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Association of methylenetetrahydrofolate reductase (*MTHFR*) gene polymorphism with ischemic stroke in the Eastern Chinese Han population

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ABSTRACT. The association between the *MTHFR* genetic polymorphism and ischemic stroke has been reported by a number of investigators. However, the results have been controversial and conflicting. The aim of this study was to explore the association between the *MTHFR* variants C677T and A1298C and the risk of ischemic stroke in an Eastern Chinese Han population. A total of 199 patients with ischemic stroke and 241 controls were recruited. Genotyping of the *MTHFR* C677T and A1298C polymorphisms was carried out using the Taqman 7900HT Sequence Detection System. The overall estimates (odds ratio: OR) for the allele (C) and genotype (AC+CC) of the A1298C polymorphism were 1.57 [95% confidence interval (CI)

= 1.16-2.10], and 2.36 (95%CI = 1.39-4.00), respectively, establishing significant association of the *MTHFR* A1298C polymorphism with ischemic stroke. In contrast, there were no statistically significant differences compared to controls between *MTHFR* C677T polymorphic variants in the association ischemic stroke risk. Furthermore, haplotype-based analysis demonstrated that compared with the C-677-A-1298 haplotype, the C-677-C-1298 and T-677-C-1298 haplotypes showed significant increased risk of ischemic stroke (OR = 1.56; 95%CI = 1.07-2.2; P = 0.02; OR = 1.76; 95%CI = 1.17-2.65; P < 0.01, respectively). We concluded that the A1298C polymorphism and the haplotypes C-677-C-1298 and T-677-C-1298 in *MTHFR* might modulate the risk of ischemic stroke in the Eastern Chinese Han population.

Key words: Ischemic stroke; Methylenetetrahydrofolate reductase; Polymorphism

INTRODUCTION

Ischemic stroke, a major cause of mortality and morbidity across the world, is a disease with a strong genetic basis (Rubattu et al., 2013). Despite intensive research efforts, the genomic etiology of ischemic stroke remains elusive.

Hyperhomocysteinemia is an independent risk factor for stroke and other vascular events (Linnebank et al., 2012). Reduction of MTHFR enzyme activity increases the pool of 5,10-methylenetetrahydrofolate (5,10-methylene-THF) at the expense of the pool of 5-methyltetrahydrofolate (5-methyl-THF), which is used as a methyl donor in the synthesis of methionine from homocysteine (Toyoda et al., 2004). In turn, methionine provides the methyl group for the formation of S-adenosylmethionine, which is involved in numerous cellular reactions, including DNA, RNA and histone methylation. A decreased pool of methionine may therefore also impact DNA methylation, a model supported by the observation that some *MTHFR* variants are associated with DNA hypomethylation (Castro et al., 2004).

The *MTHFR* gene locus is mapped to chromosome 1p36.3 in humans (Goyette et al., 1994). A common *MTHFR* polymorphism previously investigated in ischemic stroke is a C-to-T transition at nucleotide 677 (C677T) in exon 4, which results in an alanine (Ala) to valine (Val) substitution in the MTHFR enzyme (Frosst et al., 1995). Presence of this substitution in the human *MTHFR* gene predicts phenotypic expression of a heat-sensitive variant with reduced enzymatic activity, resulting in hyperhomocysteinemia (Somarajan et al., 2011). Another important polymorphism in *MTHFR* is A1298C in exon 7. The A-to-C change at nucleotide 1298 results in an amino acid substitution of glutamate (Glu) for alanine (Ala) at codon 429, within the S-adenosylmethionine regulatory domain of the MTHFR protein. The C variant of the A1298C polymorphism generates a reduction in MTHFR enzyme activity (van der Put et al., 1998; Pereira et al., 2006), and can also lead to an elevation of plasma homocysteine (Kumar et al., 2005; Laraqui et al., 2007; Klai et al., 2011).

To further explore the overall effects of *MTHFR* polymorphisms on ischemic stroke, the C677T and A1298C polymorphisms were selected in this study for investigation of the involvement of *MTHFR* genetic variants as risk factors for the pathogenesis of ischemic stroke, as part of our ongoing hospital-based case-control study in an Eastern Chinese Han population.

MATERIAL AND METHODS

Study design and subject recruitment

This study was approved by the institutional review board of Nanjing Medical University. Written informed consent was obtained from each subject who donated 5 mL blood used for DNA extraction. For comatose patients, this consent was obtained from their next of kin. The study design used has been described in our previous report (Sun et al., 2011). Briefly, in the present study, 199 genetically unrelated ethnic Han Chinese patients from Jiangsu Province and surrounding regions in Eastern China, who had been hospitalized with ischemic stroke at the First Affiliated Hospital of Nanjing Medical University (Nanjing) and the Brain Hospital Affiliated to Nanjing Medical University (Nanjing), were recruited from June 2011 to June 2012. Matched controls (N = 241) were enrolled from the same demographic area at the same time.

Ischemic stroke was defined as a focal or global neurological deficit of sudden onset, lasting more than 24 h. Diagnosis of ischemic stroke was based on the results of strict neurological examination: computed tomography (23.1%), magnetic resonance image (27.1%), or both (49.7%), according to the International Classification of Diseases, Ninth Revision (ICD-9) (American Medical Association, 2005). The exclusion criterion was a past history of stroke or related illness. In addition to neurological history and family history of coronary artery disease (CAD), cerebrovascular disease, hypertension, and diabetes mellitus (DM), the following vascular risk factors for each individual were also recorded: body mass index (BMI), cigarette smoking, alcohol consumption, systolic blood pressure (SBP), diastolic blood pressure (DBP), and blood glucose level.

Diagnostic criteria

A history of hypertension was defined as SBP or DBP readings >140 or 90 mmHg, respectively, or both, taken as an average of 3 independent measures, or use of anti-hypertensive treatment prior to the index event. DM was characterized by recurrent or persistent hyperglycemia, and was diagnosed when a patient demonstrated any one of the following: 1) fasting plasma glucose ≥7.0 mM; 2) plasma glucose ≥11.1 mM at 2 h after an oral glucose challenge; or 3) random plasma glucose ≥11.1 mM. Current smokers or subjects who ceased within the last 6 months were included. Alcohol consumption was defined as drinking alcohol at least 12 times over the last year (Kelly et al., 2008). Family history was obtained through self-report of first-degree relatives (parent or sibling).

MTHFR C677T and A1298C genotyping

Genomic DNA was extracted from peripheral white blood cells using the phenol/chloroform method. Genotyping of the *MTHFR* C677T and A1298C polymorphisms was performed using the Taqman 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). For quality control, genotyping was performed blinded to the case/control status of the subjects. In addition, a randomly chosen 10% of the samples were genotyped again by different individuals; the results of these assays were 100% concordant.

Statistical analysis

Categorical variables are reported as percentages and continuous or discrete variables are reported as means ± standard deviation (SD). The unpaired Student *t*-test for continuous variables and the chi-square test or the Fisher exact test for categorical variables was used for comparisons between the patient and control groups. Conditional univariate and multivariate logistic regression models were utilized to obtain the odds ratios (ORs) for the risk of ischemic stroke and their 95% confidence intervals (CIs), with adjustment for age, gender, BMI, past history of CAD, hypertension, and DM. The statistical package Stata 12.0 (Stata, College Station, TX, USA) was used for all analyses performed herein. Unless otherwise stated, all statistical evaluations were made assuming a two-sided test with a significance level of value $P < 0.05$.

RESULTS

Clinical characteristics of the subjects

The baseline characteristics of the two groups are presented in Table 1. Briefly, the mean age was 68.78 ± 10.63 years and 67.19 ± 9.49 years for the patients and controls, respectively; 58.3% of patients and 53.5% of controls were male. There was no significant difference in the distribution of age ($P = 0.10$) and gender ($P = 0.32$) between the patients and controls. As expected, patients had a higher prevalence of risk factors for vascular diseases, including high BMI, history of CAD, hypertension, and DM ($P < 0.05$). However, the differences in smoking and alcohol consumption between the two groups did not reach statistical significance ($P > 0.05$).

Table 1. Baseline characteristics in ischemic stroke patients and controls.

Variables	Controls (N = 241)	Patients (N = 199)	P value
Gender [N (%)]			
Male	129 (53.53)	116 (58.29)	
Female	112 (46.47)	83 (41.71)	0.32
Age (years, mean ± SD)	67.19 ± 9.49	68.78 ± 10.63	0.10
BMI (kg/m^2, mean ± SD)	23.33 ± 2.09	24.58 ± 3.10	<0.01
Smoking status [N (%)]			
Never smoker	167 (69.29)	153 (76.88)	
Ever smoker	74 (30.71)	46 (23.12)	0.08
Drinking status [N (%)]			
Never drinker	194 (80.50)	162 (81.41)	
Ever drinker	47 (19.50)	37 (18.59)	0.81
CAD history [N (%)]			
Yes	7 (2.90)	35 (17.59)	
No	234 (97.10)	164 (82.41)	<0.01
Hypertension history [N (%)]			
Yes	133 (55.19)	155 (77.89)	
No	108 (44.81)	44 (22.11)	<0.01
Diabetes history [N (%)]			
Yes	47 (19.50)	75 (37.69)	
No	194 (80.50)	124 (62.31)	<0.01

BMI = body mass index; CAD = coronary artery disease.

Analysis of *MTHFR* polymorphism association with ischemic stroke

Genotyping by direct sequencing of the DNA samples from all individuals was under-

taken for the C677T and A1298C polymorphisms. Genotype data distributions in the controls were consistent with the Hardy-Weinberg equilibrium expectations.

The allele frequencies and genotype distributions for *MTHFR* C677T and A1298C polymorphisms among the patient and control groups are shown in Table 2. We observed that for the allele frequencies of the A1298C polymorphism, the variant allele (C) was more frequently detected in patients (60.1%) in comparison to controls (47.7%), and the difference was statistically significant (P < 0.01) with an OR of 1.57 (95%CI = 1.16-2.10). These results suggest a strong association of the variant allele with ischemic stroke. Similarly, the homozygous variant genotype CC (OR = 2.55; 95%CI = 1.40-4.67; P < 0.01) and heterozygous genotype AC (OR = 2.24; 95%CI = 1.28-3.93; P < 0.01) were also shown to confer increased ischemic stroke risk.

Overall, all variant genotypes (AC+CC) were present more often in ischemic stroke individuals when compared with controls, and the difference was statistically significant (P < 0.01) with an OR of 2.36 (95%CI = 1.39-4.00). In contrast, there was no statistically significant association between the *MTHFR* C677T polymorphism and ischemic stroke in this sample.

Table 2. Association between *MTHFR* polymorphisms and risk of ischemic stroke.

Genetic model		Controls (%)	Patients (%)	Unadjusted		Adjusted	
		(N = 241)	(N = 199)	OR (95%CI)	P	OR (95%CI)	P
C677T	Genotype						
	CC	88 (36.51)	70 (35.18)				
	CT	116 (48.13)	98 (49.25)	1.06 (0.70-1.61)	0.78	1.19 (0.76-1.88)	0.44
	TT	37 (15.35)	31 (15.58)	1.05 (0.59-1.86)	0.86	1.16 (0.62-2.16)	0.64
	CT+TT	153 (63.49)	129 (64.82)	1.06 (0.72-1.57)	0.78	1.21 (0.78-1.88)	0.38
	Allele						
	C-Allele	292 (60.58)	238 (59.80)				
	T-Allele	190 (39.42)	160 (40.20)	1.03 (0.79-1.35)	0.81	1.10 (0.82-1.47)	0.54
A1298C	Genotype						
	AA	71 (29.46)	31 (15.58)				
	AC	110 (45.64)	97 (48.74)	2.02 (1.22-3.34)	<0.01	2.24 (1.28-3.93)	<0.01
	CC	60 (24.90)	71 (35.68)	2.71 (1.57-4.67)	<0.01	2.55 (1.40-4.67)	<0.01
	AC+CC	170 (70.54)	168 (84.42)	2.26 (1.41-3.63)	<0.01	2.36 (1.39-4.00)	<0.01
	Allele						
	A-Allele	252 (52.28)	159 (39.95)				
	C-Allele	230 (47.72)	239 (60.05)	1.64 (1.26-2.15)	<0.01	1.57 (1.16-2.10)	<0.01

Adjusted for gender, age, body mass index, past history of coronary artery disease, hypertension, and diabetes. OR = odds ratio; 95%CI = 95% confidence interval; bold values indicate significant findings (P < 0.05).

Haplotype analysis

Combined genotype frequencies were calculated for the combination of the C677T and A1298C loci in the total patient and control groups. Four common haplotypes composing these two single nucleotide polymorphisms were observed in this study population. As shown in Table 3, the C-677-C-1298 and T-677-C-1298 haplotypes conferred a significantly increased risk of ischemic stroke, as compared with the C-677-A-1298 haplotype. (OR = 1.56; 95%CI = 1.07-2.26; P = 0.02; OR = 1.76; 95%CI = 1.17-2.65; P < 0.01, respectively).

Table 3. Haplotype frequency distribution of *MTHFR* and its relationship with ischemic stroke.

Haplotype	Controls (N= 241)	Patients (N = 199)	OR (95%CI)	P
C-677-A-1298	157 (32.57)	98 (24.62)	-	-
T-677-A-1298	95 (19.71)	61 (15.33)	1.01 (0.65-1.57)	0.97
C-677-C-1298	135 (28.01)	140 (35.18)	1.56 (1.07-2.26)	**0.02**
T-677-C-1298	95 (19.71)	99 (24.87)	1.76 (1.17-2.65)	**<0.01**

Adjusted for gender, age, body mass index, past history of coronary artery disease, hypertension, and diabetes. OR = odds ratio; 95%CI = 95% confidence interval; bold values indicate significant findings ($P < 0.05$).

DISCUSSION

In this study, we investigated whether variants of *MTHFR* (C677T and A1298C) were associated with risk of ischemic stroke in an Eastern Chinese Han population. We found that the variant allele C and genotypes AC and CC at *MTHFR* A1298C were associated with a significant increase in the risk of ischemic stroke. The results were consistent with a previous report (Fekih-Mrissa et al., 2013) that found a significant association of the *MTHFR* A1298C genotype with ischemic stroke risk among Tunisians. In addition, the present findings were in agreement with our previously conducted meta-analyses where this polymorphism was found to be potentially involved in the development of ischemic stroke in Asian populations (Lv et al., 2013). Furthermore, we observed that the compound genotypes C-677-C-1298 and T-677-C-1298 were also significantly associated with ischemic stroke. Nevertheless, in contrast to the studies conducted by Cronin et al. (2005) and Li and Qin (2014), no evidence of association between the *MTHFR* C677T polymorphism and ischemic stroke was observed in our study. Together, these findings suggested that the *MTHFR* polymorphism might be useful as a genetic susceptibility marker for ischemic stroke in the Eastern Chinese Han population.

Although the exact mechanism by which *MTHFR* polymorphism affects ischemic stroke has not yet been fully elucidated, some possible mechanisms have been put forward. The MTHFR protein is a folate-dependent enzyme that catalyzes the rate-limiting step in the methylation of homocysteine to methionine (Xin et al., 2009). It is responsible for elastin degradation in the vascular wall and the calcification process. Incorporation of homocysteine can modify protein structure and function by disulfide or amide linkages (S-homocysteinylation or N-homocysteinylation mechanisms). Protein N-homocysteinylation causes cellular toxicity that may contribute to vascular inflammation, atherogenesis, hypercoagulation status, and vulnerability to establishment of atherosclerotic plaques (Kopyta et al., 2014). Experimental and clinical data suggest that *in vivo* auto-oxidation of the homocysteine sulfhydryl group results in the formation of reactive oxygen species promoting peroxidation of lipids bound to low-density lipoproteins (Mach et al., 1997; Somarajan et al., 2011). From meta-analyses of large cohorts, it has been estimated that for every rise in plasma homocysteine levels of 5 μM, the risk for cerebrovascular disease increases by 50% (Low et al., 2011). All the above taken into account partially explains the association between this polymorphism and ischemic stroke.

Several limitations of our study warrant consideration. First, a potential weakness in this study was the lack of determination of homocysteine levels. This was primarily because we intended to evaluate a simple association of the two genotypes with the presence of cerebral ischemic stroke, and not to also assess the potential link between genotype and thrombosis, which could be explained by elevated homocysteine levels. Second, the lack of stroke Trial of Org 10172 in Acute Stroke Treatment (TOAST) (Adams et al., 1993) classification

among the patients hampered sub-group analysis, which is essential due to the known heterogeneous etiology of ischemic stroke sub-types. Another limitation of the present study was the relatively small sample size (199 patients and 241 controls).

In conclusion, we found that the A1298C polymorphism and the compound genotypes C-677-C-1298 and T-677-C-1298 in the *MTHFR* gene were significantly associated with ischemic stroke in the Eastern Chinese Han population. This study provides additional support for an important role of the folate metabolic pathway in the pathogenesis of ischemic stroke. Additional large-scale and functional studies are needed to confirm our findings.

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