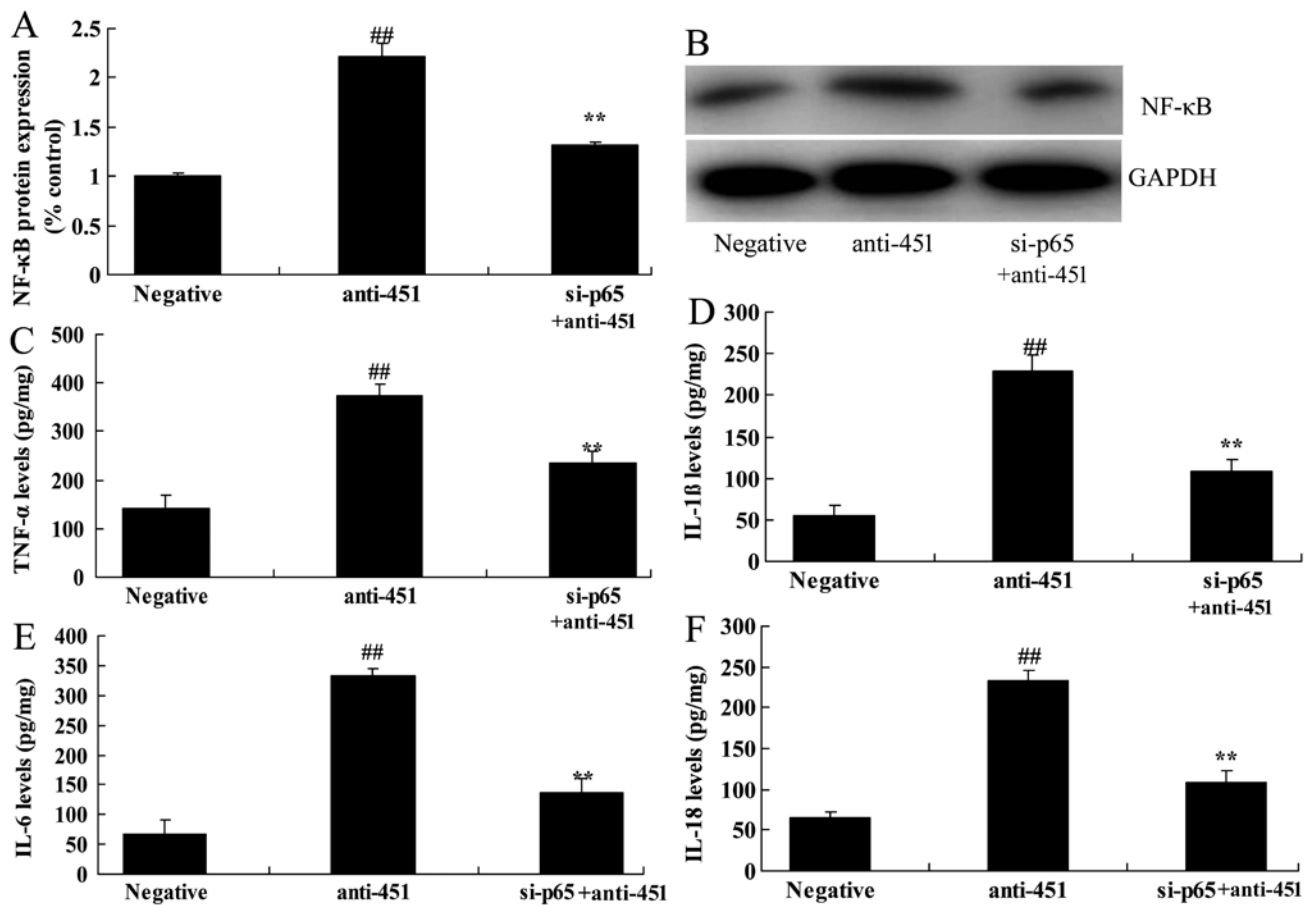


Figure 8. Knockdown of NF- κ B/p65 reduces the effect of microRNA-451 on NF- κ B/p65 protein expression of cerebral ischemia-reperfusion. The expression level of (A) NF- κ B/p65 as determined following transfection with anti-451 and depletion of NF- κ B/p65. (B) Western blot analysis of NF- κ B/p65 protein expression. The expression levels of (C) TNF- α , (D) IL-1 β , (E) IL-6 and (F) IL-18 were determined following transfection with anti-451 and the knockdown of NF- κ B/p65. ^{##} $P < 0.01$ vs. negative; ^{**} $P < 0.01$ vs. anti-451. Negative, negative control; anti-451, anti-microRNA-451; si-p65, small interfering RNA targeting NF- κ B/p65; NF- κ B/p65, nuclear factor- κ B; TNF- α , tumor necrosis factor- α ; IL, interleukin.

a reduced quality of life, which pose economic and social burdens on family and society (19,20). The development of cerebral ischemia is associated with a complicated mechanism and has diverse clinical manifestations; therefore, its pathogenesis and prevention will remain an important focus for medical research in the future. miRNAs are a type of highly conserved non-coding small RNAs that can become abnormally expressed in peripheral blood during the pathogenesis of a disease (19,20). miRNAs have been reported to play important roles in the genesis and development of ischemic cerebrovascular disease (19,20). In the present study, it was demonstrated that miRNA-451 expression was downregulated in a mouse model of cerebral ischemia-reperfusion. The downregulation of miRNA-451 increased inflammation in an *in vitro* model of cerebral ischemia-reperfusion. Wang *et al* (21) showed that miRNA-451 inhibited the proliferation of synovial fibroblasts and the secretion of inflammatory cytokines in rheumatoid arthritis.

Ischemic cerebrovascular disease results from local cerebral blood flow obstruction, which can cause death or permanent disability (10). Following a high degree of cerebral ischemia, reperfusion cannot promote the recovery of metabolism and other functions in the ischemic area, but instead may aggravate the cerebral injury (22). The mechanism of cerebral

IRI is complex, including inflammatory responses, oxidative stress-induced lipid peroxide reactions, metabolic disorders, toxic effects of excitatory amino acids and apoptosis (23). In the present study, it was demonstrated that the downregulation of miRNA-451 induced the expression of TLR4, MyD88 and NF- κ B/p65 in an *in vitro* model of cerebral ischemia-reperfusion. Sun and Zhang (24) reported that miRNA-451 elevation relieves inflammatory pain by targeting TLR4.

TLRs are members of the pathogen-related molecular pattern receptor family, which can identify and bind to conserved sequences of pathogenic micro-organisms (10). TLRs also recognize bacterial endotoxin/LPS and induce inflammatory responses (10). It has been found that TLRs are also involved in cerebral ischemia reperfusion inflammatory injury (10). The brain is a sterile organ and cerebral inflammatory injury is mainly induced by the TLR pathway (22). The recognition of DNA and protein by TLRs occurs in normal mice and mice with cerebral IRI, which suggested that DNA and protein recognition by TLR2 and TLR4 is higher in mice with cerebral IRI than normal mice (22). The activity of NF- κ B can be regulated after the activation of TLRs, and NF- κ B can upregulate the expression of inflammatory factors, thus inducing inflammatory injury (23). Furthermore, the present study demonstrated that the downregulation of

miRNA-451 induced the expression of TLR4, MyD88 and NF- κ B/p65 in an *in vitro* model of cerebral ischemia-reperfusion. Ren *et al* (25) reported that ferulic acid protected against cerebral ischemia-reperfusion-induced injury through anti-oxidant and anti-apoptotic mechanisms. Liu *et al* (26) reported that 3'-daidzein sulfonate sodium inhibited neuronal apoptosis induced by cerebral ischemia-reperfusion.

NF- κ B is a member of the Rel protein family and is an important signal transduction molecule involved in the inflammatory response (27). NF- κ B is also an important transcription factor, which can be activated by a number of factors, including inflammatory factors and cytokines, and by calcium overload in the case of cerebral ischemia (28). Activated NF- κ B can induce the expression of cytokines, adhesion molecules and inflammatory enzymes, which gives rise to an inflammatory response, resulting in brain edema and nerve cell injury (27). In the present study, the inhibition of TLR4 and MyD88, or the knockdown of NF- κ B, has been shown to reduce the effect of miRNA-451 downregulation on inflammation in an *in vitro* model of cerebral ischemia-reperfusion. Sun *et al* (29) showed that miR-451 suppressed the expression of NF- κ B-mediated pro-inflammatory molecules in diabetic nephropathy. Liu *et al* (15) reported that miRNA-451 protected neurons against IRI-induced cell death.

In conclusion, the present study found that miRNA-451 expression was downregulated in a mouse model of cerebral ischemia-reperfusion. The results from the present study suggested that the downregulation of miRNA-451 reduces inflammation in an *in vitro* model of cerebral ischemia-reperfusion through the TLR4/MyD88/NF- κ B signaling pathway. miRNA-451 may be a target for the development of therapeutic agents for use in the treatment of cerebral ischemia-reperfusion.

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Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

XS designed the experiments; WL, MD, LC and LF performed the experiments; XS analyzed the data and wrote the manuscript.

Ethics approval and consent to participate

Animal experiments were approved by the Ethics Committee of The First Affiliated Hospital of Chongqing.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Dippel DW, Majoie CB, Roos YB, van der Lugt A, van Oostenbrugge RJ, van Zwam WH, Lingsma HF, Koudstaal PJ, Treurniet KM, van den Berg LA, *et al*: Influence of device choice on the effect of intra-arterial treatment for acute ischemic stroke in MR CLEAN (multicenter randomized clinical trial of endovascular treatment for acute ischemic stroke in the Netherlands). *Stroke* 47: 2574-2581, 2016.
2. Kim SK, Jang HM and Kim DY: The promoter polymorphism of NFKB1 gene contributes to susceptibility of ischemic stroke in Korean population. *J Exerc Rehabil* 14: 1096-1100, 2018.
3. Ceulemans AG, Zgavc T, Kooijman R, Hachimi-Idrissi S, Sarre S and Michotte Y: The dual role of the neuroinflammatory response after ischemic stroke: Modulatory effects of hypothermia. *J Neuroinflammation* 7: 74, 2010.
4. Candelario-Jalil E: Injury and repair mechanisms in ischemic stroke: Considerations for the development of novel neurotherapeutics. *Curr Opin Investig Drugs* 10: 644-654, 2009.
5. Kawakami S, Tahara Y, Noguchi T, Yagi N, Kataoka Y, Asaumi Y, Nakanishi M, Goto Y, Yokoyama H, Nonogi H, *et al*: Time to reperfusion in ST-segment elevation myocardial infarction patients with vs. without pre-hospital mobile telemedicine 12-lead electrocardiogram transmission. *Circ J* 80: 1624-1633, 2016.
6. Zhao GC, Yuan YL, Chai FR and Ji FJ: Effect of Melilotus officinalis extract on the apoptosis of brain tissues by altering cerebral thrombosis and inflammatory mediators in acute cerebral ischemia. *Biomed Pharmacother* 89: 1346-1352, 2017.
7. Zhou L, Zhang J, Wang C and Sun Q: Tanshinone inhibits neuronal cell apoptosis and inflammatory response in cerebral infarction rat model. *Int J Immunopathol Pharmacol* 30: 123-129, 2017.
8. Bonestroo HJ, Nijboer CH, van Velthoven CT, Kavelaars A, Hack CE, van Bel F and Heijnen CJ: Cerebral and hepatic inflammatory response after neonatal hypoxia-ischemia in newborn rats. *Dev Neurosci* 35: 197-211, 2013.
9. Naderi Y, Sabetkasaei M, Parvardeh S and Zanjani TM: Neuroprotective effect of minocycline on cognitive impairments induced by transient cerebral ischemia/reperfusion through its anti-inflammatory and anti-oxidant properties in male rat. *Brain Res Bull* 131: 207-213, 2017.
10. Li X, Su L, Zhang X, Zhang C, Wang L, Li Y, Zhang Y, He T, Zhu X and Cui L: Ulinastatin downregulates TLR4 and NF- κ B expression and protects mouse brains against ischemia/reperfusion injury. *Neurol Res* 39: 367-373, 2017.
11. Zhou J and Zhang J: Identification of miRNA-21 and miRNA-24 in plasma as potential early stage markers of acute cerebral infarction. *Mol Med Rep* 10: 971-976, 2014.
12. Liu X, Li F, Zhao S, Luo Y, Kang J, Zhao H, Yan F, Li S and Ji X: MicroRNA-124-mediated regulation of inhibitory member of apoptosis-stimulating protein of p53 family in experimental stroke. *Stroke* 44: 1973-1980, 2013.
13. Chen S, Wang M, Yang H, Mao L, He Q, Jin H, Ye ZM, Luo XY, Xia YP and Hu B: LncRNA TUG1 sponges microRNA-9 to promote neurons apoptosis by up-regulated Bcl2l11 under ischemia. *Biochem Biophys Res Commun* 485: 167-173, 2017.
14. Truettner JS, Alonso OF, Bramlett HM and Dietrich WD: Therapeutic hypothermia alters microRNA responses to traumatic brain injury in rats. *J Cereb Blood Flow Metab* 31: 1897-1907, 2011.
15. Liu Q, Hu Y, Zhang M, Yan Y, Yu H and Ge L: microRNA-451 protects neurons against ischemia/reperfusion injury-induced cell death by targeting CELF2. *Neuropsychiatr Dis Treat* 14: 2773-2782, 2018.
16. Leecharoenkiet K, Tanaka Y, Harada Y, Chaichompoo P, Sarakul O, Abe Y, Smith DR, Fucharoen S, Svasti S and Umemura T: Plasma microRNA-451 as a novel hemolytic marker for β -thalassemia/HbE disease. *Mol Med Rep* 15: 2495-2502, 2017.
17. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25: 402-408, 2001.
18. Hill MD, Demchuk AM, Goyal M, Jovin TG, Foster LD, Tomsick TA, von Kummer R, Yeatts SD, Palesch YY and Broderick JP: IMS3 Investigators: Alberta Stroke Program early computed tomography score to select patients for endovascular treatment: Interventional Management of Stroke (IMS)-III Trial. *Stroke* 45: 444-449, 2014.

19. Zhu Z, Fu Y, Tian D, Sun N, Han W, Chang G, Dong Y, Xu X, Liu Q, Huang D and Shi FD: Combination of the immune modulator fingolimod with alteplase in acute ischemic stroke: A pilot trial. *Circulation* 132: 1104-1112, 2015.
20. Shan J, Sun L, Wang D and Li X: Comparison of the neuroprotective effects and recovery profiles of isoflurane, sevoflurane and desflurane as neurosurgical pre-conditioning on ischemia/reperfusion cerebral injury. *Int J Clin Exp Pathol* 8: 2001-2009, 2015.
21. Wang ZC, Lu H, Zhou Q, Yu SM, Mao YL, Zhang HJ, Zhang PC and Yan WJ: MiR-451 inhibits synovial fibroblasts proliferation and inflammatory cytokines secretion in rheumatoid arthritis through mediating p38MAPK signaling pathway. *Int J Clin Exp Pathol* 8: 14562-14567, 2015.
22. Zhang P, Guo ZF, Xu YM, Li YS and Song JG: N-Butylphthalide (NBP) ameliorated cerebral ischemia reperfusion-induced brain injury via HGF-regulated TLR4/NF- κ B signaling pathway. *Biomed Pharmacother* 83: 658-666, 2016.
23. Zhou H, Yang WS, Li Y, Ren T, Peng L, Guo H, Liu JF, Zhou Y, Zhao Y, Yang LC and Jin X: Oleoylethanolamide attenuates apoptosis by inhibiting the TLR4/NF- κ B and ERK1/2 signaling pathways in mice with acute ischemic stroke. *Naunyn Schmiedeberg Arch Pharmacol* 390: 77-84, 2017.
24. Sun X and Zhang H: miR-451 elevation relieves inflammatory pain by suppressing microglial activation-evoked inflammatory response via targeting TLR4. *Cell Tissue Res* 374: 487-495, 2018.
25. Ren Z, Zhang R, Li Y, Li Y, Yang Z and Yang H: Ferulic acid exerts neuroprotective effects against cerebral ischemia/reperfusion-induced injury via antioxidant and anti-apoptotic mechanisms *in vitro* and *in vivo*. *Int J Mol Med* 40: 1444-1456, 2017.
26. Liu R, Zhong X, Zeng J, Huang Z, Li X, Xiao H, Chen Q and Li D: 3'-Daidzein sulfonate sodium inhibits neuronal apoptosis induced by cerebral ischemia-reperfusion. *Int J Mol Med* 39: 1021-1028, 2017.
27. Kacimi R, Giffard RG and Yenari MA: Endotoxin-activated microglia injure brain derived endothelial cells via NF- κ B, JAK-STAT and JNK stress kinase pathways. *J Inflamm (Lond)* 8: 7, 2011.
28. Feola J, Barton A, Akbar A, Keillor J and Johnson GVW: Transglutaminase 2 modulation of NF- κ B signaling in astrocytes is independent of its ability to mediate astrocytic viability in ischemic injury. *Brain Res* 1668: 1-11, 2017.
29. Sun Y, Peng R, Peng H, Liu H, Wen L, Wu T, Yi H, Li A and Zhang Z: miR-451 suppresses the NF-kappaB-mediated proinflammatory molecules expression through inhibiting LMP7 in diabetic nephropathy. *Mol Cell Endocrinol* 433: 75-86, 2016.



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