The roles of methylenetetrahydrofolate reductase 677C>T and 1298A>C polymorphisms in moyamoya disease patients

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Abstract

Purpose The methylenetetrahydrofolate reductase (*MTHFR*) 677C>T and 1298A>C polymorphisms, which are associated with hyperhomocysteinemia and nitric oxide (NO) deficiency (which is related to atherothrombosis and cerebral ischemia), have not been studied in moyamoya disease. A case-control study was performed to investigate whether the *MTHFR* 677C>T and 1298A>C polymorphisms contribute to moyamoya disease (MMD).

Methods One hundred and seven Korean patients with MMD (mean age, 20.85±15.89 years; 66.4 % female) and 232 healthy control subjects (mean age, 23.99±16.16 years; 56.8 % female) were included. Genotyping for the *MTHFR* 677C>T and 1298A>C polymorphisms and measurements of homocysteine, folate, vitamin B₁₂, and NO in the cerebrospinal fluid (CSF) were performed. The statistical analysis was performed by multivariate linear regression and logistic regression.

Result The *MTHFR* 677CT+TT genotype frequency was significantly increased with early-onset MMD (<10 years) compared with late-onset MMD (≥10 years) (adjusted odds ratio, 3.392; 95 % confidence interval, 1.294–8.893, *P*=0.013). The *MTHFR* 677C-1298C/677T-1298A diplotype (1.71±1.23 arbitrary units) presented significantly lower NO levels in the CSF compared with the 677C-1298A/677C-1298A diplotype (11.40±12.24 arbitrary units).

Conclusion The *MTHFR* 677C>T and 1298A>C polymorphisms have restricted roles in the Korean MMD population. Therefore, further studies involving larger and more heterogeneous cohorts are needed to extend our understanding of the influence of polymorphisms in *MTHFR* and other thrombophilic genes on MMD.

Keywords Moyamoya disease (MMD) · Methylenetetrahydrofolate reductase (*MTHFR*) · Nitric oxide · Early onset · Polymorphism

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Introduction

Moyamoya disease (MMD) is characterized by the progressive occlusion of the supraclinoid portion of bilateral internal carotid arteries (ICAs), the development of an abnormal vascular network called moyamoya, and the presence of extensive transdural and leptomeningeal collateral vessels [1]. Most children with MMD develop ischemic complications, such as transient ischemic attack and cerebral infarction. Approximately 50 % of adult patients suffer an intracranial hemorrhage, and the other patients suffer ischemic complications [2]. Genetic associations between MMD and loci on chromosomes 3, 6, 8, and 17 have been reported [3–8]. Several studies have investigated the prothrombotic risk factors in patients with MMD [9, 10]. The methylenetetrahydrofolate reductase (*MTHFR*) gene, which is located

on chromosome 1, is a key genetic risk factor for thrombophilia [11]. Reduced MTHFR enzyme activity is significantly associated with hyperhomocysteinemia [12] and nitric oxide (NO) deficiency [13]. Therefore, the associations of *MTHFR* with hyperhomocysteinemia and NO deficiency are important to elucidate the etiology of thromboembolic disorders [14].

Kang et al. first described a thermolabile variant of the MTHFR enzyme [15]. Subsequently, a polymorphism designated 677C>T (A222V) was reported [16]. Thermolability and reduced enzyme activity were found when lymphocyte extracts from 677CT and 677TT subjects were assayed [16]. Numerous subsequent studies have shown that the T-allele is associated with increased total plasma homocysteine and decreased serum folate levels, although this was mainly observed in 677TT homozygotes [17]. Serum folate consists of 5-methyltetrahydrofolate, which is the product of the MTHFR-catalyzed reaction, and is a cosubstrate for the conversion of homocysteine to methionine [17]. This provides a rationale for the effects of the 677T allele on the levels of folate and homocysteine in serum/plasma.

Another functional polymorphism designated 1298A>C (E429A) was described by van der Put et al. [18] and Weisberg et al. [19]. This variant is also associated with reduced enzyme activity but not with thermolability. The impact of the 1298A>C polymorphism on enzyme activity is less than that of 677C>T, and most studies have not detected significant differences in total plasma homocysteine or serum folate levels between 1298A>C genotypes [20]. However, elevated total plasma homocysteine levels have been observed in 677CT/1298AC heterozygotes [18, 21].

The aim of this study was to determine whether *MTHFR* 677C>T and 1298A>C contribute to moyamoya disease risk and the levels of related prothrombotic factors, such as homocysteine and NO, in the cerebrospinal fluid (CSF).

Materials and methods

Study population

Korean patients with MMD ($n=107$; mean age, 20.85 ± 15.89 years; 66.4 % female) and healthy control subjects ($n=232$; mean age, 23.99 ± 16.16 years; 63.4 % female) from January 2008 through January 2012 were included. The study subjects were recruited from outpatient clinics at Severance Hospital (Seoul, South Korea) and CHA Bundang Medical Center (Seongnam, South Korea). The control subjects were patients who visited the hospitals during the same period for health examinations, including biochemical testing, electrocardiogram, and brain magnetic resonance

imaging (MRI). Control subjects did not have a medical history of cerebrovascular disease or myocardial infarction. We further divided the pathological subtypes of MMD into ischemic and hemorrhagic groups based on clinical and MRI findings. Ninety-two patients had a history of cerebral ischemic attacks, and 15 patients had a history of cerebral hemorrhage. We performed indirect bypass surgery in 64 patients and graded newly developed collateral vessels according to the method of Matsushima et al. [22]. Briefly, grade A represented synangiosis-induced filling of greater than two thirds of the middle cerebral artery circulation, grade B represented between one third and two thirds, and grade C represented less than one third. The control subjects were free from a history of cerebrovascular disease or myocardial infarction. Baseline demographic data and a history of conventional vascular risk factors were obtained for each control subject. The other exclusion criteria were the same as those applied to selection of the ischemic stroke patients. All participants gave written informed consent prior to enrollment in this study. This study was conducted with the approval of the institutional review boards of Severance Hospital (4-2008-0308) and CHA Bundang Medical Center (PBC09-103).

Genotyping

Venous blood (7 mL) was collected into tubes containing 50 mmol/L ethylenediaminetetraacetic acid (EDTA), and DNA was extracted from leukocytes using a G-DEX™ II Genomic DNA Extraction Kit (iNtRON Biotechnology, Seongnam, South Korea) according to the manufacturer's instructions. The genotyping of *MTHFR* 677C>T and 1298A>C polymorphisms by the polymerase chain reaction-restriction fragment length polymorphism assay has been previously described [18].

Measurements of homocysteine, folate, vitamin B₁₂, and NO in the CSF

The CSF from moyamoya patients was placed into a plastic tube and stored at -80°C . The concentration of homocysteine was measured by the fluorescence polarizing immunoassay (FPIA) with IMx (Abbott Laboratories, Chicago, IL). The concentrations of folate and vitamin B₁₂ were determined using a radioassay kit (ACS 180; Bayer, Tarrytown, NY). NO production was measured using the Griess Reagent System (G2930, Promega, Madison, WI), which detects nitrite and nitrate levels using a diazotization reaction that was originally described by Griess in 1879 [23]. NO levels were assayed using a modified Griess reaction [24], and the absorbance was measured at

550 nm. Nitrite standard reference curves were used to calculate nitrite levels.

Statistical analysis

To analyze the demographic characteristics of moyamoya disease patients, we used the chi-square test for categorical data and the two-sided *t* test for continuous data. Patients and controls were consistent with the Hardy-Weinberg equilibrium. The comparisons of *MTHFR* 677C>T and 1298A>C genotype frequencies were calculated using multivariate logistic regression. Adjusted odds ratios (AORs), 95 % confidence intervals (CIs), and *P* values indicated statistical strengths of genetic associations. Analyses of homocysteine, folate, vitamin B₁₂, and NO levels in the CSF of patients with *MTHFR* 677C>T and 1298A>C polymorphisms were performed using multivariate linear regression. *P*<0.05 was considered significant for all tests. Statistical analysis was performed using SPSS 15.0 (Chicago, IL, USA).

Results

Table 1 shows the demographic characteristics of MMD patients and control subjects. There were no statistically significant differences in age and gender for each case-control comparison (*P*>0.05). Moreover, the levels of homocysteine, folate, vitamin B₁₂, and NO in the CSF were not different between ischemic and hemorrhagic subtypes (*P*>0.05).

The genotype frequencies of *MTHFR* 677C>T and 1298A>C in MMD patients and control subjects are shown in Table 2. Although the genotype frequency of *MTHFR* 677TT was relatively higher in the MMD group (control, 15.5 %; MMD, 22.4 %), no significant differences in *MTHFR* polymorphisms were observed between control and MMD groups. The results of stratified analyses for onset age and pathological subtypes are presented in Tables 3, 4, and 5. A significant reduction in the frequency of the *MTHFR* 677C-1298A/677T-1298A diplotype was observed in the case-control comparison for the late-onset MMD (≥10 years; AOR, 0.309; 95 % CI, 0.134–0.174; *P*=0.006) and hemorrhagic (AOR, 0.156; 95 % CI, 0.034–0.719; *P*=0.017) subgroups. In the comparison between the ages of MMD onset, the *MTHFR* 677CT+TT genotype frequency was significantly higher in patients with early-onset MMD (<10 years; AOR, 3.392; 95 % CI, 1.294–8.893, *P*=0.013). We also investigated the frequency differences of *MTHFR* 677C>T and 1298A>C polymorphisms related to collateral vessel formation scores after indirect bypass surgery (Table 6). However, no associations between *MTHFR* and collateral

Table 1 Demographic characteristics of MMD patients and control subjects

Characteristics	Controls (<i>n</i> =232)	MMD patients (<i>n</i> =107)	<i>P</i> value ^a
Age (mean±SD)	23.99±16.16	20.85±15.89	0.085
Sex (F/M)	147:85	71:36	0.627
Ischemic subtype	–	92	
Hemorrhagic subtype	–	15	
Surgery	–	64	
Collateral grade A	–	40	
Collateral grade B	–	22	
Collateral grade C	–	2	
Suzuki stage			
Right	–	3.17±0.78	
Left	–	3.89±1.27	
Homocysteine in CSF (μmol/L)	–	10.19±2.77	
Ischemic MMD	–	10.10±2.72	
Hemorrhagic MMD	–	10.80±3.35	
Folate in CSF (ng/mL)	–	10.13±3.50	
Ischemic MMD	–	10.20±3.80	
Hemorrhagic MMD	–	9.73±1.25	
Vitamin B ₁₂ in CSF (pg/mL)	–	845.47±315.01	
Ischemic MMD	–	827.52±329.95	
Hemorrhagic MMD	–	956.80±183.75	
Nitric oxide in CSF (AU)	–	5.18±5.86	
Ischemic MMD	–	4.98±5.47	
Hemorrhagic MMD	–	6.43±6.70	

There were no statistically significant differences in biochemical parameters (homocysteine, folate, vitamin B₁₂, and nitric oxide) between ischemic and hemorrhagic MMD subgroups

MMD moyamoya disease, *SD* standard deviation, *CSF* cerebrospinal fluid, *AU* arbitrary unit

^a *P* values were calculated using the two-sided *t* test for continuous data and the chi-square test for categorical data

vessel formation score were observed. Table 7 shows the impact of *MTHFR* 677C>T and 1298A>C polymorphisms on the levels of homocysteine, folate, vitamin B₁₂, and NO in the CSF. The *MTHFR* 677C-1298C/677T-1298A diplotype presented significantly lower vitamin B₁₂ (565.00±152.73 pg/mL) and NO (1.71±1.23 arbitrary units) levels compared with the 677C-1298A/677C-1298A diplotype (*P*<0.05).

Discussion

In our study of a Korean population cohort, we found that the *MTHFR* 677CT+TT genotype frequency was significantly

Table 2 Genotype frequencies of *MTHFR* 677C>T and 1298A>C between MMD patients and control subjects

Characteristics	Controls (<i>n</i> =232)	MMD patients (<i>n</i> =107)	AOR (95 % CI)	<i>P</i> value ^a
<i>MTHFR</i> 677C>T				
CC	70 (30.2)	35 (32.7)	1.000	
CT	126 (54.3)	48 (44.9)	0.713 (0.419–1.216)	0.214
TT	36 (15.5)	24 (22.4)	1.346 (0.688–2.634)	0.386
Dominant model			0.840 (0.510–1.384)	0.494
Recessive model			1.496 (0.837–2.676)	0.174
HWE <i>P</i>	0.094	0.335		
<i>MTHFR</i> 1298A>C				
AA	160 (69.0)	79 (73.9)	1.000	
AC	66 (28.4)	25 (23.4)	0.802 (0.468–1.374)	0.422
CC	6 (2.6)	3 (2.8)	0.985 (0.239–4.062)	0.983
Dominant model			0.821 (0.490–1.378)	0.455
Recessive model			1.091 (0.266–4.472)	0.903
HWE <i>P</i>	0.794	0.556		
<i>MTHFR</i> 677/1298 diplotype				
677C-1298A/677C-1298A	33 (14.2)	22 (20.6)	1.000	
677C-1298A/677C-1298C	31 (13.4)	10 (9.3)	0.489 (0.198–1.205)	0.120
677C-1298A/677T-1298A	91 (39.2)	33 (30.8)	0.524 (0.266–1.033)	0.062
677C-1298C/677C-1298C	6 (2.6)	3 (2.8)	0.727 (0.162–3.267)	0.678
677C-1298C/677T-1298A	35 (15.1)	15 (14.0)	0.643 (0.285–1.449)	0.286
677T-1298A/677T-1298A	36 (15.5)	24 (22.4)	1.052 (0.489–2.263)	0.898

MTHFR methylenetetrahydrofolate reductase, *MMD* moyamoya disease, *AOR* adjusted odds ratio, *HWE* Hardy-Weinberg equilibrium

^a Adjusted for age and gender

increased with early-onset MMD (<10 years) compared with late-onset MMD (≥10 years). The *MTHFR* 677C-1298C/677T-1298A diplotype presented significantly lower NO levels in the CSF compared with the 677C-1298A/677C-1298A diplotype. Only the restricted roles of *MTHFR* 677C>T and 1298A>C were observed in the Korean MMD population.

The role of surgical treatment, including direct or indirect extracranial-intracranial bypass surgery for MMD, is to prevent hemodynamic instability rather than thromboembolic cerebral ischemia. The progressive narrowing of ICAs leads to hemodynamic instability, which is the common cause of cerebral infarcts in patients with MMD. However, thromboembolism steadily contributes to the MMD etiology [25–28], and an autopsy study of MMD patients showed positive correlations of increased thrombus formation in the diseased arteries [29]. Moreover, prothrombotic disorders are observed in up to 40 % of pediatric MMD patients [27].

Generally, *MTHFR* 677C>T and 1298A>C polymorphisms are believed to be predictive markers for reduced enzyme activity and increased homocysteine levels [16–19]. However, our results demonstrate a genetic association with reduced NO concentration. *MTHFR* deficiency has been proposed to induce endothelial NO synthase

uncoupling [13]. Treatment of endothelial progenitor cells with sepiapterin, which is a precursor of the endothelial NO synthase cofactor, tetrahydrobiopterin, significantly reduces reactive oxygen species levels and improves NO production [13]. In addition, the *MTHFR* deficiency-induced impairment of the differentiation of endothelial progenitor cells correlates with increased cellular senescence, decreased telomere length, and reduced sirtuin1 expression [13]. NO is a principal vasoregulatory molecule that ameliorates ischemic injury and promotes recovery from cerebral ischemia. It plays both protective and cytotoxic roles after cerebral ischemia [30, 31]. Endothelial NO is responsible for endothelium-dependent vasorelaxation, the inhibition of leukocyte and platelet adhesion, and the attenuation of inflammatory mediators. Moreover, it plays a key role in the vasodilatory regulation of vascular smooth cells [32, 33].

Noda et al. reported that NO levels in the CSF are chronically elevated in MMD patients compared with hemifacial spasm and unruptured aneurysm patients, and the levels of NO decrease after cerebral revascularization by surgery [34]. They concluded that NO concentrations in the CSF are chronically elevated in this disease, thus possibly reflecting the development of abnormal collateral circulation through the moyamoya vessels [34]. However,

Table 3 AOR values of *MTHFR* 677C>T and 1298A>C for case-control comparison by stratifying age-related subgroups

Characteristics	Control <10 years (n=63)	MMD <10 years (n=39)	AOR (95 % CI)	P value ^a	Control ≥10 years (n=169)	MMD ≥10 years (n=68)	AOR (95 % CI)	P value ^a
<i>MTHFR</i> 677C>T								
CC	17 (27.0)	7 (17.9)	1.000		53 (31.4)	28 (41.2)	1.000	
CT	33 (52.4)	24 (61.5)	1.766 (0.632–4.935)	0.278	93 (55.0)	24 (35.3)	0.488 (0.257–0.927)	0.028
TT	13 (20.6)	8 (20.5)	1.430 (0.403–5.082)	0.580	23 (13.6)	16 (23.5)	1.314 (0.598–2.887)	0.497
Dominant model			1.682 (0.619–4.569)	0.308			0.652 (0.365–1.168)	0.151
Recessive model			0.952 (0.350–2.590)	0.923			1.953 (0.958–3.981)	0.066
<i>MTHFR</i> 1298A>C								
AA	48 (76.2)	30 (76.9)	1.000		112 (66.3)	49 (72.1)	1.000	
AC	14 (22.2)	8 (20.5)	1.014 (0.372–2.762)	0.979	52 (30.8)	17 (25.0)	0.748 (0.393–1.421)	0.375
CC	1 (1.6)	1 (2.6)	3.250 (0.182–58.065)	0.423	5 (3.0)	2 (2.9)	0.894 (0.165–4.841)	0.897
Dominant model			1.085 (0.412–2.858)	0.869			0.763 (0.411–1.416)	0.391
Recessive model			2.357 (0.138–40.404)	0.554			1.004 (0.188–5.367)	0.996
<i>MTHFR</i> 677/1298 diplotype								
677C-1298A/677C-1298A	11 (17.5)	5 (12.8)	1.000		22 (13.0)	17 (25.0)	1.000	
677C-1298A/677C-1298C	5 (7.9)	1 (2.6)	0.452 (0.040–5.044)	0.518	26 (15.4)	9 (13.2)	0.437 (0.161–1.182)	0.103
677C-1298A/677T-1298A	24 (38.1)	17 (43.6)	1.478 (0.424–5.151)	0.540	67 (39.6)	16 (23.5)	0.309 (0.134–0.714)	0.006
677C-1298C/677C-1298C	1 (1.6)	1 (2.6)	3.000 (0.122–73.647)	0.501	5 (3.0)	2 (2.9)	0.500 (0.082–3.046)	0.452
677C-1298C/677T-1298A	9 (14.3)	7 (17.9)	1.773 (0.404–7.780)	0.448	26 (15.4)	8 (11.8)	0.388 (0.140–1.078)	0.069
677T-1298A/677T-1298A	13 (20.6)	8 (20.5)	1.256 (0.292–5.408)	0.760	23 (13.6)	16 (23.5)	0.900 (0.367–2.212)	0.819

MTHFR methylenetetrahydrofolate reductase, *MMD* moyamoya disease, *AOR* adjusted odds ratio, *CI* confidence interval

^a Adjusted for age and gender

Table 4 Differences in the genotype frequency of *MTHFR* 677C>T and 1298A>C according to the age of MMD onset

Characteristics	MMD ≥ 10 years (<i>n</i> =68)	MMD <10 years (<i>n</i> =39)	AOR (95 % CI)	<i>P</i> value ^a	Control ≥ 10 years (<i>n</i> =169)	Control <10 years (<i>n</i> =63)	AOR (95 % CI)	<i>P</i> value ^a
<i>MTHFR</i> 677C>T								
CC	28 (41.2)	7 (17.9)	1.000		53 (31.4)	17 (27.0)	1.000	
CT	24 (35.3)	24 (61.5)	4.434 (1.574–12.492)	0.005	93 (55.0)	33 (52.4)	1.040 (0.521–2.073)	0.912
TT	16 (23.5)	8 (20.5)	2.026 (0.609–6.746)	0.250	23 (13.6)	13 (20.6)	1.621 (0.659–3.986)	0.293
Dominant model			3.392 (1.294–8.893)	0.013			1.153 (0.596–2.232)	0.673
Recessive model			0.869 (0.331–2.282)	0.776			1.592 (0.735–3.447)	0.239
<i>MTHFR</i> 1298A>C								
AA	49 (72.1)	30 (76.9)	1.000		112 (66.3)	48 (76.2)	1.000	
AC	17 (25.0)	8 (20.5)	0.729 (0.276–1.928)	0.524	52 (30.8)	14 (22.2)	0.650 (0.325–1.300)	0.223
CC	2 (2.9)	1 (2.6)	0.817 (0.071–9.409)	0.871	5 (3.0)	1 (1.6)	0.581 (0.064–5.295)	0.630
Dominant model			0.721 (0.284–1.831)	0.492			0.645 (0.328–1.269)	0.204
Recessive model			0.869 (0.076–9.971)	0.910			0.650 (0.072–5.872)	0.702
<i>MTHFR</i> 677/1298 diplotype								
677C-1298A/677C-1298A	17 (25.0)	5 (12.8)	1.000		22 (13.0)	11 (17.5)	1.000	
677C-1298A/677C-1298C	9 (13.2)	1 (2.6)	0.300 (0.028–3.278)	0.324	26 (15.4)	5 (7.9)	0.410 (0.122–1.378)	0.150
677C-1298A/677T-1298A	16 (23.5)	17 (43.6)	3.620 (1.080–12.131)	0.037	67 (39.6)	24 (38.1)	0.721 (0.300–1.729)	0.463
677C-1298C/677C-1298C	2 (2.9)	1 (2.6)	1.719 (0.119–24.912)	0.691	5 (3.0)	1 (1.6)	0.530 (0.051–5.560)	0.597
677C-1298C/677T-1298A	8 (11.8)	7 (17.9)	2.977 (0.656–13.520)	0.158	26 (15.4)	9 (14.3)	0.667 (0.224–1.985)	0.467
677T-1298A/677T-1298A	16 (23.5)	8 (20.5)	1.667 (0.448–6.204)	0.446	23 (13.6)	13 (20.6)	1.110 (0.396–3.107)	0.843

MTHFR methylenetetrahydrofolate reductase, MMD moyamoya disease, AOR adjusted odds ratio, CI confidence interval^a Adjusted for age and gender

Table 5 AOR values of *MTHFR* 677C>T and 1298A>C polymorphisms for case-control comparison by stratifying pathological subgroups

Characteristics	Controls (<i>n</i> =232)	Ischemic MMD (<i>n</i> =92)	AOR (95 % CI)	<i>P</i> value ^a	Hemorrhagic MMD (<i>n</i> =15)	AOR (95 % CI)	<i>P</i> value ^a
<i>MTHFR</i> 677C>T							
CC	70 (30.2)	29 (31.5)	1.000		6 (40.0)	1.000	
CT	126 (54.3)	43 (46.7)	0.754 (0.427–1.330)	0.329	5 (33.3)	0.510 (0.148–1.757)	0.286
TT	36 (15.5)	20 (21.7)	1.292 (0.635–2.629)	0.479	4 (26.7)	1.844 (0.448–7.588)	0.397
Dominant model			0.870 (0.512–1.478)	0.606		0.737 (0.249–2.180)	0.582
Recessive model			1.413 (0.763–2.617)	0.272		2.576 (0.733–9.044)	0.140
<i>MTHFR</i> 1298A>C							
AA	160 (69.0)	66 (71.7)	1.000		13 (86.7)	1.000	
AC	66 (28.4)	23 (25.0)	0.918 (0.522–1.612)	0.765	2 (13.3)	0.323 (0.070–1.493)	0.148
CC	6 (2.6)	3 (3.3)	1.243 (0.299–5.169)	0.765	0 (0.0)	–	–
Dominant model			0.948 (0.552–1.629)	0.848		0.295 (0.064–1.358)	0.117
Recessive model			1.325 (0.320–5.485)	0.698		–	–
<i>MTHFR</i> 677/1298 diplotype							
677C-1298A/677C-1298A	33 (14.2)	16 (17.4)	1.000		6 (40.0)	1.000	
677C-1298A/677C-1298C	31 (13.4)	10 (10.9)	0.744 (0.286–1.939)	0.545	0 (0.0)	–	–
677C-1298A/677T-1298A	91 (39.2)	30 (32.6)	0.654 (0.312–1.371)	0.261	3 (20.0)	0.156 (0.034–0.719)	0.017
677C-1298C/677C-1298C	6 (2.6)	3 (3.3)	1.050 (0.229–4.815)	0.950	0 (0.0)	–	–
677C-1298C/677T-1298A	35 (15.1)	13 (14.1)	0.773 (0.321–1.862)	0.565	2 (13.3)	0.305 (0.055–1.698)	0.175
677T-1298A/677T-1298A	36 (15.5)	20 (21.7)	1.151 (0.509–2.606)	0.735	4 (26.7)	0.732 (0.172–3.118)	0.673

MTHFR methylenetetrahydrofolate reductase, *MMD* moyamoya disease, *AOR* adjusted odds ratio, *CI* confidence interval

^a Adjusted for age and gender

Table 6 *MTHFR* 677C>T and 1298A>C genotype frequencies, according to collateral vessel formation scores

Characteristics	Collateral grade A (<i>n</i> =40)	Collateral grade B or C (<i>n</i> =24)	AOR (95 % CI)	<i>P</i> value ^a
<i>MTHFR</i> 677C>T				
CC	11 (27.5)	9 (37.5)	1.000	
CT	20 (50.0)	10 (41.7)	0.742 (0.217–2.540)	0.634
TT	9 (22.5)	5 (20.8)	0.775 (0.162–3.701)	0.749
Dominant model			0.665 (0.214–2.066)	0.480
Recessive model			0.860 (0.237–3.116)	0.818
<i>MTHFR</i> 1298A>C				
AA	29 (72.5)	17 (70.8)	1.000	
AC	10 (25.0)	6 (25.0)	0.982 (0.280–3.448)	0.977
CC	1 (2.5)	1 (4.2)	2.192 (0.115–41.738)	0.602
Dominant model			1.109 (0.335–3.678)	0.866
Recessive model			1.854 (0.108–31.808)	0.671
<i>MTHFR</i> 677/1298 diplotype				
677C-1298A/677C-1298A	6 (15.0)	6 (25.0)	1.000	
677C-1298A/677C-1298C	4 (10.0)	2 (8.3)	0.247 (0.019–3.210)	0.285
677C-1298A/677T-1298A	14 (35.0)	6 (25.0)	0.528 (0.104–2.677)	0.440
677C-1298C/677C-1298C	1 (2.5)	1 (4.2)	1.738 (0.070–42.945)	0.736
677C-1298C/677T-1298A	6 (15.0)	4 (16.7)	0.478 (0.066–3.461)	0.465
677T-1298A/677T-1298A	9 (22.5)	5 (20.8)	0.683 (0.121–3.846)	0.666

MTHFR methylenetetrahydrofolate reductase, *AOR* adjusted odds ratio, *CI* confidence interval

^a Adjusted for age and gender

Table 7 Differences in the levels of biochemical factors in the CSF between the *MTHFR* 677C>T and 1298A>C polymorphisms

Characteristics	Homocysteine ($\mu\text{mol/L}$)	Folate (ng/mL)	Vitamin B ₁₂ (pg/mL)	Nitric oxide (AU)
<i>MTHFR</i> 677C>T				
CC	10.28 $\pm$ 1.84	9.62 $\pm$ 3.02	792.50 $\pm$ 270.69	5.83 $\pm$ 6.38
CT	9.67 $\pm$ 3.51	11.19 $\pm$ 4.25	884.29 $\pm$ 408.39	4.65 $\pm$ 5.54
TT	10.88 $\pm$ 2.63	8.73 $\pm$ 1.77	854.70 $\pm$ 221.95	4.80 $\pm$ 3.40
<i>MTHFR</i> 1298A>C				
AA	10.11 $\pm$ 2.94	10.47 $\pm$ 3.63	887.38 $\pm$ 335.29	6.65 $\pm$ 6.93
AC	10.76 $\pm$ 2.31	9.72 $\pm$ 2.91	648.40 $\pm$ 114.62*	2.85 $\pm$ 3.14*
CC	10.10 $\pm$ 0.71	6.71 $\pm$ 0.29	730.50 $\pm$ 34.65	0.63 $\pm$ 0.53
<i>MTHFR</i> 677/1298 diplotype				
677C-1298A/677C-1298A	10.09 $\pm$ 2.19	9.76 $\pm$ 3.24	848.14 $\pm$ 352.58	11.40 $\pm$ 12.24
677C-1298A/677C-1298C	10.90 $\pm$ 1.57	12.11 $\pm$ 0.37	704.00 $\pm$ 54.95	3.34 $\pm$ 3.66
677C-1298A/677T-1298A	9.53 $\pm$ 3.58	11.83 $\pm$ 4.24	937.50 $\pm$ 416.36	5.76 $\pm$ 5.99*
677C-1298C/677C-1298C	10.10 $\pm$ 0.71	6.71 $\pm$ 0.29	730.50 $\pm$ 34.65	0.63 $\pm$ 0.53
677C-1298C/677T-1298A	10.55 $\pm$ 4.03	7.34 $\pm$ 1.59	565.00 $\pm$ 152.73*	1.71 $\pm$ 1.23*
677T-1298A/677T-1298A	10.88 $\pm$ 2.63	8.73 $\pm$ 1.77	854.70 $\pm$ 221.95	4.80 $\pm$ 3.40*

Data are presented as mean $\pm$ standard deviation

MTHFR methylenetetrahydrofolate reductase, *CSF* cerebrospinal fluid, *AU* arbitrary unit

* $P<0.05$ after multivariate linear regression, including age, gender, and pathological subtypes of MMD, compared with the reference types (*MTHFR* 677CC, 1298AA, and 677C-1298A/677C-1298A)

a recent study demonstrated the neuroprotective effects of sildenafil citrate, which enhances NO levels, in neonatal hypoxic-ischemic encephalopathy [35]. Sildenafil citrate induces collateral patency and reduces the number of terminal dUTP nick-end labeling (TUNEL)-positive cells, reactive astrogliosis, and macrophage/microglial activation. The numbers of TUNEL-positive endothelial cells within the lesion site are also reduced by sildenafil. Seven days after sildenafil treatment, tissue loss is significantly reduced and motor coordination is recovered [35]. Because NO has been shown to alleviate hypoxic-ischemic damage [35], the chronic increase in NO levels in MMD patients may be a result of the body's natural defense mechanism against ischemia.

The present association study has several limitations. First, this study was a hospital-based case-control study with a relatively small sample size. Second, our study population had a relatively homogenous genetic background. Third, we were unable to measure other thromboembolic parameters, such as prothrombin time and activated partial thromboplastin time, so further studies, with larger and more heterogeneous cohorts, are needed to extend our understanding of the influence of polymorphisms in *MTHFR* and other thrombophilic genes on moyamoya disease. If additional research identifies a definitive causative role for the *MTHFR* and related pathway in moyamoya disease pathogenesis, regulation of *MTHFR*

expression or activity may provide preventive options for moyamoya disease.

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Conflict of interest The authors have no conflicts of interest to disclose.

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