Intravenous antagomiR-494 lessens brain-infiltrating neutrophils by increasing HDAC2-mediated repression of multiple MMPs in experimental stroke

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Abstract

Background: Neutrophil infiltration and phenotypic transformation are believed to contribute to neuronal damage and clinical outcome in ischemic stroke. Emerging evidence suggests that HDAC2 is an epigenetic regulator of inflammatory cells. Here, we investigated whether miR-494 affects HDAC2-mediated neutrophil infiltration and phenotypic shift.

Methods: The miR-494 levels in neutrophils from AIS patients were detected by real-time PCR. C57BL/6J mice were subjected to transient middle cerebral artery occlusion, and the N1/N2 neutrophil shift was examined. Cortical neurons were subjected to oxygen-glucose deprivation and stimulated with supernatant from differently treated neutrophils or were cocultured with neutrophils; neuronal injury was detected, and ChIP-Seq was performed to clarify which genes are the binding targets of HDAC2. Finally, a transwell assay was conducted to examine neutrophil migration.

Results: Compared to the control subjects, AIS patients had increased neutrophil expression of miR-494, and in AIS patients, elevated miR-494 expression in neutrophils was a predictor of worse neurological outcomes. MiR-494 correlates with the upregulation of adhesion molecules in neutrophils of AIS patients. Systemically administered antagomiR-494 partly shifts neutrophils into the N2 phenotype in MCAO mice. AntagomiR-494-treated neutrophils exert a neuroprotective role in vitro. ChIP-seq revealed that HDAC2 targets multiple MMP genes in neutrophils of AIS patients. Further in vitro and in vivo experiments showed that antagomiR-494 repressed expression of MMP genes, including MMP7, MMP10, MMP13, and MMP16, to reduce the number of brain-infiltrating neutrophils by regulating HDAC2.

Conclusion: MiR-494 may serve as an alternative predictive biomarker of the outcome of AIS patients, and antagomiR-494 treatment decreased the expression of multiple MMPs and the infiltration of neutrophils partly by targeting HDAC2.

Introduction

Ischemic stroke is an acute and severe neurological disease that results in disability and death. Except for recanalization therapy, there are no effective pharmacotherapies available [1–3], which is due to two major reasons. First, single agent pharmacotherapy may be insufficient, and effective treatment of stroke requires targeting multiple complementary targets. Second, most therapeutics could not efficiently penetrate the brain. Therefore, a treatment that can regulate multiple pathological processes and alleviate brain injury via intravenous administration has the advantages of being easily translated into clinical use. The continuous inflammatory cascade reaction is a major factor in cerebral ischemia pathobiology and clinical outcomes [4]. Systemic immunomodulatory therapy based on intravenous cells is attracting increasing attention.

In particular, neutrophils are among the first circulating leukocytes to invade the ischemic tissue, which then attract other immune cells and exacerbate blood-brain barrier destruction and lesions by releasing a number of inflammatory factors, including metallomatrix proteases (MMPs) and reactive oxygen species

[5, 6]. Moreover, the population of infiltrated neutrophils in the brain is heterogeneous, and the peroxisome proliferator-activated receptor-γ agonist rosiglitazone or a toll-like receptor 4 deficiency was suggested to have the ability to reprogram neutrophils toward an anti-inflammatory (N2) neutrophil phenotype, which may assist in resolving inflammation and may provide neuroprotection in ischemic mice [7–9]. However, the mechanisms involved in neutrophil transmigration and phenotypic shift in the ischemic brain are not fully elucidated. This makes it critical to find a molecule that can simultaneously target these two important neutrophil-related pathological processes.

As a promising novel class of small regulatory molecules, microRNAs have been found to play pivotal roles in the inflammatory process associated with ischemic stroke [10], and accumulating evidence has indicated the value of miRNAs in diagnosis, prognosis and treatment of ischemic stroke [11, 12]. According to the results of our previous research, miR–494 in lymphocytes was robustly upregulated and was related to lymphocyte polarization in ischemic stroke patients, and these findings were further confirmed by our animal experiments [13]. Thus, whether miR–494 has an effect on neutrophil phenotypic transformation and infiltration is not clear. Histone deacetylase 2 (HDAC2), a class I HDAC, is known to act as epigenetic and transcriptional regulators. HDAC2 was shown to be a target of miR–494 and plays a part in the T helper cell phenotype and microglia shift in ischemic stroke [13, 14] and in MMP–9-mediated macrophages [15]; therefore, whether HDAC2 is implicated in neutrophil phenotypic transformation following ischemic stroke is not clear. The goal of our current research was to 1) evaluate the clinical significance of miR–494 changes in neutrophils of acute ischemic stroke (AIS) patients and 2) assess whether miR–494 was involved in the N1/N2 phenotypic transformation and infiltration of neutrophils by targeting HDAC2.

Materials And Methods

Clinical subjects

The first-ever AIS patients were recruited between November 2017 and October 2018 and were approved for study participation by the Medical Ethics Committee of Xuanwu Hospital of Capital Medical University (trial registration no. ChiCTR-NCT-03577093). Written informed consent was obtained from all included patients or their relatives before enrolment. The inclusion criteria were as follows: (1) admitted within 6 hours of symptom onset, (2) brain MRI or repeated CT verified the diagnosis of AIS, and (3) blood samples were collected no more than 6 hours after stroke attack. Patients were excluded if they had previous stroke, malignant tumors, renal disease, acute myocardial infarction, severe heart failure, immune diseases or other neurological diseases. Finally, AIS patients were included in our study. The patients underwent standard neurological and general medical evaluations and assessments with the National Institute of Health Stroke Scale (NIHSS) score at admission, and ischemic stroke was diagnosed in accordance with the guidelines. Age- and sex-matched healthy controls were recruited from the Medical Examination Center. Some patients were examined at a follow-up visit one year later, and a modified Rankin Scale (mRS) score was used to investigate the patient’s functional independence.

Separation of neutrophils

Venous blood samples from AIS patients and healthy controls were collected into K3EDTA tubes, and neutrophils were separated by a standard Ficoll-Paque Plus gradient method as previously reported [13]. Then, neutrophils were suspended in 0.5 ml of TRIzol and frozen at −80°C prior to the test.

Real-time reverse transcription PCR (RT-PCR)

Total RNA from neutrophils was isolated using oligo-d (T) primers and SuperScript III /RNaseOUT Enzyme Mix (Invitrogen, Carlsbad, CA, USA). For miR and mRNA quantification, total RNA was reverse transcribed using the RNeasy Mini Kit (Qiagen, Gaithersburg, MD, USA) according to the manufacturer's protocol. MiRs and mRNA abundance was assessed by RT-PCR using All-in-One miR RT-PCR Reagent Kits. Values were normalized to U6 or β-actin via the $2^{-\Delta\Delta CT}$ method. RT-PCR was performed in triplicate. Primers are listed in Table 1.

ChIP sequencing

Neutrophils of AIS patients were cross-linked in 1% formaldehyde for 20 minutes at room temperature. Then, Chip-Seq samples were pooled for library construction and NGS by Illumina Genome Analyzer IIx. Next, we sonicated whole-cell extracts before immunoprecipitation with the anti-HDAC2 antibody. The purity and concentration of DNA samples were assessed with a Qubit® Fluorometer. DNA samples were end-repaired, A-tailed, and adaptor-ligated with a TruSeq Nano DNA Sample Prep Kit (FC-121-4002, Illumina). Fragments of approximately 200–1500 bp were size-selected using AMPure XP beads. The final size of the library was confirmed by an Agilent 2100 Bioanalyzer. Then, the samples were diluted to a final concentration of 8 pM, and cluster generation was performed on the Illumina cBot using a HiSeq 3000/4000 PE Cluster Kit (PE-410-1001, Illumina). Sequencing was carried out on an Illumina HiSeq 4000 using a HiSeq 3000/4000 SBS Kit (FC-410-1003, Illumina). Image and base calling, Solexa CHASTITY quality filter, human genome (UCSC hg19) alignment, peak detection, and peak annotation were performed, and the data were analyzed. The ChIP samples from each sample served as negative inputs for peak calling.

Animals

Adult male C57BL/6J mice weighing 20 to 22 g were obtained from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and housed under designated environmental conditions of 50-60%

humidity, 22-24°C with a 12 hour:12 hour light:dark cycle at Xuanwu Hospital. The animals had free access to water and a standard laboratory chow diet before and after surgery. All animal studies were approved by the Institutional Animal Care and Use Committee of Capital Medical University and carried out according to the international and national law and policies (ARRIVE guidelines and the Basel Declaration including the 3Rs concept) [16]. Animal groups were randomized and blinded to experimenters.

Transient middle cerebral artery occlusion (tMCAO) surgery and groups

The MCAO model was induced through intraluminal occlusion of the right middle cerebral artery as published previously [17]. Briefly, mice were anesthetized with isoflurane, and their body temperature was maintained at 37.0 ± 0.5°C with a heat lamp during the surgery. A silicone rubber-coated nylon filament with a 0.19 mm diameter silicon tip (Catalog number: 701956PK5Re; Doccol Corporation, Sharon, MA, USA) was inserted into the right middle cerebral artery for 45 minutes to obstruct the flow of blood, and then the suture was removed to allow for reperfusion. Local cerebral blood flow was monitored using a transcranial laser Doppler (LDF, PeriFlux System 5000; Perimed, Sweden) to verify the occlusion of the middle cerebral artery. Sham-operated mice underwent a similar procedure without suture insertion. All attempts were made to minimize animal suffering and the number of animals used.

Mice were randomly grouped into three groups with a lottery drawing box: (1) sham group: mice accepted similar procedures without inserting the suture; (2) MCAO + control group: mice underwent MCAO surgery and were injected with negative control miRNA; (3) MCAO + antagomiR-494 group: mice underwent MCAO surgery and were injected with antagomiR-494. AntagomiR-494 and negative control miRNA (100 μM, GenePharma, Suzhou, China) were administered to mice via right intracerebroventricular injection over 20 minutes, and the needle was held in situ for 10 minutes before it was slowly withdrawn. The sequence of the negative control miRNA was: 5’-CAGUACUUUUGUGUAGUACAA-3’, and the sequence of antagomiR-494 was 5’-GAGGUUUCCCGUGUAUGUUUCA-3’. Three to four mice in each group were used for flow cytometry analysis (bone marrow and blood, respectively), and six mice in each group were used for immunofluorescence staining at 3 days poststroke.

Immunofluorescence staining

Brain tissues for immunofluorescence analysis were obtained 3 days after MCAO and fixed in 4% paraformaldehyde for at least 48 hours. Mouse brains were transferred to a 30% sucrose solution and allowed to dehydrate. Then, 20-mm-thick coronal brain slices were acquired on a cryostat vibratome and incubated with a primary antibody against Ly-6G (1:200, Santa Cruz Biotechnology, CA, USA) at 4°C overnight followed by incubation with a donkey anti-rat antibody conjugated to Alexa 488 (1:200, Jackson ImmunoResearch) for an hour at room temperature. Images were then captured using a

fluorescence microscope (Olympus, Japan), and the number of Ly-6G-positive cells in the infarction border cortex was counted and analyzed with ImageJ software.

Flow cytometry analysis

For flow cytometry analysis, blood cell lysis buffer (eBioscience, San Diego, CA, USA) was added to peripheral blood and bone marrow cells. Then, the cells were centrifuged and suspended in 2% fetal bovine serum (FBS) in PBS. Next, cells were stained with Ly-6G-PE, CD11b-PerCP/PECy5.5, CD16-FITC and CD206-APC (BD Biosciences, New Jersey, USA). After filtration through a 40 μm nylon cell strainer, samples were detected on a BD Accuri™ C6 for fluorescence flow cytometry analysis.

Human HL-60 cell culture

Human HL-60 cells (ATCC, Rockville, MD, USA) were cultured in RPMI-1640 medium containing 10% heat-inactivated FBS and 1% penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO_2/95% air. Cells were divided into 3 groups: (1) Control group, (2) HL-60 + antagomiR-494 (50 nM) group, (3) HL-60 + antagomiR-494 (50 nM) + HDAC2 siRNA (50 nM) group. After cultivation for 24 hours, 200 μl supernatant was collected for further experiments, and the remaining cells were collected for the detection of HDAC2 mRNA, MMP7 mRNA, MMP10 mRNA, MMP13 mRNA, MMP16 mRNA, MMP21 mRNA, MMP25-AS1 mRNA, and MMP27 mRNA. Primers are listed in Table 1. Another set of transfected HL-60 cells was prepared for coculture with primary neurons.

Cortical primary neuron cultures and oxygen-glucose deprivation

Cortices from the brains of C57BL/6J mice of embryonic day 16-18 were dissected and minced into small pieces using a sterile scalpel and digested for 3 minutes using trypsin (Sigma-Aldrich). Then, trypsin was discarded, and fresh buffer with DNAzyme was added, followed by filtration through a 70-μm mesh cell. Single cell suspensions were suspended in Dulbecco's modified Eagle medium (DMEM) containing 10% FBS and 100 U/ml penicillin-streptomycin and seeded into culture plates precoated with poly-D-lysine (0.1 mg/mL) at a $6*10^5$ cells/mL density. Six hours later, neurons medium was replaced with Neurobasal/B27/L-glutamine/Gentamicin medium. Five days later, 80–90% confluent neurons were used for oxygen-glucose deprivation (OGD) treatment for 2 hours. After OGD, neurons were stimulated with supernatant collected from HL-60 cells or cocultured with transfected HL-60 cells. Twenty-four hours later, neurons in 48-well plates were fixed and assessed by immunofluorescence staining and a high-content screening assay for neurite outgrowth.

High-content screening assay

Neurons in 48-well plates were fixed with 4% paraformaldehyde and then blocked with 5% donkey serum and 0.3% Triton X-100 in PBS for 1 hour at room temperature. Then, the cells were incubated with primary antibodies against synaptophysin (1:150, catalog 32127, Abcam) overnight at 4°C. Then, the neurons were incubated with a donkey anti-rabbit antibody conjugated to Alexa 594 (1:200, Jackson ImmunoResearch), followed by counterstaining with DAPI. Total outgrowth, total processes, mean processes, total branches and total cell body areas were evaluated under a phase-contrast microscope using a 10× objective of ImageXpress Micro XLS System (ImageXpress Micro XLS System, Molecular Devices, USA). Sixteen areas in each well were randomly selected. Neurite length was measured using MetaXpress (RRID: SCR_016654). In this multiparameter assay, the software can segment the neuronal cell body and compute fluorescently labeled neurites with respect to their total outgrowth, branches, processes, cell body area and mean process.

Neutrophil transmigration assay

Human umbilical vein endothelial cells (HUVECs) were grown to confluence on transwell inserts (6.5 mm diameter, 8-μm pore size; Corning Inc., NY). HUVECs were subjected to 6 hours of OGD. Control HL-60 cells, antagomiR-494-treated HL-60 cells, and antagomiR-494 plus HDAC2 siRNA-treated HL-60 cells (2×10^5 in 200 μL) were placed in the upper chamber, 500 μl 10% FBS RPMI 1640 medium containing 1 μmol neutrophil attractant fMLP was added to the bottom chamber. After 3 hours of migration, the neutrophils in the lower transwell chamber were observed under a fluorescence microscope. Five randomly selected fields were captured, and the transmigrated neutrophils were quantified using ImageJ software.

Statistics

Data are presented as the mean±SD. Independent sample t-test was used for comparisons between two groups, while one-way ANOVA with Tukey's post hoc test was applied for comparisons between multiple groups. Correlation analysis was conducted using the Spearman correlation test. Receiver operator characteristic (ROC) curve analysis was adopted to calculate the predictive power of the sensitivity and specificity for the diagnosis of ischemic stroke, and the area under the ROC curve (AUC) was calculated. Differences were regarded as significant if $p<0.05$. All statistical analyses were implemented using SPSS Statistics 21.0 software (IBM Corp., Armonk, NY, USA) and GraphPad Prism v7 (San Diego, CA, USA).

Results

Elevated miR-494 in neutrophils of AIS patients predicts worse neurological outcomes

First, we assessed whether miR-494 was abnormally expressed in neutrophils of AIS patients, and RT-PCR analysis revealed that miR-494 was obviously elevated in AIS patients compared to that in healthy controls (Fig. 1A; $p<0.05$). To explore the clinical significance of the rising miR-494 levels in neutrophils of ischemic stroke patients at the hyperacute phase, an ROC curve was calculated to determine the diagnostic value of neutrophil miR-494 expression in distinguishing healthy controls from AIS patients. Our results suggested that neutrophil miR-494 expression had good diagnostic power for ischemic stroke, with an area under the ROC curves of 0.683 (Fig. 1B). To further preliminarily establish the relationship between rising miR-494 expression in neutrophils and neurological function and prognosis, we performed a correlation analysis and found that miR-494 expression in neutrophils was positively correlated with the key indicator of the degree of neurological deficit (NIHSS score) at admission and the key clinical outcome indicator (mRS score) one year after symptom onset (Fig. 1C, D; $p<0.05$). These findings indicate that increased miR-494 expression in neutrophils of AIS patients is related to serious neurological deficits and predicts worse neurological outcomes.

MiR-494 correlates with the upregulation of adhesion molecules in neutrophils of AIS patients

To further characterize the phenotypic shift of peripheral neutrophils of AIS patients, we analyzed the relevance between expression of N1 and N2 markers of neutrophils and neutrophil miR-494 expression. First, the RT-PCR results demonstrated that both the CD16 and CD206 levels in neutrophils were significantly decreased in AIS patients compared with those in healthy controls (Fig. 2A, B; $p<0.05$). However, our correlation analysis showed no obvious linear correlation between the CD16 level and miR-494 expression in neutrophils, nor a correlation between the CD206 level and miR-494 expression in neutrophils (Fig. 2C, D).

Given that our preceding bioinformatics analysis indicated that miR-494 target genes are involved in the focal adhesion molecule pathway [13], we investigated the expression of the classical adhesion molecules CD11b and MMP9 in the peripheral neutrophils of AIS patients and analyzed their relationship with the miR-494 levels. Consistent with bioinformatics analysis, both CD11b and MMP9 were prominently increased in neutrophils of AIS patients and were negatively associated with miR-494 expression in neutrophils (Fig. 2E-H; $p<0.05$). These results demonstrate that miR-494 may target the regulation of neutrophil infiltration after ischemic stroke.

Intravenous antagomiR-494 partly shifts neutrophils into the N2 phenotype in MCAO mice

To further explore the immunomodulatory effect of miR-494 on neutrophils *in vivo*, we implemented an MCAO mouse model. Flow cytometry analysis revealed that the ratio of both N1 and N2 neutrophils in the peripheral blood was increased (Fig. 3A-C) and that the ratio of N2 neutrophils was decreased in the bone marrow of ischemic mice (Fig. 3D-F); in addition, antagomiR-494 reversed the reduction in N2 neutrophils

in the bone marrow remarkably (Fig. 3F; $p<0.05$). However, the ratio of N2 neutrophils in peripheral blood in MCAO+antagomiR-494 mice was not obviously changed compared with that in MCAO mice (Fig. 3A, C). Noticeably, the alteration of N1 neutrophils in both peripheral blood and bone marrow was not evident after antagomiR-494 treatment (Fig. 3A, B, D, E). These results suggested that ischemia led to distinct changes in N2 neutrophils and that miR-494 could induce an apparent increase in N2 neutrophils in bone marrow after MCAO.

AntagomiR-494-treated neutrophils exert a neuroprotective role *in vitro*

To explicate whether antagomiR-494-treated neutrophils could directly or indirectly influence hypoxia-induced neuronal injury, we treated neurons subjected to OGD with conditioned medium from differently treated HL-60 cells (Fig. 4A) or cocultured neurons with differently treated HL-60 cells (Fig. 4B) for 24 hours. High-content analysis for the neurite marker synaptophysin showed that there was a substantial increase in axonal injury in OGD-treated neurons, and treating HL-60 cell supernatant with antagomiR-494 partly mitigated axonal injury, as indicated by an increased number of neuron processes (Fig. 4A; $p<0.05$). However, antagomiR-494-treated HL-60 cells seemingly did not show any protective effect on the hypoxia-associated neurotoxic response when cocultured with neurons (Fig. 4B). Thus, we demonstrated that antagomiR-494 might exert a slightly neuroprotective effect on ischemic neurons by shifting neutrophils toward a beneficial N2 phenotype.

HDAC2 targets multiple MMP genes in neutrophils of AIS patients

Previously, we showed that miR-494 regulates the Th1/Th2 shift by directly targeting HDAC2. In light of this, we assessed expression of HDAC2 in neutrophils and found that expression was obviously downregulated in AIS patients compared to that in healthy controls; these results are contrary to those of miR-494 expression (Fig. 5A). To identify HDAC2 target genes in neutrophils of AIS patients, we performed ChIP-seq and revealed that HDAC2 bound to various DNA-binding sites of several MMPs. Among them, HDAC2 was found to bind to the upstream region of the MMP21 DNA and MMP25-AS1 genes, the intergenic region of the MMP16, MMP13 and MMP10 genes, and the intron region of the MMP7 gene. Noticeably, HDAC2 could bind to both the upstream and intergenic regions of the MMP27 gene at different loci (Fig. 5B, C, D). These data indicate that downregulated HDAC2 potentially regulates the expression of multiple MMP genes in neutrophils of AIS patients.

AntagomiR-494 repressed MMP expression and brain-infiltrating neutrophils by regulating HDAC2

To verify whether miR-494 affects MMPs by targeting HDAC2, we transfected HL-60 cells with antagomiR-494 or HDAC2-specific siRNA and examined the mRNA levels of MMPs in neutrophils. We

found that antagomiR-494 significantly reduced the expression of MMP10, MMP13, MMP16 and MMP7 in neutrophils (Fig. 6A-D; $p<0.05$); this effect was partly counteracted by HDAC2-specific siRNA and was indicated by the upregulated expression of MMP10, MMP13 and MMP7 (Fig. 6A-C; $p<0.05$). In addition, our *in vitro* transendothelial migration experiment showed that OGD induced obvious migration of neutrophils, which was significantly decreased by antagomiR-494 treatment. Moreover, HDAC2 siRNA abolished neuroprotection provided by antagomiR-494 (Fig. 6H, I; $p<0.05$). To further test the effect of miR-494 on neutrophil infiltration in experimental stroke, we established a MCAO mouse model and detected the infiltration of neutrophils in the ischemic brain. Immunofluorescence staining demonstrated that ischemia led to an evident increase in neutrophils in the ischemic brain; however, antagomiR-494 administration through the tail vein largely reversed this situation (Fig. 6). These results illustrate that antagomiR-494 could reduce the expression of MMPs and neutrophil infiltration in ischemic brains by regulating HDAC2.

Discussion

The present study demonstrates that miR–494 plays a crucial role in mediating both brain infiltration of neutrophils and N1/N2 neutrophil programming after ischemic stroke. Here, we show that elevated neutrophil miR–494 levels in AIS patients were correlated with worse clinical neurological outcomes. Moreover, antagomiR–494 partly shifted neutrophils into the N2 phenotype *in vivo* and mediated certain neuroprotective effects *in vitro.* More importantly, we discovered that miR–494 levels correlated with the upregulation oftheadhesion molecules CD11b and MMP9 in neutrophils of AIS patients. Further, HDAC2 binds to multiple MMP genes in neutrophils of AIS patients, and antagomiR–494 represses MMP expression and brain-infiltrating neutrophils by regulating HDAC2. Overall, our study provides the first evidence of neutrophil reprogramming in neuroinflammation following ischemic stroke by miR–494-induced modulation of HDAC2.

Combined with our previous studies, miR–494 was found to be increased in peripheral neutrophils as well as plasma and lymphocytes [13] of AIS patients and reflects both neurological deficits at admission and long-term neurological outcome scores. As there is no ideal biomarker to predict the long-term prognosis of AIS, we speculate that miR–494 in the peripheral blood may provide an alternative choice. After stroke, the population of neutrophils is heterogeneous, including pro- and anti-inflammatory neutrophils (N1/N2). Our clinical data demonstrated that both N1 and N2 neutrophils were decreased in the blood of AIS patients but did not show a linear correlation with miR–494 levels. In MCAO mice, ischemia led to an obvious increase in N2 neutrophils in the blood and a decrease in N2 neutrophils in the bone marrow, and these results are consistent with those of other studies [7]; antagomiR–494 treatment via the tail vein partly reduced the number of N1 neutrophils in the peripheral blood and markedly elevated the number of N2 neutrophils in the bone marrow. In accordance with these results, an *in vitro* study showed that antagomiR–494-treated neutrophils or the supernatant could reverse OGD-induced primary neuronal damage. Taken together, it is highly likely that systemically administered antagomiR–494 confers a neuroprotective effect by mediating the N2 shift and Th2 shift [13] as well as by mitigating brain-infiltrated neutrophils after ischemic stroke.

Except for the regulation of the phenotypic shift of immune cells, our prior bioinformatics analysis revealed that miR–494 could modulate the focal cell adhesion pathway [13]; we then inspected two important adhesion molecules involved in the infiltration of neutrophils [18, 19] and found that CD11b and MMP9 were significantly increased in neutrophils of AIS patients and were associated with miR–494 levels. Subsequently, both *in vitro* and *in vivo* experiments confirmed that antagomiR–494 could reduce the migration and infiltration of neutrophils following stroke.

Our laboratory has already proven that miR–494 can directly bind to the 3' noncoding region of HDAC2 and inhibit HDAC2 expression by a luciferase reporter gene assay. Thus, we examined the expression of HDAC2 in neutrophils and found that HDAC2 was remarkably decreased in AIS patients compared with that in healthy controls, and these results are in direct contrast to the change in miR–494 expression. Therefore, we further applied ChIP sequencing to study the regulation of HDAC2 involved in the expression of genes in neutrophils in patients with AIS. Interestingly, we found that HDAC2 has a broad regulatory effect on MMPs in neutrophils. Among them, HDAC2 was upregulated when bound to the MMP16, MMP13, MMP10 and MMP21 genes, whereas HDAC2 was downregulated when bound to the MMP–25AS1 and MMP7 genes. Noticeably, HDAC2 could bind to different loci on chromosome 11 and can modulate MMP27 bidirectionally. Previous studies suggested that the HDAC family could regulate multiple MMPs, including MMP1, MMP2, MMP3, MMP7, MMP9, MMP10, MMP13, MMP14, and MMP28 [20–25], and that MMP10 could be regulated by HDAC3 and HDAC7 [25, 26]. For the first time, we found that HDAC2 could potentially modulate the MMP10, MMP16, MMP21, MMP25-AS1, and MMP27 genes in neutrophils of AIS patients.

Then, through our *in vitro* experiments, we further verified that prescribing neutrophils with antagomiR–494 could decrease expression of MMP7, MMP10, MMP13 and MMP16. Among them, MMP7 was reported to be involved in neutrophil migration and activation [27, 28]. Moreover, MMP13 deficiency was involved in a reduced infarction area, reduced hemorrhagic events and improved neurological outcome in the acute phase of ischemic stroke [29], and it was found to be related to neutrophil infiltration in bladder cancer [30]. Moreover, MMP10 was significantly increased in the brains of mice subjected to ischemic stroke [31] and was associated with macrophage-mediated neuroinflammation and atherosclerosis [32]. MMP16 was found to be associated with the migration of HUVECs [33], with no reports about its effects on neutrophil migration. Here, we found that MMPs may be involved in miR–494-mediated neutrophil migration in ischemic stroke. However, only MMP7, MMP10 and MMP13 could be upregulated after miR–494 plus HDAC2 siRNA treatment but could not be upregulated with antagomiR–494 treatment. Therefore, although antagomiR–494 could regulate MMPs by targeting HDAC2, it might not be the only modulatory pathway. The administration of HDAC2-specific siRNA did not completely reverse the downregulation of MMPs. Hence, our results suggested that miR–494 could regulate the expression of MMPs partly by targeting HDAC2.

However, some flaws exist in our study. Our study found that not all downregulated MMPs in antagomiR–494-treated neutrophils could be reversed by HDAC2 siRNA administration. As discussed above, multiple MMPs could be regulated by the HDAC family, including HDAC1, HDAC2, HDAC3, and HDAC7. Our

previous study suggested that HDAC3 was a direct target of miR–494 [34]. It is possible that miR–494 could regulate these MMPs by targeting HDAC3 or other members of the HDAC family. Further studies are warranted to dissect the complex pathways for miR–494 in regulating the expression of MMPs.

Conclusions

Our present study suggested that miR–494 in neutrophils might provide an excellent choice for the diagnosis and prediction of ischemic stroke. In addition, miR–494 had a slight regulatory effect on the neutrophil phenotypic shift and the N2 neutrophil-mediated neuroprotective effect. Most importantly, we showed for the first time that miR–494 could modulate expression of MMP7, MMP10 and MMP13 and that miR–494 is involved in the infiltration of neutrophils partly by targeting HDAC2.

Abbreviations

HDAC: histone deacetylase; MMP: matrix metalloprotein; NIHSS: National Institute of Health stroke scale; mRS: modified Rankin Scale; ROC: Receiver operator characteristic; AUC: area under ROC curve; HUVECs: Human umbilical vein endothelial cells; OGD: oxygen-glucose deprivation; MCAO: Middle cerebral artery occlusion; ChIP-Seq: Chromatin immunoprecipitation sequencing; RT-PCR: real-time reverse transcription PCR.

Declarations

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Not applicable.

Authors' contributions

FFL, HPZ, GWL, SJZ, RLW, ZT, QFM, ZPH and PL performed the experiment. FL and HZ analyzed and interpreted the data, and wrote the manuscript. YML conceived of the study and supervised the project. All authors read and approved the final manuscript.

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Availability of data and materials

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

Ethics approval and consent to participate

All animal studies were approved by the Institutional Animal Care and Use Committee of Capital Medical University and carried out in line with the international and national law and policies (ARRIVE guidelines and the Basel declaration including the 3Rs concept). And patients' studies were approved by the Medical Ethics Committee of Xuanwu Hospital of Capital Medical University (trial registration no. ChiCTR-NCT-03577093).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Table

Table 1. Primers for RT-PCR.

Gene	Primers (Forward)	Primers (Reverse)
MiR-494	5’-GGGAGGTTGTCCGTGTTGT-3’	5’-GTGCGTGTCGTGGAGTCG-3’
CD16	5’-TTTACAGAATGGCAAAGGCA-3’	5’-CATCACCAAGCAGAAAGAGAC-3’
CD206	5’-TCAATAACAAAGAGGAACAGC-3’	5’-ACCATCACT CCAAGTAAAACC-3’
CD11b	5’-ACCTCGCATAACCACCTCCTG -3’	5’-GTCCTTGTATTGCCGCTTGAA-3’
MMP9	5’-TCGAACTTTGACAGCGACAAGA-3’	5’-TTCAGGGCGAGGACCATAGA -3’
HDAC2	5’-CTGCTACTACTACGACGGTGA-3’	5’-ATTTAACAGCAAGTTATGGGTC-3’
β-actin	5’-GTGGCCGAGGACTTTGATTG-3’	5’-CCTGTAACAACGCATCTCATATT-3’
GAPDH	5’-GGGAAACTGTGGCGTGAT-3’	5’-GAGTGGGTGTCGCTGTTGA-3’
HDAC2	5’-CTGCTACTACTACGACGGTGA-3’	5’-ATTTAACAGCAAGTTATGGGTC-3’
MMP27	5’-AAGGATGGAGCAGGATTCAA-3’	5’-AAAGTCAAGTCAGGGTCACAGG-3’
MMP13	5’-TTGGGAGAGCCATACATAAAAGA-3’	5’-TGTCTGTGTGAAGAAGGGCAC-3’
MMP10	5’-GGCGAGATAGGGGGAAGAC-3’	5’-AGAACATGCAGGAAAAATTAACC-3’
MMP21	5’-CTGGCTTCCTGCTAATGGC-3’	5’-AAGTTCAACTAAGGCTTTTCCC-3’
MMP7	5’-CTCCTTTATGGTGTGACTGTGTC-3’	5’-GTAACATTTATTGACATCTACCCAC-3’
MMP25-AS1	5’-CAGGACCCAGAGGATTCACAC-3’	5’-CAGAGCAAGAGGCAGGAGGT-3’
MMP16	5’-TTCTTTCCACCTTACTCCTCCA-3’	5’-GACATACTGCTTCTTGCTCTGC-3’

Figures

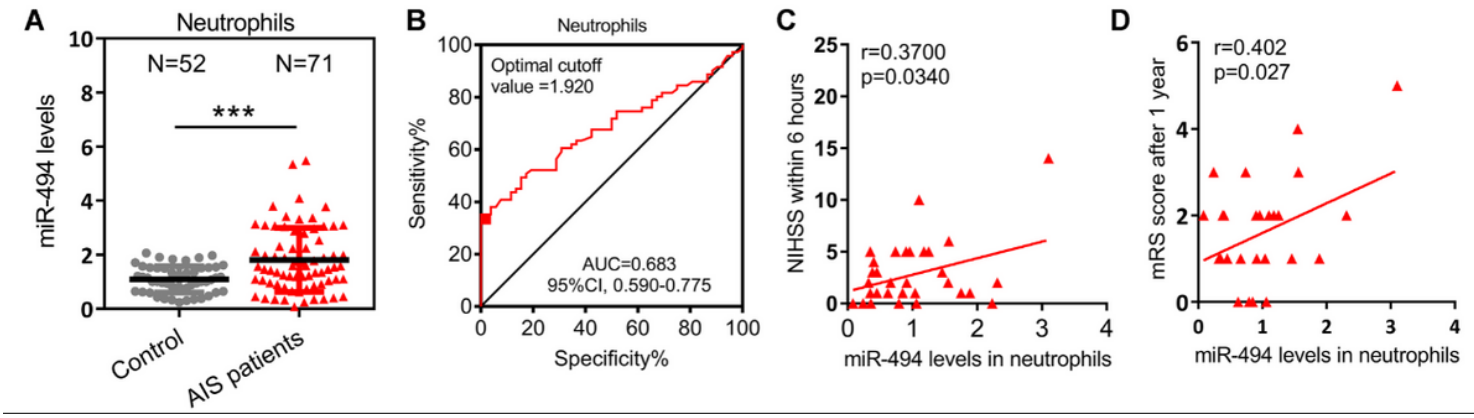


Figure 1

Elevated miR-494 levels in neutrophils of AIS patients predict worse neurological outcomes. (A). RT-PCR for miR-494 expression in neutrophils of healthy controls (n=52) and AIS patients (n=71). (B). The ROC curve of miR-494 in neutrophils for predicting AIS (n=52 in the control group, n=71 in the AIS group). (C). Correlation analysis between miR-494 in neutrophils and NIHSS score within 6 hours after symptom onset (n=33). (D) Correlation analysis between miR-494 in neutrophils and mRS score one year after symptom onset (n=30). ***p<0.001 vs. control group.

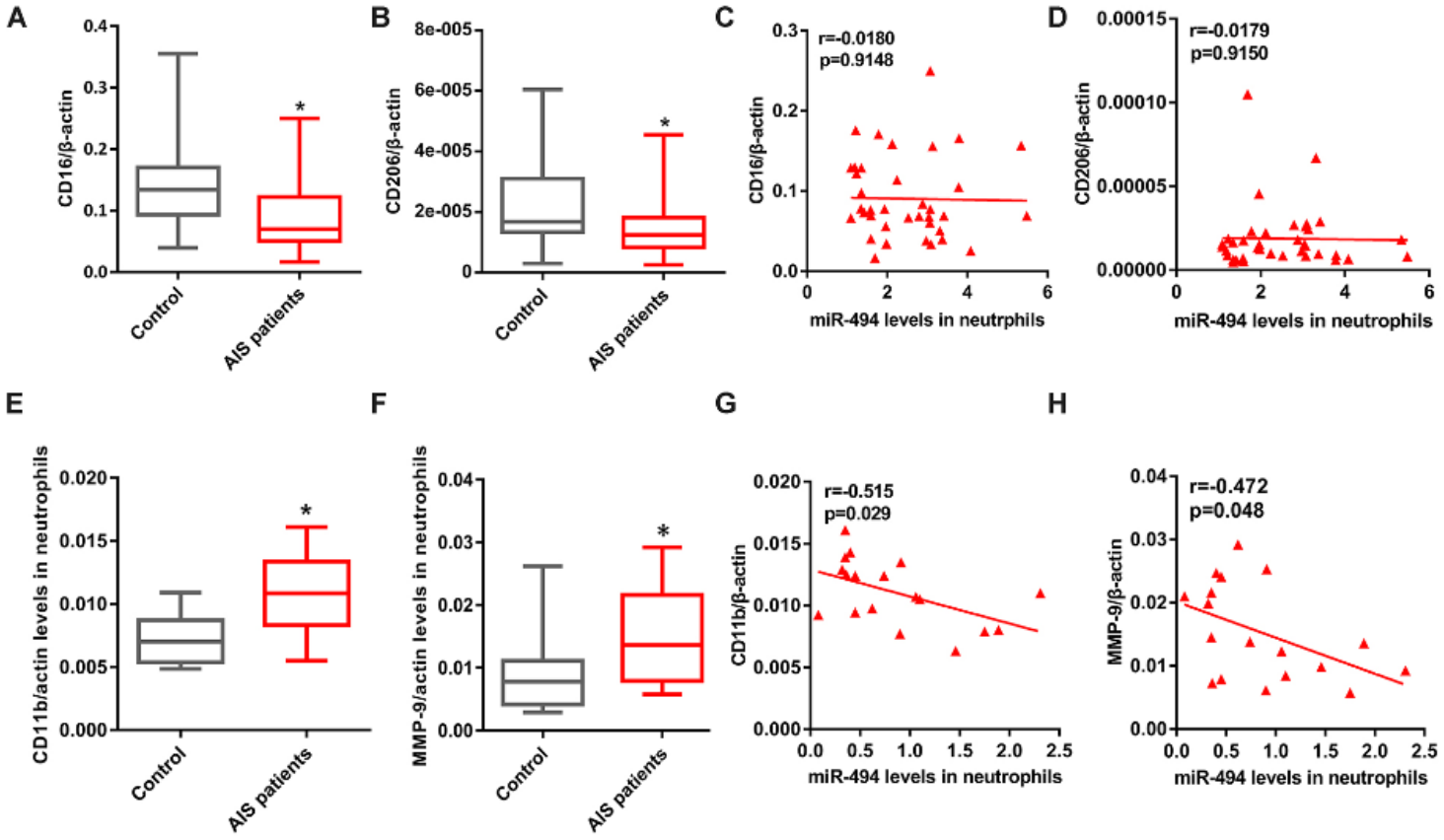


Figure 2

MiR-494 correlates with the upregulation of adhesion molecules in neutrophils of AIS patients. (A-B) Using RT-PCR to detect the mRNA expression of CD16 and CD206 in neutrophils in controls (n=27) and AIS patients (n=43). (C) Correlation between CD16 and miR-494 levels in neutrophils. (D) Correlation between CD206 and miR-494 levels in neutrophils. (E-F) Using RT-PCR to detect the mRNA expression of CD11b in neutrophils in control (n=17) and AIS patients (n=20). (G) Correlation between CD11b and miR-494 in neutrophils in AIS patients. (H) Correlation between MMP9 and miR-494 in neutrophils in AIS patients. *p<0.05 vs. control group.

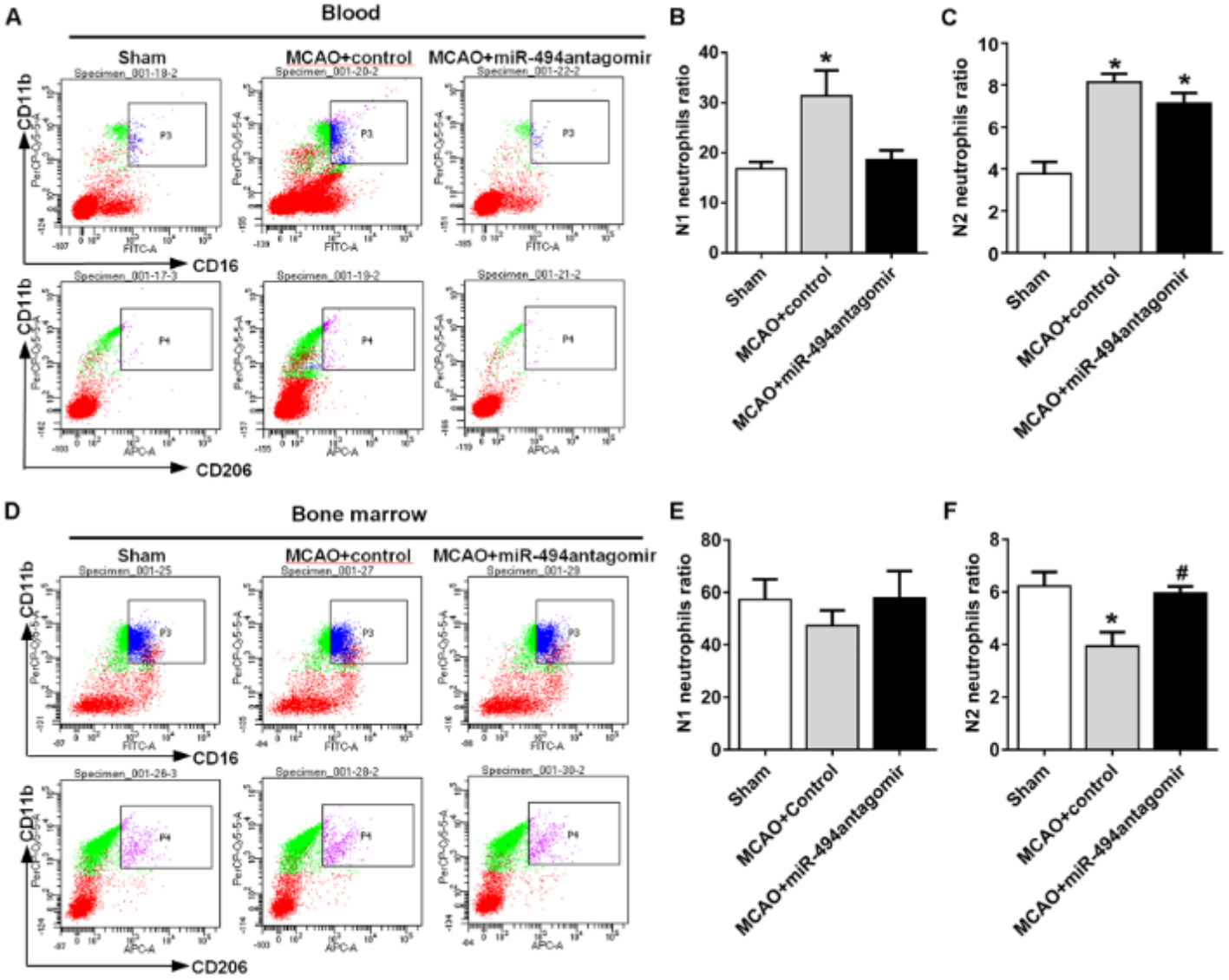


Figure 3

AntagomiR-494 partly shifts neutrophils into the N2 phenotype in MCAO mice. Flow cytometry analysis was used to detect the N1/N2 neutrophil ratio. Blood (A) and bone marrow (D) neutrophils were stained for CD11b, Gr-1, and CD16 (or CD206) 3 days after MCAO. Quantification of Gr-1+CD11b+CD16+ neutrophils in peripheral blood (B) and bone marrow (E) and Gr-1+CD11b+CD206+ neutrophils in peripheral blood (C) and bone marrow (F) in sham and MCAO animals with or without antagomiR-494 treatment (n=3-4). *p<0.05 vs sham group, #p<0.05 vs MCAO + control group.

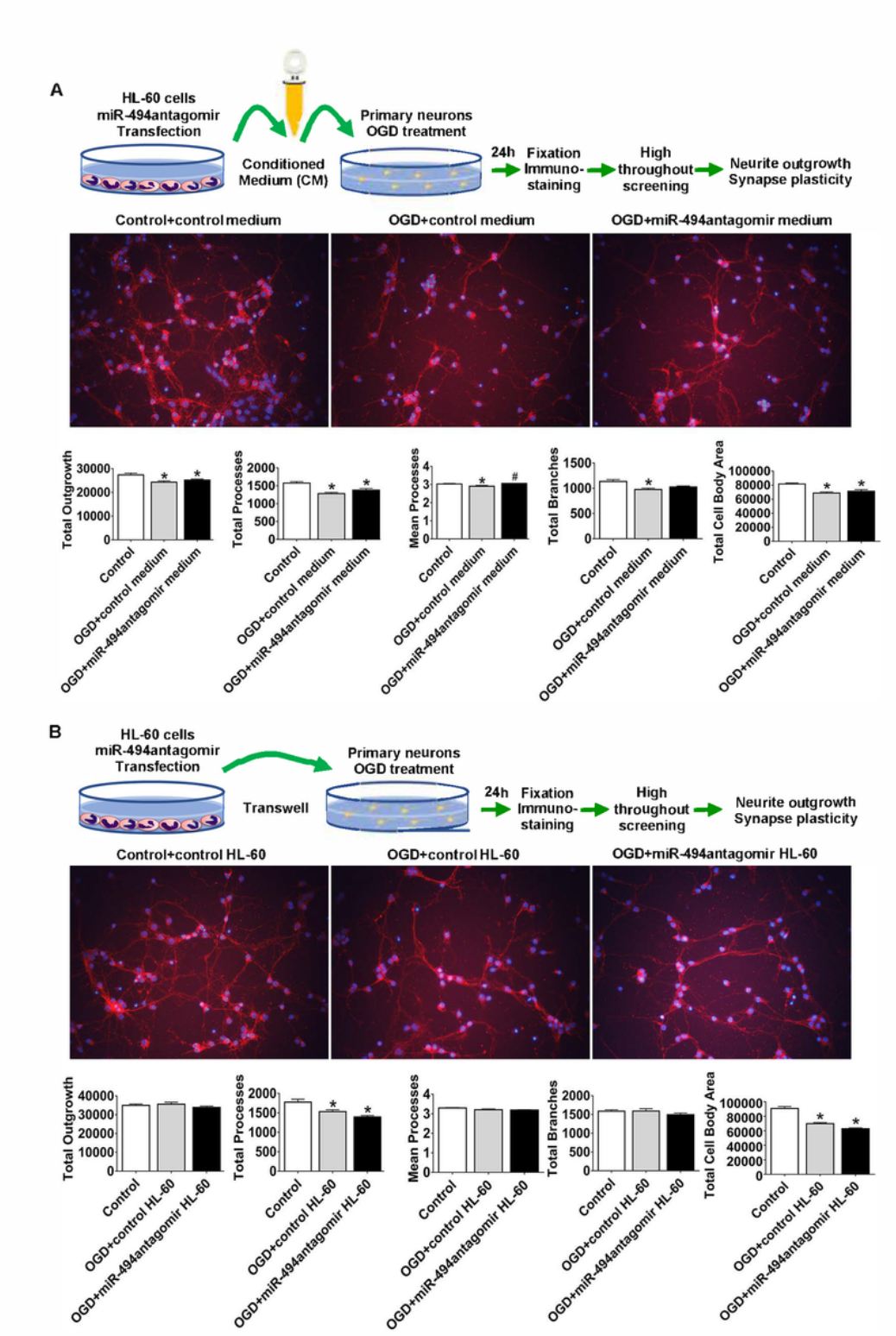
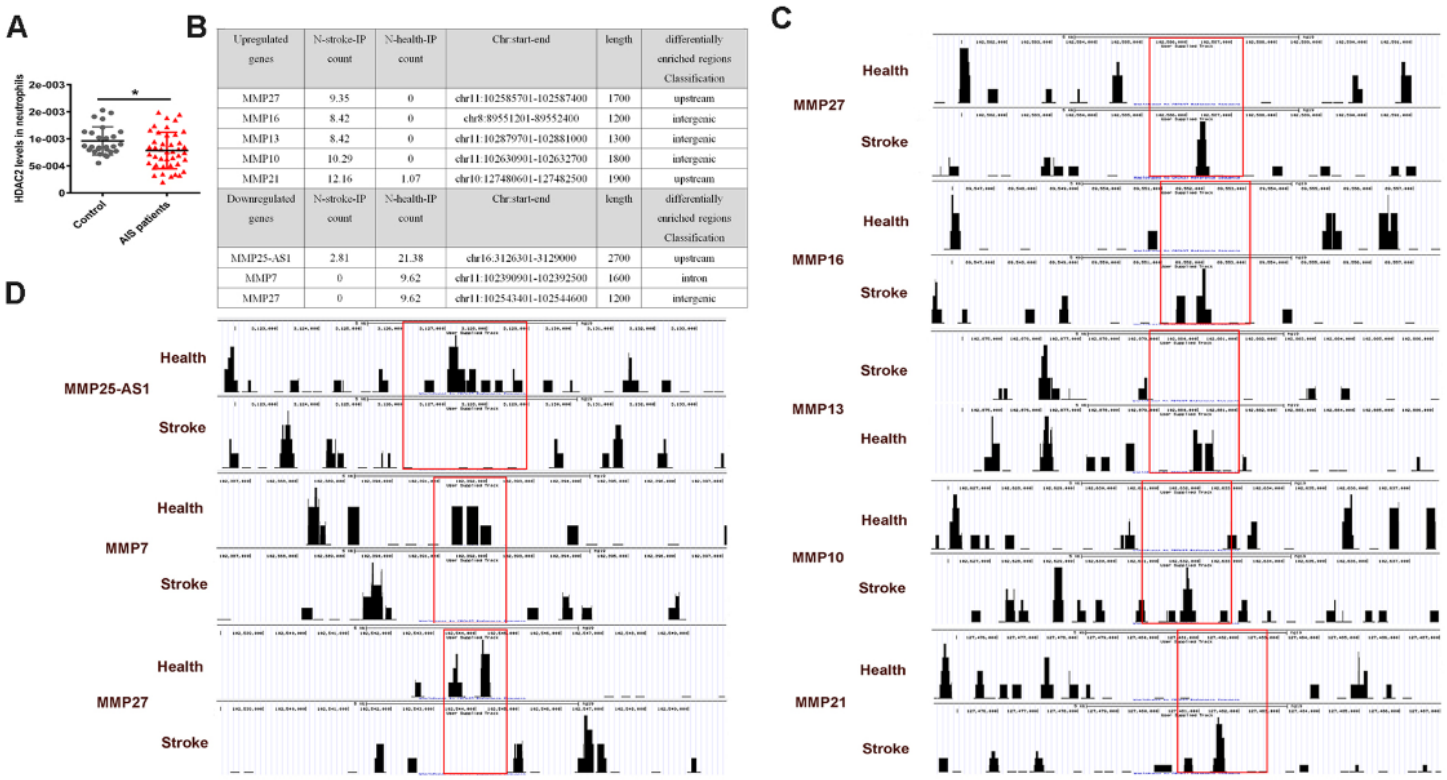


Figure 4

AntagomiR-494-treated neutrophils exert a neuroprotective role in vitro. HL-60 cells were transfected with control (50 nM) and antagomiR-494 (50 nM) and cultivated for 24 hours. Primary mouse cortical neurons following 2 hours of OGD were cultured with supernatant from transfected HL-60 cells or coculture with transfected HL-60 cells for 24 hours. Immunostaining and high-content screening analysis for synaptophysin (red) and DAPI (blue) are shown. (A) Primary neurons treated with supernatant from

transfected HL-60 cells for 24 hours. (B) Primary neurons were cocultured with transfected HL-60 cells for 24 hours. Images were taken with a 10× objective. n=6/group. *p<0.05 vs control group. #p<0.05 vs OGD + conditioned medium or OGD + control HL-60.



Upregulated genes	N-stroke-IP count	N-health-IP count	Chr:start-end	length	differentially enriched regions Classification
MMP27	9.35	0	chr11:102585701-102587400	1700	upstream
MMP16	8.42	0	chr8:89551201-89552400	1200	intergenic
MMP13	8.42	0	chr11:102879701-102881000	1300	intergenic
MMP10	10.29	0	chr11:102630901-102632700	1800	intergenic
MMP21	12.16	1.07	chr10:127480601-127482500	1900	upstream
Downregulated genes	N-stroke-IP count	N-health-IP count	Chr:start-end	length	differentially enriched regions Classification
MMP25-AS1	2.81	21.38	chr16:3126301-3129000	2700	upstream
MMP7	0	9.62	chr11:102390901-102392500	1600	intron
MMP27	0	9.62	chr11:102543401-102544600	1200	intergenic

Figure 5

HDAC2 targets multiple MMP genes in neutrophils of AIS patients. (A) Expression of HDAC2 in neutrophils of control and AIS patients. (B-D) ChIP-Seq profiles of HDAC2 binding sites include MMP genes such as upregulated MMP27, MMP16, MMP13, MMP10, and MMP21 and downregulated MMP25-AS, MMP7, and MMP27 in neutrophils of AIS patients versus controls. *p<0.05 vs control group.

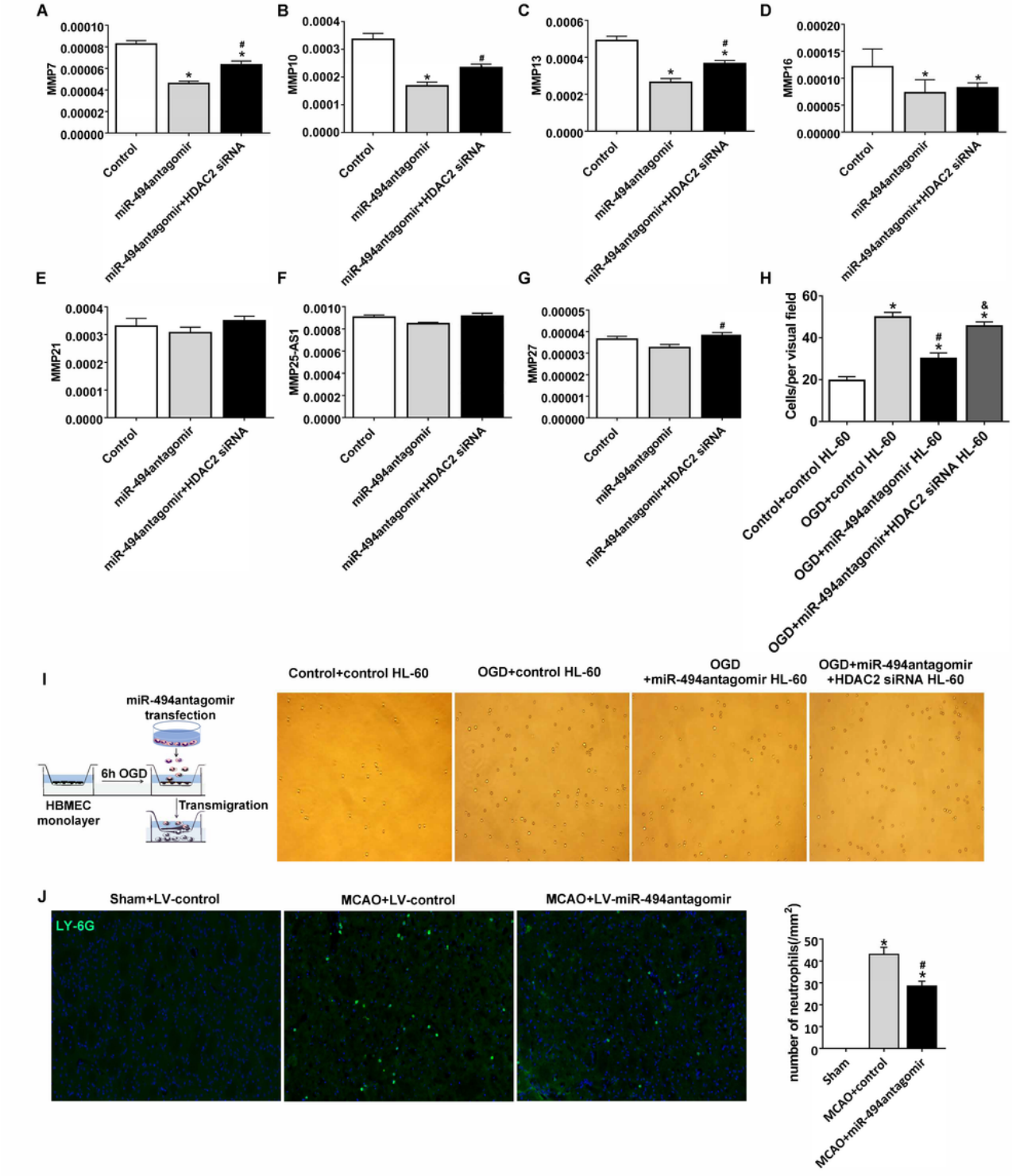


Figure 6

AntagomiR-494 repressed MMP expression and brain-infiltrating neutrophils by regulating HDAC2. HL-60 cells were transfected with control (50 nM), antagomiR-494 (50 nM), or antagomiR-494 (50 nM) + HDAC2 siRNA (50 nM). After cultivation for 24 hours, the cells were obtained and examined for the expression of MMP7, MMP10, MMP13, MMP16, MMP21, MMP25-AS1 and MMP27 by real-time PCR. (A-G) Expression of MMPs in HL-60 cells after different treatments. (I) Illustration of the in vitro migration assay. Human

bone marrow endothelial cells (HBMECs) were seeded at the top of the transwell until a monolayer was formed. Neutrophils (HL-60 cells) were transfected with control (50 nM), antagomiR-494 (50 nM), or antagomiR-494 (50 nM) + HDAC2 siRNA (50 nM) for 24 hours and plated on top of the HBMEC monolayer, which was pre-exposed to 6 hours of OGD or normal conditions (without OGD). Cells were allowed to migrate for 3 hours, and then the migrated cells in the lower chamber were collected and counted. Representative images of migratory cells are shown. (H) Histogram shows the migrated cells in the four groups (n=6). *p<0.05 vs control group, #p <0.05 vs OGD + control HL-60 group, &p<0.05 vs OGD + antagomiR-494 HL-60 group. (J) Effect of systemically administered antagomiR-494 on neutrophil infiltration in vivo. Mice were subjected to MCAO or sham surgery, and then the brain-infiltrated neutrophils were examined. Representative images for the infiltration of neutrophils (green) in different treated groups at 3 days postsurgery (n=4). Data are presented as the mean ± SEM. *p<0.05 vs sham group, #p<0.05 vs MCAO + control group.

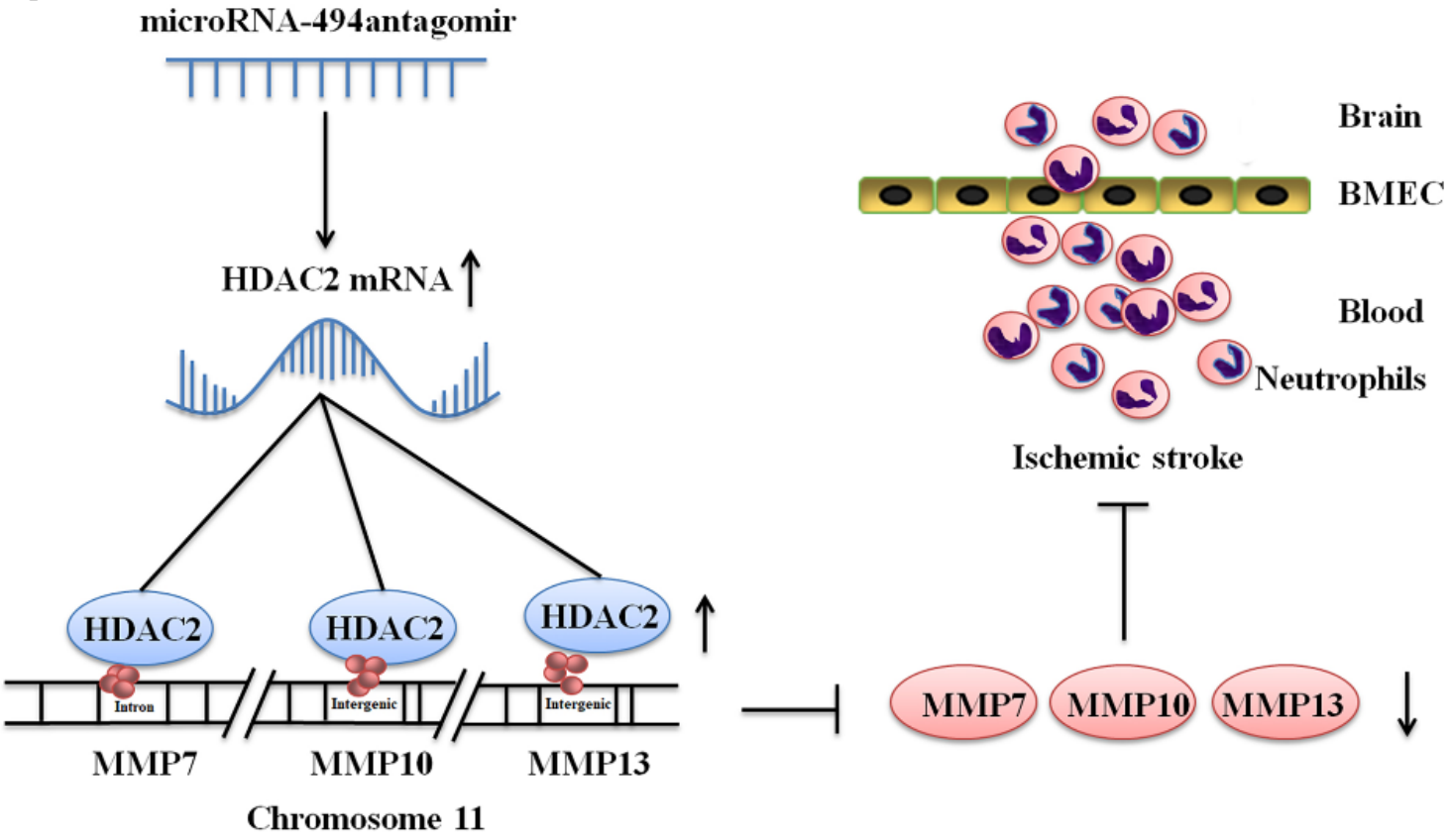


Figure 7

Schematic diagram of the proposed molecular mechanism. Our study demonstrates that antagomiR-494 treatment decreased the expression of multiple MMPs and the infiltration of neutrophils partly by targeting HDAC2. BMEC: Brain microvascular endothelial cells.