Original Paper

Methylene Tetrahydrofolate Reductase (*MTHFR*) rs868014 Polymorphism Regulated by miR-1203 Associates with Risk and Short Term Outcome of Ischemic Stroke

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Key Words

Stroke • Methylene tetrahydrofolate reductase (*MTHFR*) • SNPs • miR-1203 • Homocysteine • Outcome

Abstract

Background/Aims: Genetic polymorphisms of methylene tetrahydrofolate reductase (*MTHFR*) were associated with ischemic stroke risk. This study analyzed *MTHFR* polymorphisms at the 3'-untranslated region for association with risk and outcome of ischemic stroke in a Chinese Han population. **Methods:** 500 patients and 600 healthy volunteers were enrolled for *MTHFR* rs868014 genotyping identified bioinformatically. The binding of miR-1203 to *MTHFR* rs868014 was determined by luciferase assay, *MTHFR* expression was assessed using qRT-PCR, and plasma homocysteine levels were assayed by ELISA. **Results:** Cigarette smoking, alcohol consumption, diabetes, hypertension (all $P < 0.001$), low levels of serum high-density lipoprotein-C ($P = 0.01$), and high levels of serum low-density lipoprotein-C ($P = 0.005$) were associated with an increased risk of developing ischemic stroke. BMI and total serum cholesterol concentration was not associated with ischemic stroke. *MTHFR* rs868014 TC and CC genotypes were significantly associated with increased risk of ischemic stroke compared with the TT genotype (OR: 1.52; 95% CI: 1.01-3.39 for TC genotype, while OR: 1.99; 95% CI: 1.29-3.88 for CC genotype). Furthermore, the *MTHFR* rs868014 SNP was associated with a poor short-term ischemic stroke outcome. qRT-PCR confirmed that *MTHFR* rs868014 TC or CC genotypes could facilitate miR-1203 binding leading to low *MTHFR* levels in cells. In addition, patients carrying the *MTHFR* rs868014 TC or CC genotypes were associated with accumulation of serum tHcy and a poor ischemic stroke outcome. Linkage disequilibrium analysis indicated that the newly identified SNP rs868014 was strongly linked with the *MTHFR* A1298C polymorphism. **Conclusion:** This study demonstrates that the *MTHFR* rs868014 SNP

is associated with increased risk in developing ischemic stroke, miR-1203 binding, low *MTHFR* levels in cells, and poor short term outcome of patients.

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Introduction

Stroke is a major cause of mortality in the elderly population worldwide. Clinically, stroke can be divided into ischemic and hemorrhagic stroke. Ischemic stroke is usually caused by blockage of a blood vessel, whereas hemorrhagic stroke is caused by rupture of a vessel leading to bleeding in the brain [1, 2]. According to recent statistics, there were approximately 6.9 million patients diagnosed with ischemic stroke and 3.4 million patients with a hemorrhagic stroke in 2013 [3]. In China, the annual stroke mortality rate is approximately 157 per 100,000, which is the leading cause of death and adult disability [4-6]. The major stroke risk factors include high blood pressure, tobacco smoking, obesity, high blood cholesterol, diabetes mellitus, and atrial fibrillation [7], indicating that genetic factors also contribute to stroke.

Methylene tetrahydrofolate reductase (*MTHFR*) is the key enzyme involved in plasma homocysteine (tHcy) metabolism by catalyzing the conversion of 5,10-methyltetrahydrofolate to 5-methyltetrahydrofolate, a methyl donor during tHcy remethylation [8]. tHcy is a crucial intermediate in methionine metabolism and causes excessive production of reactive oxygen species (ROS) [9, 10]. This plays an important role in the regulation of cell signaling and homeostasis. During stress, ROS levels can be increased dramatically, resulting in cell structure damage. For example, upregulated tHcy can induce cell apoptosis by increased ROS levels through activation of the p38 MAPK pathway [11]. In the context of stroke, the elevated levels of plasma tHcy were shown to associate with increased risk of ischemic stroke [12, 13]. *MTHFR* is localized at chromosome 1 p36.3 in the human genome, and to date there are over 40 point mutations or SNPs in the *MTHFR* gene identified [14]. Of these, C677T (rs1801133) and A1298C (rs1801131) are the most significant mutations that associate with ischemic stroke [15, 16]. The *MTHFR* rs1801133 polymorphism involves substitution of C to T at position 677 (C677T), causing the conversion of alanine to valine. This missense mutation will result in approximately 70% and 35% reductions of normal *MTHFR* enzymatic activity in TT and CT genotype carriers, respectively [17]. The A1298C variant results in substitution of glutamate (Glu) to alanine (Ala) at codon 429 in the S-adenosylmethionine regulatory domain of the *MTHFR* protein [18, 19]. The *MTHFR* C677T genetic variant can lead to elevated levels of plasma tHcy and, thus, increased ischemic stroke risk [20, 21], data of which have been further confirmed in other studies [12, 13, 22]. tHcy is thought to increase thrombotic risk through induction of endothelial injury in the venous and arterial vasculature [23]. Thus, further study of genetic variants of *MTHFR* could help to better understand ischemic stroke risk and underlying molecular mechanisms.

MicroRNAs (miRNA) are small, non-coding RNA molecules. Through binding to the 3' untranslated region (3'-UTR) of messenger RNA (mRNA), miRNA can cause degradation of mRNA, thus regulating the expression of mRNA at post-transcription level. Genetic variations in the 3'-UTR of certain genes are able to change the expression of the genes and alter the risk of a variety of human cancers [24-27]. Thus, in this study, we assessed genetic variants in the *MTHFR* 3'-UTR in ischemic stroke patients vs. healthy control individuals, and then associated these genetic variants with miRNA levels using a case-control study design in a Chinese Han population. This study provides novel insightful information regarding *MTHFR* genetic variants and miR-1023 expression in changes in ischemic stroke risk in this population.

Materials and Methods

Study subjects

In this study, we enrolled 500 patients diagnosed with ischemic stroke from Jiangyin People's Hospital (Jiangyin, China) between June 2009 and August 2015. Diagnosis of patients with ischemic stroke was

established clinically and confirmed by using X-ray computed tomography and/or magnetic resonance imaging scans of the brain. We also recruited 600 healthy volunteers from this hospital during the same period of time and they were matched to the cases by age and gender. All control individuals visited our hospital for annual health check-ups and were free of ischemic stroke. This study was approved by the Ethics Committee of Jiangyin People's Hospital and each participant signed a written informed consent. All case and control individuals filled out a study questionnaire, which included demographical information, medical history (hypertension or diabetes mellitus), alcohol consumption, tobacco smoke (daily number and years), obesity, and hypercholesterolemia. Hypertension was defined as blood pressure > 140/90 mmHg (average of three independent measures) or the use of antihypertensive drugs. Diabetes mellitus was defined as a fasting glucose level ≥ 7.0 mmol/L and/or ≥ 11.1 mmol/L 2 h after oral glucose challenge, or receiving anti-diabetic medication. Subjects who had smoked more than 10 cigarettes daily for 5 years were considered as smokers. Subjects who drank more than 50 ml of alcoholic beverages daily for 5 years were considered as alcohol drinkers. The National Institutes of Health Stroke Scale (NIHSS) score was used to quantify stroke severity in patients at the time of presentation and discharge.

Genomic DNA extraction and genotyping

Blood samples were collected after a 12 h overnight fasting period and then separated into serum, red blood cells, and buffy coat. Genomic DNA from peripheral whole blood of every validation subject was extracted by using QIAamp DNA blood mini kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After quantification, these DNA samples were subjected to DNA genotyping using the TaqMan SNP Genotyping Assay. Briefly, genomic DNA samples of 10 ng each were amplified using PCR in a total volume of 5 μ L containing TaqMan Universal Master Mix, 80x SNP Genotyping Assay Mix, DNase-free water and 10-ng DNA. The PCR conditions were 50 °C for 2 min and then 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min in a 384-well ABI 7900HT Real Time PCR System as described previously [28].

Bioinformatic analysis of miRNAs binding to MTHFR SNP and linkage disequilibrium analysis among MTHFR polymorphisms

Bioinformatics software (<http://www.bioguo.org/miRNASNP/>) was used to detect the candidate SNPs which could affect *MTHFR* gene regulation via miRNAs. The Hapmap database and Haploview software were used to analyze the linkage disequilibrium among *MTHFR* polymorphisms.

Cell line, culture, and miR-1203 transfection

Embryonic Kidney 293T cells were obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China) and cultured in RPMI-1640 (Gibco, Gaithersburg, MD, USA) supplemented with 10 % fetal bovine serum (Invitrogen, Carlsbad, CA, USA) in a humidified incubator with 5 % CO₂ at 37 °C. miR-1203 mimics and control were obtained from Genepharma (Shanghai, China) and transfected into 293T cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Construction of luciferase-based reporter plasmids and luciferase reporter assay

MTHFR 3'-UTR fragments containing either C or T alleles were amplified using PCR from genomic DNA and cloned into pGL3-promoterless luciferase-based plasmids (Promega, Madison, WI, USA) multiple cloning sites, BamHI and XhoI. *MTHFR* 3'-UTR fragments potentially binding to miR-1203, predicted by bioinformatical analysis or a mutated sequence with the predicted target sites, were cloned using PCR from genomic DNA and inserted into the pGL3 promoter vector (Genscript, Nanjing, China). The cloned plasmids were amplified and verified by DNA sequencing.

For the dual-luciferase reporter assay, 293T cells were plated onto 24-well plates and transfected with 100 ng of pGL3-*MTHFR* wild, pGL3-*MTHFR* mutant, and miR-1203 mimics, respectively, using Lipofectamine 2000 (Invitrogen). A Renilla luciferase vector pRL-SV40 (5 ng) was also co-transfected as a normalization and transfection efficiency control. Each experiment was performed in triplicate and luciferase activity was assessed 48 h after transfection using the dual-luciferase reporter assay system (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity according to a previous study [29].

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

qRT-PCR was performed to determine levels of *MTHFR* mRNA. In brief, total RNA was isolated from 293T cells using a Trizol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed into cDNA using

the TaqMan® Reverse Transcription Kit, according to the manufacturers' instructions (ABI, CA, USA). The cDNA samples were then subjected to qPCR amplification under conditions of 95 °C for 10 min followed by 40 cycles of 95 °C for 30 s, 55 °C for 40 s, and 72 °C for 30 s, and finally 4 °C for 30 min using the Realmaster Mix (SYBR Green I) (TAKARA, Dalian, China).

ELISA of plasma homocysteine levels

Plasma levels of tHcy in patients was assessed using a tHcy ELISA kit (Green Stone, Bern, Switzerland), according to the manufacturer's protocol. The level of plasma tHcy was normalized to the kit standard and the data were summarized as mean $\pm$ sd and then associated with outcome in ischemic stroke patients.

Statistical analysis

The association between different genetic variants and ischemic stroke risk was evaluated by calculation of the odds ratios (ORs) and 95% confidence intervals (CIs) using univariate and multivariate logistic regression analysis. The difference in association of *MTHFR* mRNA levels with three *MTHFR* genotypes and of the relative luciferase activity between the wild and mutant genotype were evaluated by using an independent-sample *t* test. All statistical analyses were two-sided and $P < 0.05$ was considered statistically significant using SPSS 13.0 (SPSS, Chicago, IL, USA) or SAS software (version 9.1.3; SAS Institute, Cary, NC, USA).

Results

Characteristics of participants

Characteristics of patients with ischemic stroke and healthy controls are shown in Table 1. Specifically, the controls were matched with cases for age and gender. Cigarette smoking ($P < 0.001$), alcohol consumption ($P < 0.001$), diabetes and hypertension ($P < 0.001$), a low level of serum high-density lipoprotein-C (HDL-C; $P = 0.01$), and high level of serum low-density lipoprotein-C (LDL-C; $P = 0.005$) were significantly associated with ischemic stroke. However, BMI and total serum cholesterol concentration were not associated with ischemic stroke (Table 1).

Identification of MTHFR polymorphisms in 3'-UTR

Previous studies demonstrated an association of *MTHFR* genetic variants in gene coding regions with ischemic stroke risk [15, 18, 19]. In this study, we mainly focused on the relationship of the SNPs in the *MTHFR* 3'-UTR to ischemic stroke risk and outcome. We first searched the GenBank of Single Nucleotide Polymorphism (SNP) database (<https://www.ncbi.nlm.nih.gov/snp>) to identify potential *MTHFR* genetic variants in the 3'-UTR using the following parameters: Organism (Homo sapiens); Function Class (3'-UTR); Global MAF (0.05-0.1); Validation Status (by-1000 Genomes). We identified five *MTHFR* polymorphisms (Table 2).

We then assessed genotype frequencies of these five *MTHFR* SNPs in 500 ischemic stroke patients and 600 healthy controls. We found that the *MTHFR* SNP rs868014 had a statistically different distribution between ischemic stroke patients and healthy controls. The minor allele frequency (MAF) of rs868014 was 0.365 in controls. There were no differences in other *MTHFR* SNPs between cases and controls (data not shown). The MAF of rs2184226, rs3737967, rs2077360 and rs4845884 were 0.02, 0.16, 0.03 and 0.08 in controls respectively. The Chi-square test confirmed that the genotype of rs868014 was in Hardy-Weinberg equilibrium distribution pattern in the healthy control group ($P = 0.22$). The logistic regression analysis revealed that *MTHFR* rs868014 TC and CC genotypes significantly associated with increased risk of developing of ischemic stroke compared with the TT genotype (OR: 1.52; 95 %CI: 1.01-3.39 for TC genotype, while OR: 1.99; 95 %CI: 1.29-3.88 for CC genotype; Table 3). A higher number of C-alleles were also associated with an increased risk of ischemic stroke (OR: 1.71; 95 %CI: 1.33-3.56).

Table 1. Frequency distributions of selected variables in patients and healthy controls

Characteristics	Cases(n=500)	Control(n=600)	p value
Age (year)	65(51-77)	64(50-78)	0.66
Sex (Male/Female)	333/167	402/198	0.89
Smoking (Yes/No)	321/179	285/315	<0.001
Drinking (Yes/No)	299/201	278/322	<0.001
Diabetes (Yes/No)	159/341	101/499	<0.001
Hypertension (Yes/No)	221/279	119/471	<0.001
BMI (kg/m ²)	24.1(21.2-25.2)	24.3(21.5-25.3)	0.47
Total cholesterol (mmol/L)	4.61(4.01-5.41)	4.51(3.99-5.39)	0.19
HDL-C(mmol/L)	1.30(1.01-1.50)	1.41(1.08-1.55)	0.01
LDL-C(mmol/L)	2.88(2.44-3.11)	2.35(2.12-3.01)	0.5

Table2. SNPs located in the *MTHFR* gene 3'-UTR and the predicted miRNAs

Gene	SNP	Chromosome	HGVS Names
<i>MTHFR</i>	rs2184226	1:11787379	XM_005263461.1:c.*3301A>G
<i>MTHFR</i>	rs3737967	1:11787392	XM_005263460.1:c.*3288C>T
<i>MTHFR</i>	rs868014	1:11789390	XM_005263459.1:c.*1150T>C
<i>MTHFR</i>	rs2077360	1:11788822	XM_005263461.1:c.*1858T>C
<i>MTHFR</i>	rs4845884	1:11786390	XM_005263461.1:c.*4290C>T

Association of the MTHFR rs868014 SNP with a poor short-term ischemic stroke outcome

Next, we assessed the association of the *MTHFR* rs868014 SNP with short-term ischemic stroke outcome. The latter was assessed by using two independent scales: 1) the Functional Independence Measure (FIM); and 2) the National Institutes of Health Scale Score (NIHSS). The FIM instrument is an 18-item scale, which measures independence involved in feeding, grooming, dressing, toileting, mobility, and cognition. Subjects are scored from 7 (totally independent) to 1 (totally dependent) on each item, with a score of 126 indicating total functional independence. The NIHSS is composed of 11 items, each of which scores a specific ability between a 0 and 4. For each item, a score of 0 typically indicates normal function in that specific ability, while a higher score is indicative of some level of impairment. We found that patients carrying either the *MTHFR* rs868014 TC or CC genotype had a decreased FIM score in both motor and cognitive assessments. Moreover, initial stroke severity in the acute phase, as measured by NIHSS, was significantly different among the different genotypes (TC/TT, $P = 0.03$ and CC/TT, $P = 0.02$). Recovery from ischemic stroke was measured as the change in NIHSS score between the acute phase and three months post-stroke. A negative value indicated clinical improvement in ischemic stroke outcome. Our data showed that the minor alleles of rs868014 had a trend towards a poor outcome of ischemic stroke (TC/TT, $P = 0.01$ and CC/TT, $P = 0.004$; Table 4).

MTHFR rs868014 SNP alteration of miR-1203 binding

Thus far, we revealed that the *MTHFR* rs868014 SNP was associated with risk and poor ischemic stroke outcome. We then performed bioinformatical analysis to determine whether this *MTHFR* rs868014 SNP could have miRNA binding sites. We found that miR-1203 could bind to this particular SNP of the minor allele (Fig. 1A). We therefore performed a luciferase assay to confirm such binding and found that luciferase activity of C-allele-specific pGL3

Table 3. Genotype frequencies of the *MTHFR* rs868014 polymorphism among IS group and control group. ^aThe ORs, 95% CIs and *P* value were calculated after adjusting for age, gender, parental smoking, drinking and other characteristics listed in Table 1

Genotype	Cases (<i>n</i> = 500)		Controls (<i>n</i> = 600)		OR (95% CI) ^a	<i>P</i> Value ^a
	<i>N</i>	%	<i>N</i>	%		
rs868014						
TT	188	37.6	299	49.8	1.00	
TC	170	34.0	164	27.3	1.52 (1.01-3.39)	<0.001
CC	142	28.4	137	22.8	1.99 (1.29-3.88)	0.001
C carrier	312	62.4	301	50.1	1.71 (1.33-3.56)	<0.001

Table 4. Stratified analysis of rs868014 genotype with clinicopathological parameters of IS patients. *Student *t* test for either genotype distributions or allele frequencies between cases and controls. NIHSS ac, initial stroke severity during the acute phase; NIHSS 3m, stroke severity after 3 months; ΔNIHSS, recovery defined as change in NIHSS from acute to 3-month post-stroke, with a negative value indicating clinical improvement. *MTHFR*: Relative expression of *MTHFR* mRNA in IS patients

Feature	Genotype			TC vs TT* <i>P</i> Value	CC vs TT* <i>P</i> Value
	TT	TC	CC		
FIM score (Mean/SD)					
Total	65.2/22.1	62.9/21.9	60.1/22.7	0.02	0.01
Motor	44.2/19.9	42.1/18.8	40.5/14.8	0.04	0.02
Cognitive	22.9/10.1	20.8/9.1	19.7/8.1	0.001	0.02
NIHSS ac (Mean/SD)	2.8/1.2	3.2/1.9	3.8/2.2	0.03	0.02
NIHSS 3m (Mean/SD)	0.7/0.3	0.9/0.4	1.1/0.5	0.004	0.001
ΔNIHSS (Mean/SD)	-1.9/0.7	-2.2/0.8	-2.9/1.1	0.01	0.004
tHcy (mmol/L)	12.2/2.2	14.2/3.8	15.9/2.1	0.01	0.01
<i>MTHFR</i> (Mean/SD)	0.06/0.21	0.05/0.11	0.02/0.12	0.03	0.01

construct was significantly suppressed by miR-1203. Luciferase activity of the T-allele-specific pGL3 construct was not altered by miR-1203 transfection in 293T cells (Fig. 1B).

We next assessed levels of *MTHFR* mRNA in patients carrying the *MTHFR* rs868014 TT, TC, and CC genotypes. Our data showed that patients carrying the *MTHFR* rs868014 TC or CC genotypes had a decreased level of *MTHFR* compared with patients with the TT genotype (Fig. 1C and Table 4). We then assayed levels of serum tHcy in patients and found that patients carrying the *MTHFR* rs868014 TC or CC genotype were associated with the accumulation of serum tHcy and with a poor ischemic stroke outcome (Table 4).

Linkage equilibrium analysis of *MTHFR* rs868014 vs. other *MTHFR* SNPs

The linkage disequilibrium was defined as the coinheritance of alleles in a haplotype manifesting at the population level. We analyzed the linkage disequilibrium between rs868014 and other *MTHFR* SNPs using the Hapmap database and Haploview software. We found that there was no link between rs868014 and C677T (rs1801133, $r^2 = 0.27$), but a positive link between rs868014 with A1298C (rs1801131) and some other SNPs (rs6541003, rs1994798, rs1476413, rs3818762, rs4346048, $r^2 \geq 0.8$) (Fig. 2).

Discussion

In the current study, we analyzed the *MTHFR* rs868014 polymorphism in blood samples obtained from ischemic stroke patients vs. healthy controls and then associated this polymorphism with ischemic stroke risk and outcome in a Chinese Han population. We

Fig. 1. Binding of miR-1203 to *MTHFR* rs868014 minor alleles. A: Bioinformatics analysis of potential miR-1203 binding to *MTHFR* rs868014 polymorphisms. B: Luciferase assay. 293T cells were co-transfected with miR-1203 mimics or control renilla luciferase vector pRL-SV40 for 48 h. Both firefly and renilla luciferase activities were measured in the same sample using a luciferase assay kit. Firefly luciferase signals were normalized with renilla luciferase signals. C: qRT-PCR. Level of *MTHFR* mRNA was determined by qRT-PCR in patients carrying *MTHFR* rs868014 TT, TC, and CC genotypes. Data were presented as mean $\pm$ SE. * $P < 0.05$.

Fig. 2. Results of linkage disequilibrium analysis between rs868014 and other *MTHFR* SNPs. The numbers in the scheme stand for $r^2 \times 100$. The r^2 threshold was set as 0.8, and the color schemes without number mean the $r^2 \times 100 > 80$. $r^2 > 0.8$ was generally considered as linkage disequilibrium. This figure indicated a positive link between rs868014 with rs1801131 and some other SNPs (rs6541003, rs1994798, rs1476413, rs3818762, rs4346048). Color scheme: Red - $D' < 1$, $LOD > 2$; lilac - $D' = 1$, $LOD < 2$; White - $D' < 1$, $LOD < 2$. r^2 : the correlation coefficient between the two loci. D' : the value of D prime between the two loci. LOD: the log of the likelihood odds ratio, a measure of confidence in the value of D' .

found that cigarette smoking, alcohol consumption, diabetes and hypertension, low levels of serum high-density lipoprotein-C, and high levels of serum low-density lipoprotein-C were all associated with an increased risk of developing ischemic stroke. BMI and total serum cholesterol concentration were not associated with ischemic stroke. Moreover, we also found that *MTHFR* rs868014 TC and CC genotypes were significantly associated with increased risk of ischemic stroke compared with the TT genotype. *MTHFR* rs868014 TC and CC genotypes were also associated with a poor short-term ischemic stroke outcome. In addition, our data was first to show that *MTHFR* rs868014 TC or CC genotypes could facilitate miR-1203

binding, leading to low *MTHFR* levels in cells. To our knowledge, the binding of miR-1203 to *MTHFR* rs1801133 or rs1801131 has not been investigated yet. *MTHFR* rs868014 TC or CC genotypes were also associated with increased serum tHcy and a poor ischemic stroke outcome. The data from our current study demonstrated the importance of the *MTHFR* rs868014 SNP in *MTHFR* expression, miR-1203 binding, development of ischemic stroke, and poor short term outcomes of patients. Our current data are novel, although a previous study showed association of *MTHFR* rs868014 with choline/1-carbon metabolism and inversely with steatosis [30].

MTHFR is an important enzyme in the generation of ROS and subsequent cell damage [23-25]. Indeed, in ischemic conditions, ROS generation is enhanced and can lead to damage to various types of cells, including brain endothelial cells, leading to formation of thrombosis and loss of brain function [31]. Our current study provides direct evidence showing that this novel *MTHFR* rs868014 polymorphism increases the risk of developing ischemic stroke compared with healthy controls. Mechanistically, the *MTHFR* rs868014 polymorphism alters the binding of miR-1203 to negatively regulate *MTHFR* expression, providing an epigenetic mechanism of gene regulation and expression.

There is accumulating evidence demonstrating that SNPs localized at miRNA binding sites (miRSNPs) could affect the binding of miRNAs to the target genes and in turn result in reduction or increase in translation of the target mRNA and altered susceptibility to cancer [28, 32]. For example, previous studies showed that the rs2910164 polymorphism harboring the sequence for miR-146a could influence susceptibility to gastric cancer in a Chinese population, while rs4143815 and rs4819388 SNPs in the 3'-UTR of B7-H1 and B7-H2 genes, respectively, associated with development of gastric cancer [33-35]. As for miR-1203, a previous study showed that altered miR-1203 expression was a biomarker predicting prostate cancer [36]. Our current study is the first to demonstrate the association between miR-1203 and *MTHFR*. Further study is needed to reveal the role of miR-1203 in ischemic stroke development and outcome.

Furthermore, we utilized the Hapmap database and Haploview software to analyze the linkage disequilibrium among *MTHFR* polymorphisms and found that there was a positive link of rs868014 with A1298C (rs1801131) and some other SNPs (rs6541003, rs1994798, rs1476413, rs3818762, rs4346048), although there was no link between rs868014 and C677T (rs1801133). Our current data indicated that the function of rs868014 could be linked to these SNPs. Future studies are needed to confirm such a link and investigate the underlying mechanisms.

Our current study does have some limitations. For example, a total of five *MTHFR* polymorphisms were assessed in this study, we only found an association of the *MTHFR* rs868014 polymorphism with ischemic stroke risk and outcome. Statistically, type 1 error may occur in our study; thus, independent samples or samples from another ethnic population could be used to confirm our study. The MAF of rs868014 observed in our study is much higher than reported by previous study [29]. It is probably due to the fact that our study population is Southern Chinese Han population. Future study with larger samples may be needed to confirm our study. Moreover, the linkage disequilibrium analysis of rs868014 and other *MTHFR* SNPs showed that there was linkage disequilibrium between rs868014 and other SNPs including rs1801131, indicating that the effects of rs868014 polymorphism may be confounded with these *MTHFR* SNPs, however, we did not further assess their effects on regulation of ischemic stroke risk and outcome. Further studies may be needed to clarify the links between these *MTHFR* SNPs.

In summary, our current study demonstrated: 1) cigarette smoking, alcohol consumption, diabetes, hypertension, low levels of serum high-density lipoprotein-C, and high levels of serum low-density lipoprotein-C were all associated with ischemic stroke risk; 2) *MTHFR* rs868014 TC and CC genotypes were associated with increased risk of ischemic stroke compared with the TT genotype; 3) miR-1203 is able to bind to *MTHFR* rs868014 TC or CC genotypes and downregulate *MTHFR* expression; 4) patients carrying *MTHFR* rs868014 TC or CC genotypes had an increased level of serum tHcy and a poor ischemic stroke outcome.

Disclosure Statement

The authors declare that there are no conflicts of interest in this work.

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