

Inflammation and Endothelial Function Relevant Genetic Polymorphisms and Carotid Plaque in Chinese Population

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Aim: To examine the association between carotid plaque and variants in genes involved in inflammation and endothelial function.

Methods: This was a multicenter, cross-sectional survey in southwestern China. The residents aged ≥ 40 years volunteered to participate in the face-to-face survey in eight communities. A total of 2,377 subjects with high stroke risk were enrolled. Carotid plaque and plaque phenotype were assessed by carotid ultrasound. Genotypes of 19 variants in 10 genes related to inflammation and endothelial function were examined. Gene-gene interaction was analyzed by generalized multifactor dimensionality reduction (GMDR).

Results: Carotid plaques were found in 852 (35.8%) subjects, and 454 (53.3%) had stable plaques, whereas 398 (46.7%) had vulnerable plaques. *PPARA* rs4253655, *HABP2* rs7923349, and *IL1A* rs1609682 were associated with the presence of carotid plaque, and *NOS2A* rs2297518 and *PPARA* rs4253655 were associated with vulnerable plaque in univariate analysis. The GMDR analysis revealed that there was a significant gene-gene interaction among *HABP2* rs7923349, *ITGA2* rs1991013, *IL1A* rs1609682, and *NOS2A* rs8081248, and the high-risk interactive genotype among the four variants was independently associated with a higher risk of carotid vulnerable plaque after adjusting the covariates (OR, 2.86, 95% CI: 1.32–7.13, $P=0.003$).

Conclusion: The prevalence of carotid plaque was very high in the high-risk stroke population in southwestern China. Variants in genes involved in the endothelial function and inflammation were associated with the carotid plaque. The high-risk interactive genotype among rs7923349, rs1991013, rs1609682, and rs8081248 was independently associated with a higher risk of vulnerable plaque.

Key words: High-risk stroke population, Inflammation, Carotid plaque, Plaque vulnerability, Genetic polymorphism

Introduction

Stroke is the leading cause of mortality and adult disability in the world, especially in China¹. Carotid atherosclerosis is associated with an increased risk of stroke, coronary events, and cardiovascular mortality

as a result of both plaque rupture and luminal stenosis²⁻⁴. Carotid atherosclerotic plaque is an important subclinical precursor of stroke and other vascular diseases⁵. Therefore, the identification of etiology for carotid plaque, including genetic etiology, is very important for the prevention of atherosclerosis and

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stroke⁵). However, until now, such a genetic influence on carotid atherosclerotic plaque is not fully understood.

Atherosclerosis is a chronic inflammatory disease⁶. Several different mechanisms play key roles in the pathogenesis of atherosclerosis, including endothelial injury, recruitment and activation of immune-inflammatory cells, influx of lipoproteins through the vessel injury space, and smooth muscle cell proliferation^{6, 7}. In addition, substantial heritability of subclinical carotid atherosclerosis has been reported^{8, 9}. A different risk of atherosclerosis in the population may reflect variants in genes that modulate the inflammatory response to oxidized lipids in the arterial walls⁷. Therefore, genetic variants in inflammation and endothelial function relevant genes may influence metabolism of lipids and affect atherogenesis. Several studies have investigated the association between carotid atherosclerosis and inflammation- and endothelial function-related genetic variants¹⁰⁻¹³. However, information about specific genetic determinants of carotid plaque is lacking, particularly among Chinese populations.

It is known that atherosclerosis as a common complex trait does not follow the Mendelian pattern of inheritance¹⁴. Gene–gene and gene–environment interactions may be responsible for the complex trait^{12, 13}. It has been emphasized that assessment of multiple genes is necessary to identify the genetic mechanisms for carotid plaque using the generalized multifactor dimensionality reduction (GMDR) approach¹⁵. However, few studies used the GMDR method to investigate complex genetic risk factors for carotid plaque. Therefore, the aim of this study was to investigate (1) the prevalence of carotid plaque in high-risk stroke population in southwestern China and (2) the association of 19 single nucleotide polymorphisms (SNPs) in genes involved in inflammation and endothelial function with carotid plaque among individuals in southwestern China.

Materials and Methods

Study Design and Participants

This population-based cross-sectional survey was part of the China National Stroke Screening Survey (CNSSS) program of the National Health and Family Planning Commission of China (grant No. 2011BAI08B01), which was supervised by the Chinese National Centre for Stroke Care Quality Control and Management^{1, 16, 17}. The survey protocol was reviewed and approved by the ethics committee of the participating hospitals (People's Hospital of Deyang City, Suining Central Hospital, the Third Affiliated

Hospital of Wenzhou Medical University, and the Affiliated Hospital of Southwest Medical University), and informed consent was obtained from all participants during recruitment.

This study was conducted in eight communities of Sichuan Province in southwestern China and Wenzhou City from May 2015 to September 2015. The eight communities were randomly selected, and a cluster survey method was used. Details on the organization and implementation of the CNSSS can be found at the official website¹⁷. Briefly, we screened permanent residents aged 40 years or older who had lived in the area for more than 6 months in each community. All participants were initially screened using a structured face-to-face questionnaire by investigators. The questionnaire included demographic characteristics, behavioral factors, personal and family medical history of stroke and chronic diseases, and physical examination. More detailed information of laboratory examinations (such as fasting blood glucose [FBG], lipid, electrocardiogram [ECG], and carotid ultrasonography) was obtained from the individuals who were identified to be at a high risk for stroke. The investigators were physicians or neurologists from community hospitals who had at least 5 years of education in medicine.

Definitions of Stroke and Evaluation of Risk Factors

In this survey, stroke history and stroke types were established by a combination of self-reporting and the judgment of a physician or neurologist according to neuroimaging (brain computed tomography scan and magnetic resonance imaging). By definition, patients with a history of transient ischemic attack only were excluded.

The eight stroke-related risk factors were assessed, including overweight/obesity (body mass index ≥ 26 kg/m²), smoking, physical inactivity (physical exercise less than three times a week for less than 30 min each time), family history of stroke, and hypertension, diabetes mellitus, dyslipidemia, and atrial fibrillation. Hypertension was defined as using antihypertensive drugs or the average of two resting systolic blood pressure readings of ≥ 140 mm Hg and/or diastolic blood pressure ≥ 90 mm Hg in the field survey. Diabetes mellitus was defined as using insulin and/or oral hypoglycemic medications or fasting blood glucose ≥ 7.0 mmol/L in the field survey. Dyslipidemia was defined as using a lipid-lowering medication or having one or more of the following in the field survey: triglycerides ≥ 1.70 mmol/L, cholesterol ≥ 5.18 mmol/L, and low-density lipoprotein cholesterol ≥ 3.37 mmol/L. Atrial fibrillation was defined as reported by the respondent or diagnosed by electro-

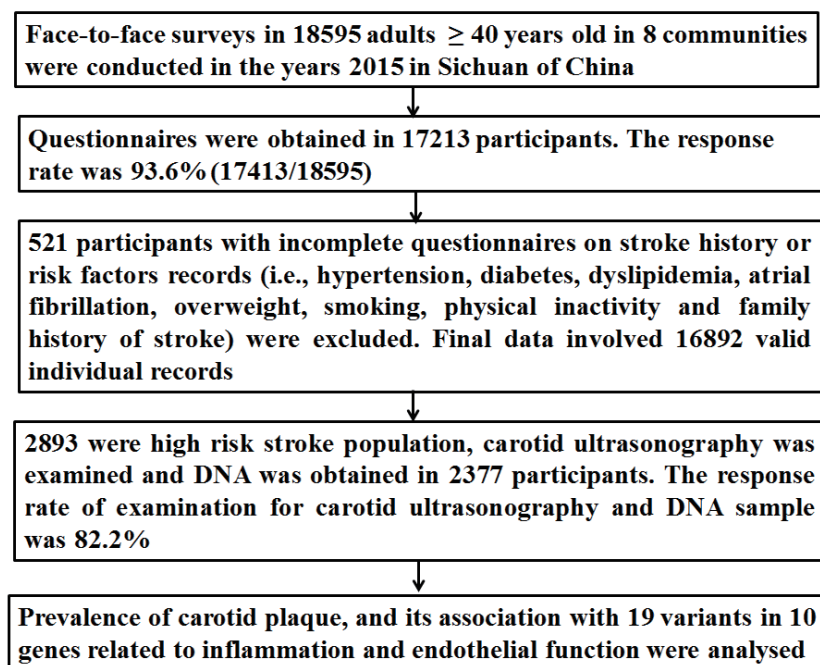


Fig. 1. Data preparing and cleaning process in this survey

cardiogram in the field survey. Subjects with at least three of the aforementioned eight stroke-related risk factors or a history of stroke were classified as the high-risk population for stroke^{17, 18}.

Data Cleaning Procedures and Quality Control

The detailed data cleaning procedure and quality control according to the CNSSS is presented in **Fig. 1**. Briefly, 18595 participants volunteered to participate in the face-to-face survey, and questionnaires were obtained in 17213 participants. The response rate was 93.6% (17413/18595). Five hundred twenty-one participants with incomplete questionnaires on stroke history or risk factor records were excluded. Finally, 16892 valid individual records were completed, including 2893 high-risk stroke population. Among 2893 high-risk stroke population, carotid ultrasonography was examined, and DNA from peripheral blood was obtained in 2377 participants (516 were unwilling to accept examination for carotid ultrasonography and offer DNA sample). The response rate of examination for carotid ultrasonography and DNA sample was 82.2% (2377/2893).

Carotid Ultrasonography

Bilateral common and internal carotid arteries, as well as bifurcations, were assessed using a diagnostic ultrasound (type 512, ACUSON Sequoia Apparatus, 7.5-MHz probe, Berlin, Germany) in 2377 high-risk stroke population according to standard scanning and

reading protocols⁵. Carotid characteristics, including intima-media thickness (IMT), plaque morphology, and degree of carotid stenosis, were evaluated. The detailed procedures for evaluating plaques, types of plaques, degree of carotid stenosis, intraobserver, and interobserver coefficients were described in our previous study^{12, 13, 19}. Briefly, bilateral internal and common carotid arteries, as well as bifurcations, were examined for the presence of atherosclerotic plaque. Atherosclerotic plaque was defined as an endoluminal protrusion of at least 1.5 mm or a focal thickening > 50% of the IMT relative to the adjacent wall segment⁵. Thereafter, the participants were divided into two groups: carotid plaque and non-carotid plaque groups. Furthermore, according to plaque echogenicity and surface structure, carotid plaque was further graded from class I to class IV as echolucent, predominantly echolucent, predominantly echogenic, and echogenic, respectively²⁰. Plaque of class I or class II was defined as vulnerable plaque, and plaque of class III or class IV was defined as stable plaque^{12, 13}. Carotid characteristics were graded independently by one ultrasound imaging doctor blinded to clinical status. The main aim of this study was to investigate the prevalence of carotid plaque in high-risk stroke population and the association of 19 single nucleotide polymorphisms (SNPs) in genes involved in inflammation and endothelial function with carotid plaque. Thus, carotid stenosis and IMT in common carotid artery were not involved in this analysis.

Polymorphism Selection and Genotyping

SNPs in 10 genes involved in inflammation and endothelial function were selected from the NCBI database (<http://www.ncbi.nlm.nih.gov/SNP>), which met the following criteria: (1) these SNPs have been evaluated in previous studies^{10, 21}); (2) SNPs with minor allele frequency >0.05; (3) tagging SNPs across different human populations (<http://pga.gs.washington.edu>); and (4) the SNPs may lead to amino acid changes. According to the criteria, 19 SNPs from 10 genes implicated in inflammation and endothelial function were evaluated, including *PPARA* (rs4253655, rs4253778), *NOS2A* (rs2297518, rs8081248), *TNF* rs3093662, *IL6R* (rs1386821, rs4845625), *TNFSF4* (rs1234313, rs11811788), *TLR4* (rs752998, rs1927911), *IL1A* (rs1800587, rs1609682), *VCAM1* (rs3783615, rs2392221), *ITGA2* (rs1991013, rs4865756), and *HABP2* (rs932650, rs7923349).

DNA from peripheral blood was extracted using a modified phenol/chloroform method and purified using the UNIQ-10 kit (Sangon Biotech Co., Ltd. Shanghai, China). The genotyping of the 19 SNPs was performed by investigators blinded to the clinical data of participants, using the matrix-assisted laser desorption/ionization time of flight mass spectrometry method, as previously described^{12, 13, 19}.

Statistical Analysis

The data were analyzed using SPSS 17.0 (SPSS Inc. New York, New York, USA). The results are expressed as percentages for categorical variables, and continuous variables are expressed as mean \pm standard deviation. Baseline clinical characteristics and genotype distributions of the 19 variants were compared using χ^2 test (categorical variables) and Student's *t*-test (continuous variables) between subjects with and without carotid plaque.

The allele frequencies for Hardy–Weinberg equilibrium were assessed using χ^2 test. The GMDR software (β version 0.7, www.healthsystem.virginia.edu/internet/addiction-genomics/Software) was used to assess gene–gene interaction among the 19 variants under various scenarios as previously reported^{15, 19}. The prevalence of carotid plaque between subjects with and without high-risk interactive genotype was compared by χ^2 test. Furthermore, we evaluated the risk of carotid vulnerable plaque conferred by the high-risk interactive genotype using multivariate logistic regression analysis, and reported as the hazard ratio (HR) with the 95% confidence interval (CI). The other variables that were statistically significant at *P* value <0.05 in the univariate analysis were entered into the multivariate logistic regression analyses to

adjust. All tests were two-sided, and *P* value <0.05 was considered statistically significant.

Results

The Baseline Characteristics of Subjects and Prevalence of Carotid Plaque

Among 2377 participants in the high-risk stroke population, carotid plaque was present in 852 (35.8%) participants, and 454 (53.3%) participants had stable plaque, whereas 398 (46.7%) had vulnerable plaque. As presented in **Table 1**, compared with individuals with no plaque, older age, male, hypertension and intake of antihypertensive drugs, and current smoker were significantly more frequent, and the levels of total cholesterol, fasting blood glucose, and homocysteine were higher in subjects with plaque.

Genotype Distributions in Subjects with and without Carotid Plaque

The genotype distributions of the 19 SNPs assessed in this study were consistent with the Hardy–Weinberg equilibrium (all *P* value >0.05). Three genes involved in endothelial function and inflammation had SNPs significantly associated with the presence of carotid plaque (*PPARA* rs4253655, *HABP2* rs7923349, and *IL1A* rs1609682, **Table 2**), and *NOS2A* rs2297518 and *PPARA* rs4253655 were significantly associated with vulnerable plaque in the single SNP analysis (**Table 3**). However, there were no significant differences in the genotype distributions in the 19 SNPs between individuals with stable plaque and vulnerable plaque (**Supplemental Table 1**), or in individuals with stable plaque and individuals without plaque (**Supplemental Table 2**).

Gene–Gene Interactions of the 19 Variants and Risk of Vulnerable Plaque

We evaluated the relationship between the higher-order interaction for the 19 variants and carotid vulnerable plaque using the GMDR analysis and found that there was a significant gene–gene interaction among genes involved in inflammation and endothelial function. After adjusting for confounding variables, the best interactive model for carotid plaque was interaction among *HABP2* rs7923349, *ITGA2* rs1991013, *IL1A* rs1609682, and *NOS2A* rs8081248, in which the cross-validation consistency was 10/10 and sign test was 9 (*P*=0.017, **Table 4**). Then, the one-locus model was computed for each variant of the four SNPs, and the *P* value for prediction error was 0.019 using permutation testing, indicating that the interaction among the four variants strongly synergistically contributed to a higher risk of

Table 1. Demographic characteristics of the study population

	Carotid plaque (<i>n</i> = 852)	Non-carotid plaque (<i>n</i> = 1,525)	<i>P</i> value
Age (years)	67.1 ± 16.8	61.9 ± 21.7	<0.001
Male (<i>n</i> , %)	422 (49.5)	643 (42.2)	<0.001
Weight (kg)	62.9 ± 15.4	63.5 ± 18.9	0.413
Height (cm)	157.3 ± 16.7	156.8 ± 17.9	0.462
Body mass index (kg/m ²)	26.4 ± 5.2	26.2 ± 5.6	0.411
Hypertension (<i>n</i> , %)	692 (81.2)	1111 (72.9)	<0.001
Diabetes mellitus (<i>n</i> , %)	231 (27.1)	422 (27.7)	0.386
Current smoking (<i>n</i> , %)	349 (41.0)	467 (30.6)	<0.001
History of stroke (<i>n</i> , %)	143 (16.8)	301 (19.7)	0.072
Stroke subtype			
Hemorrhagic	26 (3.1)	71 (4.7)	0.071
Ischemic stroke	117 (13.7)	230 (15.1)	0.574
Atherothromobosis	73 (8.6)	136 (8.9)	-
Cardioembolism	16 (1.9)	29 (1.9)	-
Lacunar	28 (3.3)	65 (4.3)	-
Coronary artery disease (<i>n</i> , %)	77 (9.0)	118 (7.7)	0.289
Atrial fibrillation (<i>n</i> , %)	15 (1.8)	35 (2.3)	0.367
Dyslipidemia (<i>n</i> , %)	291 (34.2)	527 (34.6)	0.972
Total cholesterol (mM)	5.4 ± 1.6	5.2 ± 1.8	0.006
LDL (mM)	3.1 ± 1.2	3.0 ± 1.3	0.072
HDL (mM)	1.6 ± 0.7	1.6 ± 0.8	0.965
Triglycerides (mM)	1.8 ± 0.9	3.5 ± 0.9	<0.001
Fasting blood glucose (mM)	6.6 ± 1.8	6.3 ± 2.1	<0.001
Homocysteine (mM)	13.9 ± 5.5	13.2 ± 5.7	<0.001
Antihypertensive drugs	387 (45.4)	563 (36.9)	<0.001
Antithrombotic medications	140 (16.4)	271 (17.8)	0.435
Statin use	64 (7.5)	110 (7.2)	0.773
Plaque characteristics (<i>n</i> , %)			
Stable plaque	454 (53.3)	-	
Vulnerable plaque	398 (46.7)	-	

LDL, low-density lipoprotein; HDL, high-density lipoprotein;

carotid vulnerable plaque compared with did each single variant alone.

Subsequently, we assessed the association between different genotype combinations of the four variants and risk of carotid vulnerable plaque. Compared with the individuals carrying wild-type genotypes rs7923349 GG, rs1991013 GG, rs1609682 GG, and rs8081248 GG, the relative risk of different genotype combinations among the four variants for vulnerable plaque was investigated. The results revealed that four genotype combinations making larger contributions to carotid vulnerable plaque were those individuals carrying rs7923349 TT, rs1991013 AA, rs1609682 TT, and rs8081248 AA (OR=2.83, 95% CI: 1.26–5.38, *P*=0.005); rs7923349 TT, rs1991013 GA, rs1609682 TT, and rs8081248 GA (OR=2.02, 95% CI: 1.12–4.33, *P*=0.008); rs7923349 GT, rs1991013 AA, rs1609682 GT, and rs8081248AA (OR=1.93,

95% CI: 1.07–3.84, *P*=0.028); and rs7923349 GT, rs1991013 GA, rs1609682 GT, and rs8081248GA (OR=1.85, 95% CI: 1.03–2.98, *P*=0.038). The aforementioned four genotype combinations were defined as the high-risk interactive genotype. The other genotype combinations did not reach statistical significance (*P*>0.05) and were defined as the low-risk interactive genotype.

Association between High-Risk Interactive Genotype and Carotid Vulnerable Plaque

There were 401 carriers of the high-risk interactive genotype among 2377 participants. The incidence of carotid vulnerable plaque was significantly higher in carriers of the high-risk interactive genotypes than in noncarriers (32.7% [131/401] vs. 13.5% [267/1976], *P*<0.001).

Furthermore, we evaluated the risk of the pres-

Table 2. Genotype distribution in individuals with and without carotid plaque (%)

	Carotid plaque (n = 852)	Non-carotid plaque (n = 1,525)	Wald χ^2 value	P value
<i>IL6R</i> (rs4845625)			3.427	0.180
TT	250 (29.4)	405 (26.6)		
CC	182 (21.2)	368 (24.1)		
CT	420 (49.4)	752 (49.3)		
<i>HABP2</i> (rs932650)			2.477	0.289
CT	372 (43.7)	658 (43.1)		
CC	72 (8.5)	159 (10.4)		
TT	408 (47.9)	708 (46.4)		
<i>TLR4</i> (rs1927911)			1.252	0.535
AG	424 (49.8)	740 (48.5)		
AA	135 (15.8)	226 (14.8)		
GG	293 (34.4)	559 (36.7)		
<i>VCAMI</i> (rs3783615)			-	-
AA	852 (100.0)	1525 (100.0)		
<i>PPARA</i> (rs4253778)			0.016	0.898
CG	2 (0.2)	4 (0.3)		
GG	850 (99.8)	1521 (99.7)		
<i>PPARA</i> (rs4253655)			7.172	0.007
AG	4 (0.5)	0 (0.0)		
GG	848 (99.5)	1525 (100)		
<i>VCAMI</i> (rs2392221)			2.560	0.278
CT	205 (24.1)	340 (22.3)		
CC	632 (74.2)	1145 (75.1)		
TT	15 (1.8)	40 (2.6)		
<i>IL1A</i> (rs1800587)			1.645	0.439
AG	121 (14.2)	193 (12.7)		
GG	727 (85.3)	1321 (86.6)		
AA	4 (0.5)	11 (0.7)		
<i>TNFSF4</i> (rs1234313)			2.229	0.328
AG	399 (46.8)	667 (43.7)		
GG	94 (11.0)	185 (12.1)		
AA	359 (42.1)	673 (44.1)		
<i>HABP2</i> (rs7923349)			6.745	0.034
TT	64 (7.5)	53 (3.5)		
GT	325 (38.1)	563 (36.9)		
GG	463 (54.3)	909 (59.6)		
<i>TNFSF4</i> (rs11811788)			0.446	0.800
CG	136 (16.0)	237 (15.5)		
GG	10 (1.2)	14 (0.9)		
CC	706 (82.9)	1274 (83.5)		
<i>TLR4</i> (rs752998)			1.875	0.392
TT	16 (0.019)	39 (2.6)		
GG	605 (71.0)	1059 (69.4)		
GT	231 (27.1)	427 (28.0)		
<i>IL1A</i> (rs1609682)			7.068	0.029
GG	392 (46.0)	641 (42.0)		
GT	378 (44.4)	791 (51.9)		
TT	82 (9.6)	93 (6.1)		
<i>NOS2A</i> (rs8081248)			1.265	0.531
AG	384 (45.1)	666 (43.7)		
AA	84 (9.9)	172 (11.3)		
GG	384 (45.1)	687 (45.0)		

(Cont. Table 2)

	Carotid plaque (n = 852)	Non-carotid plaque (n = 1,525)	Wald χ^2 value	P value
<i>TNF</i> (rs3093662)			0.536	0.464
AG	44 (5.2)	68 (4.5)		
AA	808 (94.8)	1457 (95.5)		
<i>ITGA2</i> (rs1991013)			4.407	0.110
GG	71 (8.3)	160 (10.5)		
AA	391 (45.9)	716 (47.0)		
AG	390 (45.8)	648 (42.5)		
<i>ITGA2</i> (rs4865756)			1.620	0.445
AG	333 (39.1)	556 (36.5)		
GG	464 (54.5)	868 (56.9)		
AA	55 (6.5)	101 (6.6)		
<i>IL6R</i> (rs1386821)			1.739	0.419
GT	58 (6.8)	123 (8.1)		
GG	3 (0.4)	4 (0.3)		
TT	791 (92.8)	1398 (91.7)		
<i>NOS2A</i> (rs2297518)			1.070	0.586
AG	241 (28.3)	409 (26.8)		
AA	20 (2.3)	30 (2.0)		
GG	591 (69.4)	1086 (71.2)		

ence of carotid vulnerable plaque conferred by the high-risk interactive genotype using multivariate logistic regression analysis. The high-risk interactive genotype was assigned as one, and the low-risk interactive genotype was assigned as zero. The other variables that were statistically significant at P value < 0.05 in the univariate analysis were entered into the multivariate logistic regression analyses to adjust. After adjusting the covariates, the high-risk interactive genotype among rs7923349, rs1991013, rs1609682, and rs8081248 was independently associated with a higher risk of carotid vulnerable plaque (OR, 2.86, 95% CI: 1.32–7.13, $P=0.003$, [Table 5](#)).

Discussion

In this study, we have identified a high prevalence of carotid plaque (35.8%) in the high-risk stroke population in southwestern China and found significant associations of three genetic variants (*PPARA* rs4253655, *IL1A* rs1609682, and *NOS2A* rs2297518) in three genes related to inflammation and one variant (*NOS2A* rs2297518) in one gene related to endothelial function with carotid plaque phenotypes in the single SNP analysis. In addition, the GMDR analysis revealed that there was a significant gene–gene interaction among *HABP2* rs7923349, *ITGA2* rs1991013, *IL1A* rs1609682, and *NOS2A* rs8081248, and the high-risk interactive genotype among the four variants was independently associated with a higher risk of carotid vulnerable plaque.

Numerous studies have investigated the association of inflammatory genes and endothelial function relevant genes with ischemic stroke^{22, 23}, but few studies have focused on subclinical atherosclerosis. Carotid plaque is an important subclinical precursor of stroke and other vascular diseases⁵. Certain plaque phenotypes, such as irregular plaques and maximal carotid plaque thickness, may be important markers of vulnerable plaques susceptible to rupture leading to stroke²⁴. To our knowledge, our study is the first to investigate the association between variants in genes related to endothelial function and inflammatory processes and possible markers of vulnerable plaque in Chinese population.

Inflammation plays a key role in increased migration of inflammatory cells and development of atherosclerosis^{6, 7}. The “response to injury” model in atherosclerosis highlights the role of cytokines such as IL-1 β and tumor necrosis factor- α in the response of endothelial cells²⁵. Polymorphisms in inflammatory genes may directly or indirectly interact with vascular risk factors to influence the progression and development of atherosclerosis²⁰. Previous studies have demonstrated overrepresentation of *IL1A* gene in patients with coronary artery disease²⁶. Furthermore, other data and our current results support an association between *IL1A* and carotid plaque²¹. The *IL1A* gene represents a susceptibility factor for the development of carotid atherosclerosis²¹. The *IL1A* alleles may affect the inflammatory environment in the vascular endothelium²⁷. *NOS2A* was associated with the pres-

Table 3. Genotype distribution between vulnerable plaque group and non-plaque group (%)

	Vulnerable plaque (n = 398)	Non- plaque (n = 1,525)	Wald χ^2 value	P value
<i>IL6R</i> (rs4845625)			1.7719	0.4123
TT	113 (28.4)	405 (26.6)		
CC	85 (21.4)	368 (24.1)		
CT	200 (50.3)	752 (49.3)		
<i>HABP2</i> (rs932650)			0.1649	0.9209
CT	170 (42.5)	658 (43.1)		
CC	40 (10.0)	159 (10.4)		
TT	188 (47.5)	708 (46.4)		
<i>TLR4</i> (rs1927911)			1.3796	0.5017
AG	202 (50.7)	740 (48.5)		
AA	63 (15.8)	226 (14.8)		
GG	133 (33.5)	559 (36.7)		
<i>VCAM1</i> (rs3783615)				
AA	398 (100.0)	1525 (100.0)		
<i>PPARA</i> (rs4253778)			0.0018	0.9657
CG	1 (0.2)	4 (0.3)		
GG	397 (99.8)	1521 (99.7)		
<i>PPARA</i> (rs4253655)			11.4554	0.007
AG	3 (0.8)	0 (0.0)		
GG	395 (99.2)	1525 (100)		
<i>VCAM1</i> (rs2392221)			1.1946	0.5503
CT	98 (24.6)	340 (22.3)		
CC	291 (73.1)	1145 (75.1)		
TT	9 (2.3)	40 (2.6)		
<i>IL1A</i> (rs1800587)			2.6445	0.2665
AG	62 (15.6)	193 (12.7)		
GG	333 (83.7)	1321 (86.6)		
AA	3 (0.8)	11 (0.7)		
<i>TNFSF4</i> (rs1234313)			1.526	0.4663
AG	188 (47.2)	667 (43.7)		
GG	43 (10.8)	185 (12.1)		
AA	167 (42.0)	673 (44.1)		
<i>HABP2</i> (rs7923349)			4.0035	0.1351
TT	33 (8.3)	53 (3.5)		
GT	154 (38.7)	563 (36.9)		
GG	211 (53.0)	909 (59.6)		
<i>TNFSF4</i> (rs11811788)			2.2025	0.3325
CG	65 (16.3)	237 (15.5)		
GG	7 (1.8)	7 (1.8)		
CC	326 (81.9)	326 (81.9)		
<i>TLR4</i> (rs752998)			2.5483	0.2797
TT	6 (1.5)	6 (1.5)		
GG	276 (69.3)	276 (69.3)		
GT	116 (29.1)	116 (29.1)		
<i>IL1A</i> (rs1609682)			0.4909	0.7824
GG	183 (46.0)	183 (46.0)		
GT	174 (43.7)	174 (43.7)		
TT	41 (10.3)	41 (10.3)		
<i>NOS2A</i> (rs8081248)			1.6235	0.4441
AG	170 (42.7)	170 (42.7)		
AA	38 (9.5)	38 (9.5)		
GG	190 (48.7)	190 (48.7)		

(Cont. Table 3)

	Vulnerable plaque (n = 398)	Non- plaque (n = 1,525)	Wald χ^2 value	P value
<i>TNF</i> (rs3093662)			0.0068	0.9345
AG	19 (4.8)	19 (4.8)		
AA	379 (95.2)	379 (95.2)		
<i>ITGA2</i> (rs1991013)			1.1302	0.5683
GG	36 (9.0)	36 (9.0)		
AA	183 (46.0)	183 (46.0)		
AG	179 (45.0)	179 (45.0)		
<i>ITGA2</i> (rs4865756)			0.9263	0.6293
AG	153 (38.4)	153 (38.4)		
GG	223 (56.0)	223 (56.0)		
AA	22 (5.5)	22 (5.5)		
<i>IL6R</i> (rs1386821)			2.1854	0.3353
GT	26 (6.5)	26 (6.5)		
GG	2 (0.5)	2 (0.5)		
TT	370 (93.0)	370 (93.0)		
<i>NOS2A</i> (rs2297518)			7.3901	0.0375
AG	119 (29.9)	119 (29.9)		
AA	8 (2.0)	8 (2.0)		
GG	271 (68.1)	271 (68.1)		

Table 4. GMDR analysis of the best models, prediction accuracies, cross-validation consistencies, and P values for carotid vulnerable plaque

Best model*	Training balanced accuracy	Testing balanced accuracy	Cross- validation consistency	Sign test (P value)
1	0.453	0.386	6/10	5 (0.635)
1,2	0.531	0.494	7/10	7 (0.347)
1, 2, 3	0.608	0.597	9/10	9 (0.082)
1, 2, 3, 4	0.642	0.626	10/10	9 (0.017)
1, 2, 3, 4, 5	0.359	0.572	7/10	7 (0.685)
1, 2, 3, 4, 5, 6	0.467	0.546	8/10	6 (0.721)
1, 2, 3, 4, 5, 6, 7	0.398	0.435	7/10	6 (0.796)
1, 2, 3, 4, 5, 6, 7, 8	0.612	0.625	9/10	8 (0.267)
1, 2, 3, 4, 5, 6, 7, 8, 9	0.513	0.524	8/10	7 (0.535)
1, 2, 3, 4, 5, 6, 7, 8, 9, 10	0.596	0.537	5/10	6 (0.689)
1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11	0.572	0.499	8/10	8 (0.267)
1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12	0.515	0.488	6/10	7 (0.496)
1, 2,3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13	0.433	0.397	5/10	6 (0.734)
1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14	0.582	0.605	8/10	6 (0.317)
1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15	0.631	0.538	7/10	7 (0.296)
1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16	0.382	0.418	4/10	6 (0.698)
1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17	0.507	0.553	5/10	5 (0.723)
1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18	0.468	0.486	7/10	7 (0.467)
1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19	0.523	0.545	8/10	8 (0.485)

GMDR, generalized multifactor dimensionality reduction

*Numbers 1-19 represent rs7923349, rs1991013, rs1609682, rs8081248, rs4253655, rs4253778, rs2297518, rs3093662, rs1386821, rs4845625, rs1234313, rs11811788, rs752998, rs1927911, rs1800587, rs3783615, rs2392221, rs4865756, and rs932650, respectively.

ence of plaque¹⁰), and this was in accordance with our current results. *NOS2A* gene regulates inducible nitric oxide synthase to produce nitric oxide and is involved in vascular tone regulation, immune response, and

neurotransmission. In humans, inducible nitric oxide synthase has been observed in the core of carotid plaques, and its inhibitor can slow the development and progression of atherosclerosis in experimental rab-

Table 5. Multivariate analysis of the major risk factors for vulnerable plaque

Risk factor	OR*	95% CI	P value
Age	1.75	1.07-4.58	0.042
Male	0.89	0.76-1.66	0.368
Hypertension	2.01	1.08-6.68	0.019
Current smoking	1.87	1.16-5.76	0.028
Total cholesterol	1.76	1.13-5.14	0.033
Triglycerides	0.89	0.83-1.99	0.432
Fasting blood glucose	1.21	0.92-2.57	0.235
Homocysteine	1.32	0.95-2.89	0.132
<i>PPARA</i> rs4253655AG	0.86	0.73-1.64	0.553
<i>HABP2</i> rs7923349TT	1.13	0.99-4.02	0.186
<i>IL1A</i> rs1609682TT	1.08	0.95-3.48	0.232
High-risk interactive genotype	2.86	1.32-7.13	0.003

OR, odds ratios; CI, confidence interval.

*OR for continuous variables means per 1- Standard Deviation increase.

bits²⁸). One study from carotid endarterectomy specimens revealed that the non-ruptured plaques had inducible nitric oxide synthase mRNA and protein, whereas the ruptured plaques did not, indicating that inducible nitric oxide synthase might contribute to the carotid plaque instability²⁹). *PPARA* has been shown to affect lipid metabolism and oxidative stress, and association between *PPARA* SNPs and myocardial infarction has been reported³⁰). However, few studies to investigate the potential relationship between *PPARA* and plaque. In a cohort of Finnish men, *PPARA* polymorphisms were associated with the progression of coronary atherosclerosis³¹). In this study, *PPARA* gene was associated with the presence of carotid plaque, implying its important role in different stages of atherosclerotic plaque.

HABP2 gene encodes a protein involved in cell adhesion and regulates vascular integrity. *HABP2* is observed to affect vascular smooth muscle cell proliferation and atherosclerotic plaque vulnerability³²). A *HABP2* SNP was associated with carotid stenosis progression³³). A significant association between *HABP2* variants and venous thromboembolic disease has also been reported³⁴). *ITGA2* regulates cell adhesion and cell surface-mediated signaling. Its polymorphisms have been associated with the risk of ischemic stroke and carotid IMT and plaque in patients with type 2 diabetes^{35, 36}). An *ITGA2* SNP (rs1991013) was related with carotid calcified plaque, a surrogate measure of an increased risk of carotid atherosclerosis¹⁰).

Besides these inspiring findings, we evaluated the relationship of gene-gene interaction among the 19 variants with carotid vulnerable plaque using the GMDR analysis. The most noteworthy finding in this study was that there was a significant gene-gene interaction among *HABP2* rs7923349, *ITGA2* rs1991013,

IL1A rs1609682, and *NOS2A* rs8081248, and the high-risk interactive genotype among the four variants was independently associated with a higher risk of carotid vulnerable plaque, indicating that interaction among the four variants synergistically contributes to vulnerable plaque. The GMDR analysis underscores the complex nature of the genetic effects and the potentially synergistic role of variants in conferring an increased risk of carotid vulnerability. Furthermore, previous studies have also examined the role of various genes in plaque etiology^{12, 19}).

It has become more evident that many common phenotypes are polygenic in nature. It is necessary to evaluate gene-gene interaction when studying the genetic etiology of plaque phenotypes³⁷). However, the nature of the gene-gene interactions among the four variants assessed in this study is not clear. As is known to all, atherosclerosis is a complex inflammatory disease. Endothelial injury, recruitment and activation of immune-inflammatory cells, influx of lipoproteins through the vessel injury space, and smooth muscle cell proliferation play important roles in the pathogenesis mechanisms of atherosclerosis^{6, 7}). A number of studies have explored variants in *HABP2*, *ITGA2*, *IL1A*, and *NOS2A* are associated with inflammation and endothelial function^{10, 29, 31, 33, 36}). Thus, one possible explanation for the interaction among four variants is that the four genes encode and regulate for inflammation and endothelial function relevant enzymes that participate in the principal pathogenic mechanisms of atherosclerosis. However, further studies are necessary in future. In the next study, we will plan to use the primary cultured neurons or animal models to explain the molecular mechanisms of interaction among the four variants.

Despite our inspiring findings, this study has sev-

eral limitations. First, this study only sampled residents aged ≥ 40 years; therefore, our results cannot be generalized to all population groups in southwestern China. Second, this study was a cross-sectional study, and recall bias may exist due to the self-reported questionnaire. Third, carotid plaque and plaque vulnerability were evaluated by ultrasound. Although ultrasound can identify carotid plaques and determine the extent of stenosis, high-resolution magnetic resonance imaging (HR-MRI) may provide more information regarding plaque composition and morphology³⁸. Thus, it is necessary to assess carotid plaque using HR-MRI and confirm our current findings in future. In addition, the main aim of this study was to evaluate the association of 19 SNPs in genes involved in inflammation and endothelial function with carotid plaque. Thus, carotid stenosis and IMT in the common carotid artery were not involved in this analysis. Fourth, although we examined the role of several known important genes involved in endothelial function and inflammation, other known and unknown genes were not captured in this study. Future studies involving a larger set of genetic variants should be conducted to elucidate further the gene–gene interaction effects on plaque phenotypes. Finally, anti-inflammatory drugs (i.e., statins) and antiplatelet drugs may affect carotid plaque characteristics. In this study, we did not investigate the effect of statins or antiplatelet drugs on carotid plaque stability. Furthermore, a lack of an independent sample for replication was also a limitation in this study.

In conclusion, the prevalence of carotid plaque was very high in the high-risk stroke population in southwestern China. Variants in genes involved in endothelial function and inflammation were associated with carotid plaque and plaque vulnerability in the single SNP analysis. The GMDR analysis revealed that there was a significant gene–gene interaction among *HABP2* rs7923349, *ITGA2* rs1991013, *IL1A* rs1609682, and *NOS2A* rs8081248. The high-risk interactive genotype among the four variants was independently associated with a higher risk of carotid vulnerable plaque and might be potential markers for plaque vulnerability. The GMDR analysis may provide further insight into the complex genetic etiology of carotid plaque vulnerability. However, further studies are needed to validate our findings.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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Supplemental Table 1. Genotype distribution comparison between stable plaque and vulnerable plaque (%)

	Stable plaque (454)	vulnerable plaque (398)	Wald χ^2 value	<i>P</i> value
rs4845625			0.424	0.809
TT	137 (30.2)	113 (28.4)		
CT	220 (48.5)	200 (50.3)		
CC	97 (21.4)	85 (21.4)		
rs932650			2.1474	0.3418
TT	220 (48.2)	188 (47.5)		
CC	32 (7.3)	40 (10.0)		
CT	202 (44.5)	170 (42.5)		
rs1927911			0.3481	0.8403
AA	72 (15.9)	63 (15.8)		
GG	160 (35.2)	133 (33.5)		
AG	222 (48.9)	202 (50.7)		
rs3783615				
AA	454 (100.0)	398 (100.0)		
rs4253778			0.0089	0.9248
CG	1 (0.2)	1 (0.2)		
GG	453 (99.8)	397 (99.8)		
rs4253655			1.2955	0.255
AG	1 (0.2)	3 (0.8)		
GG	453 (99.8)	395 (99.2)		
rs2392221			1.302	0.5215
CC	341 (75.1)	291 (73.1)		
CT	107 (23.6)	98 (24.6)		
TT	6 (1.3)	9 (2.3)		
rs1800587			2.5591	0.2782
AG	59 (13.0)	62 (15.6)		
GG	394 (86.8)	333 (83.7)		
AA	1 (0.2)	3 (0.8)		
rs1234313			0.0931	0.9545
AG	211 (46.5)	188 (47.2)		
AA	192 (42.3)	167 (42.0)		
GG	51 (11.2)	43 (10.8)		
rs7923349			0.9447	0.6235
GT	171 (37.7)	154 (38.7)		
TT	31 (6.8)	33 (8.3)		
GG	252 (55.5)	211 (53.0)		
rs11811788			2.2837	0.3192
GG	3 (0.7)	7 (1.8)		
CG	71 (15.6)	65 (16.3)		
CC	380 (83.7)	326 (81.9)		
rs752998			2.5488	0.2796
TT	10 (0.022)	6 (1.5)		
GG	329 (0.724)	276 (69.3)		
GT	115 (0.254)	116 (29.1)		
rs1609682			0.6008	0.7405
GT	204 (44.9)	174 (43.7)		
TT	41 (9.0)	41 (10.3)		
GG	209 (46.0)	183 (46.0)		

(Cont. Supplemental Table 1)

	Stable plaque (454)	vulnerable plaque (398)	Wald χ^2 value	<i>P</i> value
rs8081248			2.4557	0.2929
AG	214 (47.1)	170 (42.7)		
AA	46 (10.1)	38 (9.5)		
GG	194 (42.7)	190 (48.7)		
rs3093662			0.4506	0.502
AG	25 (5.5)	19 (4.8)		
AA	429 (94.5)	379 (95.2)		
rs1991013			0.5878	0.7454
AG	211 (46.5)	179 (45.0)		
GG	35 (7.7)	36 (9.0)		
AA	208 (45.8)	183 (46.0)		
rs4865756			1.4549	0.4831
AA	33 (0.072)	22 (5.5)		
GG	241 (0.532)	223 (56.0)		
AG	180 (0.396)	153 (38.4)		
rs1386821			0.5624	0.7549
GT	32 (7.0)	26 (6.5)		
TT	421 (92.7)	370 (93.0)		
GG	1 (0.2)	2 (0.5)		
rs2297518			1.2464	0.5362
GG	320 (70.5)	271 (68.1)		
AA	12 (2.6)	8 (2.0)		
AG	122 (26.9)	119 (29.9)		

Supplemental Table 2. Genotype distribution between stable plaque group and non-plaque group (%)

	Stable plaque (n = 454)	Non- plaque (n = 1,525)	Wald χ^2 value	P value
<i>IL6R</i> (rs4845625)			3.1825	0.2037
TT	137 (30.2)	405 (26.6)		
CC	97 (21.4)	368 (24.1)		
CT	220 (48.5)	752 (49.3)		
<i>HABP2</i> (rs932650)			4.1404	0.1262
CT	202 (44.5)	658 (43.1)		
CC	32 (7.3)	159 (10.4)		
TT	220 (48.2)	708 (46.4)		
<i>TLR4</i> (rs1927911)			0.4055	0.8165
AG	222 (48.9)	740 (48.5)		
AA	72 (15.9)	226 (14.8)		
GG	160 (35.2)	559 (36.7)		
<i>VCAM1</i> (rs3783615)				
AA	454 (100.0)	1525 (100.0)		
<i>PPARA</i> (rs4253778)			0.0264	0.8709
CG	1 (0.2)	4 (0.3)		
GG	453 (99.8)	1521 (99.7)		
<i>PPARA</i> (rs4253655)			3.3387	0.0677
AG	1 (0.2)	0 (0.0)		
GG	453 (99.8)	1525 (100)		
<i>VCAM1</i> (rs2392221)			2.8784	0.2371
CT	107 (23.6)	340 (22.3)		
CC	341 (75.1)	1145 (75.1)		
TT	6 (1.3)	40 (2.6)		
<i>IL1A</i> (rs1800587)			1.5282	0.4658
AG	59 (13.0)	193 (12.7)		
GG	394 (86.8)	1321 (86.6)		
AA	1 (0.2)	11 (0.7)		
<i>TNFSF4</i> (rs1234313)			1.0144	0.6022
AG	211 (46.5)	667 (43.7)		
GG	51 (11.2)	185 (12.1)		
AA	192 (42.3)	673 (44.1)		
<i>HABP2</i> (rs7923349)			0.6553	0.7206
TT	31 (6.8)	53 (3.5)		
GT	171 (37.7)	563 (36.9)		
GG	252 (55.5)	909 (59.6)		
<i>TNFSF4</i> (rs11811788)			0.2909	0.8646
CG	71 (15.6)	237 (15.5)		
GG	3 (0.7)	14 (0.9)		
CC	380 (83.7)	1274 (83.5)		
<i>TLR4</i> (rs752998)			1.52	0.4677
TT	10 (0.022)	39 (2.6)		
GG	329 (0.724)	1059 (69.4)		
GT	115 (0.254)	427 (28.0)		
<i>IL1A</i> (rs1609682)			1.108	0.5746
GG	209 (46.0)	641 (42.0)		
GT	204 (44.9)	791 (51.9)		
TT	41 (9.0)	93 (6.1)		

(Cont. Supplemental Table 2)

	Stable plaque (n = 454)	Non- plaque (n = 1,525)	Wald χ^2 value	P value
<i>NOS2A</i> (rs8081248)			1.6624	0.4355
AG	214 (47.1)	666 (43.7)		
AA	46 (10.1)	172 (11.3)		
GG	194 (42.7)	687 (45.0)		
<i>TNF</i> (rs3093662)			0.9554	0.3284
AG	25 (5.5)	68 (4.5)		
AA	429 (94.5)	1457 (95.5)		
<i>ITGA2</i> (rs1991013)			4.1734	0.1241
GG	35 (7.7)	160 (10.5)		
AA	208 (45.8)	716 (47.0)		
AG	211 (46.5)	648 (42.5)		
<i>ITGA2</i> (rs4865756)			2.0025	0.3674
AG	180 (0.396)	556 (36.5)		
GG	241 (0.532)	868 (56.9)		
AA	33 (0.072)	101 (6.6)		
<i>IL6R</i> (rs1386821)			0.5635	0.7545
GT	32 (7.0)	123 (8.1)		
GG	1 (0.2)	4 (0.3)		
TT	421 (92.7)	1398 (91.7)		
<i>NOS2A</i> (rs2297518)			0.7359	0.6921
AG	122 (26.9)	409 (26.8)		
AA	12 (2.6)	30 (2.0)		
GG	320 (70.5)	1086 (71.2)		