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Expression of the C677T Polymorphism of the 5, 10-Methylenetetrahydrofolate Reductase (MTHFR) Gene in Patients with Carotid Artery Atherosclerosis

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: The C677T polymorphism of the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene polymorphism has been associated with hypertension and coronary heart disease, but its relationship with carotid artery remains unknown. This study aimed to investigate the association between the C677T polymorphism of the MTHFR gene in patients with confirmed carotid artery atherosclerosis.

Material/Methods: This retrospective study included 210 patients with carotid artery atherosclerosis (the patient group) and 210 controls (the control group). Color Doppler ultrasound was used to identify carotid artery intimo-medial thickness and atherosclerotic plaques. Sanger sequencing using the polymerase chain reaction (PCR) was used to detect the MTHFR C677T gene polymorphism. Systolic blood pressure (SBP), diastolic blood pressure (DBP), fasting plasma glucose (FPG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglyceride (TG), glycosylated hemoglobin (HbA1c), and other laboratory indicators were measured.

Results: SBP, DBP, FPG, TC, LDL-C, HbA1c, and intimo-medial thickness were significantly increased in the patient group compared with the control group, and HDL-C was significantly lower. The allele frequencies of the C667T locus of MTHFR gene were significantly different between the two groups ($P<0.05$), and the TT genotype and the T allele frequencies in the patient group were higher than in the control group. Logistic regression analysis showed that SBP, TC, LDL-C, and the C667T MTHFR gene polymorphism were risk factors for carotid artery atherosclerosis.

Conclusions: The C677T polymorphism of the MTHFR gene was expressed in patients with carotid artery atherosclerosis.

MeSH Keywords: 5,10-Methylenetetrahydrofolate Reductase (FADH2) • Atherosclerosis • Polymorphism, Genetic • Statistics as Topic

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Background

With the improvement of living standards and the aging of the population, cerebrovascular diseases have become important diseases that endanger human health and life. Carotid artery atherosclerosis is a risk factor for ischemic stroke, particularly in older adults [1–3]. Atherosclerosis is a disease of muscular arteries and the aorta that begins in childhood and increases with age. Carotid artery atherosclerosis causes carotid artery stenosis, and when thrombosis is associated with the advanced atherosclerotic plaque, the artery can occlude, resulting in the loss of blood supply to the brain [4-6]. Previous studies have shown that smoking, stress, lifestyle, hypertension, diabetes, and abnormal lipid metabolism are associated with the pathogenesis of carotid artery atherosclerosis [7]. With the development of genetic testing, gene polymorphisms have been shown to affect the occurrence and development of atherosclerosis by affecting several risk factors. The susceptibility genes associated with atherosclerosis include genes associated with the renin-angiotensin system, including angiotensin-converting enzyme (ACE), apolipoprotein-related genes such as apolipoprotein L1 (APOLl), and interleukin-6 (IL-6) [8].

The 5, 10-methylenetetrahydrofolate reductase (MTHFR) gene encodes a key enzyme involved in folate metabolism. MTHFR gene polymorphisms affect the activity and thermal stability of the MTHFR protein, which changes the concentration of homocysteine and folic acid *in vivo* [9,10]. The cytosine (C) on the fourth exon of the MTHFR gene is replaced by thymidine (T), which results in the C677T polymorphism. Also, alanine (Ala) is replaced by proline, which reduces the activity of MTHFR, leading to an increase in cysteine content in the cytosol and a decrease in folic acid content [11]. The C677T polymorphism of the MTHFR gene has previously been shown to be associated with hypertension, coronary heart disease, and stroke [12–14]. However, the association between this gene polymorphism and carotid artery atherosclerosis remains to be determined, even though some studies have reported that MTHFR gene polymorphism may play a role in the development of carotid artery atherosclerosis [15–17]. Therefore, this study aimed to investigate the association between the C677T polymorphism of the MTHFR gene in patients with confirmed carotid artery atherosclerosis.

Material and Methods

Patients

A total of 210 patients who were diagnosed with carotid artery atherosclerosis were enrolled in this study from the Department of Neurology of our hospital from February 2017 to September 2018. The study group included 103 men and 107 women with a mean age of 65.54±9.23 years. All patients were diagnosed with carotid artery atherosclerosis by ultrasonography. The control group included 210 healthy adults who underwent routine medical examinations in our hospital with no cardiovascular and cerebrovascular disease, liver, or kidney disease. The control group included 106 men and 104 women with a mean age of 64.83±10.81 years who had normal carotid arteries on ultrasound examination. The Ethics Committee of the Third Xiangya Hospital, Changsha, China, approved the study, and informed consent was obtained from all study participants.

Reagents and instruments

The Eppendorf gradient polymerase chain reaction (PCR) instrument (Eppendorf, Hamburg, Germany), a cryogenic 5424 high-speed centrifuge (Eppendorf, Hamburg, Germany), and the Nanodrop2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) were used. The S2000 Color Doppler ultrasound system (Siemens, Munich, Germany) was used for carotid artery imaging. An immunoassay analyzer (Beckman Coulter, Brea, CA, USA), an AU 600 automatic biochemical analyzer (Olympus, Tokyo, Japan), and LH750 blood cell analyzer (Beckman Coulter, Brea, CA, USA) were used. The polymerase chain reaction (PCR) was performed using the KOD-FX Neo (Toyobo Co. Ltd., Osaka, Japan). A blood genomic DNA extraction kit and agarose gel were obtained from Beijing Tiangen (Beijing, China). Primer synthesis and Sanger sequencing were performed by Shanghai Shenggong Biotechnology Co., Ltd. (Shanghai, China).

Patient clinical and demographic data

Clinical and demographic data were collected for all subjects, including gender, age, family history, smoking history, alcohol consumption, diet, and exercise. Clinical measurements included systolic blood pressure (SBP), diastolic blood pressure (DBP), height, weight, and body mass index (BMI). Then, 4 mL of EDTA anticoagulated fasting venous blood was collected in the early morning. The fasting plasma glucose level was determined by the glucose oxidase method. Total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglyceride (TG), and glycosylated hemoglobin (HbA1c) were measured using an AU 600 fully automatic biochemical analyzer (Olympus, Tokyo, Japan).

Carotid ultrasound for carotid artery atherosclerosis

Carotid artery imaging was performed using color Doppler ultrasound to detect the distal part of the common carotid arteries below the bifurcation, the common carotid artery at the bifurcation, and the proximal part of the internal carotid artery to determine the intimo-medial thickness and the diameter of the vascular lumen. The intimo-medial thickness was

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Table 1. Analysis of general data and biochemical indicators.

	Patients group	Control group	t/χ^2	*P*
Agen	65.54±9.23	64.83±10.41	0.740	0.460
Gender (M/F)	103/107	106/104	0.086	0.770
Smoking (%)	138 (65.71)	124 (59.05)	1.471	0.241
Hypertension (%)	121 (57.62)	133 (63.33)	1.827	0.087
Diabetes (%)	118 (56.19)	125 (59.52)	1.581	0.281
Hcy (μmol/L)	12.4±2.14	11.2±3.01	1.281	0.162
BMI (Kg/cm^2)	25.46±3.57	24.87±2.93	1.851	0.065
SBP (mmHg)	143.08±19.41	130.74±18.02	6.752	<0.001
DBP (mmHg)	88.24±13.93	84.68±11.58	2.848	0.005
FPG (mmol/L)	5.81±1.76	4.93±0.91	6.436	<0.001
TG (mmol/L)	1.62±0.81	1.53±0.73	1.196	0.232
TC (mmol/L)	4.71±1.10	4.38±1.14	3.019	0.003
HDL-C (mmol/L)	1.26±0.53	1.57±0.59	5.664	<0.001
LDL-C (mmol/L)	2.72±0.83	2.39±0.67	11.742	<0.001
HbA1c (%)	6.92±1.54	6.24±1.37	4.781	<0.001

the vertical distance between the lumen and the endocardial junction. The presence or absence of intimo-medial thickening was determined, and the shape, size, and properties of the atherosclerotic plaque were determined.

Diagnosis of carotid atherosclerosis and plaque characteristics

An intimo-medial thickness of <1.0 mm was normal. The intimo-medial thickness was considered to be increased at between 1.0 mm and 1.2 mm. An intimo-medial thickness of >1.2 mm represented the formation of an atherosclerotic plaque. An intimo-medial thickness of ≥1.0 mm represented carotid atherosclerosis. The mixed plaques with low echogenic lipid content and uneven echogenicity were regarded as unstable plaques. The fibrous plaques with strong echogenicity or calcification were identified as stable plaques.

DNA extraction

Anticoagulated blood (500 μL) was collected, and DNA extraction was performed according to the standard genomic DNA extraction method. Briefly, 1000 μL of red blood cell lysate was added to 500 μL of anticoagulant, mixed by inversion, incubated at room temperature for 5 min, and centrifuged at 3,000 rpm for 5 min. After discarding the supernatant, 200 μL of buffer solution, and 20 μL of proteinase K were added and mixed by shaking, followed by the addition of 200 μL of buffer solution, and incubation at 70°C for 10 min until the solution became clear. Then, 200 μL of absolute ethanol was added and mixed for 15 s followed by transfer to the CB3 adsorption column and centrifuged at 30°C for 30 sec. The column was washed twice, and the DNA was eluted with 50 μL of the buffer. The DNA purity was determined using a NanoDrop 2000 ultraviolet spectrophotometer (Thermofisher Scientific, Waltham, MA, USA). DNA was stored at -20°C for further study.

Polymerase chain reaction (PCR) amplification and sequencing

The PCR reaction mixture contained 2×KOD PCR buffer (12.5 μL), dNTPs (4 μL), buffer F (0.5 μL), buffer R (0.5 μL), DNA (2 μL), and double-distilled H_2O (5.5 μL). The PCR amplification cycles included denaturing at 94°C for 2 min, 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, and 35 cycles at 72°C for 10 min. According to the MTHFR gene sequence (NG_013351.1), PCR primers were designed using Primer Premier version 5.0 software. The primer sequences used for PCR were:
MTHFR, forward: 5'-CATCCCTCGCCTTGAACAG-3';
MTHFR, reverse: 5'-GGACGATGGGGCAAGTGAT-3'.

The PCR amplification product size was 233 bp and was analyzed by agarose gel electrophoresis and sent to Shanghai Biotech (Shanghai, China) for Sanger sequencing.

Statistical analysis

Data were analyzed using SPSS version 17.0 software (IBM Corp., Armonk, NY, USA). Categorical data were presented as the mean±standard deviation (SD). Comparison between two

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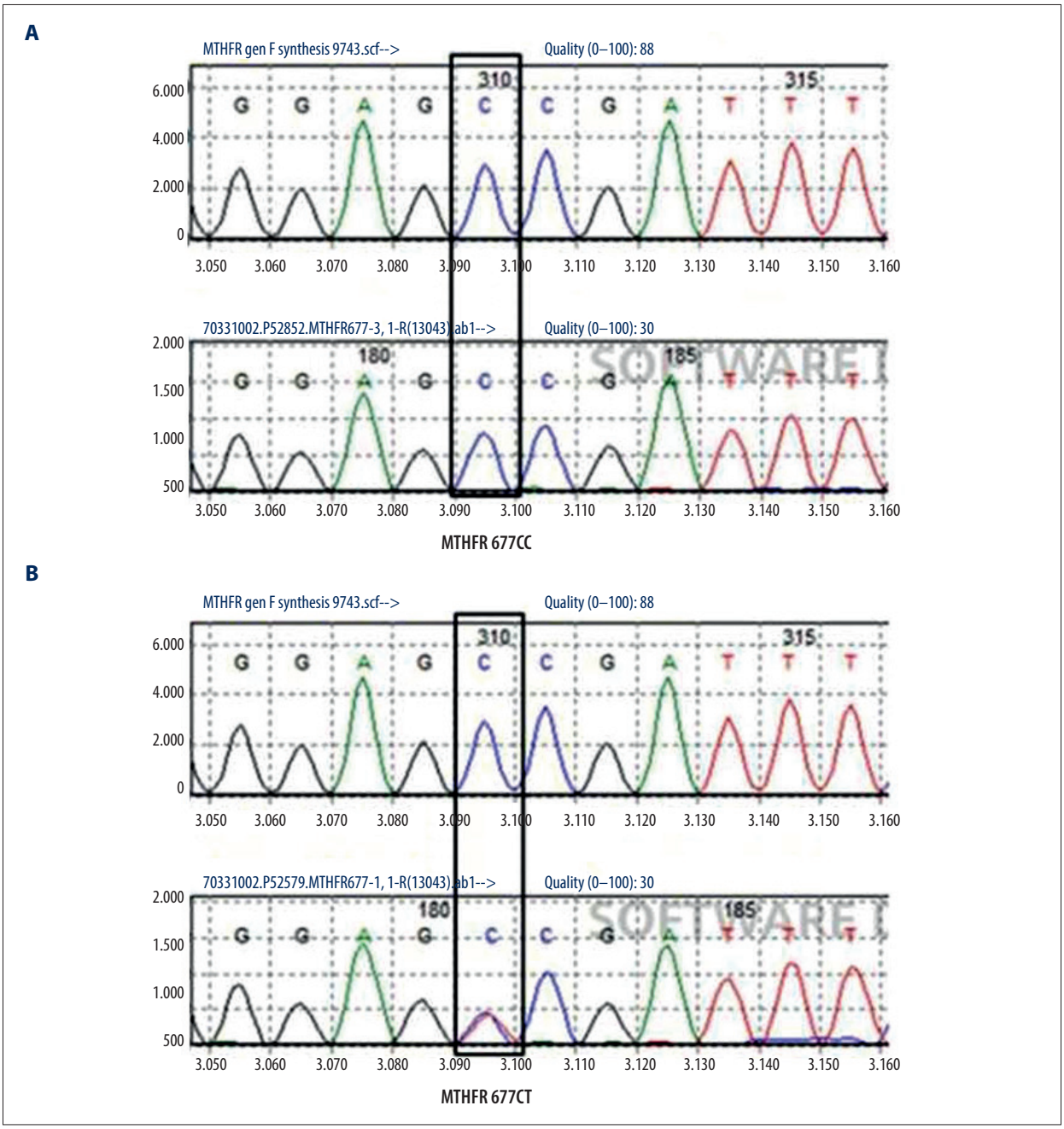
groups was performed using the Student's t-test. For two comparisons within the same group, one-way analysis of variance (ANOVA) with Bonferroni post hoc analysis were performed. The data included the number of cases of carotid artery atherosclerosis. The chi-squared (χ^2) test was used for statistical analysis. The allele frequency=(2×homozygous+heterozygous)/(2×number of subjects) was analyzed. The balance of the MTHFR gene distribution was detected by the Hardy-Weinberg method. The risk factors for carotid atherosclerosis were analyzed by multiple logistic regression analysis of the binomial classification. A p-value <0.05 was considered to be statistically significant.

Results

Comparison of general and biochemical indicators between the patient group and the control group

There were no significant differences in age, gender, body mass index (BMI), and plasma triglyceride (TG) levels between the patient group and the control group (p>0.05). However, the systolic blood pressure (SBP), diastolic blood pressure (DBP), fasting plasma glucose (FPG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and glycosylated



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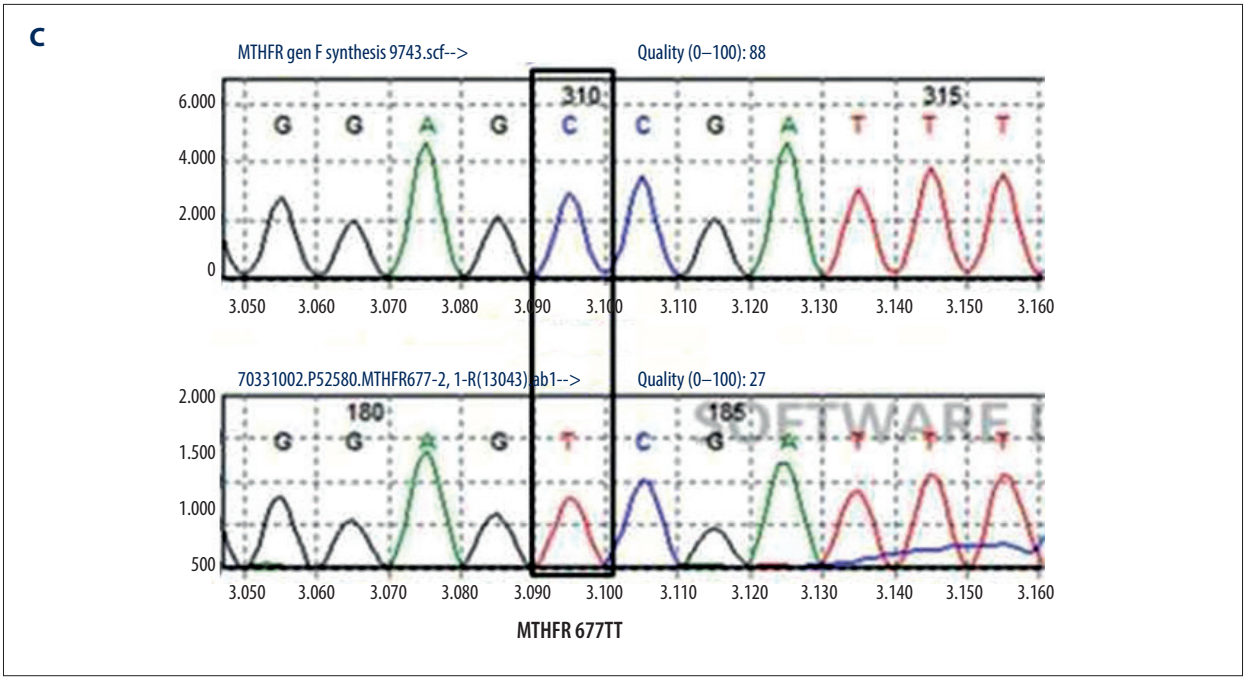

Figure 1. The MTHFR gene polymorphism sequencing results (**A**) MTHFR wild type; the 677CC genotype. (**B**) MTHFR heterozygous mutant; the 677CT genotype. (**C**) MTHFR homozygous mutant; the 677TT genotype.

hemoglobin (HbA1c) in the patient group were significantly higher than those in the control group. In contrast, plasma levels of high-density lipoprotein cholesterol (HDL-C) were lower in the control group (Table 1).

MTHFR gene polymorphisms

Three genotypes of the MTHFR gene were detected by Sanger sequencing: the wild type, CC genotype (Figure 1A); the heterozygous mutant, CT genotype (Figure 1B); and the homozygous mutant, TT genotype (Figure 1C). The gene distribution frequency of MTHFR gene C677T polymorphism was tested with the Hardy–Weinberg equilibrium and showed was consistent with genetic balance (P>0.05) (Table 2), suggesting that the selected samples were representative.

Comparison of allele frequencies and genotype frequencies between study groups

The frequencies of three genotypes and allele frequencies of the C667 locus of the MTHFR gene were significantly different between the two groups (P<0.05). The frequency of the TT genotype and T allele in the patient group were significantly higher than in the control group (Table 3). In the patient group, 83 patients had stable carotid artery atherosclerotic plaques, and 127 patients had unstable carotid artery atherosclerotic plaques. The genotype frequencies and allele frequencies of the MTHFR gene C667 locus were compared between the two plaque types. The frequency of the TT and T alleles in unstable plaque group were significantly higher than those in stable plaque group (P<0.05) (Table 4).

Logistic regression analysis of risk factors for carotid artery atherosclerosis

The independent variables used were hypertension, TC, TG, HDL, and LDL to perform stepwise regression analysis to determine whether carotid artery atherosclerosis was a dependent variable. Hypertension and diabetes were not independent risk factors for carotid artery atherosclerosis (P>0.05), as shown by univariate analysis (Table 5). For 420 study participants, when carotid artery atherosclerosis was selected as the dependent variable, using atherosclerosis as a factor in the overall population, comparing atherosclerosis to non-atherosclerosis, age, gender, BMI, SBP, DBP, FPG, TG, TC, HDL-C, LDL-C, HbA1c, and the MTHFR gene C667T polymorphism were taken as independent variables. Logistic regression analysis of locus polymorphisms showed that SBP, TC, LDL-C, and the MTHFR gene C667T polymorphism were risk factors for carotid artery atherosclerosis (Table 6).

Discussion

Timely diagnosis and intervention for patients with carotid artery atherosclerosis have a positive clinical effect in the prevention of stroke and cardiovascular and cerebrovascular disease [18]. Carotid artery atherosclerosis is a vascular disease

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Table 2. Hardy-Weinberg equilibrium test for polymorphic *loci*.

Group		Genotype			Alleles		χ^2	*P*
		CC (%)	CT (%)	TT (%)	C (%)	T (%)		
Control	C/T	98 (23.33)	179 (42.62)	143 (34.05)	375 (44.64)	465 (55.36)	7.966	>0.05
All cases	C/T	112 (21.87)	172 (44.23)	137 (31.92)	359 (41.81)	451 (59.25)		

Table 3. Comparison of MTHFR C667 allele frequency and genotype frequency.

	Genotype			χ^2	*P*	Alleles		χ^2	*P*
	CC (%)	CT (%)	TT (%)			C (%)	T (%)		
Patients	40 (19.05)	68 (32.38)	102 (48.57)			148 (35.24)	272 (64.76)	30.06	<0.01
Control	58 (27.62)	111 (52.86)	41 (19.52)			227 (12.00)	193 (88.00)		

Table 4. Comparison of MTHFR C667 allele frequency and genotype frequency.

	Genotype			χ^2	*P*	Alleles		χ^2	*P*
	CC (%)	CT (%)	TT (%)			C (%)	T (%)		
Stable plaque	12 (14.46)	44 (53.01)	27 (32.53)			68 (40.96)	98 (59.04)	3.943	0.047
Unstable plaque	28 (22.05)	24 (18.90)	75 (59.05)			80 (31.50)	174 (68.50)		
Folate level (ng/L)	7.45±2.34	7.25±1.21	6.94±1.31			6.81±1.28	6.79±1.31	4.129	0.129
B12 (pg/L)	235.25±137.21	203.51±136.25	211.25±129.55			165.85±128.58	166.95±130.55	2.142	0.081
Hcy (μmol/L)	24.24±2.04	23.24±2.15	23.35±2.21			22.24±2.04*	20.85±1.92	3.212	0.522

Table 5. Regression analysis of CAS risk factors.

Variables	β	SE	Wald χ^2	p	OR	95% CI
Hypertension (mmHg)	0.478	0.213	6.271	>0.05	1.621	0.928–2.187
Diabetes (mmol/L)	0.481	0.211	6.382	>0.05	1.438	0.791–2.381
TC (mmol/L)	0.517	0.301	7.238	>0.05	1.582	0.892–2.193
TG (mmol/L)	0.523	0.249	6.389	>0.05	1.639	0.987–2.357
HDL-C (mmol/L)	0.486	0.252	7.393	>0.05	1.538	0.892–2.452
LDL-C (mmol/L)	0.472	0.262	6.938	>0.05	1.638	0.731–2.324

Table 6. Logistic regression analysis of risk factors for carotid atherosclerosis.

	B	SE	Wals	P	OR	95% CI
SBP (mmol/L)	1.762	0.543	8.745	0.001	3.514	1.372–4.826
TC (mmol/L)	1.638	0.509	7.804	0.006	2.376	1.408–3.822
LDL-C (mmol/L)	1.204	0.463	5.317	0.017	1.925	1.124–3.017
MTHFR polymorphism	2.043	0.628	9.737	0.001	6.143	3.872–11.526

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caused by a combination of genetic and acquired factors, including diet and lifestyle, hypertension, obesity, diabetes, and hyperlipidemia [19]. This study compared the clinical indicators of patients with carotid artery atherosclerosis and healthy control and showed that SBP, DBP, FPG, TC, LDL-C, HbA1c and the intimo-medial thickness were higher in the carotid artery atherosclerosis group than in the healthy control group, while HDL-C was lower than in the control group, which was consistent with previous studies [20]. However, there were no significant differences in body mass index (BMI) between the two groups in this study, which may be related to the selected population. All subjects in this study were generally older, with an average age of over 60 years.

Previous studies have shown that MTHFR is a key rate-limiting enzyme for homocysteine metabolism, and normal MTHFR activity can maintain normal metabolism of folic acid and homocysteine, maintaining its stable plasma concentration [21]. The C677T locus in the MTHFR gene occurs in the folate binding region, which increases the thermal instability of the MTHFR enzyme and reduces its enzymatic activity, resulting in abnormal homocysteine and folate metabolism [22]. Several studies have demonstrated the close association of folate metabolism in the pathogenesis of cardiovascular diseases with potential mechanisms include antioxidant actions, effects on cofactor availability, or direct interactions with the enzyme endothelial nitric oxide synthase (NOS) [23]. The MTHFR C677 locus gene polymorphism is closely related to the occurrence of several diseases [24,25]. McNulty et al. [26] showed that MTHFR 677C>T can increase the risk of hypertension by between 24% to 87%, and the authors showed that vitamin B2 supplementation had a better therapeutic effect on hypertensive patients with the MTHFR 677TT genotype. Ponomarenko et al. [27] showed that MTHFR C677T polymorphism was a new risk factor for atherosclerosis, in addition to traditional risk factors such as LDL-C, HDL-C, and smoking.

In 2015, a meta-analysis study by Zhu et al. identified an association of the MTHFR gene C677T polymorphism with ischemic stroke in the Chinese population [15]. Also, an association between plasma homocysteine and the C677T gene polymorphism and carotid intimal thickness in South Asian, Chinese, and European Canadians has been shown [16]. In the present study, the frequencies of the three genotypes and the allele frequencies of the C667 locus of MTHFR gene were significantly different between the patient group and the control group. The frequency of the TT genotype and T allele genotype in the carotid artery in the atherosclerosis group were significantly higher than in the control population. Comparing the genotype frequency and allele frequency of the MTHFR gene C667T locus in patients with stable plaque and unstable plaque, it was found that the frequency of TT genotype and T allele were significantly higher in patients with an unstable plaque than those in the stable plaque group. Further logistic regression analysis found that SBP increased the risk of carotid artery atherosclerosis by 3.514 times, suggesting that hypertension is an independent risk factor for carotid artery atherosclerosis, which is consistent with the literature [28]. TC and LDL-C are also risk factors for carotid artery atherosclerosis, and high levels of LDL can damage arterial endothelial cells, and lipid abnormalities make lipids easily deposited in blood vessels to form atheromatous plaques [29].

The findings from the present study showed that the C667T polymorphism of the MTHFR gene is closely related to the occurrence of carotid atherosclerosis, which can increase the risk of carotid artery atherosclerosis by 6.143 times, which is an independent risk factor for carotid artery atherosclerosis. High levels of homocysteine can damage endothelial cells, promote the proliferation of smooth muscle cells, and develop atherosclerosis, which may be because the MTHFR gene C667T polymorphism affects the pathogenesis of carotid atherosclerosis [30].

This study had several limitations. This study did not include an investigation into the molecular mechanisms involved in carotid atherosclerosis, and further investigation into how the C677T polymorphism of the MTHFR gene affects the carotid artery atherosclerotic plaque is required in the future. Also, this study included a small patient number from one center, which may have introduced bias into the study. Due to the limited number of patients enrolled, large cohort clinical studies are required to support the findings from this preliminary study.

Conclusions

This study aimed to investigate the association between the C677T polymorphism of the MTHFR gene in patients with confirmed carotid artery atherosclerosis. The findings showed that the C677T polymorphism of the MTHFR gene was expressed in patients with carotid artery atherosclerosis.

Conflict of interest

None.

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