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# M6A modification promotes blood-brain barrier breakdown during cerebral ischemia/reperfusion injury through increasing matrix metalloproteinase 3 expression

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ABSTRACT

Blood-brain barrier (BBB) breakdown is a critical event in cerebral ischemia-reperfusion (I/R) injury, and matrix metalloproteinases (MMPs), which are proteolytic enzymes, play essential roles in BBB breakdown through degrading the extracellular matrix. N6-Methyladenosine (m6A), the most common and reversible mRNA modification, has an important role in the progression of cerebral I/R injury. However, whether m6A is related to BBB breakdown and MMPs expression in cerebral I/R injury is still not clear. In this study, we explored the potential effects of m6A modification on BBB breakdown in cerebral I/R injury and its underlying mechanisms using mice subjected to transient middle cerebral artery occlusion and reperfusion (MCAO/R), and mouse brain endothelial cells treated with oxygen-glucose deprivation and reoxygenation (OGD/R). We find that MMP3 expression is highly expressed and positively associated with the m6A writer CBLL1 (Cbl proto-oncogene like 1) in cerebral I/R injury *in vivo* and *in vitro*. Furthermore, MMP3 mRNA occurs m6A modification in mouse brain endothelial cells, and the m6A modification level of MMP3 mRNA is significantly increased in cerebral I/R injury. Moreover, inhibition of m6A modification reduces MMP3 expression and ameliorates BBB breakdown in cerebral I/R *in vivo* and *in vitro*. In conclusion, m6A modification promotes BBB breakdown in cerebral I/R injury through increasing MMP3 expression, indicating that m6A may be a potential therapeutic target for cerebral I/R injury.

## 1. Introduction

Ischemic stroke is still one of the major causes of disability and mortality worldwide, and causes serious sociodemographic and socioeconomic burden [1]. Although thrombolysis and thrombectomy are wildly used in clinical for patients with cerebral ischemia,

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the therapeutic effect is not very ideal, owing to the narrow therapeutic window and hemorrhagic transformation risk [2,3]. Besides, rapid recanalization of occluded arteries generally leads to ischemia-reperfusion (I/R) injury [4]. Increasing studies report that many factors participate in the cerebral I/R injury, such as blood-brain barrier (BBB) disruption, neural apoptosis, neuroinflammation, oxidative stress, excitatory toxicity, and so on [5–7]. Thus, elucidating the pathological mechanisms of cerebral I/R injury is essential for stroke therapy.

The BBB, an important physical barrier between the brain tissue and blood to maintain central nervous system homeostasis, mainly includes brain microvascular endothelial cells and tight junctions [8,9]. By lining the entire microvasculature and forming tight junctions with occludin and junctional adhesion molecules, brain microvascular endothelial cells are considered as the crucial ingredient of the BBB [10]. Several studies report that cerebral I/R triggers BBB breakdown by inducing matrix metalloproteinases (MMPs) to destroy the BBB structure [11–13]. However, the detailed mechanisms of how MMPs are regulated in cerebral I/R have not fully clear.

*N*6-methyladenosine (m6A), the most common modification in eukaryotic mRNA, participates in various cellular processes, such as mRNA metabolism, RNA splicing, and protein translation [14]. M6A modification is catalyzed by the methyltransferases (Writer), and could be removed by the demethylases (Eraser) [15,16]. In addition, m6A is recognized by "reader" proteins to regulate mRNA transcription, decoy, or translation [17]. Recent studies have shown the significance of m6A in cerebral I/R injury [18]. The methyltransferase, Methyltransferase like 3 (Mettl3) promotes stress granule formation in the early phase of ischemic stroke through regulating miR-335 maturation via m6A modification [19]. Hypothermia protects neurons from pyroptosis in cerebral I/R injury through activation hosphatase and tensin homolog via m6A [20]. However, whether m6A is related to MMPs expression in cerebral I/R injury is unknown.

Here, we analyzed the correlation between MMP-related genes and m6A-related genes in cerebral I/R using the Gene Expression Omnibus (GEO) databases, and investigated the potential effects of m6A modification on BBB breakdown in cerebral I/R injury and its underlying mechanisms *in vivo* and *in vitro*.

## 2. Materials and methods

### 2.1. Dataset acquisition and data preprocessing

The gene expression profiling dataset of GSE112348 of three 24 h post-ischemic mouse cortices and two Sham-mouse cortices (controls), and the dataset of GSE30655 including seven 24 h post-ischemic mouse cortices and three Sham-mouse cortices (controls) were downloaded from GEO. The raw data were preprocessed by the following means: 1) merging GSE112348 and GSE30655 into a metadata cohort to enlarge sample size; 2) carrying out batch normalization to offset the deviations between two datasets using R's "sva (v3.34.0)" package [21]. A total of 56 MMP-related genes were got from The PathCards Pathway Unification Database (https://pathcards.genecards.org/Card/matrix_metalloproteinases?queryString=matrix%20metalloproteinases).

### 2.2. Differentially expressed genes (DEGs) screening

Using R-packages limma [22], DEGs were screened with threshold parameters defined as $|\log2FC| > 1$ and FDR $<0.05$ (FC: fold change, FDR: false discovery rate). At the same time, the cluster heatmaps and volcano plots were executed to visualize the difference by R's"pheatmap (v1.0.12)"and"ggplot2 (v3.3.0)"packages, respectively.

### 2.3. Correlation analysis

The R software "pheatmap" package was used to draw multi-gene correlation. Used Spearman's correlation analysis to describe the correlation between quantitative variables without a normal distribution. Asterisks (*) stand for significance levels, ** for $p < 0.01$, * for $p < 0.05$.

### 2.4. Establishment of middle cerebral artery occlusion (MCAO) model in mice

All experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals established by the National Institutes of Health, and permitted by institutional ethics board of Guangzhou University of Chinese Medicine (No.20200810001). Male C57BL/6 J mice (7–8 week old, weighing 20–25 g) were purchased from the Animal Center of Guangzhou University of Chinese Medicine (Guangzhou, China), and housed in an environment with standard lighting conditions (12 h light/dark cycle), controlled temperature (20–25 °C) and humidity (40–60%), and with freely accessible food and water. All operations and treatments in the animal experiments were carried out in accordance with the Declaration of Helsinki and the "3 R" principles. To explore the changes of MMP3 expression during MCAO/R injury, mice were randomized into three groups, including Sham group ($n = 6$), MCAO/R-12 h group ($n = 6$), and MCAO/R-24 h group ($n = 6$). MCAO surgery was carried following our previous description [23]. In short, mice were anesthetized with 2% isoflurane in a 70% $N_2$/30% $O_2$ mixture. With a servo-controlled heating blanket, a body core temperature close to 37 °C was maintained throughout surgery. The focal cerebral ischemia was produced by intraluminal occlusion of the right middle cerebral artery using a silicone coated nylon (6.0) monofilament. After 60 min, the occluding filament was withdrawn to allow blood reperfusion for 12 or 24 h. The blood flow was observed using a laser Doppler perfusion imager (PeriCam PSI ZR, Sweden). A decrease in ipsilateral regional cerebral blood flow (CBF) below 30% relative to baseline was considered to represent

sufficient induction of focal cerebral ischemia. After withdrawal of the filament, an increase in ipsilateral CBF up to 70% of baseline was considered to represent successful reperfusion. Sham-operated mice underwent the same surgical procedure with the exception that their origin of the middle cerebral artery was not occluded. All animals were operated on by the same operator under the same conditions to reduce infarct variability. The operation time per animal did not exceed 15 min. The mice were sacrificed under deep anesthesia.

### 2.5. Drug administration

All operations and treatments in the animal experiments were carried out in accordance with the Declaration of Helsinki and the "3 R" principles, and permitted by institutional ethics board of Guangzhou University of Chinese Medicine (No.20200810001). *S*-Adenosyl-L-homocysteine (SAH), an inhibitor of Mettl3-Mettl14 heterodimer complex, was purchased from Selleck (S7868, Selleck). To investigate whether m6A modification affects BBB integrity in MCAO/R injury, mice were randomly divided into Sham group (n = 6), DMSO + MCAO/R-24 h group (n = 6), and SAH + MCAO/R-24 h group (n = 6). SAH (20 mg/kg) or DMSO (20 mg/kg, as control) were intraperitoneally injected into the mice at the time of reperfusion.

### 2.6. Neurobehavioral assessment

Neurological function was assessed by the modification of the Bederson neurological scale. Neurological scores were recorded as follows: 0, no neurological deficit; 1, failure to fully extend left forepaw or flexion of torso and contralateral forelimb when mouse was lifted by the tail; 2, reduced resistance to lateral push or circling to the contralateral side when mouse was held by the tail on a flat surface, but normal posture at rest; 3, spontaneous circling to left; 4, the absence of spontaneous movement or unconsciousness.

### 2.7. Measurement of infarct size

All operations and treatments in the animal experiments were carried out in accordance with the Declaration of Helsinki and the "3 R" principles, and permitted by institutional ethics board of Guangzhou University of Chinese Medicine (No.20200810001). 2% 2,3,5-triphenyltetrazolium chloride (TTC; Amresco Inc., Solon, OH, USA) staining was used to evaluate the infarct volume, as previously described.[23] In brief, mice were sacrificed 12 or 24 h after MCAO and the brains were cut in four 2 mm-thick coronal sections. The slices were stained for 20 min at 37 °C with 2% TTC to visualize the infarction. Infarction volumes were determined from the unstained areas, measured using an image analysis software (ImageJ). The infarct volume was calculated as a percentage using the following equation: infarct percentage=((contralateral hemisphere area - non-infarcted region in the ipsilateral hemisphere)/contralateral hemisphere area) × 100%.

### 2.8. Evans blue extravasation assay

All operations and treatments in the animal experiments were carried out in accordance with the Declaration of Helsinki and the"3 R″principles, and permitted by institutional ethics board of Guangzhou University of Chinese Medicine (No.20200810001). After 1 h of injection of Evans blue dye (2% in PBS; Sigma-Aldrich, E2129) via the lateral tail vein, mice brain was collected and sliced. Then, the brain slices were homogenized in 1.5 mL 50% trichloroacetic acid solution and centrifuged for 20 min at 13,600 g, and the supernatants were collected for spectrophotometric quantification at 620 nm (BioTek Synergy HT microplate reader).

### 2.9. Immunofluorescent staining

Mice were perfused with ice-cold PBS, and brain samples were dissected and fixed with 4% paraformaldehyde in PBS at 4 °C overnight. After sucrose dehydration, brain samples were embedded in optimal cutting temperature and cut into 40-μm cryosections. After permeabilization, brain sections were stained with primary m6A antibody (ab286164, Abcam) overnight at 4 °C followed by secondary Alexa Flour 488 anti-rabbit IgG antibody at room temperature for 2 h, followed by staining with DAPI. Then, the sections were imaged by microscope.

### 2.10. Quantification of the m6A level in total RNA

The m6A level in total RNA was detected using a commercial m6A RNA methylation quantification kit (P-9005, EpiGentek, United States). Briefly, 200 ng of total RNA was added into each well, and the capture antibody solution and detection antibody solution were added according to the manufacturer's protocol. The absorbance of each well at a wavelength of 450 nm was colorimetrically measured for the m6A level. The result is calculated using the following equation: Relative m6A level= (average absorbance of sample - average absorbance of the blank group)/(average absorbance of the Sham group - average absorbance of the blank group).

### 2.11. Cell culture and oxygen-glucose deprivation/reoxygenation (OGD/R) treatment

Mouse brain endothelial cells (bEnd.3) were purchased from NEWGAINBIO (Guangzhou, China) and cultured in DMEM supplemented with 10% fetal bovine serum at 37 °C and 5% $CO_2$ in a humidified incubator. For OGD/R treatment, bEnd.3 cells were

incubated in glucose-free DMEM (Gibco, USA) in an anaerobic chamber containing 95% $N_2$ and 5% $CO_2$ at 37 °C for 4 h, and then were returned to DMEM containing serum and glucose in normal conditions for 6, or 12 h, as previous description [23].

### 2.12. Cell viability assay

Cell viability was detected using the CCK-8 Cell Counting Kit (VAZYME, China), according to the manufacturers' instructions. Briefly, bEnd.3 cells were cultured in 96-well plates and subjected to OGD/R treatment as described above. Then, 10 μL of CCK-8 was added and continuously incubated at 37 °C for another 1 h in the dark, and the absorbance of each well was measured at 450 nm with a microplate reader. Relative cell viability (%) = (average absorbance of sample - average absorbance of the blank group)/(average absorbance of the normal group - average absorbance of the blank group) × 100%.

### 2.13. Small interfering RNA (siRNA) transfection

SiRNA against Mettl3 (Mettl3-RNAi, 5′-GGCACUUGGAUUUAAGGAATT-3′), siRNA against CBLL1 (CBLL1-RNAi, 5′-ACUAUUAGUGCCUUGUAACUC-3′) and siRNA negative control (*N*C-RNAi, 5′-UUCUCCGAACGUGUCACGUTT-3′) were synthesized by RiboBio (Guangzhou, China). SiRNAs were transfected into bEnd.3 cells with Lipo3000 (Invitrogen), as the manufacturers' instructions described. In brief, siRNAs and Lipo3000 were diluted with serum-free medium respectively, and were mixed for 10 min incubation at room temperature. The mixture was added to corresponding well and the plate was placed in the incubator for 4 h. The serum-free medium was replaced with complete growth medium for the following incubation.

### 2.14. Quantitative real-time PCR (qPCR)

Total RNA of the bEnd.3 cells or the mouse cortices was isolated with TRIzol, and then was reverse-transcribed into cDNA using a reverse transcriptase kit (Promega, USA). Then, qPCR was performed using SYBR green PCR Master Mix (Applied Biosystems, USA). The relative mRNA expressions were calculated by $2^{-\Delta\Delta Ct}$ method. The qPCR primers were as following: Mettl3, Forward 5′-GAGTTGATTGAGGTAAAGCGAGG-3′, Reverse 5′-GGAGTGGTCAGCGTAAGTTACA-3'; MMP3, Forward 5′-ACATGGAGACTTTGTCCCTTTTG-3′, Reverse 5′-TTGGCTGAGTGGTAGAGTCCC-3'; CBLL1, Forward 5′-TCAGCCCGTGGTATCTCAC-3′, Reverse 5′-GGTGGTGCGTAATGTTGCT-3'; GAPDH, Forward 5′-GCCAAGGCTGTGGGCAAGGT-3′, Reverse 5′-TCTCCAGGCGGCACGTCAGA-3'.

### 2.15. Transendothelial electrical resistance (TEER) assay

bEnd.3 cells were cultured plated on the gelatin-coated transwell inserts in 24-well plates until confluent. After treatments, the TEER of bEnd.3 cells was monitored using a Millicell-ERS-2 voltohmmeter (Millipore, USA). Inserts with no cells were used as blank controls, and the results of TEERs for blank controls were subtracted from the results for the measured TEERs.

### 2.16. RNA-binding protein immunoprecipitation (RIP)

RIP was performed as previously described [24]. Briefly, total RNA was extracted from $1 \times 10^7$ cells using TRIZOL (Thermo). mRNA was isolated using the PolyATtract® mRNA Isolation Systems (Promega, Z5310) following the manufacturer's instructions. IP mixture was composed by 6 μg rabbit anti m6A antibody (ab286164, Abcam), 3 μg mRNA, IP buffer (50 mmol/L Tris-HCl, pH 7.4, 750 mmol/L NaCl, and 0.5% NP-40), RNA inhibitor (Thermo) and RNase-free water up to 500 μL in total volume. After being mixed by rotating for 2 h at 4 °C, the IP mixture was incubated with the protein A beads which have been washed for three times and blocked by 0.5 mg/mL BSA, followed by rotating overnight at 4 °C. Precipitated mRNA was eluted using elution buffer (1 × IP buffer, 6.7 mM m6A). For the detection of relative m6A level, 40 ng of precipitated mRNA and input RNA was subjected to cDNA synthesis and quantitative PCR, respectively.

### 2.17. In vitro permeability assay

bEnd.3 cells were plated in the Millicell™ cell culture inserts until confluent. After transfection and OGD/R treatments, 200 μL of evans blue (EB) solution were added into the Transwell inserts for 1 h. Thereafter, 100 μL of medium from the lower compartment was transferred to a 96-well microplate, and EB that passed though the bEnd.3 cell sheet was measured spectrophotometrically at 620 nm.

### 2.18. Western blot

Western blot was carried following our previous description [23]. Briefly, after treatments, bEnd.3 cells or the mouse cortices were collected and lysed using NP40 lysis buffer (Beyotime Biotechnology, China) on ice. Lysates were quantified by the BCA kit (Beyotime Biotechnology, China), separated by 10% SDS-PAGE gel and then transferred to onto polyvinylidene difluoride membranes. Membranes were blocked, incubated sequentially with primary and secondary antibodies. The immunoblots were detected using chemiluminescence (ECL Plus detection system). Bands were quantified using Image J software. The primary antibodies used were as follows: MMP3 (ab52915, Abcam), Occludin (66378-1-Ig, ProteinTech), GAPDH (60004-1-Ig, ProteinTech).

### 2.19. Statistical analysis

Differences between two groups or multiple groups were analyzed by Student's t-test or one-way analysis of variance (ANOVA), respectively. Statistical analyses were performed using R software (v 3.4.2) and GraphPad Prism software (v 8.00). P value $< 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Correlation analysis of the differentially expressed MMP-related genes and m6A-related genes in cerebral I/R

A total of 15 samples (10 ischemic mouse cortices and 5 sham-mouse cortices) were included in this study. When $|\log2\ (FC)| > 1$ and $p < 0.05$ were used as cut-off thresholds, 286 upregulated DEGs and 41 downregulated DEGs were identified in the Stroke group compared to Sham group (Fig. 1A). The top 20 DEGs between Stroke and Sham groups were presented in heatmap (Fig. 1B). Moreover, the venn diagram showed 4 intersected genes (tissue inhibitor of metalloproteinase 1, TIMP1; MMP3; alpha-2-macroglobulin, A2M; tissue factor pathway inhibitor 2, TFPI2) between upregulated DEGs and MMP-related genes (Fig. 1C). In addition, box plots showed the 4 genes were all significantly and highly expressed in the Stroke group, compared to the Sham group (Fig. 1D).

Next, we analyzed the expression patterns of 20 m6A-related genes between the Stroke group and Sham group, and found that the expressions of the m6A writer Rbm15 (RNA binding motif protein 15) and the reader protein Hnrnpc (heterogeneous nuclear ribonucleoprotein C) were significantly higher in the Stroke group, compared to the Sham group (Fig. 2A and B). Furthermore, correlation analysis showed that only MMP3 expression was positively associated with the m6A writer CBLL1 (Cbl proto-oncogene like 1), but TFPI2 expression was negatively related to the m6A writer ZC3H13 (zinc finger CCCH-type containing 13) (Fig. 2C). Overall, these

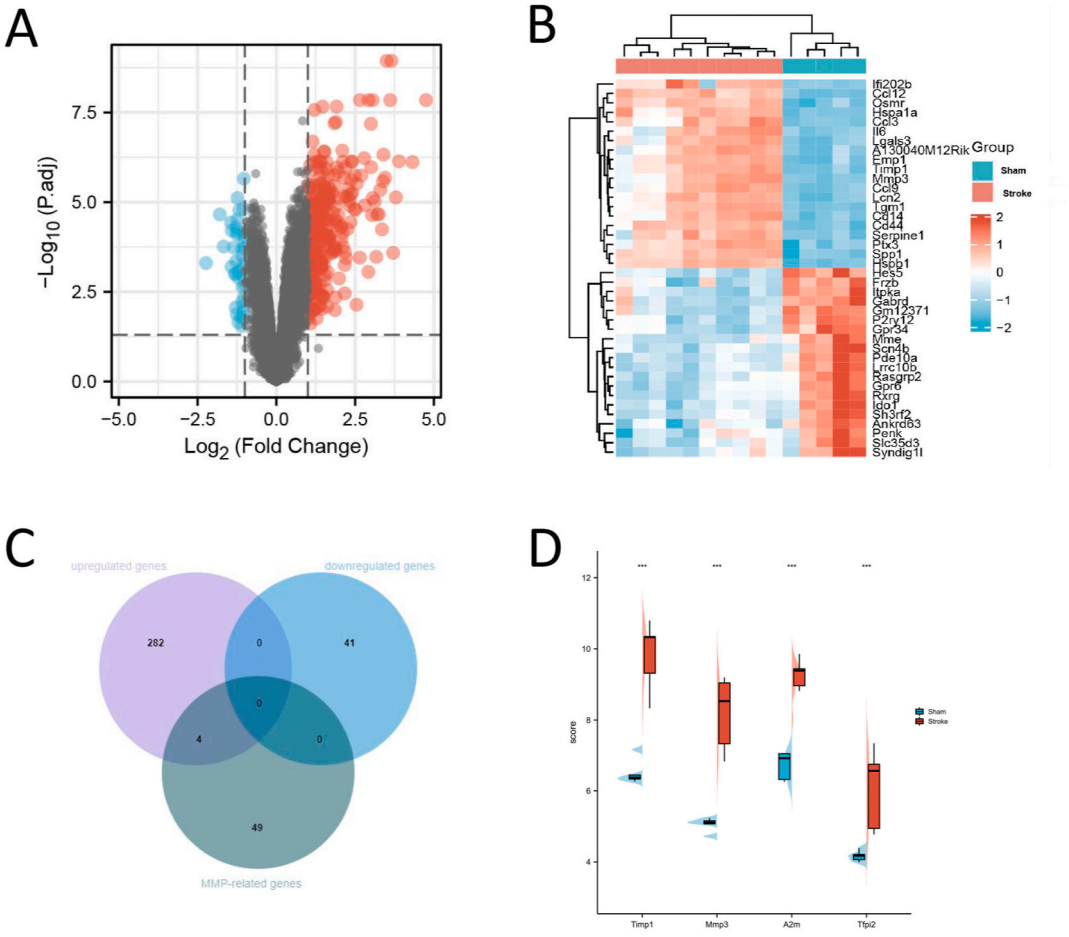


**Fig. 1. Differentially expressed MMP-related genes in the mice brain with MCAO/R injury.** (A) Volcano plot of the differentially expressed genes of GSE112348 and GSE30655 datasets between Stroke and Sham groups. (B) Heat map of the top DEGs between Stroke and Sham groups. (C) The Venn diagram depicts DEGs shared by the DEGs and MMPs-related genes. (D) The boxplot of 4 intersected genes in Stroke and Sham groups. *represents $P < 0.05$, **represents $P < 0.01$, and ***represents $P < 0.001$.

results indicate that MMP3 expression is positively associated with the m6A writer CBLL1 in cerebral I/R injury.

### 3.2. MMP3 and CBLL1 are both highly expressed in the mice brain subjected to MCAO/R injury and in the brain endothelial cells subjected to OGD/R injury

Considering that MMP3 is a critical factor in BBB damage by inducing degradation of the BBB extracellular [25,26], and MMP3 is identified in the top 20 upregulated DEGs (Fig. 1C), and is the only protein positively associated with the m6A writer expression (Fig. 2C), we pay attention to MMP3 in the following studies. Firstly, we examined the mRNA and protein expressions of MMP3 and CBLL1 in the mice brain subjected to MCAO/R injury *in vivo*, and found that MMP3 and CBLL1 mRNAs were both significantly increased in MCAO/R injury (Fig. 3A–F, and supplementary Fig. 1). Consistently, MMP3 and CBLL1 mRNAs and proteins were also significantly increased in the brain endothelial cells subjected to OGD/R injury (Fig. 3G–J). These results indicate that MMP3 expression may be associated with m6A modification in cerebral I/R injury.

### 3.3. Inhibition of m6A modification reduces MMP3 expression and ameliorates the loss of endothelial barrier integrity induced by OGD/R injury

To explore whether m6A modification affect MMP3 expression in cerebral I/R injury, we analyzed the potential m6A site in MMP3 mRNA by SRAMP, and found the 173 site with a high confidence (Fig. 4A). Furthermore, RIP assay showed that OGD/R injury promoted m6A modification of MMP3 mRNA in brain endothelial cells (Fig. 4B). Knockdown of the m6A writer Mettl3 or CBLL1 notably reduced m6A modification of MMP3 mRNA, and decreased MMP3 mRNA (Fig. 4C and D). Subsequently, we examined the effects of m6A modification on endothelial barrier integrity with brain endothelial cells subjected to OGD/R *in vitro* using TEER and EB-albumin assay. As expected, knockdown of Mettl3 or CBLL1 significantly suppressed the reduction of TEER induced by OGD/R, and inhibited

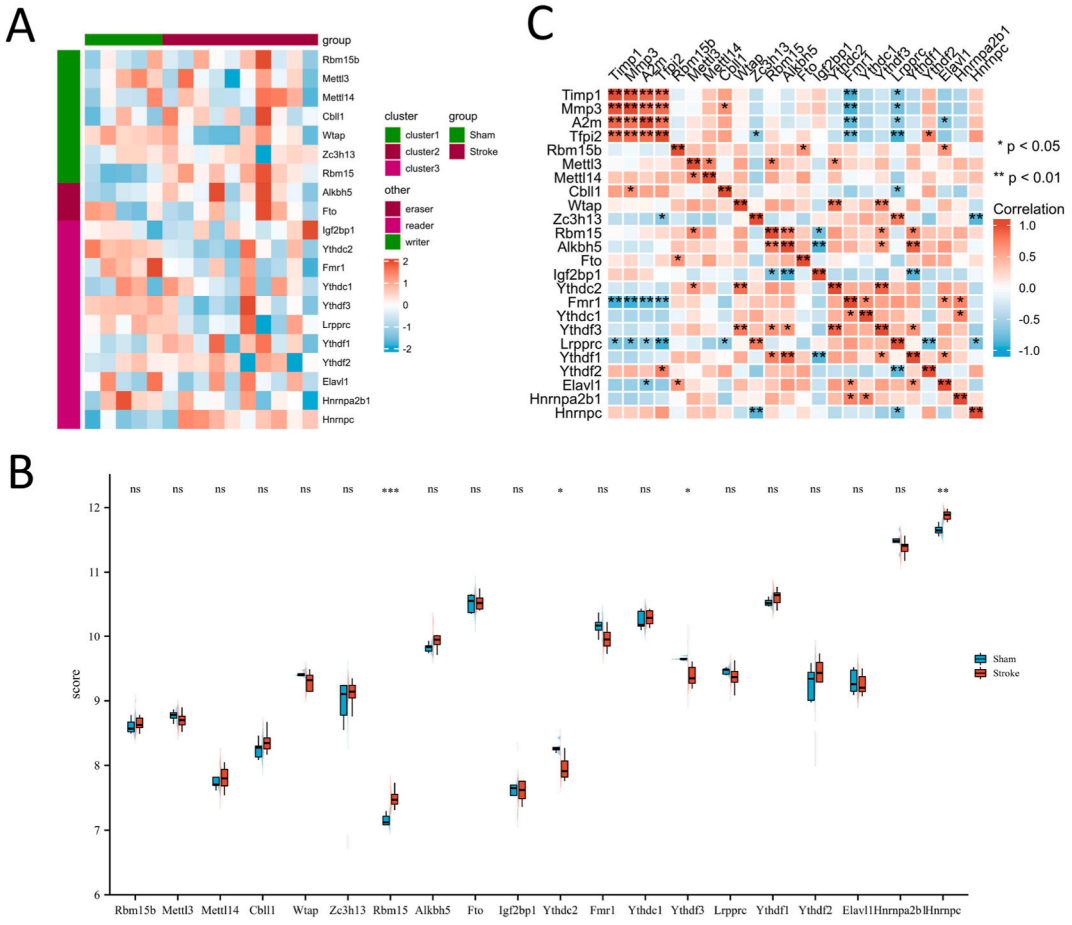


**Fig. 2. Correlation analysis between the differentially expressed MMP-related genes and m6A-related genes in cerebral I/R.** (A) Heatmap of the m6A-related genes in Stroke and Sham groups. (B) The boxplot of m6A-related genes in Stroke and Sham groups. (C) Correlation analysis between the differentially expressed MMP-related genes and m6A-related genes. *represents $P < 0.05$, **represents $P < 0.01$, and ***represents $P < 0.001$.

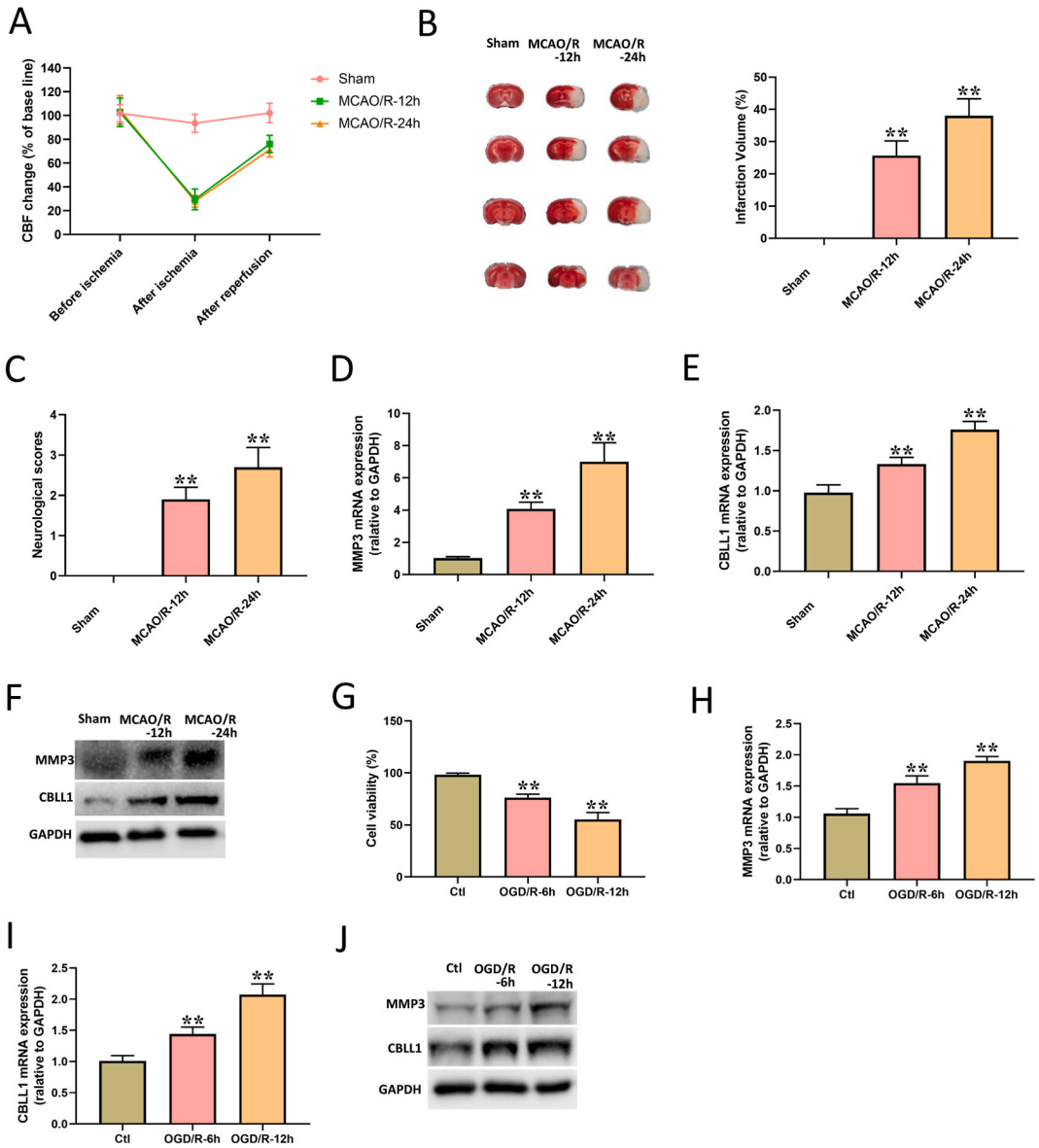


**Fig. 3. MMP3 and CBLL1 are both highly expressed in the mice brain subjected to MCAO/R injury and in the brain endothelial cells subjected to OGD/R injury.** (A) Cerebral blood flow (CBF) was measured by Laser-doppler flowmetry. (B) Representative photographs of brain sections stained with TTC (Left). Pale areas represent infarction. Statistic analysis of infarct volumes in each group (Right). N = 6. (C) Neurological Bederson score of each group. N = 6. (D and E) qRT-PCR analysis of MMP3 and CBLL1 mRNA expressions in the mice brain tissue subjected to MCAO/R injury. (F) Protein expressions of MMP3 and CBLL1 were examined by Western blot in the brain ischemic penumbra tissues. (G) Cell viability analysis of the brain endothelial cells subjected to OGD/R injury. (H and I) qRT-PCR analysis of MMP3 and CBLL1 mRNA expressions in the brain endothelial cells subjected to OGD/R injury. (J) Protein expressions of MMP3 and CBLL1 were examined by Western blot in the brain endothelial cells subjected to OGD/R injury. *represents $P < 0.05$, and **represents $P < 0.01$.

the increase of cell permeability induced by OGD/R (Fig. 4E and F). In addition, we examined the effects of m6A modification on the expressions of tight junction protein Occludin, and found that knockdown of Mettl3 or CBLL1 evidently suppressed the increase of MMP3 protein expression induced by OGD/R, and alleviated the reduction of Occludin protein expression (Fig. 4G, H and I). Collectively, these data indicate that inhibition of m6A modification reduces MMP3 expression and ameliorates the loss of endothelial barrier integrity induced by OGD/R injury.

### *3.4. Inhibition of m6A modification suppresses MMP3 expression and BBB breakdown induced by MCAO/R injury*

Finally, we explored whether inhibition of m6A modification protected BBB integrity against MCAO/R injury. *S*-Adenosyl-L-

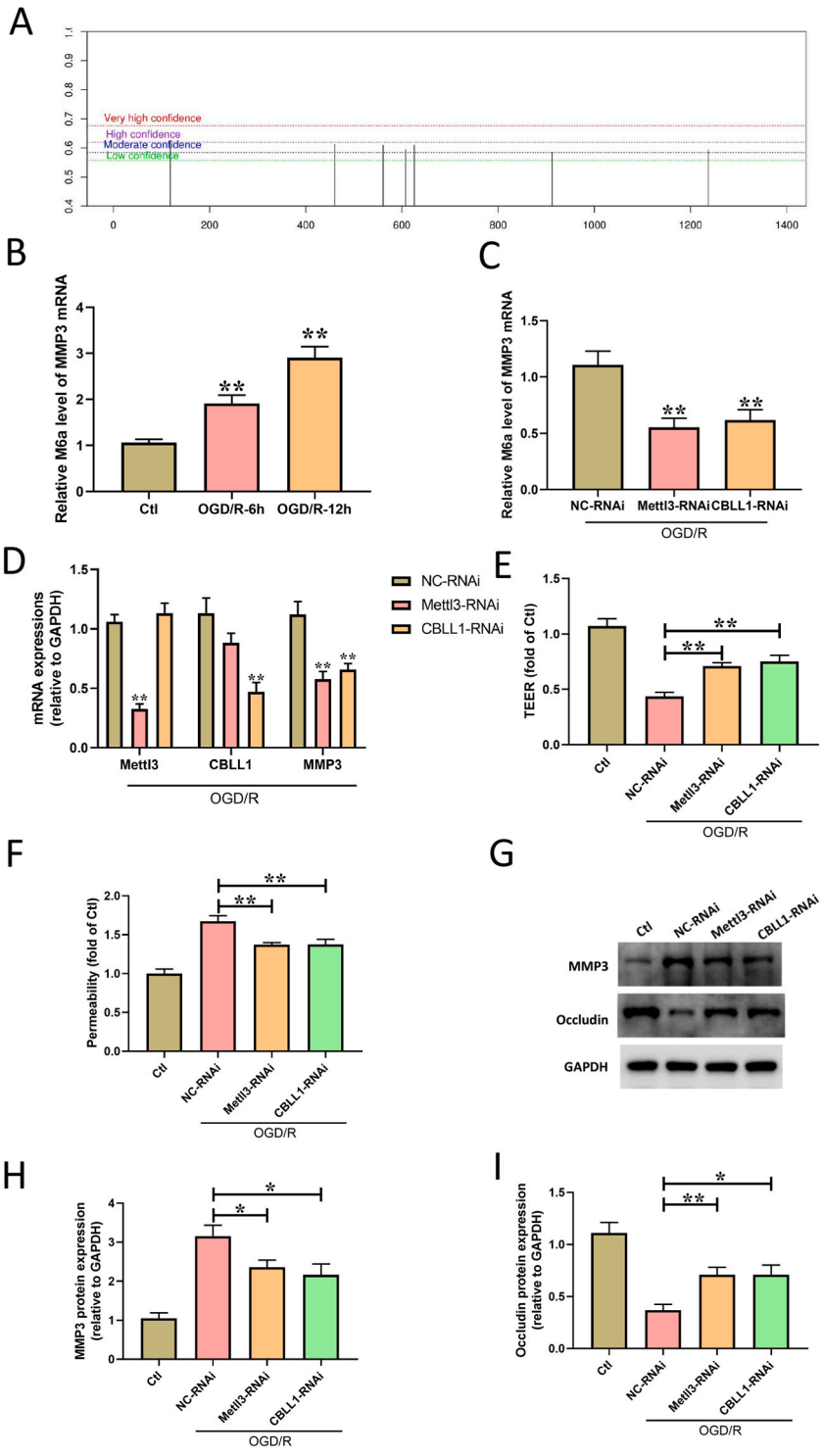


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**Fig. 4. Inhibition of m6A modification reduces MMP3 expression and ameliorates the loss of endothelial barrier integrity induced by OGD/R injury.** (A) Analysis of the potential m6A site in MMP3 mRNA by SRAMP. (B) M6A level of MMP3 mRNA was detected in the brain endothelial cells subjected to OGD/R injury using m6A-IP-qPCR. (C) M6A level of MMP3 mRNA was detected in the brain endothelial cells transfected with *N*C-RNAi, Mettl3-RNAi or CBLL1-RNAi, and then treated with ODG/R. (D) qRT-PCR analysis of MMP3, Mettl3 and CBLL1 mRNA expressions in the brain endothelial cells transfected with *N*C-RNAi, Mettl3-RNAi or CBLL1-RNAi, and then treated with ODG/R. (E and F) The protective effect of Mettl3 or CBLL1 knockdown on the endothelial cell barrier was detected using TEER and EB assays. (G) Protein expressions of MMP3, and Occludin were examined by Western blot in the brain endothelial cells transfected with *N*C-RNAi, Mettl3-RNAi or CBLL1-RNAi, and then treated with ODG/R. (H and I) Quantitative analysis of MMP3 and Occludin expressions. *represents $P < 0.05$, and **represents $P < 0.01$.

homocysteine (SAH), an inhibitor of Mettl3-Mettl14 heterodimer complex [27], was intraperitoneally injected into the mice at the time of reperfusion. As shown in Fig. 5A–D, the administration of SAH significantly inhibited MCAO/R-induced up-regulation of the m6A level, alleviated neurological deficit and brain infarction, compared to DMSO (as control) treatment. Furthermore, SAH administration also significantly reduced EB leakage (Fig. 5E) and MMP3 protein expression (Fig. 5F and G), and increased Occludin protein expressions in the brain ischemic penumbra tissues (Fig. 5F and H). Taken together, these data indicate that inhibition of m6A modification suppresses MMP3 expression and BBB breakdown induced by MCAO/R injury.

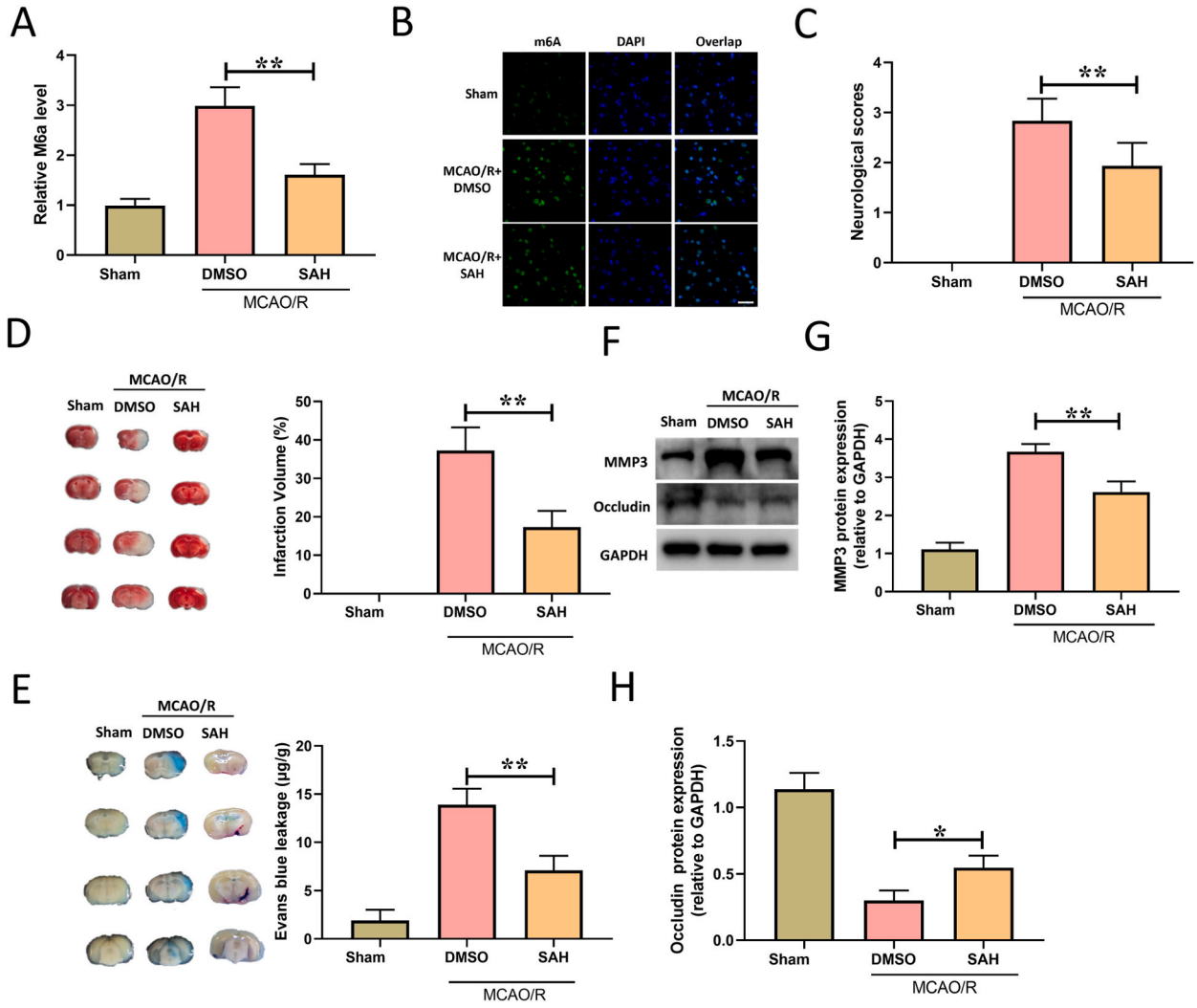


**Fig. 5. Inhibition of m6A modification suppresses MMP3 expression and BBB breakdown induced by MCAO/R injury.** (A) Detection of the total m6A level in the brain tissues. (B) Immunofluorescence staining of m6A (grenn) in the brain tissues. Nuclei were stained with DAPI (blue). Scale bar, 30 μm. (C) Neurological Bederson score of each group. N = 6. (D) Representative photographs of brain sections stained with TTC. Pale areas represent infarction. Quantitation of the infarction volume. N = 6. (E) Representative photographs of the Evans blue leakage in brain sections. Quantitation of the Evans blue leakage in the ipsilateral hemispheres. N = 6. (F) Protein expressions of MMP3, and Occludin were examined by Western blot in the brain ischemic penumbra tissues. (G and H) Quantitative analysis of MMP3 and Occludin expressions. *represents $P < 0.05$, and **represents $P < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

## 4. Results

### 4.1. Correlation analysis of the differentially expressed MMP-related genes and m6A-related genes in cerebral I/R

A total of 15 samples (10 ischemic mouse cortices and 5 sham-mouse cortices) were included in this study. When $|\log2\ (FC)| > 1$ and $p < 0.05$ were used as cut-off thresholds, 286 upregulated DEGs and 41 downregulated DEGs were identified in the Stroke group compared to Sham group (Fig. 1A). The top 20 DEGs between Stroke and Sham groups were presented in heatmap (Fig. 1B). Moreover, the venn diagram showed 4 intersected genes (tissue inhibitor of metalloproteinase 1, TIMP1; MMP3; alpha-2-macroglobulin, A2M; tissue factor pathway inhibitor 2, TFPI2) between upregulated DEGs and MMP-related genes (Fig. 1C). In addition, box plots showed the 4 genes were all significantly and highly expressed in the Stroke group, compared to the Sham group (Fig. 1D).

Next, we analyzed the expression patterns of 20 m6A-related genes between the Stroke group and Sham group, and found that the expressions of the m6A writer Rbm15 (RNA binding motif protein 15) and the reader protein Hnrnpc (heterogeneous nuclear ribonucleoprotein C) were significantly higher in the Stroke group, compared to the Sham group (Fig. 2A and B). Furthermore, correlation analysis showed that only MMP3 expression was positively associated with the m6A writer CBLL1 (Cbl proto-oncogene like 1), but TFPI2 expression was negatively related to the m6A writer ZC3H13 (zinc finger CCCH-type containing 13) (Fig. 2C). Overall, these results indicate that MMP3 expression is positively associated with the m6A writer CBLL1 in cerebral I/R injury.

### 4.2. MMP3 and CBLL1 are both highly expressed in the mice brain subjected to MCAO/R injury and in the brain endothelial cells subjected to OGD/R injury

Considering that MMP3 is a critical factor in BBB damage by inducing degradation of the BBB extracellular [25,26], and MMP3 is identified in the top 20 upregulated DEGs (Fig. 1C), and is the only protein positively associated with the m6A writer expression (Fig. 2C), we pay attention to MMP3 in the following studies. Firstly, we examined the mRNA and protein expressions of MMP3 and CBLL1 in the mice brain subjected to MCAO/R injury *in vivo*, and found that MMP3 and CBLL1 mRNAs were both significantly increased in MCAO/R injury (Fig. 3A–F, and supplementary Fig. 1). Consistently, MMP3 and CBLL1 mRNAs and proteins were also significantly increased in the brain endothelial cells subjected to OGD/R injury (Fig. 3G–J). These results indicate that MMP3 expression may be associated with m6A modification in cerebral I/R injury.

### 4.3. Inhibition of m6A modification reduces MMP3 expression and ameliorates the loss of endothelial barrier integrity induced by OGD/R injury

To explore whether m6A modification affect MMP3 expression in cerebral I/R injury, we analyzed the potential m6A site in MMP3 mRNA by SRAMP, and found the 173 site with a high confidence (Fig. 4A). Furthermore, RIP assay showed that OGD/R injury promoted m6A modification of MMP3 mRNA in brain endothelial cells (Fig. 4B). Knockdown of the m6A writer Mettl3 or CBLL1 notably reduced m6A modification of MMP3 mRNA, and decreased MMP3 mRNA (Fig. 4C and D). Subsequently, we examined the effects of m6A modification on endothelial barrier integrity with brain endothelial cells subjected to OGD/R *in vitro* using TEER and EB-albumin assay. As expected, knockdown of Mettl3 or CBLL1 significantly suppressed the reduction of TEER induced by OGD/R, and inhibited the increase of cell permeability induced by OGD/R (Fig. 4E and F). In addition, we examined the effects of m6A modification on the expressions of tight junction protein Occludin, and found that knockdown of Mettl3 or CBLL1 evidently suppressed the increase of MMP3 protein expression induced by OGD/R, and alleviated the reduction of Occludin protein expression (Fig. 4G, H and I). Collectively, these data indicate that inhibition of m6A modification reduces MMP3 expression and ameliorates the loss of endothelial barrier integrity induced by OGD/R injury.

### 4.4. Inhibition of m6A modification suppresses MMP3 expression and BBB breakdown induced by MCAO/R injury

Finally, we explored whether inhibition of m6A modification protected BBB integrity against MCAO/R injury. *S*-Adenosyl-L-homocysteine (SAH), an inhibitor of Mettl3-Mettl14 heterodimer complex [27], was intraperitoneally injected into the mice at the time of reperfusion. As shown in Fig. 5A–D, the administration of SAH significantly inhibited MCAO/R-induced up-regulation of the m6A level, alleviated neurological deficit and brain infarction, compared to DMSO (as control) treatment. Furthermore, SAH administration also significantly reduced EB leakage (Fig. 5E) and MMP3 protein expression (Fig. 5F and G), and increased Occludin protein expressions in the brain ischemic penumbra tissues (Fig. 5F and H). Taken together, these data indicate that inhibition of m6A modification suppresses MMP3 expression and BBB breakdown induced by MCAO/R injury.

## 5. Discussion

Cerebral I/R injury is a serious secondary brain injury after recanalization of occluded arteries [4]. However, there is still lack of efficient treatment, partially due to not fully understanding of the mechanisms involved in BBB breakdown. In the present study, we analyzed the correlation between MMP-related genes and m6A-related genes in cerebral I/R using the GEO databases, and explored the potential effects of m6A modification on BBB breakdown in cerebral I/R injury and its underlying mechanisms using mice subjected to MCAO/R *in vivo* and the mice brain endothelial cells treated with OGD/R *in vitro*. Our study firstly finds that m6A modification is involved in the BBB breakdown during cerebral I/R injury, and inhibition of m6A modification protects BBB integrity through reducing

MMP3 expression.

MMPs are reported to degrade ECM to remodel tissue [28]. Among several MMPs, MMP3 is fast activated to inducing BBB breakdown, neuronal death and neuroinflammation following in cerebral I/R injury [29–31]. Consistently, our study also found that MMP3 mRNA and protein expression were also significantly increased in cerebral I/R injury *in vivo* and *in vitro*. Previous studies report that MMP3 transcription is regulated by the nuclear factor (NF)-κB [32,33]. Our study firstly reports m6A modification of MMP3 mRNA was gradually increased and MMP3 expression is regulated by m6A modification in cerebral I/R injury. Based on the recent studies that m6A modification also regulates NF-κB p65 mRNA stability in atherosclerosis and stroke [34,35], it is probably that MMP3 expression is regulated by different factors at multi-facets in the BBB breakdown during cerebral I/R injury, which needs further investigation in the future studies.

M6A modification on mRNA is dynamically regulated by the methyltransferases (Writers) and demethylases (Erasers) [15,16]. Recent study reports the m6A levels in the brain cortex are significantly increased after MCAO/R injury, which is mediated by reduction of the m6A demethylase FTO [36]. Decrease of FTO promotes cerebral I/R-induced oxidative stress in neurons via regulating m6A methylation of Nrf2 mRNA [37], and promoting pyroptosis by regulating m6A methylation of PTEN [19]. Whether regulation of m6A methylation on MMP3 mRNA in brain endothelial cells during I/R injury is also related to FTO needs further investigation. M6A modified mRNAs have different fates which is controlled by the different m6A reader proteins [38]. Generally, YTH domain family 1 (YTHDF1) promotes the translation of m6A mRNA and YTHDF2 promotes m6A RNA decoy [39,40]. YTHDF3 promote the translation or degradation of mRNA by interacting with YTHDF1 or YTHDF2 [41]. Hnrnpc regulates mRNA abundance by transform its secondary structure [42]. YTH domain-containing 2 (YTHDC2) improves the translation efficiency of m6A RNA [43]. Our study analyzed that YTHDC2 and YTHDF3 mRNAs were significantly decreased in the brain with I/R injury, and Hnrnpc mRNA was significantly increased, compared to the Control groups. The detailed m6A reader proteins should be identified in the following studies. Besides, we found that knockdown of the m6A writers by siRNAs against Mettl3 or CBLL1 decreased MMP3 mRNA expression in the brain endothelial cells *in vitro*, and intraperitoneal injection of m6A inhibitor SAH also significantly decreased MMP3 expression in cerebral I/R injury *in vivo*. However, up to now, there is still no specific inhibitor of CBLL3. To further confirm the regulatory role of m6A modification on MMP3 expression in cerebral I/R injury, intracranial injection of lentivirus-mediated shRNA-against CBLL3 or mice with CBLL1 knockout in brain endothelial cells should be used in our following studies.

In conclusion, our study finds that m6A modification promotes BBB breakdown in cerebral I/R injury through promoting MMP3 expression, suggesting m6A is a potential targets for treating cerebral I/R injury.

## Ethical approval and consent to participate

This study was approved by the Institutional Animal Care and Use Committee of the Guangzhou University of Chinese Medicine.

## Consent to publish

Not applicable.

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## Author contribution statement

En liang: Yu Zhang: Guanglei Fu: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Shaorong xiao: Changtong Zhao: Performed the experiments; Analyzed and interpreted the data.

## Data availability statement

Data will be made available on request.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Not Applicable.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e16905.

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