microRNA-455-5p alleviates neuroinflammation in cerebral ischemia/reperfusion injury

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Abstract

Neuroinflammation is a major pathophysiological factor that results in the development of brain injury after cerebral ischemia/reperfusion. Downregulation of microRNA (miR)-455-5p after ischemic stroke has been considered a potential biomarker and therapeutic target for neuronal injury after ischemia. However, the role of miR-455-5p in the post-ischemia/reperfusion inflammatory response and the underlying mechanism have not been evaluated. In this study, mouse models of cerebral ischemia/reperfusion injury were established by transient occlusion of the middle cerebral artery for 1 hour followed by reperfusion. Agomir-455-5p, antagonim-455-5p, and their negative controls were injected intracerebroventricularily 2 hours before or 0 and 1 hour after middle cerebral artery occlusion (MCAO). The results showed that cerebral ischemia/reperfusion decreased miR-455-5p expression in the brain tissue and the peripheral blood. Agomir-455-5p pretreatment increased miR-455-5p expression in the brain tissue, reduced the cerebral infarct volume, and improved neurological function. Furthermore, primary cultured microglia were exposed to oxygen-glucose deprivation for 3 hours followed by 21 hours of reoxygenation to mimic cerebral ischemia/reperfusion. miR-455-5p reduced C-C chemokine receptor type 5 mRNA and protein levels, inhibited microglia activation, and reduced the production of the inflammatory factors tumor necrosis factor-a and interleukin-1β. These results suggest that miR-455-5p is a potential biomarker and therapeutic target for the treatment of cerebral ischemia/reperfusion injury and that it alleviates cerebral ischemia/reperfusion injury by inhibiting C-C chemokine receptor type 5 expression and reducing the neuroinflammatory response.

Key Words: agomiR-455-5p; biomarker; blood-brain barrier; C-C chemokine receptor type 5; ischemia/reperfusion injury; ischemic stroke; microglia; microRNA-455-5p; neuroinflammation; pretreatment

Introduction

Neuroinflammation is a major pathophysiological factor that contributes to the development of brain injury after cerebral ischemia/reperfusion (I/R). Within hours after I/R, cytokines and other proinflammatory mediators are released by injured cells, and circulating leukocytes are recruited and further infiltrate into the brain parenchyma (Jin et al., 2010; Buscemi et al., 2019; Xu et al., 2020; Goebel et al., 2021). These effects aggravate the breakdown of the blood-brain barrier (BBB) and lead to further brain injury. Though hundreds of anti-inflammatory drugs have been successfully used in animal experiments, few are available to protect the human brain from I/R injury in the clinic (Zhou et al., 2018). Thus, novel therapeutic anti-inflammatory strategies are urgently needed.

MicroRNAs (miRNAs) are small non-coding RNAs that regulate
Gene expression post-transcriptionally and are widely expressed in eukaryotic cells. Numerous miRNAs have been reported to promote or inhibit neuroinflammation by interfering with their target mRNAs (van Kralingen et al., 2019; Zuo et al., 2019). As a tumor-associated miRNA, miR-455-5p is characterized by its ability to suppress tumor metastasis (Liu et al., 2016; Hu et al., 2019, 2020). Recently, several studies have revealed that miR-455-5p attenuated inflammation by suppressing inflammation predicted targets, such as C-C chemokine receptor type 5 (CCR5) and suppressor of cytokine signaling 3 (Chen et al., 2019b; Xing et al., 2019). The level of miR-455-5p was downregulated after ischemic stroke and was suggested as a potential biomarker and therapeutic target for post-ischemic neuronal damage (He et al., 2016). However, the specific role of miR-455-5p and its mechanism have not been assessed yet. In the present study, we studied whether I/R affects the expression of miR-455-5p in mice and whether miR-455-5p is a potential I/R injury biomarker. Furthermore, we investigated the signaling pathway underlying the protective effect of miR-455-5p.

Materials and Methods

In vivo experiments

Animals and experimental design

All animal experiments were conducted in agreement with the regulations of the Animal Care and Use Committee of Shanghai Jiao Tong University School of Medicine, Shanghai, China (approval No. A-2018-004, approved on July 7, 2018). Because the estrogen level affects the physiopathology of ischemic stroke (Koehlrauffer and McCullough, 2013), specific-pathogen-free male C57BL/6 mice (20–25 g, 8–10 weeks old) were purchased from Shanghai Lingchang Biological Technology Limited Company (Shanghai, China; license No. SCXK [Hu] 2018-0003). A total of 261 mice were randomly divided into 10 groups: sham (I/R-n; n = 33), I/R (n = 38), pretreatment with miR-455-5p (Agomir + I/R, n = 39) and its negative control (Agomir-NC + I/R, n = 44), pretreatment with miR-455-5p antagonist (Antagomir + I/R, n = 39) and its negative control (Antagomir-NC + I/R, n = 39), miR-455-5p post-treatment (I/R-0h + Agomir, n = 7; I/R-7h + Agomir, n = 7) and the corresponding negative control (I/R-0h + Agomir-NC, n = 7; I/R-7h + Agomir-NC, n = 7). Detailed information about the animal assignments is provided in Additional Figure 1 and Additional Table 1. The investigators were blinded to the group assignments during all outcome assessments.

Cerebral I/R model

To induce I/R injury, the middle cerebral artery was transiently occluded as previously described (Wang et al., 2020a). Briefly, the mice were anesthetized with isoflurane (4% induction concentration and 2% maintenance concentration; RWD Life Science). The body temperature (37°C) during the operation was controlled by a rectal sensor and heating pad (Harvard Apparatus, Holliston, MA, USA). Cerebral blood flow was monitored with a transcranial laser Doppler (moor VMS-LDF2, Moor Instruments, Axminster, UK). Eighty percent reduction in cerebral blood flow compared with the baseline was considered a successful middle cerebral artery occlusion (MCAO). Mice with regional cerebral blood flow during occlusion higher than 20% of the baseline were excluded. After 1 hour of occlusion, the suture was removed and the skin incision was closed. All the mice were housed separately with free access to food and water. Sham animals underwent the same surgical procedures without the MCAO.

Intracerebroventricular injection

miR-455-5p mimics [Agomir-455-5p, agomir-NC, MiR-455-5p antagonist AntagomiR-455-5p, and antagonomic-NC (all provided by Sangon Biotech, Shanghai, China)] were dissolved at a concentration of 20 nmol/μL in PBS and administered into the lateral ventricle at 0.2 μL/min. After injection, the needle was left for an additional 5 minutes to prevent possible leakage, then withdrawn slowly. The burr hole was sealed with bone wax and the incision was closed.

Neurological behavior assessment

The neurological deficits were assessed by the modified Garcia test by an experimenter blinded to the treatments. The Garcia scores include six aspects: vibrissae touch, touch of trunk, climbing wall of wire cage, movements of forelimbs, spontaneous movements of all limbs, and spontaneous activity (Hu et al., 2017). The total score is 18 (healthy), and a lower score indicates serious deficits.

2,3,5-Triphenyltetrazolium hydrochloride staining

Fresh brain samples were collected 24 hours after MCAO and sectioned coronally at 1-mm intervals using a brain matrix (RWD Life Science). The slices were stained with 2,3,5-triphenyltetrazolium hydrochloride (MTT; Sigma-Aldrich, Darmstadt, Germany) for 20 minutes, and then fixed with 2% paraformaldehyde before being photographed. To reduce the interference of brain edema with the infarct volume, the infarction ratio was expressed as follows: 1 – (nonischemic ipsilateral hemisphere volume/whole contralateral hemisphere volume) (Zuo et al., 2019).

Foot fault test

The motor coordination of mice was tested by foot faults 1, 7 and 14 days after MCAO (Zuo et al., 2019). Mice were placed on a grid for 2 minutes to observe their movements. We monitored when the mouse’s feet missed the grid and fell into the gap. The total number of mouse foot faults was recorded. The percentage of mouse foot faults was calculated and statistically analyzed.

Brain water content

Twenty-four hours after MCAO, the animals were anesthetized with isoflurane and decapitated. Brains were quickly extracted and separated into the left and right hemisphere and cerebellum. Samples were weighed before (wet weight) and after (dry weight) the brain tissue was placed in an oven at 105°C for 72 hours. The brain water content (%) was calculated as follows: 100 – dry weight/ wet weight × 100 (Zuo et al., 2019).

Evaluation of BBB disruption (Evans blue assay)

Two percent Evans blue (Sigma-Aldrich) was injected intraperitoneally (2 mL/kg of body weight) 22 hours after MCAO. The dye was allowed to circulate for 2 hours. After anesthesia with isoflurane, animals were perfused with ice-cold PBS via intracardial injection and euthanized. Brains were sectioned coronally at 1-mm intervals and photographed. Additionally, the ipsilateral hemisphere was collected and weighed. Then, 1 mL of PBS was added to the brain samples, which were homogenized, centrifuged at 12,000 × g for 20 minutes at 4°C, and the supernatants were collected. Half a milliliter of the supernatants was mixed with an equal volume of 100% trichloroacetic acid and incubated overnight at 4°C. On the next day, the samples were centrifuged and the supernatants were collected. The content of Evans blue in the ipsilateral hemisphere was calculated according to a standard curve (Zuo et al., 2019).

In vitro microglia oxygen-glucose deprivation/reoxygenation model

Primary microglia culture was performed as previously described (Chen et al., 2019a). Twenty-four neonatal 1–2-day-old C57BL/6J mice (Shanghai Lingchang Biological Technology Limited Company) were anesthetized with isoflurane and the brains were extracted rapidly. The cerebral cortices were dissected on ice and digested with 0.05% trypsin/ethylenediaminetetraacetic acid for 15 minutes at 37°C. Then, 10% fetal bovine serum in Dulbecco’s modified Eagle’s medium (Cat# 11965092, Thermo Fisher Scientific) was added to the tissue samples, which were homogenized, centrifuged at 12,000 × g for 20 minutes at 4°C, and the supernatants were collected. Half a milliliter of the supernatants was mixed with an equal volume of 100% trichloroacetic acid and incubated overnight at 4°C. On the next day, the samples were centrifuged and the supernatants were collected. The content of Evans blue in the ipsilateral hemisphere was calculated according to a standard curve (Zuo et al., 2019).

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On day 11, microglial cells were collected by shaking the flasks at 5 x g for 2 hours, then cultured for 2 days. The purity of the microglial cultures was examined by immunostaining with a mouse monoclonal anti-CD45 antibody (Caltag, Cat# 345001, Caltag, CA, USA) and a rabbit polyclonal anti-CD68 antibody (Abcam). The cells were identified as >97%.

An in vitro oxygen-glucose deprivation (OGD)/reoxygenation model was used to facilitate the experiment as previously described (Pu et al., 2019). The medium of the microglial cells was replaced with glucose-free Dulbecco’s modified Eagle’s medium (Gibco, Cat#11966025) and transferred to a ProOx C21 hypoxia chamber (BioSpherix, Parish, NY, USA) containing 94% N2/5% CO2/1% O2 for 3 hours. Reoxygenation was initiated by returning the cells to normal conditions, followed by addition of 100 nM/mL agomiR-455-5p, antagonimir-455-5p, or their negative control to the medium.

**Western blot assay**

To study the role of miR-455-5p in the regulation of inflammation, the expression of CCR5, tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) was determined by western blotting (Zuo et al., 2019). The brain samples were extracted, fixed for 24 hours with 4% paraformaldehyde, and dehydrated with 30% sucrose solution for 2 days. Coronal sections (10 μm) of the brain tissue were cut using a cryostat (Leica, Buffalo Grove, IL, USA). For permeabilization, sections were incubated for 30 minutes with 0.3% Triton X-100. After blocking with 10% bovine serum, the sections were incubated with the primary antibodies: rabbit anti-CCR5 antibody (1:1000, Cat# ab65850, Abcam), rabbit anti-Iba1 antibody (1:1000, Cat# ab104224, RbID: AB_10711040, Abcam), mouse anti-Iba1 antibody (1:200, Cat# ab156690, RbID: AB_2224403, Abcam), and mouse anti-GFAP antibody (1:200, Cat# 50-9892-82, RbID: AB_2574408, Thermo Fisher Scientific) at 4°C overnight. After three 5-minute washes with PBS, the slices were incubated with the following secondary antibodies: Alexa 647-labeled donkey anti-rabbit IgG (green) (1:1000, Cat# A11037, Thermo Fisher Scientific), and Alexa 488-labeled goat anti-mouse IgG (1:500, Cat# ab110113, RbID: AB_2576208, Abcam) for 1 hour at room temperature. Imaging was performed on a laser scanning confocal microscope (Eclipse TE2000U, Nikon Instruments, Melville, NY, USA).

**Statistical analysis**

No statistical methods were used to predetermine sample sizes; however, our sample sizes are similar to those reported in a previous study (Zuo et al., 2019). No animals or data points were excluded from the final analysis. For this study, no statistical software (GraphPad software, Inc., La Jolla, CA, USA) was used. Student’s t-test was used to analyze the difference between two groups. For comparisons of multiple groups, one-way analysis of variance followed by Tukey’s post hoc test was used. The data are presented as mean ± standard deviation (SD). Mortality was evaluated by Fisher’s exact test. For data that do not follow the homogeneity of variance, non-parametric Kruskal-Wallis rank sum test was used. P < 0.05 was considered statistically significant.

**Results**

**Pretreatment with agomiR-455-5p decreases infarct volume and improves neurological function in cerebral I/R mice**

Twenty-four hours after the MCAO, I/R induced significant brain infarction and resulted in severe neurological deficits (P < 0.01, sham group; Figure 1A–C). Treatment with agomiR-455-5p 2 hours before the MCAO effectively decreased the infarct volume (P < 0.01, vs. I/R group; Figure 1A and B) and ameliorated neurological deficits in cerebral I/R mice (P < 0.05, vs. I/R group; Figure 1C). However, no protective effects were observed when agomiR-455-5p was administrated after reperfusion (Additional Figure 2). To further investigate the involvement of miR-455-5p in the development of post I/R injury, we silenced the expression of endogenous miR-455-5p by an antagonir. The results demonstrated that antagonir-455-5p increased the infarct volume and aggravated I/R-induced neurological dysfunction (both P < 0.05, vs. I/R group; Figure 1A–C). Collectively, our findings revealed that agomiR-455-5p can be a potential therapeutic strategy to evaluate the long-term recovery of motor function. We found that animals in the Agomir + I/R group needed significantly less time to cross the beam at day 14, compared with the I/R group (P < 0.05, vs. I/R group; Figure 1A–C).
vs. I/R group; Figure 1D). Furthermore, significant improvement of motor coordination and less misplacements were observed in the Agomir + I/R group in the foot fault test (P < 0.05, vs. I/R group; Figure 1E). In contrast, pretreatment with antagoniR-455-5p prolonged the beam crossing time (P < 0.05, vs. I/R group at day 14; Figure 2D) and increased the foot faults (P < 0.05, vs. I/R groups, days 1, 7, and 14; Figure 1E). No statistically significance difference in mortality was observed between the I/R (16%) and Agomir + I/R groups (13%; Additional Figure 3). Moreover, administration of agomir-NC or antagonist-NC induced no significant difference in infarct volume and neurological deficits compared with the I/R group (P > 0.05; Figure 1).

Figure 2 | Pretreatment with agomir-455-5p enhances the blood-brain barrier integrity at 24 hours after I/R.
(A) Representative images of Evans blue extravasation in Sham, I/R, I/R + pretreatment with agomir-455-5p or antagonist-455-5p, and I/R + the corresponding negative control. Agomir-455-5p protected the BBB integrity after I/R. (B, C) Quantitative results of the content of Evans blue and of the brain water content (C). Data are presented as mean ± SD (n = 6 per group). **P < 0.01, *P < 0.05, #P < 0.01, vs. I/R group (one-way analysis of variance followed by Tukey’s post hoc test). I/R: Ischemia/reperfusion; NC: negative control.

Ccr5 is a direct downstream target of miR-455-5p
Real-time PCR showed that I/R decreased the expression of miR-455-5p in the ipsilateral cortex (P < 0.05, vs. sham group; Figure 3A) and in the peripheral blood (P < 0.01, vs. sham group; Additional Figure 4) at 24 hours. As expected, agomir-455-5p pretreatment increased the level of miR-455-5p in the brain (P < 0.01, vs. I/R group), whereas antagonist-455-5p decreased the expression of miR-455-5p (P < 0.05, vs. I/R group; Figure 3A). The luciferase assay demonstrated that cotransfection of the miR-455-5p plasmid reduced the luciferase activity of the Ccr5 3′UTR reporter in HEK293T cells (P < 0.05, vs. agomir-NC + Ccr5 3′UTR group; Figure 3B). Consistently, pretreatment with agomir-455-5p decreased the expression of Ccr5 mRNA in the brain of cerebral I/R mice (P < 0.01, vs. I/R group; Figure 3C). Conversely, administration of antagonist-455-5p increased the level of Ccr5 mRNA (P < 0.05, vs. I/R group; Figure 3C). Additionally, the western blot results of CCR5 confirmed the mRNA results, i.e., agomir-455-5p decreased the expression of CCR5 at the protein level (P < 0.05, vs. I/R group) and pretreatment with antagonist-455-5p increased the CCR5 protein expression (P < 0.01, vs. I/R group; Figure 3D and E). Therefore, these results support the idea that Ccr5 is a direct downstream target of miR-455-5p.

Agomir-455-5p prevents the activation of microglia 24 hours after cerebral I/R
Immunofluorescence was used to determine the location and expression of CCR5 in the brain tissue and examine the activation of microglia and astrocytes. CCR5 was highly expressed in neurons, astrocytes, and microglia 24 hours after I/R (Figure 4). Furthermore, the number of microglia and astrocytes was increased in the ischemic cortex compared with the sham animals (both P < 0.01 vs. sham group; Figure 5A and B); however, pretreatment with agomir-455-5p decreased the number of microglia but not of astrocytes (P < 0.01 for microglia and P = 0.9602 for astrocytes, vs. I/R group; Figure 5A-C). After ischemia, microglia in the ischemic cortex became activated and presented with an enlarged cell body (Circularity Index, P < 0.01 vs. sham group; Figure 5A and D) and a reduced ramification (Ramification Index, P < 0.01 vs. sham group; Figure 5A and E). Pretreatment with agomir-455-5p attenuated the morphological alterations of microglia to a smaller cell body (P > 0.01 vs. Agomir-NC + I/R group; Figure 5A and D) and an increased ramification (P < 0.01 vs. Agomir-NC + I/R group; Figure 5A and E).

Figure 3 | Quantitative results of the content of Evans blue and of the brain water content (A) at 24 hours. As expected, agomiR-455-5p pretreatment increased the level of miR-455-5p in the brain (P < 0.01, vs. I/R group), whereas antagonist-455-5p decreased the expression of miR-455-5p (P < 0.05, vs. I/R group; Figure 3A). The luciferase assay demonstrated that cotransfection of the miR-455-5p plasmid reduced the luciferase activity of the Ccr5 3′UTR reporter in HEK293T cells (P < 0.05, vs. agomir-NC + Ccr5 3′UTR group; Figure 3B). Consistently, pretreatment with agomir-455-5p decreased the expression of Ccr5 mRNA in the brain of cerebral I/R mice (P < 0.01, vs. I/R group; Figure 3C). Conversely, administration of antagonist-455-5p increased the level of Ccr5 mRNA (P < 0.05, vs. I/R group; Figure 3C). Additionally, the western blot results of CCR5 confirmed the mRNA results, i.e., agomir-455-5p decreased the expression of CCR5 at the protein level (P < 0.05, vs. I/R group) and pretreatment with antagonist-455-5p increased the CCR5 protein expression (P < 0.01, vs. I/R group; Figure 3D and E). Therefore, these results support the idea that Ccr5 is a direct downstream target of miR-455-5p.
I/R | P | Agomir+I/R | Agomir-NC+I/R
---|---|---|---
Agomir+ | I/R | Agomir-NC+I/R | Agomir-NC+I/R

However, Agomir+ treatment with antagomiR-455-5p increased the expression of Ccr5, TNF-α, and IL-1β (
P < 0.01, vs. sham group; Figure 6). In contrast, pretreatment with antagomiR-455-5p enhanced the TNF-α (P < 0.01, vs. I/R group) and IL-1β production in the brain tissue (P < 0.05, vs. I/R group; Figure 6).

Figure 5 | Pretreatment with agomiR-455-5p inhibits the activation of microglia at 24 hours after I/R injury in mice.

(A) Representative images of Iba1 and Gfap in the ischemic cortex of Sham, I/R, I/R + pretreatment with agomiR-455-5p and its negative control animals 24 hours after I/R. Pretreatment with agomiR-455-5p decreased the number of microglia and attenuated the morphological alterations in microglia, resulting in a smaller cell body and increased ramification. Gfap: red, Alexa Fluor® 647; Iba1: red, Alexa Fluor® 487. Scale bars: 50 μm. (B, C) Quantitative results of the number of microglia and astrocytes in the ischemic cortex. (D, E) Quantitative results of the Circularity Index and Ramification Index of microglia. For each animal, 33 cells from three sections were analyzed; cells from different animals are represented by different colors. Data are presented as mean ± SD (n = 6 per group). *P < 0.05, vs. sham group; **P < 0.01, vs. sham group; ***P < 0.001, vs. sham group; ##P < 0.01, vs. Agomir-NC + I/R group (one-way analysis of variance followed by Tukey’s post hoc test). Gfap: Glial fibrillary acidic protein; I/R: ischemia/reperfusion; Iba1: Ionized calcium binding adaptor molecule 1; NC: negative control.

Figure 6 | AgomirR-455-5p suppresses the inflammation in the ipsilateral cortex 24 hours after I/R.

(A) Representative western blots of Tnf-α and IL-1β in the ipsilateral cortex of Sham, I/R, I/R + pretreatment with agomiR-455-5p or antagomiR-455-5p, and I/R + the corresponding negative control animals. (B, C) Quantitative results of the production of proinflammatory mediators TNF-α and IL-1β in the ipsilateral cortex (P < 0.05, vs. sham group; Figure 6). However, pretreatment with agomiR-455-5p decreased the brain TNF-α and IL-1β production post I/R (P < 0.05, vs. I/R group; Figure 6). In contrast, pretreatment with antagomiR-455-5p enhanced the TNF-α (P < 0.01, vs. I/R group) and IL-1β production in the brain tissue (P < 0.05, vs. I/R group; Figure 6).

Agomir-455-5p suppresses I/R-induced inflammation in primary cultured microglia and in cerebral I/R mice

In primary cultured microglia subjected to OGD/reoxygenation, treatment with agomiR-455-5p significantly decreased the Ccr5 protein expression as well as the expression of proinflammatory factors TNF-α and IL-1β (P < 0.05, vs. Agomir-NC group). In contrast, treatment with antagomiR-455-5p increased the expression of Ccr5, TNF-α, and IL-1β (P < 0.05, vs. Antagomir-NC group, Additional Figure 5). In the cerebral I/R mice, I/R significantly increased the production of proinflammatory mediators TNF-α and IL-1β in the ipsilateral cortex (P < 0.05, vs. sham group; Figure 6). However, pretreatment with agomiR-455-5p decreased the brain TNF-α and IL-1β production post I/R (P < 0.05, vs. I/R group; Figure 6). In contrast, pretreatment with antagomiR-455-5p enhanced the TNF-α (P < 0.01, vs. I/R group) and IL-1β production in the brain tissue (P < 0.05, vs. I/R group; Figure 6).
Discussion
Reperfusion is one of the most common factors that lead to aggravation of stroke-induced brain injury. In acute ischemic reperfusion injury, inflammation greatly contributes to neuronal cell death and BBB disruption; however, few anti-inflammation therapies have been proven to be beneficial in the clinical setting (Zhou et al., 2018). There is mounting evidence indicating that miRNAs are critically involved in the regulation of inflammation. Although it has been reported that as a member of the miR-455 family, miR-455-5p is crucially involved in inflammation, its role in reperfusion-induced inflammation remained unclear (Torabi et al., 2019). Here, we demonstrated that I/R significantly downregulated miR-455-5p production in the brain tissue and in the peripheral blood in mice; however, augmentation of miR-455-5p expression with agomiR-455-5p effectively decreased the brain infarction, preserved the BBB integrity, and improved neurological function. Furthermore, the downregulation of the drug-resistant Ccr5 level remained unclear (Torabi et al., 2019). Several master regulators of the inflammatory response, including suppressor of cytokine signaling 3, Janus kinase 1, myeloid differentiation factor 88, and Ccr5, have been verified as miR-455-5p targets (Chen et al., 2019b; Torabi et al., 2019; Xing et al., 2019). MI-455-5p represses these inflammatory signaling pathways, and consequently suppresses inflammation. Furthermore, several neuronal genes have been reported to be expressed in neurons, microglia, and astrocytes, and dysregulation of miR-455-5p has been demonstrated in numerous neurological diseases (Sierksma et al., 2018). Increased expression of miR-455-5p has been reported in rat dorsal root ganglion neurons after spinal nerve ligation (Strickland et al., 2011). MI-455-5p is recognized as one of the miRNAs that are mostly affected by cerebral I/R injury and is linked to poor outcomes (He et al., 2016). A clinical study has established that miR-455-5p was greatly downregulated in the peripheral blood of ischemic stroke patients (He et al., 2016). In I/R mice, treatment with miR-455 mimics protected the brain against ischemia-reperfusion-induced brain damage, further indicating that miR-455-5p attenuated reperfusion-induced inflammation, resulting in decreased expression of inflammatory mediators Tnf-a and IL-1β. Most importantly, we verified that Ccr5 is a direct gene target of miR-455-5p, which is negatively regulated by miR-455-5p. This downregulation was accompanied by suppression of the post-I/R inflammatory response. Stroke is unpredictable, hence medical intervention prior to stroke onset is mostly impossible. However, the number of patients undergoing cardiac surgery, e.g., coronary artery bypass grafting, is increasing every year with a high risk of perioperative stroke (Jovin et al., 2019). The surgery timeline enables an intervention before the start of the drug-resistant Ccr5 level. However, the effectiveness of any potential preventive strategy, making pretreatment a promising therapeutic option. This approach is recommended by experts in the field (Salameh et al., 2016). Our study may provide a potential strategy for preventing the brain from ischemic injury and reducing perioperative complications.

Excessive and unresolved neuroinflammation is a major driver of brain damage after I/R (Jayaraj et al., 2019; Wang et al., 2020c). Ischemia leads to impaired energy metabolism immediately after insult, which eventually results in necrosis and apoptosis (Campbell et al., 2019). Furthermore, I/R leads to reactive oxygen species overproduction. The debris from injured cells and reactive oxygen species trigger an inflammatory response by recruiting systemic immune cells from the circulating blood as well as activating microglia and astrocytes. The released proinflammatory mediators from these cells increase the BBB permeability and induce neuronal cell death, leading to functional deficits. In the last decade, sufficient evidence has supported the hypothesis that miRNAs play an important role in regulating inflammatory signaling (Slota and Booth, 2019). As a member of the miR-455 family, miR-455-5p is involved in tumor suppression, and emerging evidence suggests that it negatively regulates inflammation. Furthermore, it negatively regulates inflammation, its role in I/R-induced brain injury remained unclear (Torabi et al., 2019). Several master regulators of the inflammatory response, such as suppressor of cytokine signaling 3, Janus kinase 1, myeloid differentiation factor 88, and Ccr5, have been verified as miR-455-5p targets (Chen et al., 2019b; Torabi et al., 2019; Xing et al., 2019). MI-455-5p represses these inflammatory signaling pathways, and consequently suppresses inflammation. Furthermore, several neuronal genes have been reported to be expressed in neurons, microglia, and astrocytes, and dysregulation of miR-455-5p has been demonstrated in numerous neurological diseases (Sierksma et al., 2018). Increased expression of miR-455-5p has been reported in rat dorsal root ganglion neurons after spinal nerve ligation (Strickland et al., 2011). MI-455-5p is recognized as one of the miRNAs that are mostly affected by cerebral I/R injury and is linked to poor outcomes (He et al., 2016). A clinical study has established that miR-455-5p was greatly downregulated in the peripheral blood of ischemic stroke patients (He et al., 2016). In I/R mice, treatment with miR-455 mimics protected the brain against ischemia-reperfusion-induced brain damage, further indicating that miR-455-5p attenuated reperfusion-induced inflammation and decreasing the expression of Tnf receptor associated factor 3. Interestingly, our findings are consistent with these reports that I/R decreased the level of miR-455-5p in the brain tissue as well as in the peripheral blood. However, the decrease was followed by upregulated expression of the target gene Ccr5, along with increased production of the inflammatory mediator Tnf-a and IL-1β.

The chemokine receptor, Ccr5, is a G-protein coupled receptor that is activated by β-chemokines and is widely implicated in increased neuroinflammation (Martin-Blondel et al., 2016). Previous studies have shown that Ccr5 is constitutively expressed on macrophages and immune cells, as well as microglia and neurons, and it is upregulated after cerebral I/R (Sorce et al., 2011; Victoria et al., 2017; Joy et al., 2019; Chen et al., 2020). In our study, a substantial increase in Ccr5 expression was observed after I/R. Immunofluorescence double staining confirmed that it is highly expressed in neurons, glia, and microglia after I/R, and that the Ccr5 level positively correlates with the inflammatory response. In cerebral I/R mice, the number of microglia and astrocytes in the ischemic cortex was significantly increased and the microglia presented with a highly activated retracted processes. The morphology alterations of microglia observed here highly corresponded to their typical features reported previously. I/R induced microglia activation, accompanied with decreased miR-455-5p and increased Ccr5 expression, leading to the release of proinflammatory mediators.

To date, downstream signaling pathways of Ccr5 such as mitogen-activated protein kinase, signal transducer and activator of transcription 1, phosphatidylinositol-3 kinase, and cyclooxygenase response element-binding protein have been discovered (Yang et al., 2010; Banerjee et al., 2011; Chen et al., 2018; Joy et al., 2019; Lan et al., 2019). As a co-receptor for the human immunodeficiency virus, Ccr5 is responsible for cognitive deficits induced by impairing neuronal plasticity, learning, and memory in human immunodeficiency virus patients (Vangelista and Vento, 2017). In ischemic cerebrovascular disease, the role of Ccr5 is discussed controversially. Patients with Ccr5 D32 polymorphism presented increased risk of ischemic stroke (Joy et al., 2019). Sorce et al. (Sorce et al., 2010) have found that Ccr5-deficient mice displayed more serious neutrophil invasion and greater infarction compared with wild-type mice. However, other studies have demonstrated that inhibition of Ccr5 by its antagonist or genetic silencing led to a reduced infarct volume and a better neurological outcome in animal stroke models (Li et al., 2017; Victoria et al., 2017; Joy et al., 2019). AgomiR-455-5p reduced the luciferase activity of the plasmid containing the 3’UTR of the Ccr5 gene, inhibited the miRNA and protein expression of Ccr5, suggesting that miR-455-5p decreased Ccr5 expression post-transcriptionally by binding to its 3’UTR. In primary cultured microglia, agomiR-455-5p reduced the protein expression of Ccr5 and of the proinflammatory factors Tnf-a and IL-1β, 24 hours after OGD/reoxygenation. Accordingly, in cerebral I/R mice, pretreatment with agomiR-455-5p augmented the level of miR-455-5p and inhibited microglia activation, resulting in beneficial outcomes of reduced infarct size and improved neurological deficits. Given our findings, direct targeting of Ccr5 may be the pathway responsible for the anti-inflammatory effect of miR-455-5p after I/R.

Our study has some limitations. We evaluated the anti-inflammatory properties of the treatment and its ability to protect the post-ischemic brain only in the acute stage of the disease, at 24 hours after the MCAO. However, the inflammatory response following ischemic stroke is a dynamic process. Furthermore, whether the reduction of inflammation in the acute stage is beneficial, inflammation in the chronic stage of the disease is positively associated with brain repair (Jayaraj et al., 2019). Therefore, whether short-term protection, which was established in the current study, results in decreased brain damage at later time points and contributes to long-term recovery needs to be evaluated in future studies. Additionally, given that one miRNA can interact with hundreds of target genes, it is possible that other downstream targets of miR-455-5p are involved in the neuroprotection of miR-455-5p. Several small-molecule antagonists of Ccr5, including aplaviroc, vicriviroc, and maraviroc, have been reported with detrimental consequences of liver toxicity and resistance (Clozet et al., 2007). Compared with traditional small-molecule inhibitors, miRNAs have the advantages of targeting multiple genes simultaneously and being able to easily chemically modified to improve their pharmacokinetics (Li and Rana, 2014). MiRNA-based therapeutics may be a viable option for inhibiting Ccr5 function in the future, though challenges such as off-target effects, stability, and cell-specific targeting have not been resolved.

In conclusion, we demonstrated that miR-455-5p may serve as an I/R injury biomarker and that it diminished neuroinflammation by suppressing Ccr5 expression, consequently alleviating I/R-induced injury. The miR-455-5p/Ccr5 signaling pathway may provide a novel insight into ischemic stroke management, making it a promising therapeutic target.

Author contributions: Study design and manuscript draft: QH, QYM; animal model and behavioral tests: JSZ, SS; molecular studies: PPH, YC, BZ; data analysis: JSZ, SS, ZPX, FJ; manuscript revision: AM, XHZ. All authors read, revised, and approved the final manuscript.

Conflicts of interest: The authors declare that the research was conducted...
in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Availability of data and materials: All data generated or analyzed during this study are included in this published article and its supplementary information files.

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Additional files:

- **Additional Figure 1**: Experimental design, animal groups classification and timeline.
- **Additional Figure 2**: Administration of agomir-455-Sp at 0 and 1 hour after reperfusion had no protective effect on infarct volume and neurological deficits.
- **Additional Figure 3**: The mortality of mice in the study.
- **Additional Figure 4**: I/R reduces the level of mir-455-Sp in the peripheral blood in cerebral/IR mice.
- **Additional Figure 5**: Agomir-455-Sp inhibits the expression of CCRL3 and pro-inflammatory factors TNF-a and IL-18 24 hours after OGD/reoxygenation (OGD/R), and antagonism-455-Sp reverses the effects.
- **Additional Table 1**: Animal assignment in each group.
- **Additional file 1**: Open peer review report 1.

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Experiment 1, animal study.

| Treatment (i.c.v) | MCAO | Reperfusion | Infarction, Garcia scores, Foot fault, Beam walking, Evans blue content, Brain water content, WB, PCR, IF | Beam walking, Foot fault |
|------------------|------|-------------|-------------------------------------------------------------------------------------------------|--------------------------|
| C57BL/6 mice     |      |             |                                                                                                 |                          |
| -2 h 0 h         | 2 h  | 24 h        | 7 d 14 d                                                                                         |                          |

Groups:
1. Sham (n=33)
2. I/R (n=38)
3. Agomir+I/R (n=39)
4. Agomir-NC+I/R (n=44)
5. Antagomir+I/R (n=39)
6. Antagomir-NC+I/R (n=39)
7. I/R-0 h+agomir (n=7)
8. I/R-0 h+agomir-NC (n=7)
9. I/R-1h+agomir (n=7)
10. I/R-1h+agomir-NC (n=8)

Experiment 2, in vitro study.

| Primary cultured microglia | OGD | Treatment | Reoxygenation | WB |
|---------------------------|-----|-----------|---------------|----|
| 0 h 3 h 24 h              |     |           |               |    |

Groups:
1. OGD/R+Agomir (n=6)
2. OGD/R+Agomir-NC (n=6)
3. OGD/R+Antagomir (n=6)
4. OGD/R+Antagomir-NC (n=8)

Additional Figure 1 Experimental design, animal groups classification and timeline.

i.c.v: Intracerebroventricular injection; I/R: ischemia/reperfusion; IF: immunofluorescence; MCAO: middle cerebral artery occlusion; NC: negative control; OGD: oxygen–glucose deprivation; PCR: polymerase chain reaction; WB: western blot.
Additional Figure 2 Administration of agomiR-455-5p at 0 and 1 hour after reperfusion has no protective effect on infarct volume and neurological deficits.

(A) Representative 2,3,5-triphenyltetrazolium hydrochloride-stained brain slices in agomiR-455-5p or its negative control post-treated (0 hour and 1 hour after reperfusion) mice. (B) Quantitative results of infarction ratio. (C) Garcia scores at 24 hours after I/R. Data are presented as mean ± SD (n = 6 per group). Data in Garcia scores were analyzed by non-parametric Kruskal-Wallis rank sum test and data in infarction ratio were analyzed by one-way analysis of variance followed by Tukey’s post hoc test. I/R: Ischemia/reperfusion; NC: negative control.
Additional Figure 3 The mortality of mice in the study.

In the treatment groups, agomir-455-5p, antagomir-455-5p, and their negative controls (agomir-NC and antagomir-NC) were injected intracerebroventricularly 2 hours before or 0 and 1 hour after MCAO in mice.

Neither pre-treatment or post-treatment with agomir-455-5p affected the mortality after I/R. Data are presented as number. *P < 0.05, vs. sham group (Fisher exact test). I/R: Ischemia/reperfusion; NC: negative control.
Additional Figure 4 I/R reduces the level of miR-455-5p in the peripheral blood in cerebral I/R mice.

The expression of miR-455-5p in the peripheral blood decreased at 24 hours after I/R. Data are presented as mean ± SD (n = 6). *P < 0.05, vs. I/R group (Student’s t-test). I/R: Ischemia/reperfusion; snRNA: small nuclear RNA.
Additional Figure 5 AgomiR-455-5p inhibits the expression of CCR5 and pro-inflammatory factors TNF-α and IL-1β 24 hours after OGD/R, and antagomiR-455-5p reverses the effects.

(A) Representative western blot bands of CCR5, TNF-α and IL-1β in microglia after OGD/R with /without agomiR-455-5p or antagomiR-455-5p treatment. (B-D) Quantitative results of CCR5, TNF-α, and IL-1β production. Data are presented as mean ± SD (n = 6). &P < 0.05, vs. OGD/R + agomir-NC group; φP < 0.05, vs. OGD/R + antagomir-NC group (one-way analysis of variance followed by Tukey’s post hoc test). CCR5: C–C chemokine receptor type 5; IL-1β: interleukin-1β; NC: negative control; OGD/R: oxygen–glucose deprivation/reoxygenation; TNF-α: tumor necrosis factor alpha.
### Additional Table 1 Animal assignment in each group

| Groups               | Infarct volume, Garcia score | Foot fault, beam walking | Evans blue content | Brain water content | WB, PCR | Immunofluorescence | Total |
|----------------------|------------------------------|--------------------------|--------------------|---------------------|---------|-------------------|-------|
| **Pre-treatment**    |                              |                          |                    |                     |         |                   |       |
| Sham                 | 0/0/6                        | 0/0/6                    | 0/0/6              | 0/0/6               | 0/0/6   | 0/0/3             | 33    |
| I/R                  | 1/1/6                        | 2/0/6                    | 2/0/6              | 1/0/6               | 0/1/6   |                   | 38    |
| Agomir+I/R           | 2/0/6                        | 1/1/6                    | 1/0/6              | 1/0/6               | 0/0/6   | 0/0/3             | 44    |
| Agomir-NC+I/R        | 2/1/6                        | 2/0/6                    | 1/1/6              | 1/1/6               | 1/0/6   | 1/0/3             | 39    |
| Antagomir+I/R        | 3/0/6                        | 1/1/6                    | 2/1/6              | 1/0/6               | 0/0/6   |                   | 39    |
| Antagomir-NC+I/R     | 2/0/6                        | 1/1/6                    | 1/0/6              | 1/1/6               | 1/1/6   |                   | 39    |
| **Post-treatment**   |                              |                          |                    |                     |         |                   |       |
| I/R-0h+agomir        | 1/0/6                        |                          |                    |                     |         |                   | 7     |
| I/R-0h+agomir-NC     | 1/0/6                        |                          |                    |                     |         |                   | 7     |
| I/R-1h+agomir        | 1/0/6                        |                          |                    |                     |         |                   | 7     |
| I/R-1h+agomir-NC     | 1/1/6                        |                          |                    |                     |         |                   | 8     |
| **Total**            | 77                           | 46                       | 45                 | 43                  | 40      | 10                | 261   |

Data are expressed as died/excluded/survived. I/R: Ischemia/reperfusion; NC: negative control; PCR: polymerase chain reaction; WB: western blot.