Long noncoding RNA ANRIL Knockdown Attenuates Neuroinflammation Following Ischemic Stroke via Suppressing the Expression of NF- κ B In Vitro and In Vivo

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Abstract

Increasing evidence suggests that long noncoding RNAs can exert neuroprotective effects in cerebral ischemia-reperfusion injury. Levels of the long noncoding RNA ANRIL (ANRIL) are reportedly altered in ischemic stroke (IS) patients, but its role in IS requires further clarification. This study was designed to explore the mechanistic function of ANRIL in IS. *In vitro*, HT22 cells were treated with an oxygen-glucose deprivation/reperfusion (OGD/R). *In vivo*, brain ischemia/reperfusion was induced by 60-minute transient middle cerebral artery occlusion/ reperfusion (MCAO/R) IS model in C57/BL6 mice. Additionally, cells were transfected with si-ANRIL, pcDNA3.1-ANRIL, pcDNA3.1-NF- κ B, or appropriate negative controls, and si-ANRIL and pcDNA3.1-NF- κ B were administered into the lateral ventricles in MCAO/R model mice. Cell viability and apoptosis were detected via MTT and flow cytometry assays. mRNA and protein expression of NF- κ B were detected via qRT-PCR and Western blotting. IL-1 β , IL-6, TNF- α , and iNOS levels were detected via ELISA. In addition, infarcted area and neuronal injury were evaluated via TTC, Nissl, and immunofluorescent staining. We found that ANRIL knockdown increased cell viability and reduced apoptosis *in vitro*. Additionally, we found that ANRIL knockdown decreased p-P65, P65, IL-1 β , IL-6, TNF- α , and iNOS levels, whereas these effects were reversed by NF- κ B overexpression both *in vitro* and *in vivo*. Our results suggest that ANRIL knockdown attenuates neuroinflammation by suppressing the expression of NF- κ B both *in vitro* and *in vivo* model of IS, suggesting that ANRIL might be a potentially viable therapeutic target to diminish neuroinflammation in IS patients.

Introduction

Ischemic stroke (IS) is one of the most common causes of death and disability in the world, with more than two million young adults (18–50 years old) suffering from IS episodes each year (Ekker et al. 2018; Hankey 2017). After a stroke, patients are often faced with aphasia or hemiplegia, leading to significant reductions in their quality of life and mental health.

Inflammation and oxidative stress occurring secondary to IS can also cause irreversible neuronal damage, with unrestrained neuroinflammation having the potential to exacerbate this damage (Chamorro et al. 2016; Q Yang et al. 2019). Accumulating evidence indicates that inflammation plays a key role in the pathogenesis of stroke, and as such, it has become a target for therapeutic intervention (Jayaraj et al. 2019). The pro-inflammatory cytokines such as interleukin-1 β (IL-1 β), interleukin-6 (IL-6), and tumor necrosis factor (TNF- α) begin to be produced rapidly within 6 h of ischemia, potentially causing neuronal injury (Bonaventura et al. 2016). In addition, these neurons are highly sensitive to irreversible damage caused by ischemia and hypoxia (Shi et al. 2019). The therapeutic options available to treat IS are limited, and novel therapies capable of preventing IS-associated neuroinflammatory damage are urgently needed.

Long noncoding RNA over 200 nucleotides in length have been shown to play diverse roles in biological processes, controlling gene expression, mRNA splicing, and other epigenetic processes (Qiao et al. 2019). Long noncoding RNA have been shown to be highly expressed in the central nervous system (Clark et al.

2014), with some also being closely tied to IS pathogenesis (Deng et al. 2018; Dharap et al. 2012). Long noncoding RNA ANRIL (ANRIL), which is encoded by the chromosome 9p21 region in humans (Congrains et al. 2013). Previous studies have demonstrated that ANRIL is associated with metabolic disease (Kong et al. 2018), cancer, and cardiovascular disease (Aguilo et al. 2016; L L Chen 2016). ANRIL can additionally influence the pathology of coronary artery disease owing to its ability to modulate microRNA – 181b (Guo et al. 2018). ANRIL protects H9c2 cells against hypoxia-induced injury by targeting the miR-7-5p/SIRT1 axis (Shu et al. 2020). Some prior bioinformatics studies have reported that ANRIL expression is altered in IS patients (Feng et al. 2019; Tan et al. 2019; J Yang et al. 2018), but its mechanism role in IS requires further clarification.

The present study was designed to explore the underlying mechanisms whereby ANRIL influences IS-related injury. *In vitro*, HT22 mice hippocampal neuron line cells was treated with an oxygen-glucose deprivation/reperfusion (OGD/R). *In vivo*, brain ischemia/reperfusion was induced by 60-minute transient middle cerebral artery occlusion/ reperfusion (MCAO/R) IS model in C57/BL6 mice. Our results suggest that ANRIL knockdown attenuates neuroinflammation by suppressing the expression of NF- κ B both in vitro and vivo model of IS.

Materials And Methods

Animals

In total, 183 male C57BL/6 mice (20–25 g) were housed under standard conditions in a $25 \pm 2^{\circ}\text{C}$, facility with 60–70% relative humidity and a 12 h light/dark cycle. Animals had free access to food and water. The Chongqing Medical Animal Experiment Center oversaw all studies, which had additionally been approved by The Ethics Committee of Animal laboratory of Chongqing Medical University (License Number: SYXK YU 2010-001). Animal suffering was minimized wherever possible.

MCAO/R model

We developed an MCAO model of IS based upon previously published methods (H Liu et al. 2019). Briefly, animals were anesthetized using intraperitoneally injected pentobarbital sodium (40 mg/kg) (sunlidabio, China) and were then placed in a supine position. The skin was then incised, and the right common carotid artery (CCA), internal carotid artery (ICA), and external carotid artery (ECA) were exposed and dissected away from adjacent nerves. A silicone nylon suture (0.21 mm in diameter, Fengteng Biology, China) was then inserted through the CCA into the ICA such that it blocked the middle cerebral artery. After 60 minutes, this blockage was removed and the CCA was ligated at the site of the incision. The wound was then disinfected using iodine and was sutured. Animals were warmed to $37.5 \pm 0.5^{\circ}\text{C}$ during surgery and recovery using heating pads. In sham-operated animals, all surgical procedures were identical to procedure in MCAO model mice, but the silicone nylon suture was not inserted.

Animal treatment groups

For studies of ANRIL expression in MCAO/R mice, 30 mice were randomly assigned to the following 5 groups: Sham, MCAO/R 0 h, MCAO/R 8 h, MCAO/R 16 h, and MCAO/R 24 h. For studies of the mechanistic role of ANRIL in MCAO/R mice, groups as follow: Sham, MCAO/R, MCAO/R + si-ANRIL, MCAO/R + si-negative control (NC), MCAO/R + si-ANRIL + pcDNA3.1-NF- κ B, MCAO/R + pcDNA3.1-NF- κ B. A small interfering RNA specific for ANRIL [si-ANRIL: 5'-GGACACCUUUAGCUGUUGATT...UCAACAGCUAAAGGUGUCCTT-3'] (2 μ l, 20 μ M, GenePharma, China), a negative control siRNA (si-NC, GenePharma), pcDNA3.1-NF- κ B (2 μ l, 20 μ M, GenePharma), or a negative control pcDNA (pcDNA3.1-NC, GenePharma) were administered into the lateral ventricles (injected at 0.2 μ l/min with a mini-pump [RWD, China]) of different groups of mice at 72 h and 24 h prior to ischemia, respectively. Animals in the sham and MCAO model groups were administered ddH₂O as a control using the same injection conditions. Mice hippocampal samples were then collected for analysis 24 h after MCAO/R.

Cell culture

HT22 cells were grown in high-glucose DMEM (Saimike, China) containing 10% fetal calf serum (Hyclone, USA) and 1% penicillin/streptomycin in a 5% CO₂ incubator.

OGD/R treatment

Briefly, HT22 cells were grown for 24 h under normal conditions, after which they were transferred to glucose-free DMEM (Gibco, USA) for 2 h under hypoxic conditions (1.0% O₂, 93.5% N₂, 5%CO₂) at 37°C. Then cells were grown under normal culture conditions for 24 h to simulate reperfusion. For studies of ANRIL expression in OGD/R HT22 cells, we assigned groups as follow: Control, OGD/R/R 0h, OGD/R 8h, OGD/R 16h, and OGD/R 24h.

si-RNA and plasmid transfection

To explore the functional role of ANRIL in OGD/R-treated cells, we knocked down or overexpressed ANRIL or overexpressed NF- κ B in HT22 cells prior to 24 h OGD/R treatment. We designed groups as follow: Control, OGD/R, OGD/R + si-ANRIL, OGD/R + si-NC, OGD/R + pcDNA3.1-ANRIL, OGD/R + pcDNA3.1-NC. Then, to studies of the mechanistic protective role of ANRIL, we designed groups as follow: Control, OGD/R, OGD/R + si-ANRIL, OGD/R + si-ANRIL + pcDNA3.1-NF- κ B, OGD/R + pcDNA3.1-NF- κ B. si-ANRIL (50 nM), si-NC, pcDNA3.1-ANRIL (1 μ g/ml), pcDNA3.1-NC, pcDNA3.1-NF- κ B (1 μ g/ml) and pcDNA3.1-NC were transfected into appropriate HT22 cells using the riboFECT CP reagent (RIBBIO, China). Following transfection, cells were cultured in normal media for an additional 24 h after OGD treatment.

Cell viability assay

A Cell Proliferation and Cytotoxicity Assay Kit (Methylthiazolyldiphenyl-tetrazolium bromide, MTT) was used to measure cell viability according to provided directions. Cells were seeded in 96-well plates at a density of 10,000 cells per well. Subsequently, cells were incubated with 20 μ l MTT (5 mg/ml, Sigma) at 37°C for an additional 2 h. At the end of this incubation, the culture medium was removed and 150 μ l of dimethyl sulfoxide (Sigma) was added to dissolve the formazan crystals in these wells. Absorbance at 490 nm was measured using a fluorescence plate reader (Thermo Scientific, USA).

Apoptosis measurement

Briefly, HT22 cells from different treatment groups were harvested and resuspended at 6×10^5 cells, the percentage of apoptotic cells was analyzed via fluorescence-activated cell sorting following Annexin V-FITC/PI staining according to provided instructions.

Immunofluorescent staining

Cells were fixed at 24 h post-OGD/R treatment for 10 minutes with 4% paraformaldehyde. Cells were then probed with anti-P65 (Proteintech, USA). Additionally, 4% paraformaldehyde-fixed mice hippocampus tissue sections were stained with a NeuN antibody (1:100) and coralit488-conjugated affinipure goat anti-Rabbit IgG(H + L), whereas DAPI (Beyotime, China) was used for nuclear staining. A fluorescence microscope was used to image cells and samples

Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

Trizol (Sagon Biotech, China) was used to extract RNA from cell and hippocampal tissue samples based on provided directions. An all-in-one cDNA Synthesis SuperMix (Bimake, China) was used for cDNA synthesis. qRT-PCR reactions were conducted to measure ANRIL and mRNA NF- κ B expression using SYBR Green qPCR Master Mix (Bimake) with the following primers: ANRIL (Forward)-CAAGCCACGTTGGAAGATGC, ANRIL (Reverse)-AGAGTGTGTAGCAGCTGACG; NF- κ B (Forward)-TGCGATTCCGCTATAAATGCG, NF- κ B (Reverse)-ACAAGTTCATGTGGATGAGGC; β -actin (Forward)-GTGCTATGTTGCTCTAGACTTCG, β -actin (Reverse)-ATGCCACAGGATsTCCATACC. The Bio-Rad CFX Manager 3.1 system was used with the following thermocycler settings: 95°C, 7 minutes; 39 cycles of 95°C for 15 seconds, 60°C for 40 seconds, and 65°C for 30 seconds.

Western blotting

Samples were lysed using RIPA buffer (Dingguo, China) based on provided directions. The total protein was separated by PAEG gel fast preparation kit (Epizyme, China) and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with bovine serum albumin for 2 h at room temperature. Then, primary antibodies against rabbit p65 (anti-P65, Proteintech, USA), Phospho-NF- κ B p65 (Ser536) (anti-p-p65, Beyotime), and β -actin (anti- β -actin, Proteintech) were incubated overnight at 4°C. Followed, washed it with TBST and incubated with goat anti-rabbit secondary for 1 h. Finally, the membranes were exposed using ECL FemtoLightSubstrate (Epizyme). The immunoreactive protein bands were quantified using ImageJ software.

Enzyme-linked immunosorbent assay (ELISA)

RIPA buffer (Dingguo) was used to prepare proteins from cell lysates and brain homogenates. Levels of IL-1 β , IL-6, TNF- α , and iNOS in these samples were then measured using commercial ELISA kits (Beyotime) based on provided directions.

Neurological Deficits

At 24 h post-MCAO, mice neurological functions were assessed by a researcher blinded to group assignments using a modified version of a scoring system previously reported (Xue et al. 2016): 0 = no apparent deficits; 1 = difficulty with full extension of the contralateral forelimb; 2 = unable to extend the contralateral forelimb; 3 = mild contralateral circling; 4 = severe circling; 5 = falling to the contralateral side or death. Animals with scores of 0 were considered to have undergone unsuccessful IS modeling, whereas those with a score of 5 were excluded from the study and euthanized due to the severity of their illness.

Infarcted area

At 24 h post-MCAO, hippocampal tissue samples were collected from mice and used to prepare 1.5 mm-thick coronal sections. Sections were stained for 10 minutes with 2% TTC (2,3,5-Triphenyltetrazolium chloride, Sigma) at 37°C, after which 4% paraformaldehyde was used to terminate staining. In stained sections, infarcted tissue appeared white whereas all other tissue appeared red. The ImageJ software was used to measure the infarcted area as a proportion of the total area to evaluate infarction severity as follows: $\text{infarcted area (\%)} = \frac{[\text{the infarcted area} - (\text{the ipsilateral hemisphere area} - \text{the intact contralateral hemisphere area})]}{2 \times \text{the intact contralateral hemisphere area}} \times 100$ (Y A Chen et al. 2020).

Nissl staining

Briefly, three mice per treatment group were selected at random, anesthetized using pentobarbital sodium (40 mg/kg), and perfused with 4% paraformaldehyde prior to Nissl staining. Hippocampal CA1 staining was evaluated by a researcher blinded to experimental protocols.

Rota-rod Test

Murine motor coordination was evaluated using a rota-rod (Ugo Basile, Italy). Briefly, animals were trained on an accelerating (4–40 rpm over 5 minutes) rota-rod cylinder for 3 days prior to MCAO in order to obtain baseline measurements. All animals were able to remain on the rota-rod for at least 160 seconds at baseline, consistent with prior studies (Zhao et al. 2017). At 24 h post-MCAO, animals (n = 15/group) were placed on the rota-rod apparatus using the same acceleration conditions as above. A researcher blinded to group experiments then observed animal performance and recorded latency to fall during each trial, with a maximum time of 160 s. Animals were tested three times per day with 10 minutes between trials, and the mean time for each animal was then calculated.

Statistical analysis

All data are expressed as the mean $\pm$ SD. SPSS v21.0 (SPSS Inc., USA) was used for all statistical testing. The experiments were repeated three times in vitro. The number of mice in each group was noted in the legend. Data were compared via One-way ANOVAs with Tukey's post hoc test, with $p < 0.05$ as the significance threshold.

Results

ANRIL expression is elevated in IS model systems in vitro and in vivo

ANRIL expression was upregulated in hippocampal samples at 8, 16, and 24 h post-MCAO compared to Sham controls (Figure.1A). Similarly, at 0, 8, 16, and 24 h post-OGD/R, ANRIL expression was significantly increased in HT22 cells compared to untreated cells (Figure. 1B). These results revealed that ANRIL expression was persistently increased within 24 hours after MCAO. Based on these results, we selected at MCAO/R 24 h time point for subsequent studies both in vitro and in vivo.

ANRIL knockdown reduces OGD/R-induced injury and inflammatory cytokine production in HT22 cells

We confirmed that we were able to successfully knock down or overexpress ANRIL and si-NC, pcDNA3.1-NC had no impact on ANRIL expression in OGD/R-treated cells (Figure. 2A), whereas did not impact HT22 cells under normal growth conditions (Figure. 2B). However, we found that ANRIL knockdown increased OGD/R-treated cell viability (Figure. 2C) and diminished OGD/R-induced cellular apoptosis (Figure. 2D, 2E), while ANRIL overexpression had the opposite effect. In addition, our results showed that ANRIL knockdown decreased NF- κ B mRNA levels via qRT-PCR (Figure. 2F), reduced p-P65 and P65 protein levels via Western blotting (Figure. 2G, 2H), and suppressed the production of the pro-inflammatory cytokines IL-1 β , IL-6, TNF- α , and iNOS as assessed via ELISA (Figure. 2I).

ANRIL knockdown reduces OGD/R-induced inflammation by suppressing NF- κ B expression

Next, we successfully overexpressed NF- κ B in OGD/R-treated cells (Figure. 3B). We found that ANRIL knockdown increased OGD/R-treated cell viability, whereas NF- κ B overexpression reversed this effect (Figure. 3C). ANRIL knockdown was associated with reductions in OGD/R-induced p-P65 and P65 levels, while NF- κ B overexpression also reversed this phenotype in cells via Western blotting (Figure. 3D, 3E). ANRIL knockdown also reduced IL-1 β , IL-6, TNF- α , and iNOS levels, while NF- κ B overexpression restored the production of these cytokines (Figure. 3F). Immunofluorescence analyses also revealed that ANRIL knockdown suppressed the OGD/R-induced increases in nuclear and cytoplasmic P65 levels (Figure. 3G), whereas these cytoplasmic levels were decreased by si-ANRIL treatment.

Knockdown of ANRIL protects mice against cerebral ischemia-reperfusion injury

We found that si-ANRIL reduced ANRIL expression successfully in MCAO/R model mice, whereas si-NC had no impact on ANRIL expression in these animals (Figure. 4A). Neurological deficits scores was elevated in MCAO/R mice, and reduced by si-ANRI (Figure. 4B). We observed a significant increase in infarcted area (30.6%) in MCAO/R model animals, while the infarcted area was reduced by 12.9% upon si-

ANRIL treatment (Figure. 4C, 4D). The number of injured neurons in the CA1 region of MCAO/R group was higher than in sham controls, but these numbers were reduced by si-ANRIL treatment (Figure. 4E, 4F). Immunofluorescent NeuN staining also indicated that the survival of hippocampal neurons was significantly enhanced in MCAO/R mice with si-ANRIL treatment (Figure. 4G).

ANRIL knockdown reduces MCAO/R-induced neuroinflammation in mice by suppressing NF- κ B expression

pcDNA3.1-NF- κ B was sufficient to increase NF- κ B expression in MCAO/R mice, whereas pcDNA-NC had no impact on NF- κ B expression (Figure. 5A). ANRIL knockdown significantly reduced NF- κ B mRNA expression (Figure. 5C) and reduced p-P65 and P65 levels in the hippocampus of MCAO/R mice, whereas these effects were reversed by NF- κ B overexpression (Figure. 5D, 5E). Pro-inflammatory IL-1 β , IL-6, TNF- α , and iNOS levels were increased in MCAO/R mice (Figure. 5F), while these levels were decreased by ANRIL knockdown. However, NF- κ B overexpression reversed these effects. Neurological deficits scores was elevated in MCAO/R mice, and reduced by si-ANRIL, NF- κ B overexpression reversed these effects (Figure. 5G). We additionally found that fall latency was significantly reduced in MCAO/R model animals relative to sham controls in a rota-rod test, whereas this phenotype was significantly improved in animals in the MCAO/R + si-ANRIL treatment group, and NF- κ B overexpression reversed these effects (Figure. 5H).

ANRIL regulates NF- κ B expression at the mRNA level but is not reciprocally regulated by NF- κ B in vitro and in vivo

We found that ANRIL knockdown significantly reduced NF- κ B mRNA expression, whereas this was reversed by NF- κ B overexpression in OGD/R-treated cells (Figure. 3B) and in MCAO/R-treated mice (Figure. 5C). However, the overexpression of NF- κ B did not impact ANRIL expression levels in OGD/R-treated cells (Figure. 3A) or in MCAO/R-treated mice (Figure. 5B).

Discussion

ANRIL has previously been studied as a biomarker associated with IS risk (W Zhang et al. 2012). We observed that ANRIL expression was elevated in the hippocampus of MCAO/R mice, consistent with previous reports (Tan et al. 2019; J Yang et al. 2018). Our results revealed that ANRIL knockdown reduced OGD/R-induced injury and associated inflammation, whereas ANRIL overexpression aggravated these phenotypes. ANRIL knockdown suppressed neuroinflammation by suppressing NF- κ B expression both *in vitro* and *in vivo*. A previous study demonstrated that knockdown of circ-ANRIL improved OGD/R-induced cell damage, apoptosis, and inflammatory responses by inhibiting the NF- κ B pathway in OGD/R-induced human brain microvascular endothelial cells (Jiang et al. 2020). Our data and this study together thus confirmed the protective effect of ANRIL knockdown in IS. However, another study reported that the overexpression ANRIL alleviated OGD-induced cell injury, as evidenced by increased cell viability and reduced apoptotic cell death, through a mechanism dependent upon the negative regulation of Mcl-1

expression in the rat adrenal medulla-derived pheochromocytoma PC-12 cell line (B Liu et al. 2019). While there is thus substantial evidence that ANRIL plays a role in IS, the mechanismft basis for this role requires further clarification. Our results provide further support for a model wherein ANRIL alleviates inflammation and thus influences IS pathophysiology.

It has been shown that NF- κ B expression is increased in patients with acute ischemic stroke (Anrather et al. 2016; Du et al. 2019). Enhanced NF- κ B expression and activation in MCAO /R mice results in the uncontrolled release of the downstream pro-inflammatory cytokines such as IL-1 β , IL-6, TNF- α , and iNOS, which play vital roles in neuronal injury (W. Liang et al. 2019; Simmons et al. 2016). Suppressing NF- κ B activity is thus critical as a means of defending the neural parenchyma against brain ischemia. Our results indicated that ANRIL knockdown suppressed neuroinflammation by reducing NF- κ B expression in cell lines and animal models of IS.

The NF- κ B transcription factor can be composed of homo- or hetero-dimers of the p50, p52, p65, RelA-B, and c-Rel proteins, with p65 being essential for NF- κ B functionality. We therefore measured p65 levels as a readout for overall NF- κ B activity. Under steady-state conditions, this transcription factor is normally found in the cytoplasm bound to its inhibitor protein I κ B. The phosphorylation and degradation of I κ B ultimately result in NF- κ B activation, in turn driving the upregulation of damaging pro-inflammatory factors that aggravate IS-induced neuronal damage(Cheng et al. 2019; Weidong Liang et al. 2019). We detected significantly elevated total NF- κ B levels in OGD/R-treated HT22 cells and in hippocampal tissues from MCAO/R mice. These increases were likely attributable to a combination of hypoxia- and hypoglycemia-induced *de novo* NF- κ B transcription and the release of NF- κ B from its interaction with I κ B. However, as ANRIL is a long noncoding RNA, it is only able to directly interact with NF- κ B at the mRNA level and cannot influence this protein directly, preventing it from disrupting NF- κ B protein activity following I κ B dissociation. As ANRIL might influence total NF- κ B expression by reducing *de novo* NF- κ B transcription of mRNA NF- κ B, and because translation is governed by cytoplasmic ribosomes, we therefore studied total NF- κ B levels in our samples without analyzing the subcellular localization of this transcription factor via Western blotting. Additionally, cellular immunofluorescent staining results indicated that si-ANRIL was able to reduce cytoplasmic NF- κ B levels in HT22 cells after OGD/R.

Our results revealed that ANRIL was upregulated in OGD/R-treated HT22 cells. However, ANRIL overexpression alone in cells cultured under normal conditions did not induce cellular injury. The reasons might be that overexpressed ANRIL need to interact with other damaging mechanisms induced by OGD/R as a means of driving enhanced cellular damage.

IS is associated with damage to the cortical regions of the brain, which contain high levels of astrocytes and microglia. Following IS, these cell types produce large volumes of pro-inflammatory cytokines and chemokines (Jayaraj et al. 2019; B Zhang et al. 2019). It is possible that ANRIL may play distinct roles in cortical astrocytes and microglia in MCAO/R model, but future research will be required to support such a hypothesis.

Long noncoding RNAs are capable of promoting or suppressing the expression of specific target genes through a range of mechanisms including transcription factor recruitment, regulation of mRNA splicing, chromatin modification, the formation of heterogeneous nuclear ribonucleoprotein complexes, or via directly interacting with certain RNA or DNA targets through complementary base-pairing (Kopp et al. 2018). In the present study, we found that ANRIL knockdown reduced NF- κ B expression in MCAO/R mice. In the future, we will explore the mechanistic basis for this ANRIL-mediated regulation of NF- κ B and will establish the cause of ANRIL upregulation in IS.

Conclusion

Our results indicate that ANRIL plays a functionally important role in the pathology of cerebral ischemia reperfusion injury. ANRIL knockdown can suppress the expression of NF- κ B and consequent neuroinflammation in the hippocampus of MCAO/R mice, suggesting that ANRIL might be a potentially viable therapeutic target to diminish neuroinflammation in IS patients.

Declarations

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Compliance with Ethical Standards

Animals were treated in accordance with animal ethics standards throughout the animal experiment.

Conflict of Interest

The authors declare that they have no conflicts of interest.

Consent for Publication

All authors agree to publish.

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Figures

Figure 1

ANRIL expression in MCAO/R mice and in OGD/R HT22 cells (A) ANRIL levels in mice hippocampal samples collected at 0, 8, 16, and 24 h post-MCAO were measured by qRT-PCR, n = 6. (B) ANRIL expression in HT22 cells was measured at 0, 8, 16, and 24 h post-OGD/R by qRT-PCR in separate assays. *P < 0.05 vs. Sham or untreated control; One-way ANOVA with Tukey’s post hoc test.

Figure 2

The impact of ANRIL knockdown or overexpression in OGD/R HT22 cells (A) Successful ANRIL knockdown or overexpression in OGD/R HT22 cells was confirmed by qRT-PCR. (B) Cell viability under normal conditions was assessed via MTT assay. (C) OGD/R cell viability was assessed via MTT assay. (D, E) The percentage of apoptotic cells was assessed via Annexin V-FITC/PI staining. (F) mRNA NF-κB expression was assessed by qRT-PCR. (G, H) p-P65 and P65 protein levels were assessed via Western blotting; (I) IL-1β, IL-6, TNF-α, and iNOS levels were measured by ELISA. *P < 0.05 vs. Control; # P < 0.05 vs. OGD/R group; One-way ANOVA with Tukey's post hoc test.

Figure 3

The impact of ANRIL knockdown and NF-κB overexpression in OGD/R HT22 cells (A) ANRIL expression was quantified via qRT-PCR. (B) mRNA NF-κB expression was quantified via qRT-PCR. (C) OGD/R treatment-associated cell viability was assessed via MTT assay. (D-E) p-P65 and P65 protein levels were measured via Western blotting. (F) IL-1β, IL-6, TNF-α, and iNOS levels were quantified via ELISA. (G) ANRIL knockdown decreases P65 immunofluorescence in OGD/R cells, Scale bar: 25 μm. *P < 0.05 vs. Control; # P < 0.05 vs. OGD/R group; &P < 0.05 OGD/R+ si-ANRIL+ pcDNA-NF-κB vs. OGD/R +pcDNA- NF-κB; One-way ANOVA with Tukey's post hoc test.

Figure 4

The impact of ANRIL knockdown in MCAO/R mice (A) Successful knockdown of ANRIL expression in MCAO/R mice was assessed via qRT-PCR, while negative control vectors had no impact. (B) The scores of Neurological Deficits. (C, D) The infarction cerebral area was measured via TTC staining. (E, F) Nissl staining was used to visualize neuronal loss, Scale bar: 100 μ m and 25 μ m. (G) Immunofluorescent analysis of mice hippocampus tissue using a NeuN antibody. $n=3$; * $P < 0.05$ vs. Sham; # $P < 0.05$ vs. MCAO/R group.

Figure 5

The impact of ANRIL knockdown and NF-κB overexpression in MCAO/R mice (A) Successful overexpression of NF-κB mRNA in MCAO/R mice was assessed via qRT-PCR, while negative control vectors had no impact; (B) ANRIL expression was assessed via qRT-PCR. (C) mRNA NF-κB expression was assessed via qRT-PCR. (D, E) Relative p-P65 and P65 protein levels were assessed via Western blotting. (F) IL-1β, IL-6, TNF-α, and iNOS levels were quantified via ELISA. (G) The scores of Neurological

Deficits. (H) Fall latency seconds of Rota-rod testing results. $n = 6$; * $P < 0.05$ vs. Sham; # $P < 0.05$ vs. MCAO/R group; & $P < 0.05$ MCAO/R+ si-ANRIL+ pcDNA-NF- κ B vs. MCAO/R +pcDNA-NF- κ B.