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The *MTHFR* C677T Polymorphism and Risk of Intracerebral Hemorrhage in a Chinese Han Population

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Background: Methylenetetrahydrofolate reductase (*MTHFR*) C677T polymorphism has been speculated to be and extensively investigated as a risk factor for various vascular diseases, including intracerebral hemorrhage (ICH). However, results from published studies regarding the role of C677T polymorphism in ICH risk in Chinese populations were contradictory rather than conclusive.

Material/Methods: In this study, a total of 180 ICH patients and 180 matched controls of Chinese Han ethnicity were enrolled. The *MTHFR* C677T polymorphism was genotyped by polymerase chain reaction-ligation detection reaction (PCR-LDR). A meta-analysis was conducted by combining our data with previous relevant studies in Chinese populations.

Results: In our case-control study, similar allele frequency (p=0.492) and genotype distribution (p=0.748) of *MTHFR* C677T polymorphism were detected between ICH patients and controls. Further analysis based on hematoma location did not show a significant association. When combined with previous studies, however, C677T polymorphism was found to be significantly associated with an increased risk for ICH in Chinese populations (recessive model: OR=1.57, 95%CI=1.29–1.91). When focusing on the Han ethnicity, carriers of the TT genotype had an increased risk of ICH (recessive model: OR=1.36, 95%CI=1.05–1.75).

Conclusions: In this case-control study we did not observe that the *MTHFR* C677T polymorphism was associated with ICH risk in people of Chinese Han ethnicity. However, when combined with previous published studies, a significant association of C677T polymorphism with an increased risk of ICH was detected in Chinese populations, and also in the subgroup analysis focusing on Han ethnicity.

MeSH Keywords: Cerebral Hemorrhage • China • Methylenetetrahydrofolate Reductase (NADPH2) • Polymorphism, Single Nucleotide

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Background

Spontaneous intracerebral hemorrhage (ICH) is a devastating disease with an overall incidence of 24.6 per 100 000 person-years [1]. It accounts for 10–15% of all strokes, leading to a catastrophic consequence. The mortality of ICH at 1 month was 35–52% and only 20% regained functional independence by 6 months [2]. Hypertension, low cholesterol levels, heavy alcohol intake, advanced age, and male gender have been identified as important risk factors for ICH [3]. However, these conventional risk factors could not explain all cases of ICH. On the other hand, genome-wide association studies and large-scale genetic association studies have indicated an important role of genetic factors in ICH pathogenesis [4–6].

Homocysteine (Hcy) is a metabolite of the essential amino acid methionine. Elevated Hcy has been associated with various vascular diseases, including coronary artery disease, venous thrombosis, and stroke [7,8]. Although not fully understood, endothelial dysfunction related to hyperhomocysteinemia has been suggested as one of the mechanisms [9].

Methylenetetrahydrofolate reductase (MTHFR), encoded by *MTFHR* gene, is a crucial enzyme regulating intracellular Hcy and folate metabolism. MTHFR catalyzes the transformation of 5,10-methylentetrahydrofolate to 5-methyltetrahydrofolate, which serves as the methyl group donor for converting homocysteine into methionine [10,11]. The most investigated polymorphism in *MTHFR* gene is C677T, which can lead to the replacement at codon 222 for alanine to valine, causing increased thermolability and reduced activity of enzyme MTHFR and, subsequently, an elevated plasma level of Hcy [12]. *MTHFR* C677T polymorphism has been speculated to be and extensively investigated as a risk factor for various vascular diseases, including ICH [13,14]. However, results from published genetic-association studies were contradictory rather than conclusive. Some reported a significant impact of C677T polymorphism on ICH risk [15, 16] while some failed to replicate these findings [17,18].

Here, we conducted a case-control study to evaluate the influence of *MTHFR* C677T polymorphism on ICH risk. Furthermore, since ICH incidence is heterogeneous among different Chinese populations [19,20], this case-control study included subjects of Chinese Han ethnicity only. In addition, a meta-analysis was performed integrating data from the present samples and those available from previous studies focusing on Chinese populations.

Material and Methods

Study subjects

Consecutive adult CT-proved ICH patients of Chinese Han ethnicity admitted to West China Hospital of Sichuan University were enrolled in this study from November 2011 to September 2012. We excluded patients with secondary ICH due to brain tumor, aneurysm, vascular malformation, head trauma, hemorrhagic transformation of cerebral infarction, coagulation disorder, or concurrent use of anticoagulation. Age-, sex-, and ethnicity-matched control subjects without history of ICH were recruited from those undergoing routine health examinations in our hospital. The following demographic and clinical data were collected from medical records of each participant: age, sex, hypertension, diabetes mellitus, hyperlipidemia, alcohol consumption, and smoking. Hematoma location was classified as lobar or deep (basal ganglia, thalamus, brain stem and cerebellum). This study was approved by the Ethics Committee of West China Hospital of Sichuan University. Written informed consents were obtained from all participants or their legal surrogates.

Genotyping

DNA was isolated from peripheral whole blood using the DNA Blood Kit (Bioteke Corp., China). The C677T polymorphism was genotyped by the polymerase chain reaction- ligase detection reaction (PCR-LDR) sequencing method, as described previously [21]. Briefly, the PCR primers used were: 5'-AGGCCAGCCTCTCCTGACTGT-3' (forward) and 5'-CCATGTCGGTGCATGCCTTCA-3' (reverse). The PCR protocol was: an initial denaturation step of 5 min at 95°C, 35 cycles of 94°C for 15 s, 55°C for 15 s, and 72°C for 30 s, and a final extension step of 72°C for 10 min.

The following LDR was carried out in a total volume of 10μl, containing 3 μl PCR product, 1 μl 10×Taq DNA ligase buffer, 2 U of Taq DNA ligase (NEB), and 0.1 pmol of each probe. LDR probes were composed of 1 common probe and 2 discriminating probes. The LDR protocol was: 95°C for 2 min, 35 cycles of 94°C for 30 s, and 60°C for 2 min. The fluorescent products of LDR were differentiated using ABI 3730xl (Applied Biosystems, USA). Genotype was confirmed by DNA sequencing of randomly selected PCR products. The results were 100% concordant.

Statistical analysis

Hardy-Weinberg equilibrium (HWE) in the control subjects was tested by a goodness-of-fit chi-squared test. Differences in baseline characteristics, genotype distribution, and allele frequency between ICH patients and controls were evaluated using the *t* test or χ^2 test, as appropriate. Multiple regression

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Table 1. Baseline data in ICH patients and controls.

Characteristics	ICH patients (n=180)		Controls (n=180)		*p*
Age, mean (SD), y	57.7	(14.1)	57.0	(9.7)	0.575
Male, n (%)	126	(70.0)	120	(66.7)	0.497
Hypertension, n (%)	94	(52.2)	56	(31.1)	<0.001
Diabetes mellitus, n (%)	16	(8.9)	8	(4.4)	0.091
Hyperlipidemia, n (%)	5	(2.8)	2	(1.1)	0.449
Smoking, n (%)	59	(32.8)	37	(20.6)	0.009
Drinking, n (%)	35	(19.4)	27	(15.0)	0.264

analysis was used to assess the independent impact of C677T polymorphism after adjusting for age, sex, hypertension, diabetes, hyperlipidemia, smoking, and alcohol consumption. The alternative genetic models for the C677T polymorphism included alleles (T *vs.* C) and genotypes for codominant, dominant, and recessive models. Stratified analyses according to the hematoma location (lobar or deep) were also conducted. Statistical analysis was performed with SPSS for Windows, version 18.0 (SPSS Inc., Chicago, IL, USA). Results were considered significant when $P<0.05$ (2-sided).

Meta-analysis

A meta-analysis was performed combining our data with previous published studies to further investigate the association of *MTFHR* C677T polymorphism with ICH risk in Chinese populations. We searched PubMed, Embase, and China National Knowledge Infrastructure (CNKI) databases until July 2015 to screen for relevant genetic association articles. The following search terms were used: intracerebral hemorrhage, hemorrhagic stroke, MTHFR, methylenetetrahydrofolate reductase, polymorphism, genotype, variant, and mutation. The search result was supplemented by screening references of retrieved articles and relevant reviews.

The inclusion criteria were: (1) a case-control design; (2) conducted in a Chinese population; (3) provided information on genotype frequencies or sufficient data to calculate them; (4) genotype distribution in control group was in accordance with HWE. For studies with overlapping subjects, only the one with the most complete dataset was included. For each included study, the following data were extracted: authors, year of publication, province, age, sex, sample size, source of control, matching criteria, and genotype distribution.

The association of C677T polymorphism with ICH risk in Chinese populations was evaluated by calculating pooled ORs with 95% CIs. We used Cochran's *Q* test and I^2 statistic to assess between-study heterogeneity. When substantial heterogeneity was detected ($P<0.05$ for *Q* test or an $I^2>50\%$), we used a random-effects model to assess the influence of genotype on ICH risk [22]. Otherwise, a fixed-effects model was applied [23]. To test the robustness of the findings, we performed sensitivity analysis by sequentially omitting each study. We assessed the potential publication bias visually by estimating the possible skewness in a funnel plot [24] and statistically with the methods described by Egger [25]. The latter is a weighted linear regression of standard normal deviates against the inverse of the standard error. The intercept of the regression is applied to measure the degree of funnel plot asymmetry [23].

Results

Case-control study

In total, we enrolled 180 patients and 180 healthy controls. Baseline characteristics are summarized in Table 1. Mean age was 57.7±14.1 years for ICH patients and 57.0±9.7 years for controls.

Genotype distribution in the controls was in accordance with HWE ($p=0.903$). No significant difference was observed regarding the genotype distribution ($p=0.748$) or T allele frequency ($p=0.492$) between the ICH patients and healthy controls (Table 2). When stratified by hematoma location, no significant difference in genotype frequencies between ICH patients and controls was detected in lobar ICH ($p=0.193$) or in deep ICH ($p=0.682$). In the multiple logistic regression model adjusted for age, sex, hypertension, diabetes, hyperlipidemia, smoking, and alcohol consumption, no significant association of C677T polymorphism with ICH risk was detected in allelic, codominant, dominant, or recessive models (Table 3).

Meta-analysis

After a comprehensive literature search and records screening, full texts of 16 studies were included for further evaluation.

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Table 2. Association between *MTHFR* C677T polymorphism and ICH risk.

Genotype	Control (n=180)	Total ICH (n=180)	Lobar ICH (n=47)	Deep ICH (n=133)	OR (95% CI) Total ICH *vs.* control	OR (95% CI) Lobar ICH *vs.* control	OR (95% CI) Deep ICH *vs.* control
CC	64 (35.6)	71 (39.4)	20 (42.6)	51 (38.3)	1.00	1.00	1.00
CT	86 (47.8)	81 (45.0)	24 (51.1)	57 (42.9)	0.865 (0.54, 1.39)	0.89 (0.45, 1.76)	0.84 (0.50, 1.42)
TT	30 (16.7)	28 (15.6)	3 (6.4)	25 (18.8)	0.77 (0.41, 1.47)	0.29 (0.08, 1.10)	1.05 (0.53, 2.07)
C	214 (59.4)	223 (61.9)	64 (68.1)	159 (59.8)	1.00	1.00	1.00
T	146 (40.6)	137 (38.1)	30 (31.9)	107 (40.2)	0.87 (0.64, 1.19)	0.66 (0.41, 1.08)	0.98 (0.70, 1.38)
Dominant	116 (64.4)	109 (60.6)	27 (57.4)	82 (61.7)	0.84 (0.54, 1.31)	0.71 (0.36, 1.37)	0.89 (0.55, 1.46)
Recessive	150 (83.3)	152 (84.4)	44 (93.6)	108 (81.2)	0.84 (0.47, 1.51)	0.31 (0.09, 1.10)	1.15 (0.62, 2.14)

Dominant: TT+CT *vs.* CC; Recessive: TT *vs.* CT+CC; OR were adjusted for age, sex, hypertension, diabetes, hyperlipidemia, smoking, and alcohol consumption.

Table 3. Characteristics of studies included in the meta-analysis.

Author	Year	Province	Han ethnicity	No. (case/control)	Source of control	Match criteria	Case			Control		
							C/C	C/T	T/T	C/C	C/T	T/T
Fang [15]	2004	Jilin	Yes	27/96	PB	NA	7	10	10	40	37	19
Fu [35]	2005	Shanghai	NA	26/50	PB	Age, gender	10	13	3	22	25	3
Hu [36]	2007	Inner Mongolia	No	32/115	PB	Age, gender	11	12	9	61	42	12
Xiao [37]	2006	Shanghai	NA	61/100	PB	NA	12	33	16	49	41	10
Zhang [38]	2004	Shanghai	NA	94/100	PB	Gender	21	59	14	40	49	11
Zhang [39]	2004	Beijing	Yes	156/239	PB	Smoking, drinking	37	68	51	65	123	51
Zhang [40]	2008	Beijing	Yes	222/282	PB	Current smoking, alcohol drinking	57	103	62	74	140	68
Zhao [16]	2001	Northern	NA	202/190	NA	Age, gender, blood pressure	29	85	88	48	87	55
Zheng [34]	2000	Hunan	NA	30/122	PB	Gender	17	10	3	62	45	15
This study	2015	Sichuan	Yes	180/180	PB	Age, gender	71	81	28	64	86	30

NA – not available; PB – population-based.

Eight studies were then excluded: five for departure from HWE [26–30], one for insufficient data [31], one [32] for duplication with another study [28], and one for Mongolian population [33]. One more eligible study was detected through screening reference lists of achieved studies and reviews [34]. Finally, nine previous studies together with the current study, forming a total of 1030 ICH patients and 1474 controls, were enrolled in this meta-analysis [15,16,34–40] (Table 3). Overall, a significant association between C677T polymorphism and ICH risk in Chinese populations were observed (dominant model: OR=1.57, 95%CI=1.29–1.91, Table 4, Figure 1). When focusing on the Chinese Han population, a significant association was

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Table 4. Meta-analysis of the association between MTHFR C677T polymorphism with ICH risk in Chinese population.

Variables	N	Case/ control	T *vs.* C		TT *vs.* CC		CT *vs.* CC		TT+CT *vs.* CC		TT *vs.* CT+CC	
			OR (95% CI)	P_{het}	OR (95% CI)	P_{het}	OR (95% CI)	P_{het}	OR (95% CI)	P_{het}	OR (95% CI)	P_{het}
Total	10	1030/1474	1.42 (1.14, 1.77)	0.001	1.97 (1.31, 2.95)	0.009	1.23 (1.01, 1.49)	0.054	1.49 (1.09, 2.05)	0.005	1.57 (1.29, 1.91)	0.164
Han ethnicity	4	585/797	1.18 (0.92, 1.51)	0.080	1.31 (0.97, 1.77)	0.147	0.95 (0.74, 1.23)	0.791	1.06 (0.83, 1.34)	0.365	1.36 (1.05, 1.75)	0.177
Population-based	9	828/1284	1.39 (1.09, 1.77)	0.002	1.89 (1.20, 2.96)	0.012	1.26 (0.92, 1.73)	0.049	1.44 (1.02, 2.02)	0.008	1.49 (1.19, 1.86)	0.148
Match	8	942/1278	1.29 (1.05, 1.59)	0.018	1.57 (1.23, 2.01)	0.052	1.13 (0.92, 1.39)	0.203	1.31 (0.98, 1.74)	0.049	1.48 (1.21, 1.82)	0.227

N – number of datasets in the meta-analysis; P_{het} – *P* value of Q-test for heterogeneity test.

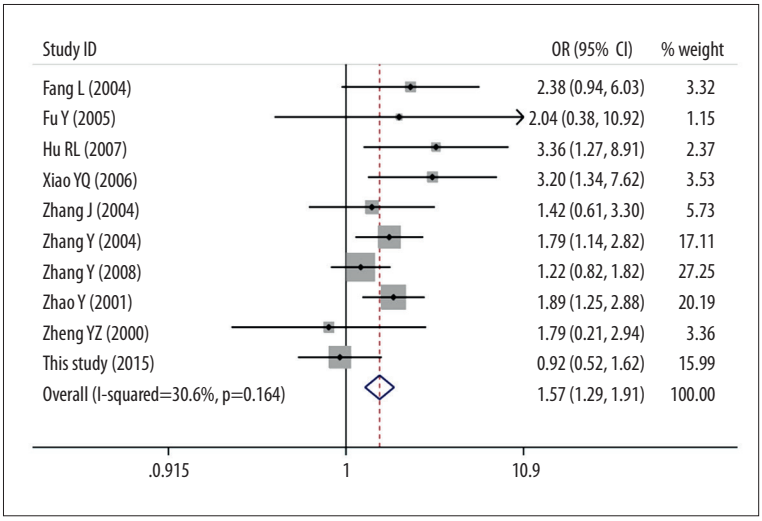

Figure 1. Forest plot for association of MTHFR C677T polymorphism with ICH risk (TT *vs.* CT+CC).

detected in the recessive model (dominant model: OR=1.36, 95%CI=1.05–1.75). When combining studies with population-based controls, C677T polymorphism was found to be significantly associated with an increased ICH risk (OR=1.39, 95%CI=1.09–1.77). When stratified by match status, we also detected a significant role of C677T polymorphism in ICH susceptibility (OR=1.29, 95%CI=1.05–1.59). In sensitivity analysis, we repeated the meta-analysis by omitting each study sequentially and found that the results did not change substantially under the genetic models except for the heterozygous co-dominant model. The funnel plots (Figure 2) and Egger's test (data not shown) suggested that there was no publication bias in the present meta-analysis.

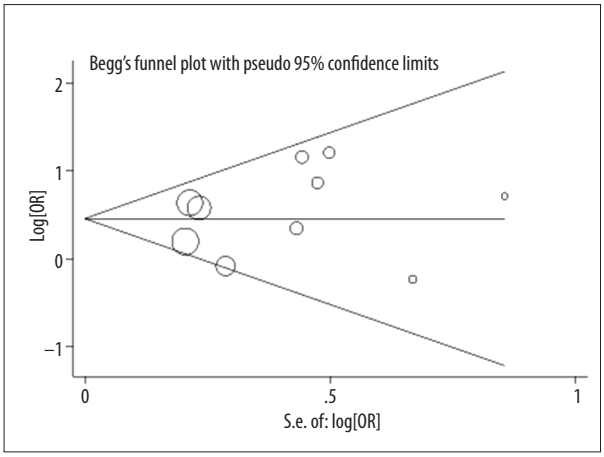

Figure 2. Funnel plot for publication bias test (TT *vs.* CT+CC).

Discussion

Despite advances in neurocritical care in recent decades, ICH remains a devastating disease causing substantial death and chronic disability. This status highlights the critical role of primary and secondary preventive strategies, which is now most promising for reducing the burden imposed by ICH. However, the current limited understanding of ICH pathogenesis remains a formidable barrier to the development of these strategies [41]. Data on familial aggregation suggests the role of genetics in ICH pathogenesis [42].

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In our case-control study, we detected a similar genotype distribution regarding *MTHFR* C677T polymorphism between ICH patients and healthy controls in the Chinese Han population. However, when combined with previously published data, we found a significant association between this polymorphism and an increased ICH risk in Chinese populations. The association remained significant when confined to Han ethnicity, studies using matched controls, and studies including population-based controls. The impact of *MTHFR* C677T polymorphism on ICH risk has also been evaluated in other populations, with conflicting results. Hultdin et al., investigating Swedish cohorts, reported that *MTHFR* TT carriers had a 3.62-fold higher risk of hemorrhagic stroke when compared with CC carriers after adjusting for other confounding factors [43]. However, Dikmen et al. did not detect any significant role of C677T polymorphism in hemorrhagic stroke risk in a Turkish population [44]. Ethnic, environmental, and socioeconomic factors might partially contribute to this discrepancy.

In vivo and *in vitro* studies suggested that subjects homozygous for the T allele have significantly elevated Hcy levels [12,45]. It is generally accepted that elevated Hcy status can induce endothelial dysfunction. Interestingly, some studies, although still controversial, observed significant associations of *MTHFR* C677T polymorphism with increased risk of ischemic stroke [46,47]. Moreover, elevated tHcy has also been associated with development of ischemic stroke [48,49]. The explanation underlying this phenomenon was suggested to be that elevated Hcy can cause either ischemic stroke through its coagulative effect or ICH through promoting plaque rupture [28].

One plausible application of genetic information on ICH susceptibility is its potential to improve risk assessment and thus to provide immediate clinical impact, such as decision-making regarding coagulation. However, the further application of *MTHFR* polymorphism, at least for coagulation adjustment, seems to be hampered by its dual effect on both ischemic stroke and ICH. Notably, ICH is a polygenic disease, with many genes thought to be involved, while a specific gene might make only a small contribution to ICH risk. We could not exclude the possibility that the ability to predict ICH would overwhelm ischemic stroke when incorporating C677T polymorphism into other specific genetic or environmental risk factors. Therefore, *MTHFR* C677T polymorphism still has the potential to contribute to the construction of future risk assessment algorithms.

Several limitations should be addressed when interpreting our results. Firstly, some studies, including one multicenter case-control study, were excluded due to deviation from HWE. Secondly, although it was mentioned in all studies that participants were Chinese, it was not clearly if they were of Han ethnicity. Thus, only four studies were combined in the subgroup analysis focusing on the Chinese Han population. Thirdly, adjusted pooled ORs could not be calculated due to the limited information available in the included studies. Fourthly, although it is suggested that cerebral amyloid angiopathy might contribute to lobar ICH, while deep ICH is related to hypertensive vasculopathy, it remains unknown whether genes play different roles in lobar *vs.* deep ICH. However, the included studies did not perform further analysis based on hematoma location. Therefore, we could not calculate pooled ORs estimating the potential role of *MTHFR* C677T polymorphism in lobar ICH and deep ICH, respectively. Fifthly, the sample size in our case-control study was relatively small. However, this study has some strengths. Firstly, this is the first meta-analysis evaluating the role of *MTHFR* C677T polymorphism in ICH risk in Chinese populations. Secondly, we performed detailed subgroup analysis, including focusing on Chinese Han ethnicity.

Conclusions

In the present case-control study, we did not observe that *MTHFR* C677T polymorphism is associated with ICH risk in the Chinese Han population. However, when combined with previously published studies, a significant association of C677T polymorphism with an increased risk of ICH was detected in Chinese populations, and also in the subgroup analysis focusing on Han ethnicity.

Competing interests

The authors declared no competing interests.

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