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Original Article

Methylenetetrahydrofolate reductase gene A1298C polymorphism and gene-environment interactions are associated with carotid plaque in a south Chinese population

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Abstract: Objective: Our primary objective was to evaluate the associations of conventional risk factors and methylenetetrahydrofolate reductase (MTHFR) gene polymorphisms with the risk of carotid plaque in a south Chinese population. Our secondary objective was to explore gene-environment interactions and potential relationship with carotid plaque. Method: We enrolled 229 patients suffer from carotid plaque and 180 controls in this case-control study. We measured the carotid intima-media thickness by ultrasound and investigated conventional risk factors, biomarkers and C677T/A1298C MTHFR genotypes. Logistic analysis was used to evaluate the association between conventional risk factors and carotid plaque. The SNPstats platform was used to investigate the association between MTHFR gene polymorphisms and carotid plaque under 5 genetic models (dominant, recessive, codominant, over dominant and additive models). Gene-environment interactions analysis was then performed by multifactor dimensionality reduction. Results: Age and hypertension were identified as independent risk factors of carotid plaque. C677T and A1298C demonstrated associations with carotid plaque under the recessive model (C677T: $P = 0.03$, odds ratio = 3.14, 95% confidence interval = 1.04-11.21; A1298C: $P = 0.018$, odds ratio = 2.40, 95% confidence interval = 1.13-5.10). Neither C677T nor A1298C polymorphism was associated with stable or vulnerable plaques. Additionally, Significant multiplicative and additive interactions were observed in terms of carotid plaque between A1298C polymorphism and diabetes, age, and smoking ($P = 0.013$). Conclusion: MTHFR gene C677T and A1298C polymorphisms may act as modifiers of carotid plaque risk in south Chinese population. In addition, the combined effect of gene-environment interactions between A1298C polymorphism and conventional risk factors may promote the progression of carotid plaque.

Keywords: A1298C, C677T, carotid plaque, gene-environment interactions, MTHFR gene, risk factor

Introduction

Carotid plaque (CP) and stenosis represent serious stages in the development of atherosclerosis [1], and are leading causes for future cardiovascular disease events [2, 3], particularly for ischemic stroke and transient ischemic attack [4, 5]. Research has shown that the global prevalence of CP is keeping high level [6]. Moreover, race and gender play important roles in the formation of plaques. Furthermore, studies have shown that the Asian race shows lower risk for CP and cardiovascular disease than Caucasians [7]. The prevalence of CP is known to be significantly higher in men than in women [8-10]. Consequently, screening individuals with CP is of vital clinical importance, particularly to those patients who require medication can be identified in a timely manner.

Hyperhomocysteinemia is known as an atherosclerotic risk factor [11], and increases the risk of cardiovascular diseases [12], particularly among hypertensive individuals [13]. The methylenetetrahydrofolate reductase (MTHFR) gene is known to be a strong predictor for hyperhomocysteinemia in general populations [14]. However, the association between MTHFR gene mutations and the risk of CP remains inconsistent. Some studies have reported that the

C677T polymorphism is independently associated with increased carotid intima-media thickness, plaque or stenosis [15, 16]. On the other hand, several studies failed to find such relationships in different ethnic populations [17-19].

Here, we hypothesized that C677T and A1298C polymorphisms are associated with CP in a south Chinese population. In the present study, we investigated conventional risk factors and MTHFR gene polymorphisms and estimated potential associations with the risk of CP. We also tried to explore the association between gene-environment interactions of these conditions in relation to the risk of CP in this study protocol.

Materials and methods

Study design and population

We used a stroke screening and prevention project, taking place between during October 2015 and December 2015, to perform a community-based study to determine whether the MTHFR gene C677T and A1298C polymorphisms are associated with CP. All subjects were genetically unrelated Han Chinese, who were stroke-free local residents aged over 40 years, and recruited from a community of Haikou city, Hainan province, China. We excluded subjects with any of the following conditions: autoimmune disease, serious infection within the previous 6 months, liver and kidney disease, malignant tumors, and heart failure. Finally, our study included 229 CP patients (bilateral and/or unilateral plaques) and 180 controls (local thickness of the carotid intima <1.0 mm), 40-93 years of age, who attended regular health checkups and subsequently underwent ultrasonic examinations and genetic analysis. The study protocol was approved by the Medical Ethics Committee of Haikou People's Hospital. All participants provided written informed consent prior to participation.

Demographic and clinical records

We collated information relating to a variety of demographic factors, including name, gender, age, height, weight, smoking status, and daily intake of vegetables. Body mass index (BMI) was calculated as the ratio of weight (in kilograms) to height squared (in meters). Subjects were also classified into groups according to whether they were current smokers or not; current smoking was defined as ≥1 cigarette per day for more than 1 year. The boundary value adopted in this study for vegetable intake was ≥300 g/day. Medical records were also analyzed for hypertension, diabetes, hypercholesterolemia, cardiovascular events, and other serious systemic diseases. Hypertension was defined as a systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg, or when the subject was currently taking anti-hypertensive treatment. Subjects with a repeated fasting bold glucose of ≥7.0 mmol/L, HbA1c ≥6.5%, or who were being treated with anti-diabetic drugs were considered to be diabetic. Hypercholesterolemia was defined as cholesterol levels ≥6.2 mmol/L.

Ultrasound assessment of CP

We used HD7 ultrasound equipment (Philips, Amsterdam, The Netherlands) equipped with a 5 to 12 MHz liner array transducer to measure the carotid intima-media thickness. All measurements of CP in this study were taken by two experienced vascular ultrasound technologists. During the scan, the patient was positioned supine with the head resting flat on the bed with a rolled-up towel under the neck. We examined the following areas for the presence of CP: the bilateral distal common carotid arteries, bulbs, and proximal internal carotid arteries. CP was defined as a clearly local thickening of the intima-media layer ≥1.5 mm but less than 50% of vessel lumen reduction (common carotid artery).

Blood sample collection and measurement of biomarkers

Double venous blood samples, drawn by trained phlebotomists from each subject's antecubital venous after overnight fasting, were stored into a red tube and a purple tube, respectively. The blood samples in red tubes were immediately sent to the central laboratory of Haikou People's Hospital for biochemical tests. Creatinine, fasting blood glucose, and total cholesterol were analyzed by enzymatic methods. Homocysteine levels were analyzed using the Enzymatic Cycling Method and normal values were determined as being no more than 15 μmol/L. All biochemical variables were measured using an auto-analyzer (AU5800, Beckman-Coulter,

Table 1. Characteristics and clinical features

Risk factors	Controls (n = 180)	CP (n = 229)	*p*
Age, years	60.45±10.41	68.34±9.63	<0.001
40-49, n (%)	26 (14.44)	10 (4.36)	
50-59, n (%)	56 (31.11)	31 (13.54)	
60-69, n (%)	66 (36.67)	84 (36.68)	
70-79, n (%)	25 (13.89)	79 (34.50)	
≥80, n (%)	7 (3.89)	25 (10.92)	
Gender			0.138
Female, n (%)	106 (58.89)	118 (51.53)	
Males, n (%)	74 (41.11)	111 (48.47)	
Body mass index, kg/m^2	25.22±3.67	24.58±3.80	0.052
Smoking, n (%)	29 (16.11)	24 (10.48)	0.090
Vegetables intake ≥300 g/day, n (%)	58 (32.22)	78 (34.06)	0.672
Hypertension, n (%)	126 (70)	198 (86.46)	<0.001
Hypercholesterolemia, n (%)	129 (71.67)	168 (73.36)	0.703
Diabetes, n (%)	42 (23.33)	82 (35.81)	0.006
Anti-hypertensive, n (%)	30 (16.67)	61 (36.64)	0.015
Anti-diabetic, n (%)	31 (17.22)	45 (19.65)	0.517
Statin treatment, n (%)	20 (11.11)	31 (13.54)	0.515
Creatinine, μmol/L	98 (75, 123)	102 (76, 122)	0.481
Total cholesterol, mmol/L	5.05 (4.30, 6.40)	5.35 (4.50, 6.10)	0.114
Homocysteine, μmol/L	11.60 (9.45, 15.95)	13.85 (11.19, 17.85)	<0.001

CP: carotid plaque. Data are shown as means ± standard deviation (SD), median (interquartile-range), or number (percentage).

Fullerton, CA, USA). Blood samples in the purple tubes, containing ethylenediaminetetraacetic acid (EDTA), were immediately placed on ice, and then centrifuged for 15 min at 3000 g and 4°C. After separation, the sediment in the tubes was stored in aliquots in a temperature of -80°C. The remaining analysis was performed at Northwest University (Xi'an City, Shannxi Province, China).

MassARRAY analysis for MTHFR genotypes

Detailed methods of DNA extraction and genotyping of the two polymorphisms were described in a previous study [20]. In brief, DNA was extracted from whole blood samples using a Blood Genomic DNA Isolation Kit (Xi'an GoldMag Biotechnology, Xi'an, China) in accordance with the manufacturer's protocol. DNA concentrations were determined by absorption at 260 nm using a Nanodrop2000 (Thermo Fisher, Waltham, MA, USA). The C677T locus was amplified with forward primer (5'-TGA AGG AGA AGG TGT CTG CGG GA-3') and reverse primer (5'-AGG ACG GTG CGG TGA GAG TG-3'); while the A1298C locus was amplified with forward primer (5'-CAA GGA GGA GCT GCT GAA GA-3') and reverse primer (5'-CCA CTC CAG CAT CAC TCA CT-3'), as reported by Yi et al. [21]. SNPs within the MTHFR gene were genotyped using the Sequenom MassARRAY RS1000 (Sequenom, San Diego, CA, USA) in accordance with a standard protocol.

Statistical analysis

Continuous variables are presented as means ± standard deviation (SD) or median (interquartile-range). Qualitative variables are presented as absolute and relative frequencies. In order to compare proportions, we used chi-square (χ^2) or Fisher's exact tests. To compare variables between different parameters, we used the Student's t-test or a non-parametric test, as appropriate. Association between conventional risk factors and CP were estimated using logistic regression analysis.

Hardy-Weinberg equilibrium in the control group was calculated by χ^2 test, and the association of C677T and A1298C polymorphisms with CP was investigated by logistic regression analy-

Table 2. Logistic regression analysis of risk factors for carotid plaque

Risk factors	Crude OR (95% CI)	Adjusted OR (95% CI)[#]
Age, years		
40-49	1.00	1.00
50-59	1.44 (0.61-3.37)	1.51 (0.59-3.84)
60-69	3.31 (1.49-7.35)	2.89 (1.20-6.98)
70-79	8.22 (3.49-19.36)	6.90 (2.65-17.97)
≥80	9.28 (3.06-28.21)	7.45 (2.25-24.60)
Female gender	0.74 (0.50-1.10)	0.59 (0.355-1.01)
Smoking	0.61 (0.34-1.09)	0.78 (0.38-1.62)
Hypertension	2.74 (1.67-4.49)	2.54 (1.45-4.42)
Diabetes	1.83 (1.18-2.84)	1.62 (0.99-2.62)
Anti-hypertensive	1.83 (1.12-2.98)	1.23 (0.71-2.11)
Homocysteine, μmol/L	1.06 (1.02-1.09)	1.01 (0.97-1.05)

OR: odds ratio; CI: confidence interval. [#]Adjusted for age, gender, smoking, hypertension, diabetes, anti-hypertensive therapy, homocysteine.

Table 3. Allele frequency and genotype of MTHFR gene polymorphisms

	Overall	Controls	CP	*p*-HWE
C677T				0.33
C	660	292	368	
T	158	68	90	
C/C	268	116	152	
C/T	124	60	64	
T/T	17	4	13	
A1298C				1
A	571	267	304	
C	247	93	154	
A/A	207	99	108	
A/C	157	69	88	
C/C	45	12	33	

CP: carotid plaque; *p*-HWE: *p* value for Hardy-Weinberg equilibrium in the control group.

sis. Genotypic effects were examined according to a dominant, recessive, codominant, over dominant, and an additive model. In addition, unconditional logistic regression analysis and multifactor dimensionality reduction (MDR) were utilized to investigate the role of gene and environment interactions for CP. Statistical significance was set at 0.05 and analyses were conducted using SPSS statistical software (version 22.0, SPSS Inc., Chicago, IL, USA), SNPstats platform (http://www.snpstats.net), and MDR software (version 3.0.2; downloaded from http://www.multifacterdimensionalityreduction.org).

Results

Characteristics of the study subjects

As shown in **Table 1**, there were 111 (48.47%) men and 118 (51.53%) women in the CP group, and 74 (41.11%) men and 106 (58.89%) women in the control group. Mean age was 68.34±9.63 years in the CP group and 60.45±10.41 years in the control group. There were significant differences for age, hypertension, diabetes, anti-hypertensive therapy, and plasma homocysteine levels between the two groups. No statistical difference was detected for gender, BMI, smoking status, vegetables intake, hypercholesterolemia, anti-diabetes therapy, statin treatment, creatinine, and total cholesterol levels (P>0.05). Original data are provided in Supplementary Table.

Conventional risk factors associated with CP

The odds ratio (OR; 95% confidence intervals [CI]) of risk factors for CP are shown in **Table 2**. Multivariate regression analysis for the risk of CP reported that increased age and hypertension represented independent risk factors after adjustment for confounding variables (gender, smoking, diabetes, anti-hypertensive treatment, and homocysteine).

Associations between C677T and A1298C polymorphisms and CP

The observed genotype frequencies of the two SNPs were in Hardy-Weinberg equilibrium in the control group (**Table 3**). The minor allele frequency distribution was calculated according to genotyping data from the CP group and the control group, and the results did not show any statistical differences. **Table 4** summarizes detailed data from the association of C677T and A1298C polymorphisms with CP risk in five genetic models after adjustment for age, gender, hypertension, diabetes, anti-hypertensive therapy, and homocysteine. Both C677T and A1298C demonstrated significant associations with CP under the recessive model (C677T: P = 0.03, OR = 3.14, 95% CI = 1.04-11.21; A1298C: P = 0.018, OR = 2.40, 95% CI = 1.13-5.10). No statistical significance was observed in the other models. Further exploration of the association between the haplotypes and CP, however, found no statistical difference (**Table 5**).

Table 4. Association of MTHFR gene polymorphisms and carotid plaque

Genetic model	Genotype	Controls (n = 180)	CP (n = 229)	OR (95% CI)	$p^{\#}$
C677T					
Codominant	C/C	116	152	1.00	
	C/T	60	64	0.73 (0.46-1.16)	0.04
	T/T	4	13	3.10 (0.93-10.28)	
Dominant	C/C	116	152	1.00	
	C/T-T/T	64	77	0.86 (0.55-1.35)	0.50
Recessive	C/C-C/T	176	216	1.00	
	T/T	4	13	3.41 (1.04-11.21)	0.03
Over dominant	C/C-T/T	120	165	1.00	
	C/T	60	64	0.68 (0.43-1.09)	0.11
Log-additive	--	--	--	1.04 (0.72-1.52)	0.82
A1298C					
Codominant	A/A	99	108	1.00	
	A/C	69	88	1.10 (0.70-1.74)	0.06
	C/C	12	33	2.50 (1.15-5.44)	
Dominant	A/A	99	108	1.00	
	A/C-C/C	81	121	1.30 (0.85-1.99)	0.22
Recessive	A/A-A/C	168	196	1.00	
	C/C	12	33	2.40 (1.13-5.10)	0.018
Over dominant	A/A-C/C	111	141	1.00	
	A/C	69	88	0.96 (0.62-1.48)	0.84
Log-additive	--	--	--	1.38 (1.00-1.90)	0.05

CP: carotid plaque; OR: odds ratio; CI: confidence interval. #adjusted for age, gender, hypertension, diabetes, anti-hypertensive therapy, and homocysteine.

Table 5. Association of haplotype with carotid plaque

Haplotype	Frequency	OR (95% CI)	$p^{\#}$
C-A	0.5189	1.0	--
C-C	0.2879	1.44 (1.00-2.08)	0.05
T-A	0.1791	1.18 (0.77-1.81)	0.44
T-C	0.0141	1.59 (0.40-6.32)	0.51

OR: odds ratio; CI: confidence interval. #adjusted for age, gender, hypertension, diabetes, anti-hypertensive therapy, and homocysteine.

In addition, we compared genotypes of two SNPs according to the properties of CP, neither C677T nor A1298C polymorphism was associated with stable or vulnerable plaques under any genetic model.

Gene-environment interaction analysis

Results arising from our gene-environment interaction analysis are shown in **Table 6**. We applied the two SNPs, along with all of the conventional risk factors mentioned above, into a gene-environment interaction analysis for CP. A significant multiplicative and additive interaction was observed in CP between four risk factors: diabetes, age, the A1298C polymorphism and smoking (Testing accuracy = 0.748; *p*-Testing = 0.013).

Discussion

We investigated the relationship between two polymorphisms in the MTHFR gene, C677T and A1298C, and CP, in a south Chinese Han population, and assessed gene-environment interactions between these conditions. Our results suggested that these two SNPs, and traditional risk factors (age and hypertension), were significantly associated with the risk of CP. Furthermore, significant gene-environment interactions were identified by MDR.

Conventional risk factors, including increased age and hypertension, were identified as independent risk factors of CP. Our findings were consistent with several previous studies 8, 10. With increasing age, the number of CPs increased and the morphology and stability varied [22]. Although the prevalence of hypertension was high in this population cohort, the proportion of subjects receiving anti-hypertensive therapy was far below expectation. Hypertension promotes the development of CP by certain mechanisms. Studies have demonstrated that current smokers were more likely to have plaques when compared to those who have never smoked [23]. Furthermore, subjects are associated with a significantly increased risk of CP in adulthood if they have been exposed to parental smoking during childhood [24]. However, we failed to identify a relationship between smoking and CP in the present study, probably due to the low proportion of

Table 6. Gene-environment interactions of carotid plaque

Risk factors involved in MDR model	Testing accuracy	CVC	*p*-Testing
Diabetes	0.729	10/10	<0.001
Diabetes, age	0.728	10/10	0.004
Diabetes, age, A1298C polymorphism	0.735	10/10	0.015
Diabetes, age, A1298C polymorphism, smoking	0.748	9/10	0.013

MDR: multifactor dimensionality reduction; CVC: cross-validation consistency; *p*-Testing: *p* value for testing χ^2.

smokers among the study population. In addition, using univariate logistic regression, we found that diabetes was inversely associated with plaque formation. However, this relationship was not valid following adjustment for conventional risk factors as potential confounding variables.

Previous studies have linked elevated levels of homocysteine to the development of CP [25]. In other words, hyperhomocysteinemia is able to cause atherosclerosis by impairing the inner vascular wall through a variety of different mechanisms [26-28]. In our present CP group, 85.2% of subjects had a mild high homocysteine level, although the mean homocysteine level was 13.85 μmol/L. In the controls, 65.4% subjects had a high homocysteine level, and the mean homocysteine level was 11.60 μmol/L, which was slightly lower than that in the CP group (data not shown in results). As homocysteine levels increases, so did the risk of advanced CP. Several previous studies have shown similar trends, thus strengthening the reliability of our present findings [25, 29]. Our present study found that females had a lower level of homocysteine compared to males (11.44 *versus* 14.82, $P<0.05$); this may be due to the higher trans-methylation rate in women [30, 31].

We observed some positive relationships between C677T and A1298C polymorphisms and the presence of CP under the recessive model after adjustment for age and gender. However, literature regarding the association between MTHFR gene variants and CP remains contradictory. The results of previous studies are summarized in **Table 7**. Briefly, some studies have reported that C677T polymorphism is independently associated with the increased carotid intima-media thickness, CP, or stenosis [15, 16]. In contrast to this standpoint, most studies have shown that the C677T polymorphism had no significant relationship with CP and stenosis in different ethnic populations. The A1298C polymorphism is strongly associated with elevated homocysteine levels [32]. However, literature regarding the association between A1298C polymorphism and CP is sparse parsing. The differences in association between MTHFR gene polymorphisms and CP risk between our study and other reports may be mostly attributed to the different genetic backgrounds of the study cohorts involved. The T allele mutation of C677T was lower, and the CC homozygote of A1298C, was higher in our present study when compared to previous studies. In addition, different confounding factors (e.g., baseline of clinical status, medication consume, lifestyle factors, and diet habits) were adopted in previous studies, which may also have contributed to the observed inconsistencies.

In addition, the established correlation of the MTHFR gene A1298C polymorphism in combination with certain conventional risk factors and CP risk could explain the observed gene-environment interactions. The best model contains four risk factors: diabetes, age, A1298C polymorphism, and smoking. Moreover, MDR consistently incorporated diabetes into different models, followed by age, A1298C polymorphism and smoking. MDR is sensitive to high-order interactions, but cannot explain the main effect of interaction. Therefore, the relative importance of these four risk factors in the etiology of CP formation still remains unknown.

There were several limitations to this study. First, this study was of a clinical case-control design; therefore, it was not impossible to infer the mechanisms underlying the relationships detected. Secondly, marine-derived food has been demonstrated to increase plaque stability by changing the components of the plaques themselves [33]. However, we had no enough data regarding dietary habits and the nutritional status of our subjects. Thirdly, the study was performed with visiting residents aged over 40 years, selection bias potentially exists. Finally, the sample size of the initial study may not be sufficiently large to screen all the potential risk-associated SNPs.

Table 7. Summary of literature on the association between the MTHFR gene and carotid atherosclerosis

Author	Nation	Design	Sample size, n	Population	C677T	CAS
Passaro, et al. [15]	Italy	Cross-sectional	120	Elderly women	yes#	CIMT
Imamoto, et al. [16]	Japan	Cross-sectional	1693 women, 1554 men	General population	Women, yes; Men, no*	CIMT, CP, Stenosis
Chutinet, et al. [17]	Thailand	Case-control	141/167	General population, age >45 years	no	Stenosis
Zuntar, et al. [18]	Croatia	Case-control	95/298	Residents, stenosis >60%	no	Stenosis
Topić, et al. [19]	Croatia	Case-control	36/124	Patients with stenosis	no	Stenosis
Mazza, et al. [34]	Italy	Cross-sectional	276	Hypertensive population	no	CIMT, CP
McQuillan, et al. [35]	Australia	Cross-sectional	1111	Random electoral roll survey	no	CIMT, CP
Spence, et al. [36]	Canada	Cross-sectional	307	Premature atherosclerosis population	no	CP
Bova, et al. [37]	Israel	Case-control	48/26	Asymptomatic severe CAS patients	yes	Stenosis

CAS: carotid atherosclerosis; CIMT: carotid intima-media thickness; CP: carotid plaque. #yes: C677T polymorphism is associated with CAS; *no: C677T polymorphism is not associated with CAS.

In conclusion, our present study shown indicated that the presence of MTHFR gene C677T and A1298C polymorphisms may act as modifiers for CP risk. In addition, combined effects of gene-environment interactions between diabetes, age, A1298C polymorphism, and smoking may promote the progression of CP. Consequently, managing these risk factors is of clinical importance for community residents with atherosclerosis.

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Disclosure of conflict of interest

None.

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