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Long non-coding RNA HCG11 silencing protects against cerebral ischemia/reperfusion injury through microRNA miR-381-3p to regulate tumour protein p53

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Abstract

Introduction: *Long non-coding RNA (LncRNA) plays a critical role in cerebral ischemia-reperfusion (CI/R) injury. The purpose of the current research was to investigate the regulatory role of the LncRNA human leukocyte antigen complex group 11 (HCG11) in CI/R injury and explore its potential mechanism.*

Material and methods: *The rat middle cerebral artery occlusion (MCAO) model was established to simulate CI/R injury in vivo. mRNA levels of HCG11, microRNA (miR)-381-3p, tumour protein p53, and neuro-inflammatory factors were detected by quantitative reverse transcription PCR (RT-qPCR). Bederson score and Longa score were assessed for neurological deficits. Triphenyl tetrazolium chloride (TTC) staining was used to examine the cerebral infarct volume. What is more, oxidative stress was evaluated by the commercial kit. Finally, the relationship between HCG11, miR-381-3p, and p53 was verified by a dual-luciferase reporter assay.*

Results: *HCG11 was elevated in MCAO rats. And it competitively bound miR-381-3p and down-regulated the expression of p53. Inhibition of HCG11 inhibited cerebral infarct volume and neurological deficits in MCAO rats, and inhibited the secretion of neuro-inflammation and the over-activation of oxidative stress, exerting the protective effect of CI/R injury. However, inhibition of miR-381-3p in rats significantly weakened the protective effect of depression of HCG11 in MCAO rats, resulting in increased cerebral infarction volume and neurological deficits, elevated neuro-inflammatory secretion, and oxidative stress activation.*

Conclusions: *The present research shows that LncRNA HCG11 silencing protects against cerebral ischemia/reperfusion injury through miR-381-3p to regulate p53.*

Key words: *HCG11, miR-381-3p, p53, CI/R injury.*

Introduction

The brain is the most sensitive organ to hypoxia and ischemia. Cerebral ischemia can cause brain cell death or necrosis [17,32]. The mortality rate due to ischemic cerebrovascular disease is as high as 60-80% [11]. Therefore, timely haemolysis treatment within an effective time, effective and rapid establishment of microvascular circulation, and restoration of blood reperfusion in the ischemic area are central to the treatment of cerebral ischemia [13]. However, previous studies have proved that reperfusion after ischemia can cause local damage to the brain tissue, and the function cannot be restored, leading to more serious brain dysfunction. And this cascade reaction is rapid [26]. Therefore, while restoring the blood perfusion in the ischemic region in time, inhibiting cerebral ischemia-reperfusion injury (CI/R injury) has become the focus of current research.

Non-coding RNA with a length of more than 200 nt is defined as long non-coding RNA (LncRNA), which participates in chromosome modification or reconstruction, transcription activation, and post-transcriptional processing

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[8]. According to reports, about 40% of abnormal LncRNAs are brain-specific, participate in the regulation of brain development, and are related to brain damage [4,10]. LncNEAT1 [28], LncMALAT1 [48], and LncSNHG12 [21] are involved in the pathological process of CI/R injury. As a member of the LncRNA family, LncRNA human leukocyte antigen complex group 11 (HCG11) has been reported to be involved in osteosarcoma [38], pancreatic cancer [42], and gastric cancer [49]. In cardiovascular and cerebrovascular disease, HCG11participates in the rupture of the atherosclerotic plaque by regulating vascular endothelial cells [12]. Atherosclerosis is the pathological basis of cerebral infarction and surrounding vascular disease. HCG11 is elevated in patients with atherosclerosis [25]. What is more, HCG11 has been reported to be involved in the expression and regulation of glioma, a malignant tumour of the central nervous system in adults [9]. However, the role of HCG11 in CI/R injury remains unclear.

microRNA (miRNA) is an endogenous non-coding small RNA molecule that partially or completely binds to the 3' untranslated region (3' UTR) of targeted mRNA, regulating the stability of the target and the process of transcription and translation. Moreover, there are reports that a variety of miRNAs are involved in the pathological process of CI/R injury, such as miR-670 [46], miR-202-3p [45], and miR-326-5p [14]. What is more, bioinformatics analysis indicated that HCG11 targets miR-381-3p. It has been reported that miR-381-3p overexpression promotes angiogenesis and inhibits inflammation to combat ischemic stroke [19]. Up-regulation of miR-381 promotes nerve repair in acute cerebral ischemia [30].

Therefore, we further explored whether miR-381-3p is involved in HCG11-mediated CI/R injury. Based on the aforementioned reports, the current research aims to explore the potential role of HCG11 in CI/R injury and reveal whether miR-381-3p and its potential target genes are involved in this regulatory process.

Material and methods

Ethics statement

All animal-related experiments were approved by the Animal Care and Use Institutional Committee of the Binzhou Medical University Hospital. In addition, the animal experiments in this study were carried out in strict accordance with the "Guidelines for the Care and Use of Laboratory Animals". In addition, the stress and pain of the animals used in this research were minimized.

Experimental animals

Adult male Sprague-Dawley (SD) rats, weighing 250 ±30 g, were purchased from the Experimental Animal Centre of the Chinese Academy of Sciences (Shanghai, China). All rats were placed in a controlled environment laboratory animal centre under a 12 h light/dark alternate cycle, they had free access to food and water. And the rats were raised in this environment for 1 week to adapt to the environment, and then the experiment was carried out.

Experimental grouping and intraventricular injection

Twenty-four SD rats were randomly divided into 4 groups (6 rats in each group): sham group, MCAO group, si-HCG11 group (MCAO + HCG11 small interfering RNA group), and si-NC group (MCAO + small interfering RNA negative control group). The first batch of rats was designed to detect the regulation of HCG11 on CI/RI. Subsequently, to examine the potential role of miR-381-3p in HCG11-interfering MCAO rats, 30 SD rats in the second batch were divided into 5 groups (6 rats in each group): MCAO group, si-HCG11 group, si-NC group, si-NC+antagomir NC group, and si-HCG11 + miR-381-3p antagomir group. The above-mentioned transfection vector was diluted with the *in vivo* transfection reagent Entranster^{TM} (Cat#186688-11-1, Engreen Biosystem, Beijing, China) and injected into the rat brain with a stereotactic apparatus (the stereotactic coordinates were 0.8 mm in the front and rear, 1.4 mm in the middle, and 3.5 mm in the back and abdomen) three days before the establishment of the MCAO model as previously studied [7,44]. The injection volume was 10 µl, including 2 µl of entranster^{TM} and 8 µl of transfection vector (including 500 pmol HCG11 siRNA or/and 8 nmol miR-381-3p antagomirs). Among them, si-HCG11 (si-HCG11 (5'-UUCUCCGAACGUGUCACGUTT-3') and negative control si-NC (5'-GCCAGAAUGUUCCUAUUUATT-3') were synthesized by Shanghai GenePharma. miR-381-3p antagomir (miR30017081-4-5), miR-381-3p agomir (miR40017081-4-5), negative control antagomir NC (miR3N0000002-4-5) and agomir NC (miR4N0000002-4-5) were purchased from Guangzhou RiboBio.

Construction of the middle cerebral artery occlusion (MCAO) rat model

The modified method of Longa *et al*. [27] was used to establish a rat middle cerebral artery occlusion (MCAO) model to induce CI/R injury. In short, the preoperative fasting for 12 h, injection of 10% chloral hydrate of 30 mg/kg was used for deep anaesthesia. Subsequently, an incision was made along the midline of the neck to separate the skin and muscle to expose the right common carotid artery (CCA) and its branches external carotid arteries (ECA), and internal carotid

arteries (ICA). Then, they were carefully separated under the operating microscope. After ligating the proximal end of the CCA and ECA, the 4-0 nylon thread was gently inserted from the right CCA through the ICA into the middle cerebral artery (MCA). After 2 h of ischemia, the nylon thread was removed. During the whole process, the rat was placed on a heating platform and a body temperature of 37 ±0.3°C was maintained, and there were no signs of peritonitis, pain, and discomfort. The rats were then reperfused for 24 h. The sham group used the same operation, that is, anesthetizing the rat, incised three arteries without ligation. The incision was sutured after 30 min. Additionally, an 80% reduction in cerebral blood flow, as measured by transcranial Doppler, confirmed the success of the model construction and that all rats were successfully modelled.

Neurological deficient assessment

After 24 h of reperfusion, two experimenters who did not know the experimental group used the Bederson [5] and Longa [27] neurological deficient scores for evaluation based on previous research reports. In summary, the Bederson score range is 0-5: 0 = no defect, 1 = abnormal forelimb bending; 2 = abnormal forelimb bending and low lateral push resistance; 3 = single hover; 4 = longitudinal rotation or epileptic activity; and 5 = loss of moto ability. The Longa score ranges from 0 to 4, where 0 represents the absence of neurological defects; 1 indicates that the contralateral forelimb of rats cannot be fully extended; 2 means that the rat's body leans to the hemiplegic side when walking; 3 indicates that the body of rats leans to the hemiplegic side when walking; and 4 indicates loss of autonomy consciousness in rats. Both scoring standards are interpreted: the higher the score, the more serious the neurological deficit.

Infarct volume assessment

After assessment of neurological deficit, cerebral infarct volume was assessed by triphenyl tetrazolium chloride (TTC) staining based on previous studies [37]. The rats were anesthetized by injecting 300 mg/kg chloral hydrate (10%). The rats were euthanized by cervical dislocation and their brain tissues were collected. And then the brain tissue was washed with phosphate-buffered saline (PBS), and then it was placed in the refrigerator at –20°C for 30 min. 6 consecutive coronary brain sections were taken from brain tissue with a thickness of 2 mm and incubated at 2% TTC (Cat#G3005, Solarbio, Beijing, China) solution for 30 min. It was then fixed with 4% paraformaldehyde. Since infarcted tissue will not stain white due to loss of mitochondrial enzyme activity, normal tissue will be stained red by TTC solution. The infarct volume was calculated by Image-Pro Plus 6.0 software. The percentage of infarct volume was calculated by the following equation: infarct volume (%) = [(normal hemisphere volume – the non-infarct volume of infarct side)/normal hemisphere volume] × 100%.

Oxidative stress index detection

RIPA lysate (Cat#A0277, Beyotime, Shanghai, China) was added to the brain sample tissue and incubated on ice for 30 min to ensure that the sample tissue was fully lysed, and the supernatant was collected after centrifugation at 13000 rpm for 30 min. Then, according to the instructions of the SOD detection kit (Cat# A001-3-2, Nanjing Built Bioengineering Institute, Nanjing, China), 20 µl of sample tissue and 0.22 ml of reagent were mixed and incubated at 37°C. After 20 min, a microplate reader was used to detect the absorbance (OD) at 450 nm. MDA detection was performed with an MDA detection kit (Cat#A003-1-2, Nanjing Built Bioengineering Institute, Nanjing, China), which was to mix 100 µl tissue supernatant with 3 ml of reagents, and incubate in a water bath at 95°C for 40 min. Subsequently, it was centrifuged at 3500 rpm for 10 min, and the OD at 532 nm was detected with the help of a microplate reader. SOD was detected by a 2'7-dichlorofluorescein diacetate probe (DCFH-DA) according to the SOD detection kit (Cat#E004-1-1, Nanjing Built Bioengineering Institute, Nanjing, China). In short, the tissue was prepared into single-cell suspension by enzyme, resuspended with DCFH-DA, incubated at 37°C for 1 h for fluorescent labelling, centrifuged at 1000 g for 10 min, and then the cell pellet was collected. After washing with PBS, the OD at 560 nm was detected.

Reverse transcription-quantitative PCR (RT-qPCR)

Trizol was used to extract total RNA from tissues, and then NanoDrop was used to detect the concentration and quality. A260/280 of RNA is 1.8-2.1. Reverse transcription of 1 µg RNA to synthesize complementary single-stranded cDNA with the help of the HiFiScript cDNA Synthesis Kit (Cat#CW2569, CW Biotech, Beijing, China) and miRNA cDNA Synthesis Kit (Cat#CW2141, CW Biotech, Beijing, China). Then cDNA, primers, UltraSYBR Mixture (Cat#CW0957, CW Biotech, Beijing, China), or miRNA qPCR Assay Kit (Cat#CW2142, CW Biotech, Beijing, China) were mixed and mRNA expression was detected under the LightCycler PCR detection system. The levels of HCG11, p53, and inflammatory factors such as tumour necrosis factor α (TNF-α), transforming growth factor β1 (TGF-β1), and interferon γ (IFN-γ) were standardized to GAPDH,

and the relative expression levels of miR-381-3p were standardized to U6. The primer sequence used was as follows: HCG11 forward, 5'-GCTCTAGCCATCCTGCTT-3' and reverse, 5'-TCCCATCTCCATCAACCC-3'; miR-381-3p forward, 5'-TACTTAAAGCGAGGTTGCCCTT-3' and reverse, 5'-GGCAAGCTCTCTGTGAGTA-3'; TNF-α forward, 5'-ATGAGCACAGAAAGCATGATCCGC-3' and reverse, 5'-CCAAAGTAGACCTGCCCGGACTC-3'; IFN-γ forward, 5'-GAAAGCCTAGAAAGTCTGAATAACT-3' and reverse, 5'-ATCAGCAGCGACTCCTTTTCGGCTT-3'; TGF-β forward, 5'-GACCGCAA CAACGCCATCTA-3' and reverse, 5'-GGCGTATCAGTGGGGGTCAG-3'; GAPDH forward, 5'-GAGTCCACTGGCGTCTTCAC-3' and reverse, 5'-ATCTTGAGGCTGTTGTTFTCATACTTCT-3'; U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'. The following program was used for RT-qPCR: 95°C for 3 min followed by 40 cycles of 95°C for 10 s, and 60°C for 30 s.

Western blot analysis

Rat brain tissue was collected and RIPA lysate was supplemented to ensure adequate cleavage of protein and centrifuged in a centrifuge, supernatant was collected and the lower precipitate was removed. Protein concentration was determined by the BCA protein assay reagent kit (Cat#P0012S, Beyotime Biotechnology, Shanghai, China) [7]. Then 30 µg protein was separated and electrophoresed with 10% sodium dodecyl sulfate-polyproacrylaminde gel (80 V, 120 min), and then transferred to PVDF membrane. It was blocked by 5% bovine serum albumin. The PVDF membrane was then paired with mouse monoclonal anti-p53 (1 : 1000, Cat#60283-2-Ig, Proteintech, Wuhan, China) or mouse monoclonal anti-GAPDH (1 : 1000, Cat# 60004-1-Ig, Proteintech, Wuhan, China) for overnight incubation at 4°C. After TBST was cleaned three times, the PVDF membrane was incubated with a horseradish peroxidase-labelled secondary antibody (goat anti-mouse IgG, 1 : 5000, Cat#SA00001-1, Proteintech, Wuhan, China) at room temperature for 1.5 h. Finally, the enhanced chemiluminescence agent was added to the PVDF membrane to observe the protein bands in the imaging system. Finally, the grey value of protein was calculated by Image J software.

Luciferase reporter assay

Online bioinformatics software DIANA tools and miRDB analysis revealed the targeting relationship between miR-381-3p and the 3'UTR of HCG11 and p53. The WT HCG11 sequence or WT p53 mRNA containing the miR-381-3p binding site was amplified and inserted into the dual-luciferase reporter gene vector pmirGLO to construct recombinant reporter plasmids pmirGLO-HCG11-wild-type (WT) and pmirGLO-p53-WT. Site-directed mutations (MUT) were performed on the binding sites of HCG11 and p53 with miR-381-3p, and they were inserted into pmirGLO vectors to construct recombinant mutant plasmids pmirGLO-HCG11-MUT and pmirGLO-p53-MUT. The commonly used tool cell, 293T was inoculated in a 96-well plate, and when it reached the logarithmic growth phase, was mixed the recombinant plasmid with miR-381-3p antagomir or miR-381-3 agomir, mixed with the transfection reagent, and dropped into the cells. After 48 h, the luciferase activity was measured by the luciferase reporter assay system (Cat#E1601, Promega, Madison, WI, USA).

Statistical analysis

GraphPad Prism 6 performed statistical analysis of data and drew figures. The results were expressed as mean ±SD after at least 3 replicates. The differences between groups were compared by one-way or two-way analysis of variance (ANOVA) followed by Tukey's post-hoc comparison test. A difference was considered significant at $p < 0.05$.

Results

Here, we found that HCG11 is involved in cerebral infarction, peripheral vascular disease, and neurological disease. We hypothesized that HCG11 must play an important role in the occurrence of CI/R injury. The objective is to detect the role of HCG11 in CI/R injury and explore its possible mechanism. The MCAO model was constructed to evaluate the effects of HCG11 on cerebral infarction volume, nerve defects, oxidative stress, and neuroinflammation. The targeting relationship between miR-381-3p and HCG11 was detected by dual-luciferase report luciferase, and the correlation between miR-381-3p and HCG11 was further verified by a rescue experiment.

HCG11 levels in the rat MCAO model were significantly increased

To investigate the potential role of HCG11 in CI/R injury, we first detected the level of HCG11 in the brain tissue of MCAO rats. The results confirmed that compared with the sham group, HCG11 in the MCAO group was dramatically increased ($p < 0.001$, Fig. 1A). However, when HCG11 small interfering (si) RNA was injected into the brain, its level was typically suppressed ($p < 0.001$, Fig. 1A).

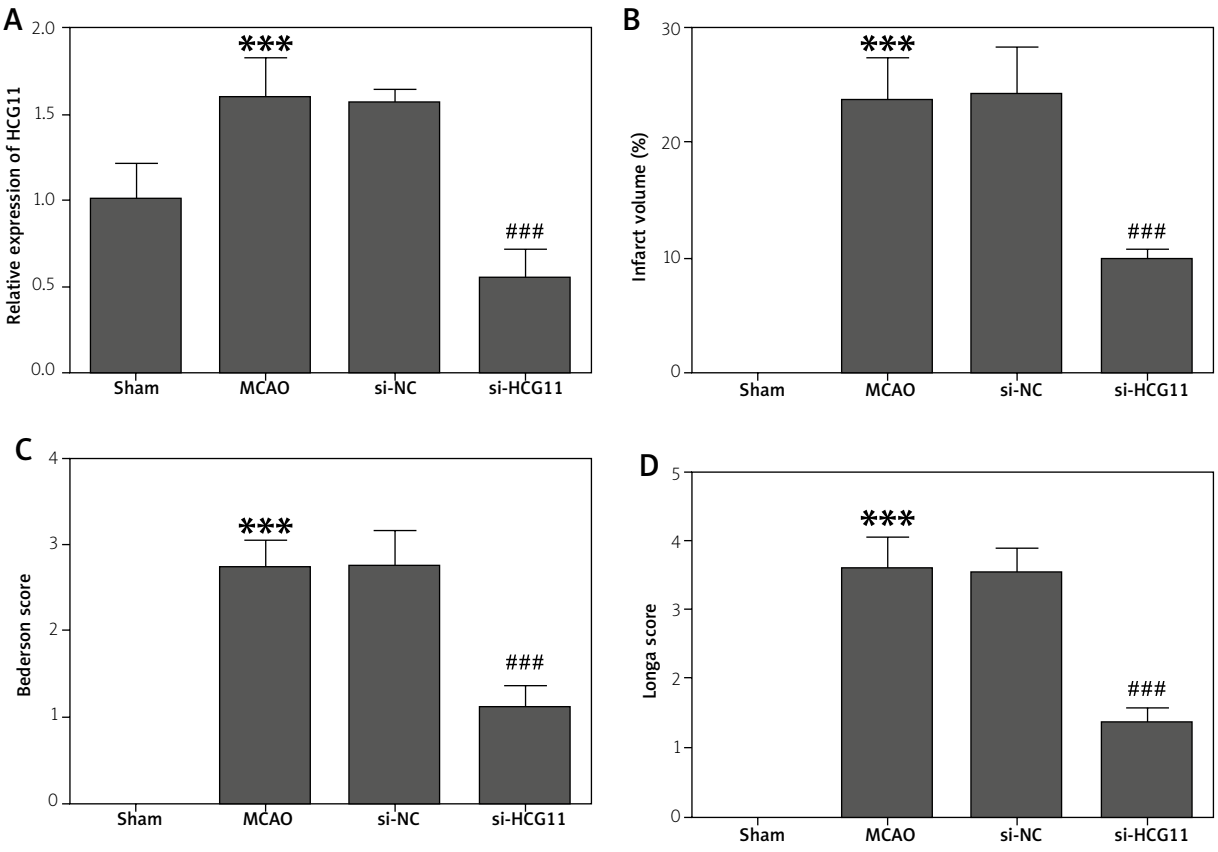

Fig. 1. Evaluation of cerebral infarction volume and nerve deficits in the middle cerebral artery occlusion (MCAO) rat model. **A**) RT-qPCR confirmed that HCG11 mRNA was increased in the MCAO rat model, and intracerebral injection of si-HCG11 decreased the levels of HCG11 in the MCAO rat model. **B**) Inhibition of HCG11 can significantly improve the increase of cerebral infarction volume in the MCAO model of rats. Bederson score (**C**) and Longa score (**D**) were used to evaluate the effect of HCG11 inhibition on the neural deficit. $N = 6$, one way ANOVA with Tukey's post-hoc analysis; $^{***}p < 0.001$ MCAO group vs. sham group; $^{###}p < 0.001$ si-HCG11 group vs. si-NC group.

Reduced HCG11 reduced cerebral infarction volume and attenuated neurological deficits in CI/R injury of MCAO rats

Subsequently, we examined the effect of HCG11 on cerebral infarct volume and neurological deficits in MCAO model rats. The cerebral infarction volume of MCAO rats was increased compared to the sham group, but low HCG11 markedly inhibited the increase of cerebral infarction volume ($p < 0.001$, Fig. 1B). Finally, the Bederson score and Longa score were used to evaluate the neurological deficits of rats in each group. During the research, it was found that the activities of the rats in the sham group were normal, while the MCAO rats showed different degrees of right-leaning or right-turning behaviour, and the neurological deficit scores were dramatically higher than those in the sham group ($p < 0.001$, Fig. 1C, D). However, when HCG11 was inhibited, a small number of rats turned to the right, the behavioural symptoms of the nerve defect were significantly improved, and the neurological deficit scores were dramatically reduced. It suggests that HCG11 may play an indispensable role in CI/R injury.

Inhibition of HCG11 alleviated oxidative stress and neuroinflammation in the rat MCAO model

Previous studies have confirmed that CI/R injury can include the secretion of neuroinflammatory and excessive production of oxidative stress, thereby aggravating brain injury [18]. The results showed that ROS and MDA levels in MCAO model rats were increased, and the levels of SOD

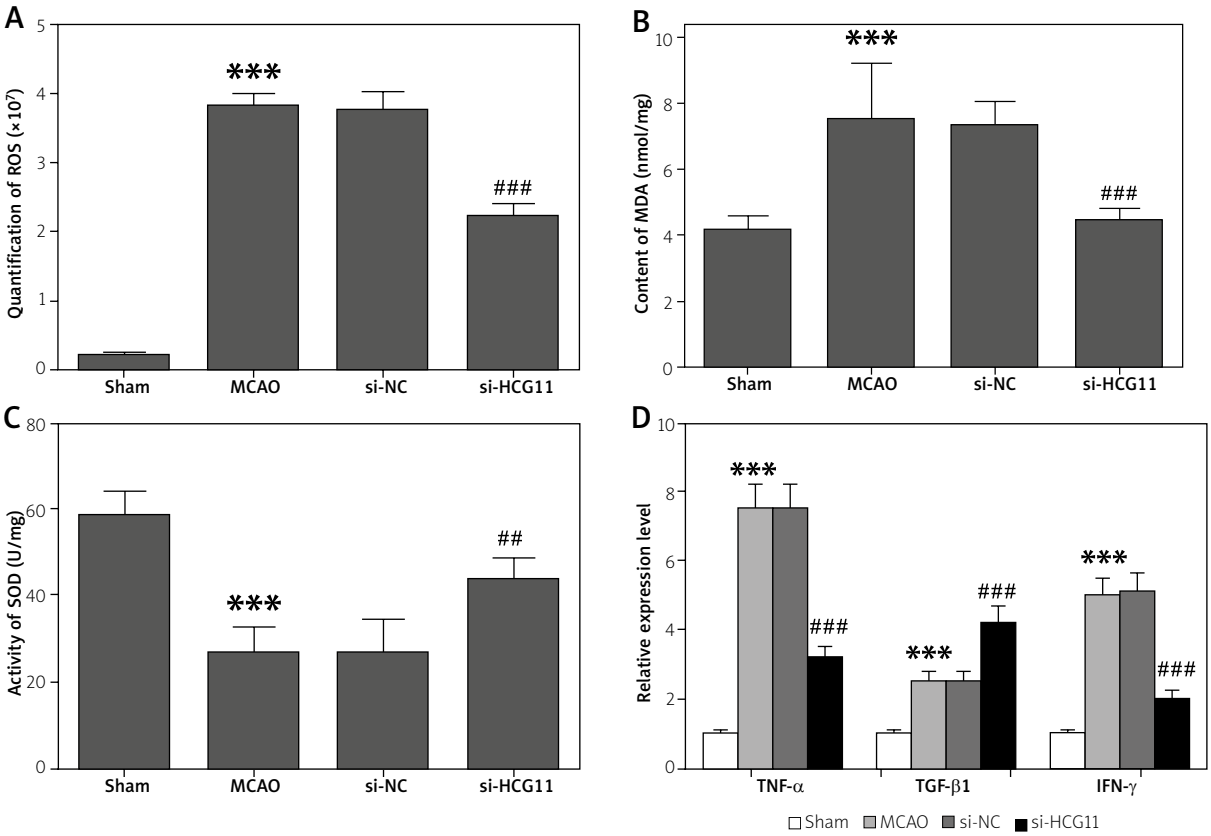

Fig. 2. Effect of HCG11 on oxidative stress and inflammation. Oxidative stress indexes ROS (**A**) and MDA (**B**) were increased in the middle cerebral artery occlusion (MCAO) model, while SOD (**C**) was significantly decreased in the rats. While the HCG11 was inhibited, these indexes changed. **D**) Decreased HCG11 markedly inhibited the secretion of proinflammatory factors and decreased anti-inflammatory factors in the MCAO model of rats. $N = 6$, one-way or two-way ANOVA followed by Tukey's post-hoc comparison test; $^{***}p < 0.001$ MCAO group vs. sham group; $^{\#\#\#}p < 0.001$ si-HCG11 group vs. si-NC group.

were decreased ($p < 0.001$, Fig. 2A-C). However, when inhibited HCG11, the increase or decrease of these indictors of oxidative stress was improved. In addition, neuroinflammatory factors tumour necrosis factor α (TNF-α), interferon γ (IFN-γ), and transforming growth factor β1 (TGF-β1) were increased in the MCAO rats. However, when HCG11 was inhibited, these indicators were significantly reduced or increased, that is, the neuroinflammatory responses were improved ($p < 0.001$, Fig. 2D). The results confirmed that HCG11 may be involved in CI/R injury through the regulation of oxidative stress and neuroinflammation.

HCG11 inhibited the expression of miR-381-3p by targeting it

LncRNA competes with miRNA response elements to bind miRNA, thereby preventing miRNA from binding to the target. Therefore, we speculated that HCG11 might act as ceRNA to regulate miRNA. To explore how HCG11 participates in CI/R injury by regulating neuroinflammation and oxidative stress, we found a binding site between miR-381-3p and HCG11 through online software (Fig. 3A). The dual-luciferase reporter assay confirmed the targeting relationship between them. miR-381-3p agomir or antagomir dramatically reduced or increased the HCG11-WT luciferase activity ($p < 0.001$, Fig. 3B), but the HCG11-MUT was unchanged. What is more, the study also found that miR-381-3p was significantly reduced in the MCAO model, and was significantly reversed when HCG11 was inhibited ($p < 0.001$, Fig. 3C). The results showed that miR-381-3p was the target of HCG11, and its expression was negatively regulated by HCG11.

Reducing miR-381-3p reverses the inhibitory effect of si-HCG11 on the infarct volume and nerve deficits in rats

We further investigated the regulatory effect of miR-381-3p on CI/R injury nerve deficits and infarct volume inhibition. As shown in Figure 4A, inhibition of HCG11 in the MCAO rat model increased the mRNA level of miR-381-3p in brain tissue, while intracranial injection of miR-

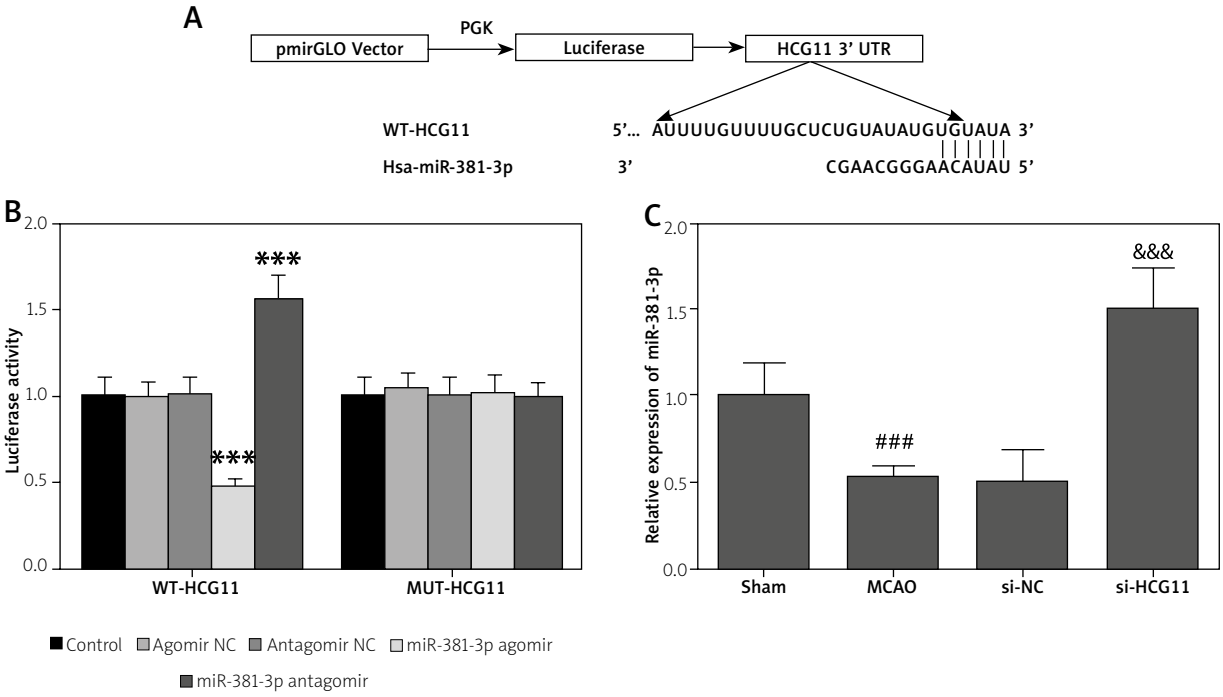

Fig. 3. The targeting relationship between HCG11 and miR-381-3p. **A**) Potential binding site of miR-382-3p on luciferase recombinant plasmid pmirGLO-HCG11-WT. **B**) Luciferase reporter assay detection confirmed the targeted binding relationship between HCG11 and miR-381-3p. **C**) Reduced HCG11 promoted the expression of miR-381-3p in the middle cerebral artery occlusion (MCAO) model of rats. $N = 6$, one-way or two-way ANOVA followed by Tukey's post-hoc comparison test; $^{***}p < 0.001$ miR-381-3p agomir or antagomir group vs. control group; $^{\#\#\#}p < 0.001$ MCAO group vs. sham group; $^{\&\&\&}p < 0.001$ si-HCG11 group vs. si-NC group.

381-3p antagomir attenuated the increase. In addition, co-injection of si-HCG11 and miR-381-3p antagomir in MCAO rats dramatically reversed the reduction of cerebral infarction volume in MCAO model rats by si-HCG11 ($p < 0.001$, Fig. 4B). Similar to the results of cerebral infarction volume, miR-381-3p antagomir also restored neurological deficits in MCAO rats ($p < 0.001$, Fig. 4C, D). The above results indicate that the reduction of miR-381-3p could eliminate the protective effect of si-HCG11 on infarct volume and nerve deficits, suggesting that HCG11 may participate in the pathological process of CI/R injury by regulation of miR-381-3p.

Down-regulation of miR-381-3p attenuates the inhibitory effect of si-HCG11 on oxidative stress and neuroinflammation in MCAO rats

Subsequently, we examined the effect of down-regulation of miR-381-3p on the inhibition of oxidative stress and neuroinflammation by si-HCG11 in rats. As shown in Figure 5, si-HCG11 significantly reduced oxidative stress and neuroinflammation in MCAO rats, playing a protective role. However, this protective effect was weakened by down-regulation of miR-381-3p, the indicators ROS and MDA were significantly increased, and SOD was significantly reduced. In neuroinflammation, the pro-inflammatory factor TNF-α, IFN-γ was increased, and the anti-inflammatory factor TGF-β1 was decreased ($p < 0.001$). These results suggest that HCG11 may be involved in the pathological process of CI/R injury by inhibiting the level of miR-381-3p, regulating oxidative stress and neuroinflammation.

miR-381-3p binds with the 3'UTR of p53

Previous studies have confirmed that p53 is related to CI/R injury and participates in its regulation [33]. Through online target gene prediction data, we found that miR-381-3p has a binding region with the 3'UTR of p53 (Fig. 6A). What is more, the luciferase reporter assay verified the targeting relationship between them, namely, miR-381-3p agomir or antagomir significantly inhibited or increased the luciferase activity of WT-p53, but did not affect the luciferase activity of Mut-p53

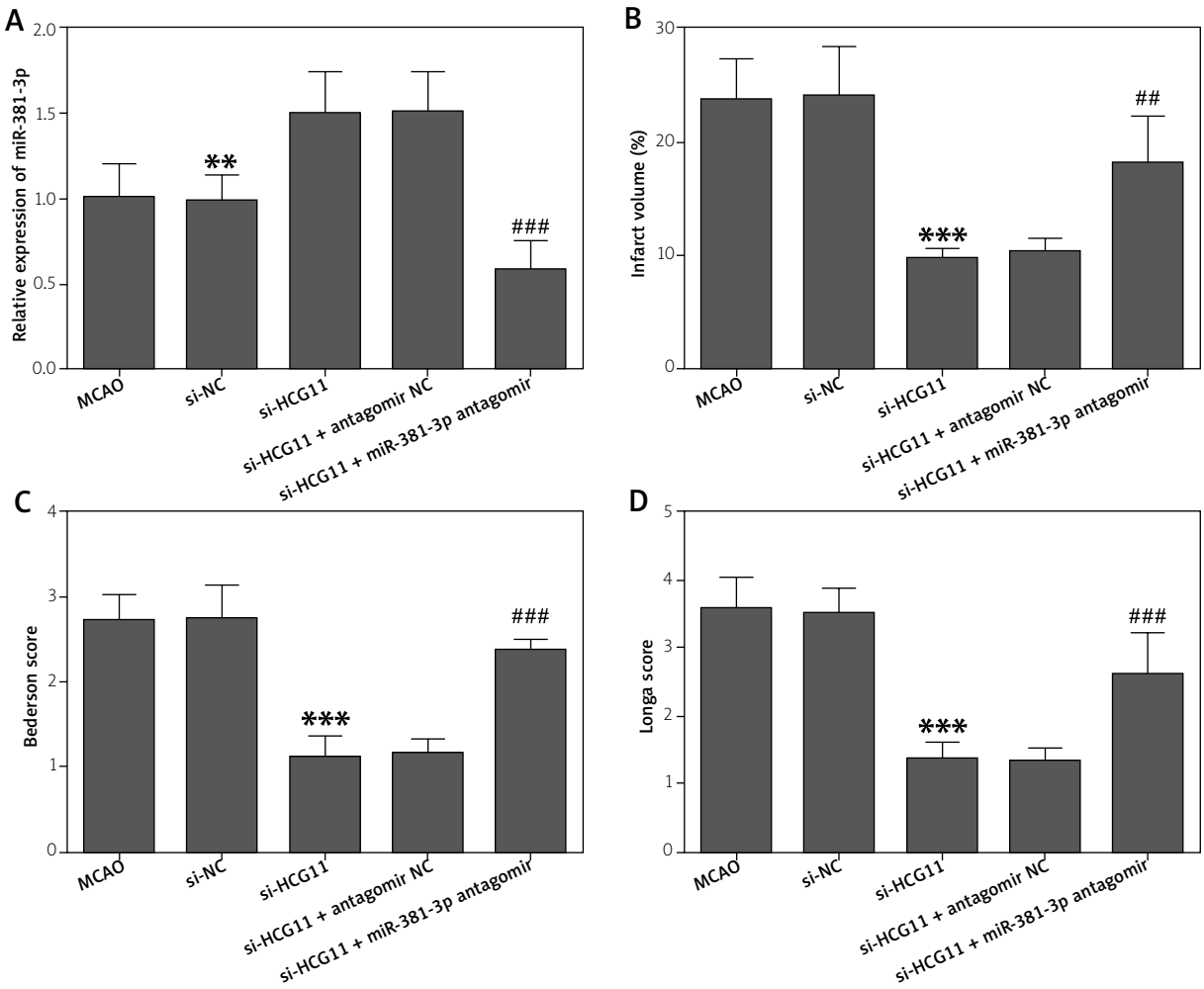

Fig. 4. Inhibition of miR-381-3p eliminated the inhibitory effects of si-HCG11 on cerebral infarct volume and nerve deficits. **A**) The level of miR-381-3p in the brain tissue of rats after intracranial injection of miR-381-3p antagomir. **B**) Effect of inhibiting miR-381-3p level on cerebral infarction volume in the middle cerebral artery occlusion (MCAO) model of rats. Bederson score (**C**) and Longa score (**D**) were used to detect the changes of nerve deficits in rats after miR-381-3p decreased. $N = 6$, one-way ANOVA followed by Tukey's post-hoc comparison test; $^{**}p < 0.05$, $^{***}p < 0.001$ si-HCG11 group vs. MCAO group; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ si-HCG11 group + antagomir NC group vs. si-HCG11 + miR-381-3p antagomir.

($p < 0.001$, Fig. 6B). Finally, we also found that si-HCG11 inhibited the mRNA and protein level of p53 in MCAO rat tissues, but the inhibition of miR-381-3p significantly reversed the inhibitory effect of si-HCG11 on p53 and promoted the mRNA and protein expression of p53 ($p < 0.001$, Fig. 6C, D). The above study results confirmed that p53 is the target of miR-381-3p and HCG11 may regulate CI/R injury through miR-381-3p/p53 axis.

Discussion

While reducing reperfusion injury, it is essential to restore blood flow in the cerebral ischemic area. At present, the potential importance of LncRNA in the physiology of cerebrovascular diseases is increasingly emerging, and it can be used as a key target for CI/R injury treatment [2]. In the current study, we confirmed that LncRNA HCG11 was dramatically up-regulated in the brain tissue of MCAO model rats. And inhibiting HCG11 in MCAO rats can significantly reduce cerebral infarction volume, decrease neurological deficits, inhibit oxidative stress injury and neuroinflammatory injury, and play as a protective role. However, inhibiting miR-381-3p dramatically weakened the protective effect of HCG11 in MCAO in rats, and increased the chances of the above-mentioned reaction. Moreover, the attenuation of miR-381-3p may be achieved through targeted regulating of p53.

Stroke is a common cause of disability and death worldwide; 80-85% of cases are caused by cerebral

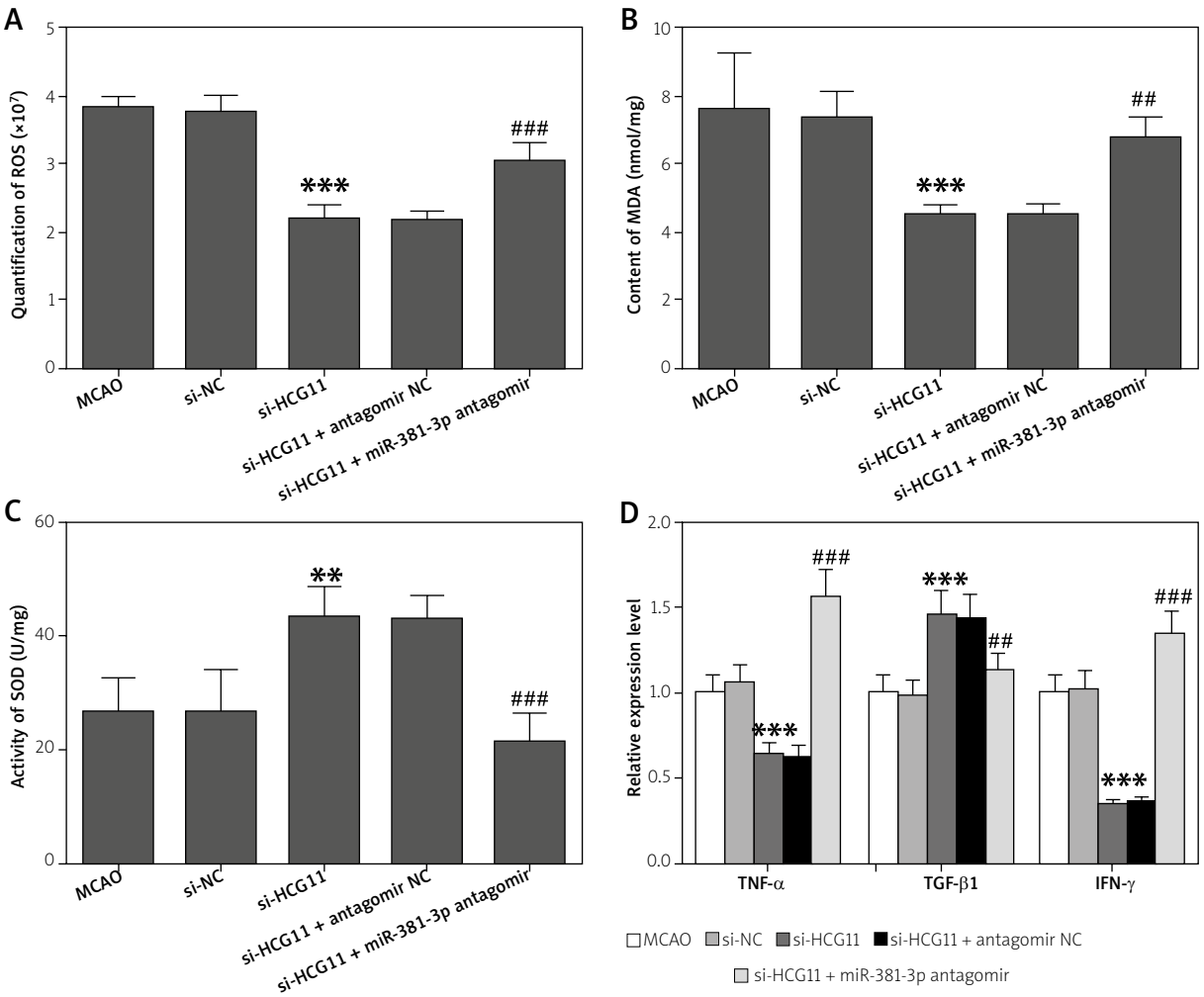

Fig. 5. Inhibition of miR-381-3p eliminated the inhibitory effects of si-HCG11 on neuroinflammation and oxidative stress in the middle cerebral artery occlusion (MCAO) model. The changes of oxidative stress indicators ROS (**A**), MDA (**B**), SOD (**C**) and neuroinflammation (**D**) after inhibition of miR-381-3p in the MCAO model. $N = 6$, one-way ANOVA followed by Tukey's post-hoc comparison test; $^{**}p < 0.05$, $^{***}p < 0.001$ si-HCG11 group vs. MCAO group; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ si-HCG11 group + antagomir NC group vs. si-HCG11 + miR-381-3p antagomir.

ischemia [6]. Although the effective period to restore blood reperfusion in the ischemic area is the key to the treatment of cerebral ischemia, it often leads to CI/R injury [16]. At present, more and more evidence suggests that LncRNA is an important regulator of various physiological and pathological responses, including CI/R injury. For example, knockdown LncXIST can reduce nerve injury and inflammatory response in MCAO mice [36]. Gastrodin reduces CI/R injury neuroinflammation and pyrolysis by regulating LncNEAT1 [50]. Upregulation of LncC2dat2 promoted CI/R injury-induced neuronal autophagy and apoptosis [43].

As a kind of LncRNA located on human chromosome 6p22.2, HCG11 has been reported in detail for its regulatory role in a variety of cancers. For example, it is related to the malignant biological characteristics of pancreatic cancer [42], can regulate the malignant phenotype of non-small cell lung cancer [34], participates in the cisplatin resistance of colorectal cancer [40], and can also accelerate the progression of hepatocytes [20]. What is worthy of our attention is that HCG11 also plays an important role in cardiovascular and cerebrovascular disease. Atheromatous plaque rupture is the main cause of most cardiovascular and cerebrovascular diseases, and HCG11 is highly expressed in patients with plaque vulnerability [12]. In addition, atherosclerosis is also the pathological basis of cerebral infarction and peripheral vascu-

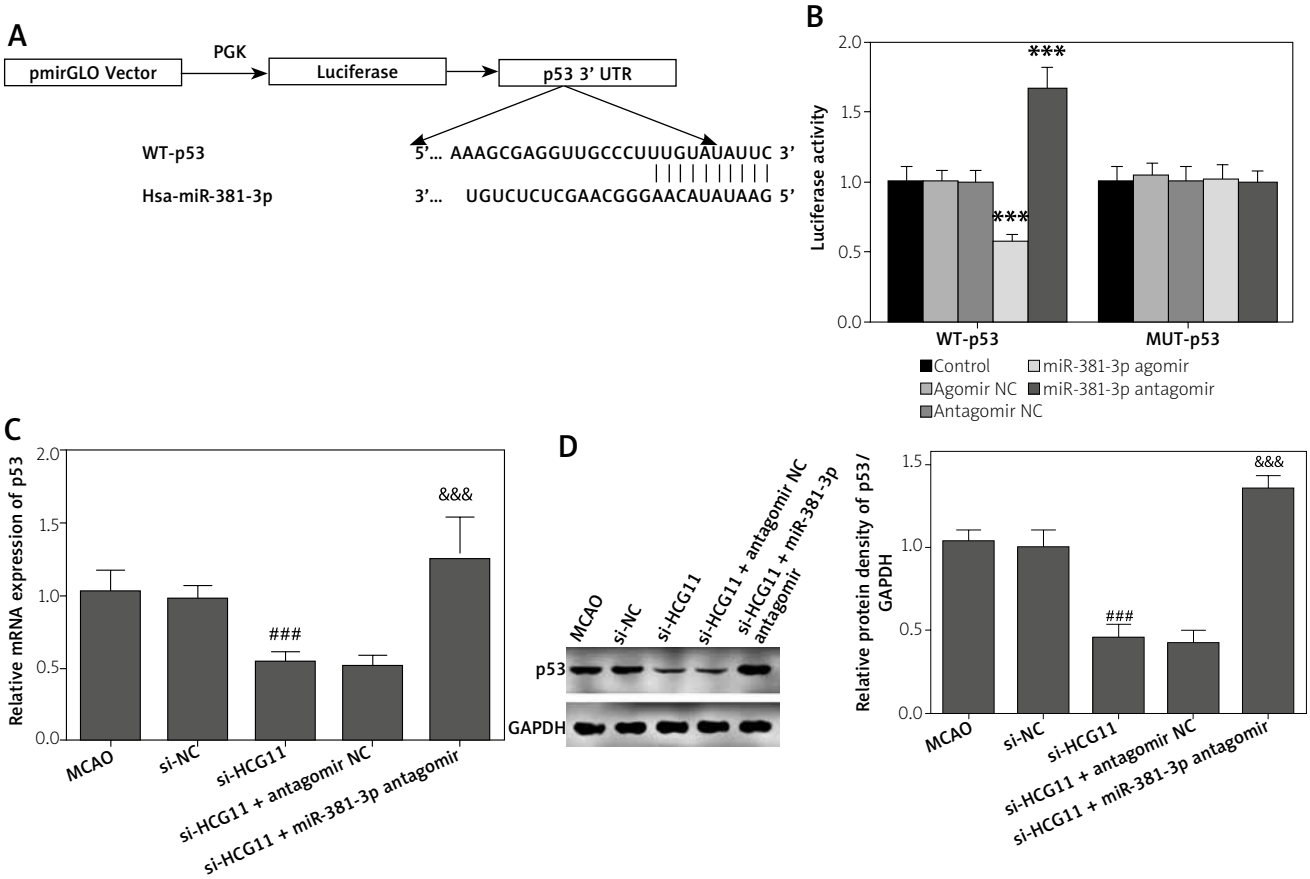

Fig. 6. miR-381-3p targeted binding with the 3'UTR of p53. **A**) The targeted binding site of p53 3'UTR and miR-381-3p. **B**) Luciferase reporter assay detection confirmed the targeted binding relationship between p53 and miR-381-3p. The levels of p53 mRNA (**C**) and protein (**D**) in the brain tissue of the middle cerebral artery occlusion (MCAO) model of rats after injection of si-HCG11 or co-injection of si-HCG11 and miR-381-3p antagomir. $N = 6$, one-way or two-way ANOVA followed by Tukey's post-hoc comparison test; $^{***}p < 0.001$ miR-381-3p agomir or antagomir group vs. control group; $^{\#\#\#}p < 0.001$ si-HCG11 group vs. MCAO group; $^{\&\&\&}p < 0.001$ si-HCG11 group + antagomir NC group vs. si-HCG11 + miR-381-3p antagomir.

lar disease, and HCG11 is dramatically increased in AS patients [25]. Human umbilical vein colorectal cells are involved in CI/R injury [24], and HCG11 can regulate the proliferation of these cells and inhibit angiogenesis [12]. However, the role of HCG11 in CI/R injury remains unclear. Based on the above research, we detected the level of HCG11 in the brain tissue of the MCAO rat model and found that the level of HCG11 was dramatically increased. In addition, the celebration infarction volume of the MCAO rat model increased, and the neurological deficit was severe. However, when HCG11 depleted, the cerebral infarction volume and neurological deficit of MCAO rats were dramatically reduced. The results suggest that HCG11 silencing attenuates CI/R injury.

Studies have confirmed that oxidative stress and neuroinflammation are important pathogenic mechanisms of CI/R injury [47]. The brain is very sensitive to hypoxia, and hypoxia caused by ischemia is an important cause of CI/R injury. SOD and MDA are markers of oxidative stress, which directly reflects the degree and speed of lipid peroxidation [31]. Our research found that ROS and MDA were dramatically increased in the MCAO rat model, and SOD was dramatically reduced. This is consistent with previous studies [22], where stimulation of CI/R injury caused severe oxidative stress. However, when HCG11 is depleted, the levels of these oxidation biomarkers were reversed. The results indicate that HCG11 may be related to oxidative stress. In addition, the excessive secretion of neuroinflammatory affects the permeability of the blood-brain barrier, the release of pro-inflammatory factors accelerated the spread of infarcts, and the delayed release of anti-inflammatory factors contrib-

utes to the infarction outcome and the initiation of repair mechanisms [3]. In addition, TGF-β1 is induced with corresponding CI/R injury and played a protective role in ischemic stroke [1]. In our research, we found that MCAO promoted neuroinflammation in which both pro-inflammatory factors, TNF-α and IFN-γ, were significantly increased, while inhibition of HCG11 significantly suppressed the neuroinflammatory response. What is more, TGF-β, a pleiotropic cytokine, had a neuroprotective effect in response to hypoxia-induced central nervous system (CNS) stress in the MCAO model, whereas inhibition of HCG11 increased the protective effect on CI/RI with a further elevation of TGF-β [1]. We demonstrated that HCG11 silencing may play a neuroprotective role in inhibiting CI/R injury through oxidative stress and neuroinflammation.

In general, LncRNA acts as a competitive endogenous RNA (ceRNA) of miRNA to regulate the miRNA-mRNA axis, which is the main regulatory mechanism. Multiple miRNAs targeting HCG11 have been found through online software, including miR-381-3p. Previous studies have confirmed its involvement in disorders including cancer, coronary heart disease, and endothelial damage. In addition, miR-381-3p prevents ischemic stroke by inhibiting angiogenesis and inflammation [19]. The upregulation of miR-381 is involved in nerve injury repair in rats with acute cerebral ischemia through LRRC4 [30]. However, its role in CI/R is unknown, which is our interest. Although we have proved that the consumption of HCG11 can reduce neurological deficits and cerebral infarction volume, reduce neuroinflammation and oxidative stress damage, and exert the neuroprotective effect of CI/R injury, the reduction of miR-381-3p weakened the protective effect of HCG11 depletion. In other words, compared with HCG11 depletion, cerebral infarction volume, nerve function deficit, neuroinflammation, and oxidative stress were increased.

As a tumour suppressor protein and transcription factor, p53 is involved in the physiological and pathological processes of many diseases and is associated with oxidative stress and inflammatory response [35]. Previous studies have repeatedly confirmed the important role of p53 in cerebral ischemia and CI/R injury. For example, Ginkgo can reduce the upregulation of p53 induced by CI/R injury [29]. Resveratrol can inhibit CI/R injury in rats by inhibiting p53 and has a neuroprotective effect [15]. NDRG4 inhibits cerebral ischemia injury by inhibiting p53-mediated apoptosis [39]. In addition, our previous studies have confirmed that p53 is involved in the repair of ischemic stroke [51]. In our study, we found that p53 is a potential target of miR-381-3p, and the reduction of HCG11 inhibited the level of p53, but the down-regulation of miR-381-3p reversed this inhibition. What is more, the Wnt signalling pathway is involved in brain development and plays an important role in CI/R injury [23]. It has been reported that CI/R injury induces neuroinflammation and apoptosis by activating the Wnt signalling pathway [53]. For example, the dihydrochloride of novel Cyclovirobuxine D (CVB-D) participates in blood-brain barrier dysfunction in MCAO/R rats through the Wnt/β-catenin signalling pathway [52]. Curcumin can inhibit the inflammatory response of I/R through the Wnt signalling pathway [53], and this signalling pathway is also associated with oxidative stress in the MCAO model of mice [41]. p53 has been proved to be a key regulatory factor in the Wnt signalling pathway. For example, p53 induces osteoblast differentiation by regulating the Wnt signalling pathway [54]. Therefore, we speculated that HCG11 might play a role in CI/R damage through regulation of the p53 mediated Wnt signalling pathway through miR-381-3p, but this requires further study and discussion.

A potential limitation of our study is the lack of immunohistochemistry, tissue staining images, and more methods for assessing nerve injury besides the Bederson and Longa scores used in this study. Another potential limitation of the current research is the lack of relevant behavioural analysis, which is a flaw in our experimental design and which we will focus on in the next research. Finally, calcium imbalance was reported to be involved in CI/RI, but whether it is associated with HCG11 will also be looked at in a later study.

Conclusions

Taking everything into account, our current study confirmed that HCG11 decreasing may protect against cerebral ischemia/reperfusion injury through miR-381-3p to regulate p53. These findings offer a potential novel strategy for CI/R injury.

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Disclosure

The authors report no conflict of interest.

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