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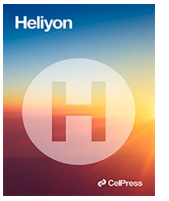

Research article

MTHFR C677T, hyperhomocysteinemia, and their interactions with traditional risk factors in early neurological deterioration in Chinese patients with ischemic stroke

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A R T I C L E I N F O

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[A B S T R A C T]

Objective: This study aimed to investigate the relationship between methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism and early neurological deterioration (END) in patients with acute ischemic stroke (AIS) and any possible interactions between specific MTHFR alleles and traditional risk factors among a Han Chinese cohort.

Methods: 434 AIS patients were consecutively recruited between January 2017 and June 2019, including 129 END and 305 non-END cases. A candidate gene association study design was used to analyze the association between MTHFR gene polymorphism and END risk. The polymerase chain reaction-restriction fragment length polymorphism (RFLP) method was employed to genotype the MTHFR C677T polymorphism. The interactional analyses were performed using the multifactor dimensionality reduction test.

Results: Hyperglycemia (odds ratio [OR]: 2.410, 95 % confidence interval [CI]: 1.436–4.046, p = 0.001), neurological function impairment (NIHSS score >5) (OR: 2.158, 95%CI: 1.337–3.484, *p* = 0.002) on admission, and hyperhomocysteinemia (HHcy) (OR: 2.570, 95%CI: 1.229–5.376, *p* = 0.012) were independently associated with END. The TT genotype (OR: 1.710, 95%CI: 1.021–2.863, p = 0.043) and T allele (OR: 1.710, 95%CI: 1.021–2.863, p = 0.043) of this C677T polymorphism were associated with susceptibility to END, and the TT genotype was more common in the subjects with HHcy (OR: 2.525, 95%CI: 1.111–5.739, P = 0.023). In addition, we also found interactions for END risk between the C677T polymorphism and traditional risk factors for END, including: hyperglycemia on admission, drinking, and moderate to severe neurological deficits (OR 1.237, 95 % CI 0.227–6.734), although the results were not statistically significant (p = 0.806).

Conclusions: Our results show a possible association between MTHFR C677T polymorphism and gene–environment interactions with END susceptibility in a Han Chinese cohort.

1. Introduction

Stroke is associated with the greatest loss of disability-adjusted life years of any disease in China, and ischemic stroke (IS) accounts

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for approximately 80 % of all strokes [1]. The term "early neurological deterioration" (END) is used when a patient shows no improvement or experiences deterioration in the severity of their neurological deficits in the early poststroke period [2]. According to different severity threshold and interval of clinical evaluation, the incidence of END ranges from 2.2 % to 37.5 % [3,4]. END is closely associated with poor outcomes after stroke [3]. It is therefore essential to identify reliable predictors of END. END shares common pathophysiological mechanisms with IS, which is a multifactorial disease with a strong genetic background. And so, the study of susceptibility genes for END has attracted researcher's attention.

The human 5, 10-methylenetetrahydrofolate reductase (MTHFR) gene, characterized by its structure of 11 exons, is genomically located at the 1p36.3 locus on the short arm of chromosome 1, denoting a precise and critical position within the human genome for its involvement in folate metabolism [5]. MTHFR plays a critical role in regulating plasma homocysteine (Hcy) levels by converting 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate [6]. The C677T polymorphism in the MTHFR gene (the substitution of cytosine (C) with thymine (T) at nucleotide position 677) results in a specific genetic alteration affecting the activity of the MTHFR [7]. The MTHFR C677T mutation is strongly associated with atherosclerotic diseases, such as stroke. A meta-analysis with a large sample size demonstrated that the MTHFR 677C > T mutation increased the risk of IS by nearly 1.5 times in people over 18 years old [8]. In people over 65 years of age, IS risk is also significantly higher in MTHFR C677T T allele carriers [7]. Moreover, Hcy levels are closely related to IS risk in the Chinese population [9]. One study also suggested that elevated levels of Hcy were predictive factors for the occurrence of END in patients with acute IS [10].

To date, few studies have been conducted into genetic biomarkers of END risk. The present study aimed to investigate the role of the MTHFR C677T polymorphism and Hcy levels in END, as well as the interaction between this polymorphism and other traditional risk factors for END in acute IS patients.

2. Materials and methods

2.1. Participants

Participants were consecutively recruited from acute IS patients who were admitted to the Department of Neurology in the Third People's Hospital of Chengdu, affiliated to Southwest Jiaotong University, between January 1, 2017, and June 30, 2019. We included only patients who meet the following criteria: 1) a diagnosis of IS as per the standards of the World Health Organization (WHO); 2) admission within 48 h of onset; and 3) agreed to participate in the research. The patients with the following conditions will be excluded from this study: 1) patients with secondary cerebral infarction with definitive reasons; 2) patients with other serious diseases, such as organ failure, severe infections; 3) patients discharged after less than 7 days in hospital; 4) patients who received intravenous thrombolysis or endovascular interventional therapy; and 5) patients with hemorrhagic transformation of the cerebral infarction. We employed a recruitment strategy combining physician's recommendations with the attitudes of patients to enroll study participants. All subjects included were divide into two groups (END and Non-END) according to definition. The Ethics Committee of the Third People's Hospital of Chengdu approved this hospital-based study (Reference number: [2021]-T-39), and all patients or their families signed an informed consent form. The study is also registered in the Chinese Clinical Trial Registry (www.chictr.org.cn): ChiCTR2000032684.

2.2. Clinical data Collection and evaluation

Within 24 h of admission, we collected the following clinical data from all included subjects: 1) demographic information (age and sex); 2) vascular risk factors (hypertension, hyperlipidemia, diabetes, ischemic heart disease, atrial fibrillation (AF), previous stroke, history of transient ischemic attack (TIA), smoking, or drinking alcohol); and 3) baseline laboratory tests (fasting blood glucose, routine blood test, coagulation function, and biochemical blood indicators including C-reactive protein (CRP), Hcy, total cholesterol, triglycerides, and low-density lipoprotein cholesterol).

2.3. Definitions of research indicators

The degree of neurological deficit was assessed using the National Institutes of Health Stroke Scale (NIHSS), and END was defined as an increase of at least 1 point in motor items or at least 2 points in the total score within 1 week after admission [10]. Hyperhomocysteinemia (HHcy) was defined as plasma Hcy >10 μmol/L [11]. High-risk CRP was defined as hypersensitive-CRP (hsCRP) > 3.0 mg/L [12]. Hyperuricemia was defined as blood uric acid >420 μmol/L on two different days, regardless of the patient's sex [13]. Severe neurological impairment was defined as NIHSS >5 (i.e., a non-minor stroke) [14]. Hyperglycemia refers to the 1998 WHO standard. Hypertension was defined as systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg, according to the 1999 WHO standard. A history of smoking was defined as more than one cigarette per day (on average) for more than 1 year, and a history of drinking was defined as drinking alcohol at least 12 times during the previous year [15]. A diagnosis of dyslipidemia was based on the 2016 Chinese guidelines for the management of dyslipidemia in adults. Overweight was defined as a body mass index ≥24 kg/m^2.

2.4. MTHFR C677T genotyping

Sample DNA was acquired by taking 2 mL of peripheral venous blood from each subject, which was anticoagulated with sodium

citrate. Next, DNA was extracted using a Blood Genomic DNA Isolation Midi Kit (DP318-02/03, TIANGEN, Beijing) according to the manufacturer's instructions. To genotype the MTHFR C677T polymorphism, we used polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) according to Frosst's method [16]. We first performed PCR amplification using the upstream primer 5′-TGA AGG AGA AGG TGT CTG CGG GA-3′ and the downstream primer 5′-AGG AGC GTG CGG TGA GAG TG-3′ (designed by Invitrogen, Waltham, MA, USA). The objective fragment for PCR amplification involved a 198 base pair (bp) segment encompassing the 677 locus within the MTHFR gene. The reaction system was as follows: primer 10 pmol each, template DNA 100 ng, Taq DNA polymerase 1 U, four dNTPs 200 μmol/L each; final volume 25 μL. The PCR conditions were as follows: 94 °C for 2 min; 30 cycles of 94 °C for 30 s, 61 °C for 30 s, and 72 °C for 30 s; and 72 °C for 5 min. The PCR products were detected using 8 % non-denaturing polyacrylamide gel electrophoresis. The amplified product was then digested using the restriction endonuclease *Hinf* I (total volume 20 μL: 10 μL amplified product, 2 μL buffer, *Hinf* I 8 U, and 7 μL deionized water) at 37 °C overnight. The product was determined using 3 % agarose gel electrophoresis. After staining with ethidium bromide, the digestion results were observed under an ultraviolet lamp. The C allele of MTHFR C677T demonstrates resistance to *Hinf* I cleavage, resulting in the retention of a 198bp fragment. Conversely, the T allele undergoes specific cleavage by the *Hinf* I enzyme, leading to the generation of a 23bp fragment and a 175bp fragment.

2.5. Statistical analysis

We used SPSS 21.0 (Chicago, IL, USA) to analyze the data. Quantitative data (normal distribution) were described by mean ± standard deviation (SD), and between-group variation was assessed using the unpaired *t*-test. The data with Non-normal distribution were represented by the median and interquartile range (IQR), and the Wilcoxon rank-sum test was employed for intergroup comparisons. The χ^2 test was used for evaluating categorical variables. The genetic association analyses were performed in three genetic models (dominant, recessive, and allelic comparison). Univariate and multivariate logistic regression analysis with adjustment of traditional risk factors for IS was performed to obtain the crude and adjusted odds ratios (ORs) and 95 % confidence intervals (CIs) for the risk genotype. Multifactor dimensionality reduction (MDR) software was used for detecting the possible interactions between genetic and traditional risk factors [17]. Unless otherwise specified, the significance level was set at $\alpha = 0.05$.

3. Results

3.1. General information analysis

A total of 434 IS patients were enrolled in this study based on the inclusion and exclusion criteria. Of whom, 129 patients developed END (29.7 %; 82 males and 47 females aged 69.78 ± 10.90 years) and 305 did not meet END diagnostic criteria (70.3 %; 171 males and

Table 1
Patients characteristics and logistic regression analysis results.

Variables	END ($n = 129$)	non-END ($n = 305$)	Univariable analysis[a]			Logistic Regression Analysis[b]		
			OR	95%CI	P_value	*OR*	95%CI	P_value
Age (years, X ± S)	69.78 ± 10.90	68.62 ± 12.08	t = 0.937		0.349	–	–	–
Sex (M/F, n)	82/47	171/134	1.367	0.895–2.089	0.148	–	–	–
Age≥65 (n)	99	200	1.732	1.081–2.777	0.022	1.631	0.979–2.716	0.060
Hyperlipidemia (n)	93	206	1.242	0.789–1.953	0.350	–	–	–
Hyperuricemia (n)	12	43	0.625	0.318–1.229	0.173	–	–	–
Hypoalbuminemia (n)	28	76	0.835	0.510–1.367	0.474	–	–	–
Abnormal renal function (n)	12	23	1.258	0.606–2.611	0.539	–	–	–
NIHSS>5 (n)	89	146	2.423	1.568–3.746	0.000	2.158	1.337–3.484	0.002
Overweight (n)	51	127	0.916	0.602–1.395	0.684	–	–	–
Diabetes (n)	36	59	1.614	1.000–2.604	0.050	1.185	0.667–2.107	0.562
Smoking (n)	58	107	1.512	0.994–2.299	0.053	1.471	0.827–2.619	0.189
Drinking (n)	45	74	1.672	1.070–2.614	0.023	1.311	0.708–2.426	0.389
AF (n)	17	36	1.134	0.612–2.103	0.689	–	–	–
History of TIA (n)	20	19	2.762	1.420–5.373	0.003	1.976	0.932–4.188	0.076
Hypertension (n)	86	218	0.798	0.513–1.242	0.318	–	–	–
Admission hypertension (n)	96	235	0.867	0.538–1.396	0.556	–	–	–
Admission Hyperglycemia (n)	65	85	2.629	1.716–4.026	0.000	2.410	1.436–4.046	0.001
Recurrent Stroke (n)	23	48	1.162	0.673–2.006	0.591	–	–	–
WBC Abnormality (n)	34	50	1.825	1.112–2.995	0.017	1.557	0.894–2.712	0.118
HHcy (n)	118	253	2.205	1.110–4.379	0.021	2.570	1.229–5.376	0.012
hsCRP >3.0 mg/L (n)	75	152	1.398	0.923–2.118	0.114	–	–	–

Notes: END: early neurological deterioration; M: male; F: female; OR: odds ratio; 95%CI: 95%confidence interval; NIHSS: the National Institutes of Health Stroke Scale; AF: atrial fibrillation; TIA: transient ischemic attack; HHcy: Hyperhomocysteinemia; hsCRP: hypersensitive-CRP.

[a] Original values of unadjusted covariates.

[b] Logistic multiple regression analysis results (dependent variable: END; adjusted variables: age ≥65 years, WBC abnormality, TIA history, drinking, smoking, diabetes history, HHcy, admission hyperglycemia, and NIHSS >5).

134 females aged 68.62 ± 12.08 years). Univariate analysis revealed no significant differences in sex or age between groups ($P > 0.05$). However, there were more subjects with over 65 years old (99 vs. 200 participants) in END group, and it was also found that the END subjects presented higher frequency of NIHSS>5, admission hyperglycemia, HHcy, drinking history, TIA history, and abnormal white blood cell ratios ($P < 0.05$). After adjusting for covariates, there were significant differences only in NIHSS>5, admission hyperglycemia and HHcy among groups ($P < 0.05$). Background characteristics of the subjects are given in Table 1.

3.2. The MTHFR C677T polymorphism analyses

3.2.1. Relationship between MTHFR C677T polymorphism and END

The C677T polymorphism was successfully genotyped in all samples. It was found that the TT genotype increased the risk of END under the recessive model (OR: 1.710, 95 % CI: 1.021–2.863, $P = 0.043$), and the risk for END also increased in subjects with the T allele with an adjusted OR (95 % CI) of 1.583 (1.181–2.121) (P = 0.002). Detailed results are presented in Table 2.

3.2.2. Relationship between MTHFR C677T polymorphism and HHcy

We also analyzed the association of MTHFR C677T of HHcy (see Table 3). The results showed that the TT genotype frequency was higher in subjects with HHcy (OR: 2.525, 95 % CI: 1.111–5.739, $P = 0.023$). However, no significant differences were found in CC genotype or allele distribution.

3.3. Interaction analysis of MTHFR C677T and traditional risk factors in END

All variables with P < 0.1 in univariable analysis on risk factors for END in 434 samples were entered into the analyses using MDR software. The MDR model identified the fourth-order interaction among MTHFR C677T and drinking, admission hyperglycemia as well as NIHSS >5 as the most optimal, with a best cross-validation consistency of 8/10. However, no statistical significance was found for this model (P = 0.2804). After adjusting for age ≥65 years, WBC abnormality, TIA history, drinking, smoking, diabetes history, HHcy, admission hyperglycemia and NIHSS >5 through multivariate logistic regression analysis, this model still had no statistical significance (OR: 1.237, 95 % CI: 0.227–6.734, P = 0.806).

4. Discussion

END incidence within 7 days after admission is reportedly 20.18%–42.5 % in Chinese hospitalized IS patients [18–20]. Our study showed that the incidence of END was 29.7 % in acute IS patients, which is consistent with previous studies.

In the present study, NIHSS >5, admission hyperglycemia and HHcy were associated with an increased risk of END. The pathophysiological mechanisms precipitating END predominantly encompass the lack of efficacious collateral circulatory pathways, thrombotic progression, cerebrovascular event recurrence, augmentation of intracranial pressure, epileptogenic activities, and hemorrhagic transmutation [21]. It has been reported that the incidence of END is proportional to stroke severity at admission (as determined by the NIHSS) [22]. A higher NIHSS score indicates more severe cerebral infarction or edema in patients, so a relatively high level of complications and oxygen-free radical release may be the fundamental causes of END [20]. Furthermore, previous studies [2,23] have demonstrated a significant association between hyperglycemia and END. Elevated fasting glucose levels can lead to an overproduction of harmful substances in cerebral tissue, such as lactate and reactive oxygen species, which exacerbate neuronal damage and contribute to the onset of END. The excessive accumulation of lactate in the brain may convert critically hypoperfused

Table 2
Distribution of the MTHFR C677T polymorphism in the END and Non-END Groups Under Different Genetic Models.

Genetic models	Distribution (n, %)		Unadjusted			Adjusted[a]		
	END ($n = 129$)	Non-END ($n = 305$)	*OR*	95%CI	P_value	*OR*	95%CI	P_value
Allelic model								
T	139 (53.9)	259 (42.5)	1.583	1.181–2.121	0.002	–	–	–
C	119 (46.1)	351 (57.7)						
Genotype								
TT	40 (31.0)	56 (18.4)						
CT	59 (45.7)	147 (48.2)						
CC	30 (23.3)	102 (33.4)						
Dominant model								
CC	30 (23.3)	102 (33.4)	0.603	0.376–0.968	0.035	0.748	0.440–1.272	0.284
TT + CT	99 (76.7)	203 (66.6)						
Recessive model								
TT	40 (31.0)	56 (18.4)	1.998	1.246–3.205	0.004	1.710	1.021–2.863	0.042
CC + CT	89 (69.0)	249 (81.6)						

Notes: END: early neurological deterioration; OR: odds ratio; 95%CI: 95%confidence interval; [a]Original values of unadjusted covariates.

[a] Logistic multiple regression analysis results (adjusted variables: sex, age, WBC abnormality, drinking, smoking, diabetes history, HHcy, admission hyperglycemia, and NIHSS >5).

Table 3
Distribution of the MTHFR C677T polymorphism across different Hcy levels groups.

Genotyping	HHcy ($n = 129$)	Non-Hcy ($n = 305$)	*OR*	95%CI	*p*_value
Genotype					
TT	89	7	2.525	1.111–5.739	0.023
CT	170	36	–	–	–
CC	112	20	0.930	0.523–1.652	0.804
Allelel					
C	348	50	1.343	0.914–1.973	0.133
T	394	76	–	–	–

Notes: HHcy: hyperhomocysteinemia; Non-Hcy: nornal-homocysteinemia.OR: odds ratio; 95%CI: 95%confidence interval.

tissues into infarcts, further impairing neurological function. Additionally, hyperglycemia has a prothrombotic effect, increasing the likelihood of thrombus extension. Hyperglycemic-induced microvascular complications can also impair collateral circulation, making patients more vulnerable to fluctuations in blood pressure and increasing the risk of hypoperfusion. Together, these changes precipitate the development of END. Hcy is a non-essential amino acid that contains sulfur and is mainly produced by the demethylation of methionine in food. Studies have shown that HHcy increases the inflammatory response, which may lead to unstable carotid atherosclerotic plaque and carotid/intracranial artery stenosis. HHcy is also a high-risk factor for cerebrovascular disease [24], and a meta-analysis demonstrated that HHcy was strongly associated with END risk [25].

Our results suggested that the MTHFR C677T TT genotype and T allele increased END risk, and in HHcy patients, there was a higher TT genotype frequency. MTHFR is a key enzyme in the folate metabolism pathway and plays an essential role in regulating blood HCY levels [6]. However, this pathway is strongly influenced by the MTHFR C677T and A1298C polymorphisms [26]. In the 677C > T mutation, where the base C is replaced by T, the encoded alanine is replaced by valine, which results in a 70 % decrease in MTHFR activity [16]. In IS patients with the 677C > T mutation, Hcy levels are usually increased [6]. However, the effects of the C677T site on enzyme activity are not affected by the 1298A > C mutation [27]. MTHFR is closely related to cardiovascular and cerebrovascular events. A meta-analysis showed that coronary artery disease risk was 1.2 times higher in T allele carriers at C677T [28]. As an independent predictor of atherosclerotic diseases, HHcy may also be a mechanism of END. It is thus presumed that MTHFR C677T may be directly involved in the pathogenesis of IS-related END by causing changes in Hcy metabolism.

We also found that interactions among the C677T polymorphism, NIHSS >5, admission hyperglycemia and drinking might be a risk factor for END. Although the subsequent validation analysis revealed no statistical significance of this finding, we speculated that the onset of END might result from interactions among many factors.

Limitations of the present study include the challenges in multivariable interaction analysis, where despite employing advanced techniques, the identified four-factor model involving traditional risk factors and MTHFR C677T polymorphism in relation to END did not reach statistical significance. Additionally, the small sample size may have constrained the analyses' statistical power, hindering the identification of significant findings. These issues highlight the necessity for future research to explore deeper into the genetic and environmental factors influencing END and to utilize larger sample sizes to enhance study robustness and findings' applicability.

5. Conclusion

The present study indicates that in the Han population of Chengdu (Sichuan, China), the MTHFR C677T polymorphism, admission hyperglycemia, HHcy, and the degree of neurological deficit are related to the onset of END after acute IS. Moreover, the interaction between traditional risk factors and susceptible genes may be involved in END onset.

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Qiang Zhou: Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Zhiyao Xu:** Writing – original draft, Formal analysis, Data curation. **Yuanyuan Duan:** Software, Investigation, Formal analysis, Data curation. **Hui Tang:** Software, Resources, Methodology, Investigation. **Haitao Zhang:** Supervision, Software, Investigation. **Hua Liu:** Writing – review & editing, Visualization, Validation, Supervision, Software, Resources, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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