


Correlation Between *MMP9* Promoter Methylation and Transient Ischemic Attack/Mild Ischemic Stroke with Early Cognitive Impairment

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Background/Objective: Dyskinesia caused by transient ischemic attack (TIA) and mild ischemic stroke (MIS) is mild and short-lived; however, cognitive impairment (CI) can occur in the acute phase and be easily overlooked. DNA methylation is an epigenetic phenomenon that can affect gene expression through gene silencing. Blood levels of matrix metalloproteinase (MMP) 9 are elevated in ischemic stroke patients and is associated with the destruction of the blood-brain barrier and the occurrence of CI. No studies have investigated the relationship between *MMP9* gene methylation and TIA/MIS with early cognitive impairment (ECI). As such, the purpose of the present study was to investigate the correlation between *MMP9* gene methylation and TIA/MIS with ECI.

Methods: Data from 112 subjects were collected, including 84 with TIA/MIS (National Institutes of Health Stroke Scale <5 points) and 28 non-stroke control subjects. Patients were evaluated within 7 days of TIA/MIS onset according to four single-domain cognitive scales. Whole blood DNA methylation was detected using MethylTarget sequencing technology. Comparison of *MMP9* gene methylation levels among subgroups was performed using statistical methods.

Results: The site S33-79 in the TIA/MIS group was hypomethylated compared with the control group, and sites S33-25 and S33-30 in TIA/MIS with ECI was hypomethylated compared with TIA/MIS without ECI. Compared with the small artery occlusion group, *MMP9* gene, S33-25, 30, 39, 53, 58, 73, 79, 113 and 131 sites in the large artery atherosclerosis group were hypomethylated.

Conclusion: *MMP9* gene hypomethylation sites were associated with TIA/MIS and TIA/MIS with ECI, and there was a strong correlation between *MMP9* gene hypomethylation and atherosclerotic TIA/MIS. *MMP9* gene methylation can reflect the severity of TIA/MIS. *MMP9* gene hypomethylation sites may be used as potential biomarkers and therapeutic targets for TIA/MIS and TIA/MIS with ECI.

Keywords: early cognitive impairment, hypomethylation, amyloid β -protein, blood-brain barrier, *MMP9* gene

Introduction

China has a high incidence of ischemic stroke. Although the dyskinetic symptoms of transient ischemic attack/minor ischemic stroke (TIA/MIS) can recover quickly, cognitive impairment (CI) in the early stages can be easily ignored. Early cognitive impairment (ECI) is highly correlated with a persistent decline in cognitive function.¹⁻⁴ As an epigenetic form, methylation can affect gene expression without altering the gene sequence, and abnormal methylation of multiple genes has been found.⁵⁻¹⁰ The blood-brain barrier (BBB) is a highly selective membrane structure composed of endothelial cells, tight connections, basement membranes, and astrocytes. It can separate blood from the brain tissue of the central nervous system, selectively pass through water, gas, and liposoluble substances, block macromolecular substances and toxic substances from entering the brain,¹¹ and maintain the stability of the central nervous system.¹² Ischemic stroke can destroy the blood-brain barrier, the degree of which is related to the severity and prognosis of the

stroke.¹³ After an ischemic stroke, brain edema appears rapidly. It leads to transport dysfunction, and the infiltration of inflammatory cells and the entry of inflammatory molecules lead to secondary inflammatory reactions, which can further aggravate the damage of the blood-brain barrier and brain injury.¹⁴

Damage to cognitive function caused by the destruction of the blood-brain barrier is likely related to amyloid β -protein (A β) transportation.¹⁵ Under normal circumstances, a large part of the outward transportation of A β is through the blood-brain barrier.^{16,17} After an ischemic stroke, the blood-brain barrier is damaged, and the deposition of A β in the brain tissue and blood vessel walls is increased because of the abnormal A β transportation. At the same time, inflammation and oxidative stress reactions are secondary after stroke and can cause cognitive function damage under joint action.¹⁵ Matrix metalloproteinases (MMP) is a large protease family and a kind of metallo-zinc-dependent protease family, which plays an important role in maintaining the stability of the blood-brain barrier. MMP mainly hydrolyzes tight junctions and matrix membranes, causing damage to the blood-brain barrier and affecting brain function.

MMP9 is a gelatinase. Research has shown that MMP9 increases in the acute stage of ischemic cerebrovascular disease and participates in blood-brain barrier destruction. The increased serum MMP9 level is related to an adverse prognosis after ischemic stroke.^{18,19} MMP9 inhibitors can reduce the volume of infarcted lesions and the symptoms of stroke^{20–22} and reduce the risk of hemorrhagic transformation.²³ Therefore, MMP9 can serve as both a biomarker and a potential target for treatment. No research has confirmed the relationship between *MMP9* methylation and TIA/MIS, and there is no research on the relationship between *MMP9* methylation and TIA/MIS with ECI. Therefore, we speculated that *MMP9* methylation relates to TIA/MIS and TIA/MIS with ECI.

Materials and Methods

Participants

This study included 84 patients with TIA and MIS (NIHSS <5 points) who were hospitalized in the Department of Neurology of Qilu Hospital (Qingdao) of Shandong University from June 2019 to July 2020. In the control group, 28 healthy volunteers or dizziness/headache patients without TIA, ischemic stroke, and cerebral hemorrhage were collected in the same period. The TIA/MIS patients enrolled meet the following criteria: age 18–75; the NIHSS score is less than 5 points, and the cognitive scale assessment is completed within 7 days after the onset; TOAST classification was large artery atherosclerosis and small artery occlusion and confirmed by magnetic resonance imaging (MRI), computed tomography (CT), or both and vascular examination; no deafness, blindness, severe hemiplegia, severe depression, and anxiety; no acute cerebral hemorrhage, epilepsy, malignant tumor, degenerative disease, severe kidney and liver dysfunction, etc.; signed the informed consent form. Basic clinical data of all subjects were collected, including age, gender, education level, history (mainly including diabetes, coronary atherosclerotic heart disease, hypertension, hyperlipidemia), blood examination results (including low-density lipoprotein, high-density lipoprotein, uric acid, homocysteine, and vitamin B12), smoking history, alcohol history, and Fazekas score.

Cognitive Scales

The enrolled TIA/MIS patients were tested using the Boston Naming Test (BNT), Audit Verbal Learning Test (AVLT), Trail Making Test (TMT)-A, and TMT-B single-domain cognitive scales, and their language, memory, visuospatial, and executive function^{24–31} respectively. The criteria for the existence of abnormalities were as follows: having been educated for > 9 years with a score ≤ 21.5 (if educated for ≤ 9 years, the score ≤ 19.5 points), indicating that there is a language barrier. An AVLT score <5 indicated memory dysfunction. TMT-A: if age is 50–64 years, visuospatial function ≥ 80.5 s; age is 65–74 years, visuospatial function ≥ 90.5 s; Age ≥ 75 years, visuospatial function ≥ 101.5 s. TMT-B: if age is 50–64 years, executive dysfunction ≥ 150.5 s; age is 65–74 years, executive dysfunction ≥ 165.5 ; Age ≥ 75 years, executive dysfunction ≥ 199.5 s. CI was determined by ≥ 1 scale abnormality.²⁴ Two neurologists with at least 5 years of experience completed the scale evaluation in this study.

The Ethics Committee of Qilu Hospital (Qingdao) of Shandong University approved the study.

Methylation Sequencing

A reagent kit (Tianjin Biotech, Beijing, China) was used to extract the frozen whole blood DNA. DNA methylation was detected by Genesky Biotechnologies Inc. (Shanghai, China) using MethylTarget sequencing technology, which is based on second-generation sequencing and can accurately calculate the methylation level of each CpG site.^{32,33} The EZ DNA Calculation™- GOLD Kit (ZYMO, CA, USA) reagent kit performed multiple sulfite treatments on the extracted DNA. GeneCpG software was used to analyze the genome of the region of interest and the bisulfite-treated sequence. Primer3 is used to design primers for the bisulfite-treated sequence (<http://primer3.ut.ee/>). The following table for primer designs (Table 1). Finally, after multiple PCR amplifications (HotStarTaq polymerase kit, TaKaRa, Dalian, China) and specific tag sequences were added to the samples, high-throughput sequencing (Illumina, CA, USA) was performed using the Illumina HiSeq platform.³⁴

Statistical Methods

R software (version; R Foundation for Statistical Computing, Vienna, Austria) was used for statistical analyses. Continuous data and categorical data are presented as mean \pm standard deviation ($x \pm s$) and percentages (n [%]) respectively. The Student's *t*-test was used for continuous variables with normal distribution and homogeneous variance to compare observations between both study groups, and the Wilcoxon test was used otherwise. The percentage test, including the chi-square test and Cochran–Armitage trend test, was used to determine whether there was a significant statistical difference between the percentage data. The chi-square test was used to test nominal classification variables, and the Cochran–Armitage trend test was used to test ordinal classification variables. Statistical significance was set at $P < 0.05$.

Results

We tested the target region (Table 2) methylation of the whole blood *MMP9* gene in 112 samples (Figures 1–3), including 84 TIA/MIS patients and 28 control samples (Supplementary Table 1). The incidence of CI in TIA/MIS patients was 65.5% (55/84), and the incidence of CI was higher in patients with large atherosclerotic TIA/MIS (78.8%, 26/33) (Supplementary Table 2). The basic clinical data of all the cases are shown in the Supplementary material.

Differences Between TIA/MIS and the Control Group in *MMP9* Gene Methylation

We found that the methylation level of *MMP9* gene in the whole blood of the TIA/MIS group (Group J) was significantly lower than that of the control group (Group C) (Groupdiff < 0) ($P < 0.05$) through sequencing methylation in the target region in 84 TIA/MIS patients and 28 control groups (Figure 4) (Supplementary Table 1). After analyzing the methylation levels at various sites between the TIA/MIS group and the control group, we found that the TIA/MIS group also had hypomethylation at sites S33-39, S33-53, S33-58, S33-73, S33-79 and S33-156 (Groupdiff < 0), and there

Table 1 Primer Information

Target	Gene	Primers
<i>MMP9-33</i>	<i>MMP9</i>	F: GGAATTTGTTTAGGTTTGGGATT R: CCCTTCATCCACAAAATACCT

Abbreviations: Target, target segment name; Gene, gene name; F, forward; R, reverse.

Table 2 Information on Target DNA Methylation Sequencing

Target	Chr	Gene	mRNA	mRNA strand	TSS	TES	Start	End	Length	Target strand	Distance 2TSS
<i>MMP9-33</i>	20	<i>MMP9</i>	NM_004994	+	44,637,546	44,645,200	44,638,688	44,638,491	198	-	1142

Abbreviations: Chr, chromosome; mRNA, mRNA closer to the product; mRNA strand, mRNA direction; TSS, transcription start site of mRNA; TES, transcriptional end site of mRNA; Start, starting position of the product on reference genomes; End, ending position of the product on reference genomes; Length, length of the product; Target strand, direction of the product; Distance2TSS, relative distance between product and TSS; a negative sign indicates that the site is upwards TSS.

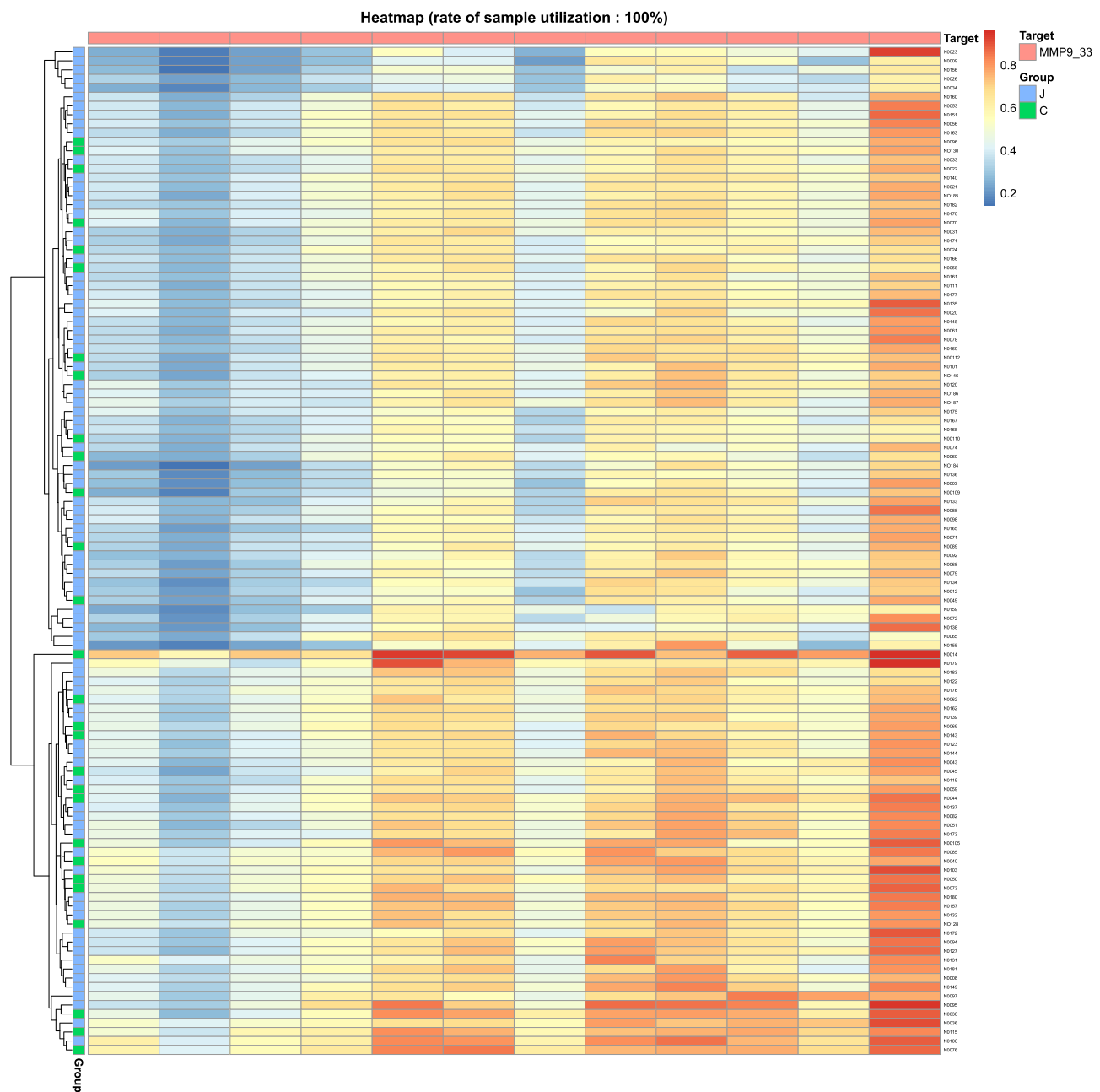


Figure 1 A heatmap based on the methylation levels of the CpG sites in all samples. Each row is a sample, and each column is a CpG site. Each cell represents the relative methylation level of the CpG site of the corresponding row of samples and reflects the change in methylation level with a color gradient. If it tends to be blue, the methylation level is lower; if it is red, it is higher. The arrangement order of the rows shows the similarity of the sample methylation levels. The more adjacent rows there are, the higher the similarity of the sample methylation levels they represent.

was a significant statistical difference compared with the control group ($P < 0.05$). However, after adjustment for age, sex, and Fazekas score (Supplementary Table 1), only S33-79 showed a statistically significant difference between the two groups ($P < 0.05$) (Table 3), respectively.

Differences Between TIA/MIS Groups with Large Atherosclerosis and Small-Artery Occlusion in *MMP9* Gene Methylation

We analyzed the methylation of the *MMP9* gene between the two groups of large atherosclerosis (Group LA) and small artery occlusion (Group SA) TIA/MIS (Supplementary Table 3). We found that the methylation of the *MMP9* gene at

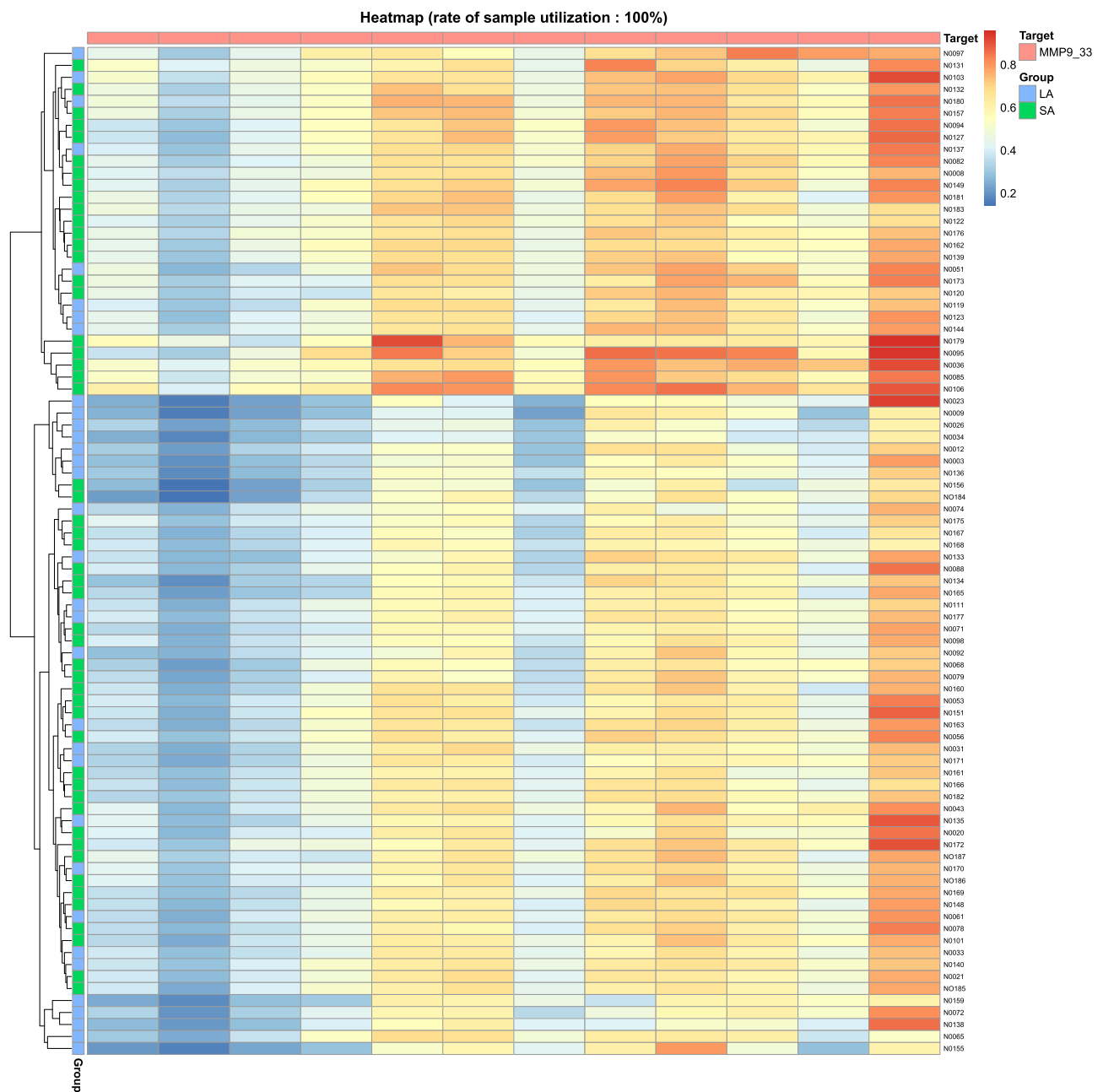


Figure 2 A heatmap based on the methylation levels of CpG sites in samples in groups LA and SA.

sites S33-25, S33-30, S33-39, S33-58, S33-73, S33-79, S33-113, and S33-131 in the LA group was significantly lower than that in the SA group (Groupdiff <0) ($P < 0.05$) (Figure 5). There were still statistical differences in gene and site methylation levels between the two groups after adjusting for age, sex, and Fazekas score (Table 4) (Supplementary Table 3), respectively.

Differences Between TIA/MIS with and without ECI in *MMP9* Gene Methylation

After sequencing the methylation of the target region of 55 TIA/MIS patients with ECI (Group A) and 29 patients without ECI (Group B) (Supplementary Table 2), we found that although the average methylation level of the group with ECI was lower than that of the group without ECI (Groupdiff <0), there was no statistically significant difference between

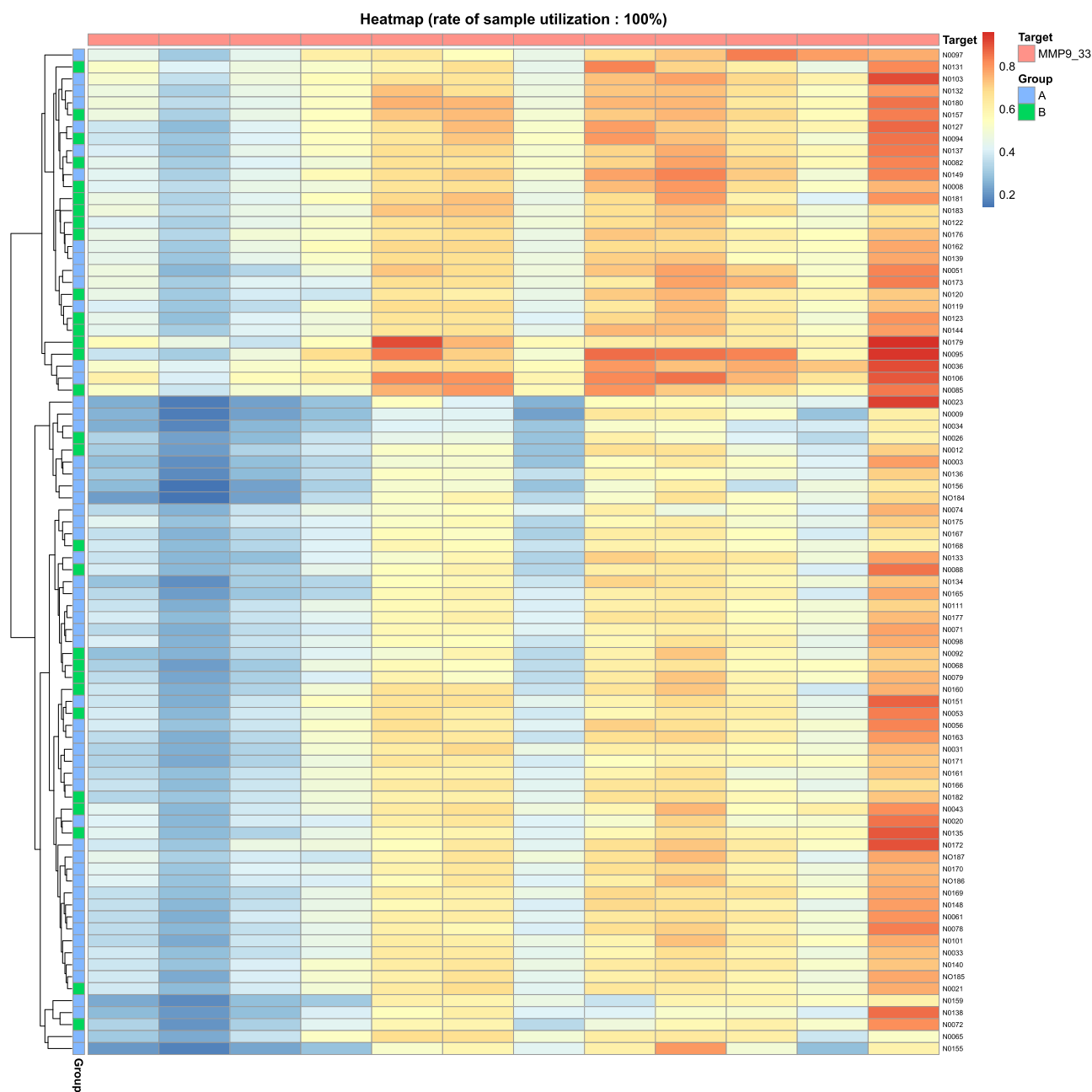


Figure 3 A heatmap based on the methylation levels of CpG sites in samples in groups A and B.

the two groups in *MMP9* gene methylation level ($P>0.05$), even after adjusting for age, sex, education level, and Fazekas score ($P>0.05$) (Table 5 and Figure 6) (Supplementary Table 2).

However, after analyzing the methylation level of the whole blood *MMP9* gene sites between the two groups, we found that the methylation levels of S33-25 and S33-30 were lower in the ECI group (Groupdiff <0), and there was a statistically significant difference between the two groups ($P<0.05$), even after adjusting for age, sex, education level, and Fazekas score ($P<0.05$) (Table 5) (Supplementary Table 2).

Discussion

The incidence of TIA/MIS with ECI was high, and our study found an incidence of 65.5%. These results are similar to those obtained by screening with MoCA and other scales.^{1,3} The cognitive function evaluation of TIA/MIS should not be

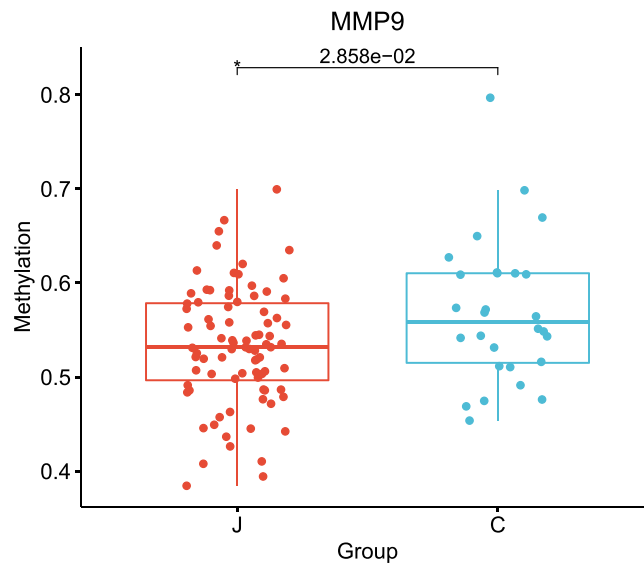


Figure 4 The difference in *MMP9* methylation levels between group J and group C. The *MMP9* gene was relatively hypomethylated in group J.

ignored. The specific scale selection can be determined according to the patient's actual situation. *MMP9* can be used to reflect the degree of blood-brain barrier damage. There has been no research on the methylation of *MMP9* after ischemic stroke. This study focused on the relationship between *MMP9* methylation and TIA/MIS in ECI.

Hypomethylation of the *MMP9* gene can lead to the upregulation of gene expression and an increase in *MMP9* levels. Our study confirmed the overall hypomethylation of the *MMP9* gene in the TIA/MIS group compared with the control group, and there was a significant statistical difference at sites S33-79. There is a clear correlation between *MMP9* and ischemic stroke,^{18,35,36} and the destruction of the blood-brain barrier mainly causes the impact on ischemic stroke.²⁰⁻²³ Studies have shown that *MMP2* and *MMP9* can increase significantly during the early stages of ischemic stroke.^{22,35} Planas et al found in animal models that the levels of *MMP2* and *MMP9* in the brain tissue increased after ischemic stroke, but *MMP9* increased more obviously in the early stage,³⁶ and *MMP9* played a greater role in the destruction of the blood-brain barrier in the later stage.³⁵ Zhong et al found that increasing *MMP9* in the serum of patients with acute

Table 3 Differences in *MMP9* Methylation Levels and Methylation Sites Between TIA/MIS and Control Groups

Target	POS	Type	Groudiff	P value	Adj. P value
<i>MMP9</i>		–	–0.036	0.029	0.147
S33-25	25	CG	–0.034	0.104	0.279
S33-30	30	CG	–0.029	0.085	0.315
S33-39	39	CG	–0.052	0.009	0.069
S33-53	53	CG	–0.049	0.004	0.111
S33-58	58	CG	–0.053	0.021	0.275
S33-73	73	CG	–0.047	0.013	0.099
S33-79	79	CG	–0.053	0.009	0.019
S33-113	113	CG	–0.023	0.212	0.365
S33-131	131	CG	–0.009	0.571	0.586
S33-141	141	CG	–0.03	0.134	0.517
S33-156	156	CG	–0.049	0.015	0.086
S33-171	171	CG	–0.01	0.598	0.846

Notes: Target, name of the site/gene; POS, position of the methylated site on the fragment; Type, type of methylation; Groudiff, difference in mean methylation between the two groups; Adj. P value, P value after adjusting for age, sex, and Fazekas score.

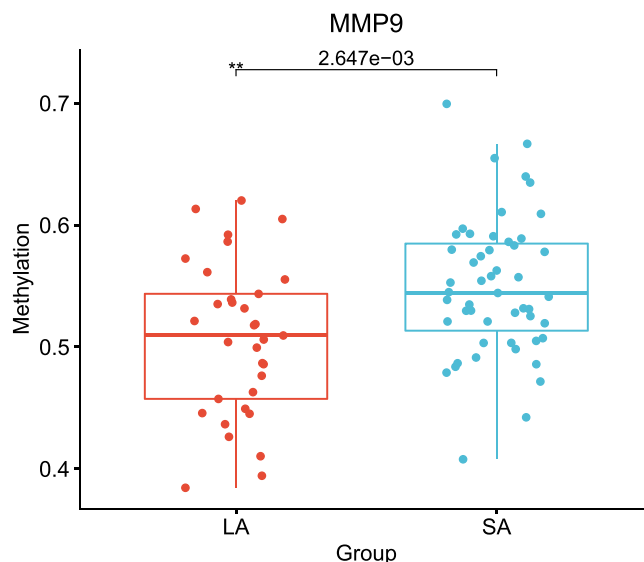


Figure 5 The difference in *MMP9* methylation levels between group LA and group SA. The *MMP9* gene was relatively hypomethylated in group LA.

ischemic stroke can increase the risk of poor prognosis.¹⁸ Some studies have confirmed that adding an *MMP9* inhibitor can reduce the lesion volume, damage the blood-brain barrier,^{20–22} and reduce the risk of bleeding,²³ showing that the intervention of *MMP9* can affect the prognosis of ischemic stroke. These findings are consistent with our findings. *MMP2* and *MMP9* are gel-like enzymes. Lin et al found that *MMP2* is hypomethylated in ischemic stroke,⁶ but there has been no research on the relationship between *MMP9* methylation and ischemic stroke. This study confirmed that *MMP9* gene site methylation relates to TIA/MIS.

After further analysis of the TIA/MIS group, we found that the whole blood *MMP9* gene methylation level of TIA/MIS patients caused by large atherosclerosis was significantly lower than that of TIA/MIS patients caused by small arterial occlusion. The high expression of *MMP9* caused by hypomethylation can aggravate damage to the blood-brain barrier. Patients with large atherosclerotic stenosis have a longer period of hypoperfusion in the brain tissue before the onset of stroke. Inflammation and oxidative stress reactions secondary to hypoperfusion can also lead to activation of the *MMP* family^{37,38} and aggravate damage to the blood-brain barrier. We consider that patients with large atherosclerotic

Table 4 Differences in *MMP9* Methylation Levels and Methylation Sites Between LA and SA Groups

Target	POS	Type	Groudiff	P value	Adj. P value
<i>MMP9</i>	–	–	–0.042	0.003	0.002
S33-25	25	CG	–0.052	0.003	0.004
S33-30	30	CG	–0.042	0.001	0.002
S33-39	39	CG	–0.052	0.001	0.002
S33-53	53	CG	–0.037	0.047	0.007
S33-58	58	CG	–0.049	0.009	0.006
S33-73	73	CG	–0.049	0.006	0.003
S33-79	79	CG	–0.042	0.009	0.005
S33-113	113	CG	–0.048	0.014	0.019
S33-131	131	CG	–0.048	0.007	0.013
S33-141	141	CG	–0.027	0.150	0.098
S33-156	156	CG	–0.028	0.142	0.050
S33-171	171	CG	–0.034	0.095	0.121

Notes: Adj. P value, P value after adjusting for age, sex, and Fazekas score.

Table 5 Differences in MMP9 Methylation Levels and Methylation Sites Between TIA/MIS with and without ECI Groups

Target	POS	Type	Groudiff	P value	Adj. P value
MMP9		–	–0.025	0.069	0.116
S33-25	25	CG	–0.038	0.023	0.029
S33-30	30	CG	–0.035	0.014	0.025
S33-39	39	CG	–0.026	0.088	0.098
S33-53	53	CG	–0.030	0.078	0.054
S33-58	58	CG	–0.034	0.100	0.102
S33-73	73	CG	–0.028	0.114	0.066
S33-79	79	CG	–0.011	0.536	0.633
S33-113	113	CG	–0.038	0.056	0.149
S33-131	131	CG	–0.023	0.164	0.383
S33-141	141	CG	–0.016	0.381	0.690
S33-156	156	CG	–0.013	0.457	0.757
S33-171	171	CG	–0.012	0.549	0.941

Notes: Adj. P value, P value after adjusting for age, sex, education level, and Fazekas score.

stenosis were more likely to have larger defects and more lesions than patients with smaller artery occlusion, resulting in more severe blood-brain barrier damage, consistent with the trend of lower methylation levels of the *MMP9* gene. In addition, in the analysis of this subgroup, we found that the statistical difference between the two groups was the most obvious, regardless of the total gene aspect or detected sites. This shows that hypomethylation of the *MMP9* gene plays an important role in the pathogenesis of large atherosclerotic TIA/MIS. They strongly correlate and indirectly reflect the extent of TIA/MIS blood-brain barrier damage. *MMP9* methylation can potentially become a biomarker for predicting the severity of TIA/MIS.

In this study, we found that the overall methylation level of *MMP9* in the TIA/MIS group with ECI was lower than that in the group without ECI. Although there was no statistical difference, these results were consistent with the trend of previous research results and the process of *MMP9* participating in pathology. We found that S33-25 and S33-30 of the *MMP9* gene showed statistically significant differences between the groups with and without ECI, showing hypomethylation. Previous studies have found that increased serum *MMP9* level in the acute phase of ischemic stroke is related to

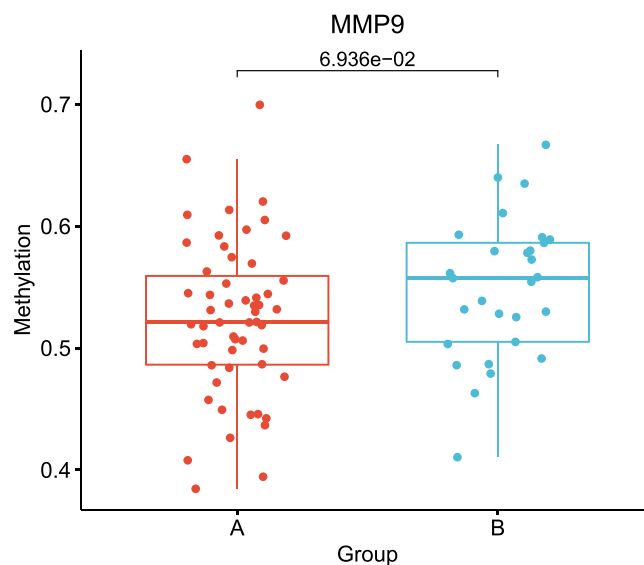


Figure 6 The difference in *MMP9* methylation levels between group A and group B. The *MMP9* gene was relatively hypomethylated in group A.

CI after 3 months.³⁹ Bruno et al also found that the level of MMP9 in the brain tissue of patients with mild cognitive impairment (MCI) increased and speculated that the cause of CI might be related to the degradation mediated by MMP9.⁴⁰ Adair et al found that the level of MMP9 in the cerebrospinal fluid of patients with vascular dementia was higher than that in patients with Alzheimer's disease and the control group.⁴¹ In another study of patients with subcortical ischemic cerebrovascular disease, the overall level of MMP9 in the case group was higher. However, there was no significant difference between the levels of MMP9 in the cerebrospinal fluid of the case and control groups.⁴² Previous research results are consistent with those of TIA/MIS with ECI. Simultaneously, we also found two sites with statistical differences, which confirmed the correlation between lower methylation of the *MMP9* gene site and TIA/MIS with ECI. The cause of CI caused by the increase in MMP9 levels is not completely clear, which may be related to the aggravation of blood-brain barrier damage. Thus affecting the transportation of toxic substances such as A β , causing A β accumulation in the brain, and the oxidative stress reaction is secondary. In general, this study showed that hypomethylation of *MMP9* gene sites was associated with TIA/MIS with ECI, which was likely to be generated through the destruction of the blood-brain barrier and the secondary influence on A β transportation. Methylation of *MMP9* gene sites can potentially become a biomarker and intervention target for TIA/MIS in patients with ECI.

Subgroup analysis of TIA/MIS with large atherosclerosis and small artery occlusion confirmed that hypomethylation of *MMP9* has a stronger correlation with TIA/MIS with large atherosclerosis. In contrast, the subgroup analysis with and without ECI found that hypomethylation of *MMP9* was associated with TIA/MIS with ECI. However, in stroke patients, the combination of large artery atherosclerotic stenosis and ECI is often associated with poor prognosis.^{1,43} The analysis between the two groups also indicated that the lower methylation of *MMP9* was related to the poor prognosis of TIA/MIS to a certain extent.

This study also has some shortcomings. First, the number of patients in this study was small and not completely matched according to age and sex. However, when we conducted statistical research, we corrected for age and sex to minimize their impact on the study. Second, because this study aimed to find biomarkers in whole blood, the methylation level of the *MMP9* gene in whole blood was detected. However, it cannot be confirmed that the methylation level of genes in whole blood is consistent with that in brain tissue. However, we consider that changes in the level of *MMP9* in whole blood can also affect the blood-brain barrier. Blood samples could be easily obtained for dynamic monitoring if it was used as a biomarker or intervention target. Third, we studied just China's population of stroke, can not full reflect the correlation between *MMP9* methylation and TIA/MIS in other ethnic groups and races. Therefore, more researches may be needed in other ethnic groups and races.

Conclusion

The incidence of TIA/MIS with ECI is high, and cognitive function should be routinely screened in such patients. Hypomethylation of *MMP9* was associated with TIA/MIS and TIA/MIS with ECI. Hypomethylation of *MMP9* was strongly associated with TIA/MIS with large artery atherosclerosis. Methylation of *MMP9* is involved in the pathogenesis of TIA/MIS and can reflect the severity of TIA/MIS. The methylation site of *MMP9* can be used as a potential biomarker and therapeutic target for TIA/MIS and TIA/MIS with ECI.

Data Sharing Statement

The sequence data had been uploaded to the National Genomics Data Center. The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive⁴⁴ in National Genomics Data Center,⁴⁵ China National Center for Bioinformation / Beijing Institute of Genomics, Chinese Academy of Sciences (GSA-Human: HRA004471) that are publicly accessible at <https://ngdc.cnbc.ac.cn/gsa-human>.

Ethics Statement

The Ethics Committee of Qilu Hospital (Qingdao) of Shandong University approved the study. All patients provided written informed consent. The study complies with the Declaration of Helsinki.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

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.

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