Original Article

CYP Genetic Variants, CYP Metabolite Levels, and Symptomatic Carotid Stenosis in Ischemic Stroke Patients

Xingyang Yi¹, Duanxiu Liao¹, Lang Wu², Hong Chen¹, Jie Li¹ and Chun Wang¹

Xingyang Yi, Duanxiu Liao and Lang Wu contributed equally to this work

¹Department of Neurology, The People's Hospital of Deyang City, Sichuan, China

²Division of Epidemiology, Department of Medicine, Vanderbilt Epidemiology Center, Vanderbilt University School of Medicine, Tennessee, USA

Aim: To evaluate the relationship between CYP genetic polymorphisms and CYP metabolite levels with carotid artery stenosis in acute ischemic stroke (IS) patients.

Methods: Eleven single nucleotide polymorphisms (SNPs) of seven CYP genes were genotyped in 136 IS patients with carotid stenosis and 158 patients without carotid stenosis. CYP plasma metabolite levels [20-hydroxyeicosatetraenoic acid (HETE), total epoxyeicosatrienoic acids (EETs), and dihydroxyeicosatrienoic acids (DiHETEs)] were assessed in a subsample of 90 patients with carotid stenosis and 96 patients without carotid stenosis. We evaluated the relationship between assessed variants and carotid stenosis risk, variants with CYP metabolite levels, and variants in mediating the differences of CYP metabolite levels between patients with carotid stenosis and those without. Additionally, gene–gene interactions were analyzed to assess the interactive role of the assessed variants in affecting CYP metabolite levels and risk of carotid stenosis.

Results: The genotypes of rs17110453CC, rs751141GG, and rs9333025GG were significantly associated with carotid stenosis risk. Also these polymorphisms were associated with CYP plasma metabolite levels in patients with carotid stenosis. There was a significant gene–gene interaction between rs17110453 and rs9333025 in affecting the risk of carotid stenosis. Patients with rs17110453CC and rs9333025GG had a significantly higher risk of carotid stenosis than those with rs17110453AA and rs9333025AA (OR=2.12, 95% CI: 1.13–7.26, $P=0.013$).

Conclusions: Specific CYP450 gene SNPs and their interactions are associated with CYP450 plasma metabolite levels, which may partially explain their associations with carotid stenosis. Further studies are needed to validate our findings.

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Key words: Carotid atherosclerosis, Carotid stenosis, Cytochrome P450, Single nucleotide polymorphisms, GMDR

Introduction

Approximately, 8% of the general population have relatively severe stenosis of the internal carotid arteries (ICA)¹, which demonstrates an increased risk

of developing stroke². Interestingly, research has also shown that only a proportion of stroke patients have ICA stenosis³, suggesting a more complicated relationship between carotid stenosis and stroke. The impact of significant carotid stenosis on stroke recurrence and cardiovascular outcomes after an ischemic stroke (IS) has been well studied and its value is well-established in stroke populations⁴⁻⁶. Thus, it is critical to clarify the etiology of carotid stenosis within the context of stroke, including the genetic etiology. Further understanding of the roles of genetic factors in

Address for correspondence: Duanxiu Liao, Department of Neurology, The People's Hospital of Deyang City, 173, North Taishan Road, Deyang, Sichuan 618000, China
E-mail: 1842942576@qq.com

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influencing the risk of carotid stenosis can provide valuable insights into the pathogenesis of this disease, which can have implications for preventing stroke and stroke recurrence. However, to date such a genetic etiology has not been satisfactorily understood.

Genes involved in inflammation and endothelial function have been suggested to be involved in the pathogenesis of atherosclerosis^{7, 8}. Arachidonic acid (AA) and metabolites of 20-hydroxyeicosatetraenoic acid (20-HETE) and epoxyeicosatrienoic acids (EETs) have inflammatory activities⁹. AA can be metabolized by CYP ω -hydroxylase into 20-HETE¹⁰ or by CYP epoxigenases into four EETs, which can then be metabolized by soluble epoxide hydrolase (sEH) to yield less biologically active dihydroxyeicosatrienoic acids (DiHETEs)¹¹. A study has suggested an association between these metabolite levels with stroke risk¹². However, till date few studies have assessed the association between these AA metabolites and carotid artery stenosis. To evaluate such a relationship is critical for understanding the etiology of carotid stenosis within the context of stroke.

Several genes have been involved in the AA metabolism pathway. For example, *CYP4A11* and *CYP4F2* genes encode CYP ω -hydroxylase; the CYP 2, 3 gene family encodes majority of epoxigenase enzymes; and the epoxide hydrolase-2 (*EPHX2*) gene encodes sEH. Some studies have shown that genetic variants of relevant genes are associated with their metabolite levels, an increased blood pressure, and a higher risk of stroke¹³⁻¹⁸. However, few studies have assessed the relationship between these genetic variants, CYP metabolite levels, and the risk of carotid stenosis in stroke patients.

Aim

There was a significant association of carotid stenosis with stroke recurrence and cardiovascular outcomes after IS⁴⁻⁶. It is critical to further understand the risk of carotid stenosis in the context of stroke, including the genetic etiology, for developing further strategies for preventing stroke and stroke recurrence. Therefore, the aim of the present study was to evaluate whether CYP gene polymorphisms and their plasma metabolite levels are associated with severe carotid artery stenosis in patients with IS. We also assessed potential gene–gene interaction effects in modulating the associations.

Materials and Methods

Study Population

In the current study, we studied 136 consecutive

acute IS patients with significant ($\geq 50\%$) symptomatic carotid stenosis and 158 acute IS patients with no carotid stenosis using carotid ultrasound examination. These subjects suffered from first IS stroke, were admitted within 3 days of the index stroke onset, and received medical care in the People's Hospital of Deyang City between August 2011 and March 2013. The diagnosis of IS was confirmed on the basis of both clinical findings and neurological examinations using computerized tomography or magnetic resonance imaging. Patients were defined as with symptomatic carotid artery stenosis if they experienced an ipsilateral (carotid territory) IS. Exclusion criteria included: (i) patients with atrial fibrillation or other cardiac sources of emboli; (ii) IS caused by unknown factors; (iii) asymptomatic carotid artery stenosis; (iv) patients that are biologically related to each other.

Patients without carotid stenosis were selected from consecutive IS patients during the same time period (between August 2011 and March 2013). Patients without carotid stenosis were defined as no carotid stenosis ($< 15\%$ stenosis)¹⁹. Exclusion criteria included: (i) symptomatic or asymptomatic carotid stenosis ($\geq 50\%$); (ii) patients with $15\%–49\%$ stenosis¹⁹; (iii) patients with atrial fibrillation or other cardiac sources of emboli; (iv) IS caused by unknown factors; (v) any relatedness between IS patients.

Standard Protocol Approvals, Registrations, and Patient Consents

The protocol of this study was reviewed and approved by the Ethics Committee of The People's Hospital of Deyang City. All patients provided written informed consent before enrollment into this study.

Echocardiography and Holter monitoring examinations were performed on all patients. Vascular risk factors including body mass index (BMI), tobacco smoking history, diabetes mellitus, and hypertension were collected. Fasting blood samples were collected for assessing blood sugar, total plasma cholesterol (TC), triglycerides (TG), and low-density lipoprotein cholesterol (LDL-C).

Carotid Ultrasonography

We performed duplex scans in duplicate with the equipment of Acuson Sequoia Apparatus type 512 (7.5-MHz probe, Berlin, Germany). The degree of stenosis was evaluated using color duplex imaging and power mode, for both longitudinal and transverse sections. The morphological lumen reduction assessment in diameter was combined with peak systolic velocities at the location of the stenosis as well as the internal carotid artery/common carotid artery ratio. The fol-

Table 1. Characteristics of ischemic stroke patients with or without carotid stenosis

Characteristics	carotid stenosis (<i>n</i> = 136)	Non-carotid stenosis (<i>n</i> = 158)	<i>P</i> value
Age (years)	68.86 ± 12.23	67.98 ± 11.82	0.618
Men (<i>n</i> , %)	82 (60.29)	93 (58.86)	0.592
Diabetes mellitus (<i>n</i> , %)	47 (34.56)	52 (32.91)	0.487
Hypertension (<i>n</i> , %)	110 (80.88)	127 (80.38)	0.998
Previous MI (<i>n</i> , %)	2 (1.47)	3 (1.89)	0.992
Previous angina pectoris	3 (2.21)	3 (1.89)	0.982
Cigarette smoking (<i>n</i> , %)	55 (40.44)	62 (39.24)	0.942
Body mass index (kg/m ²)	24.08 ± 2.36	23.95 ± 2.56	0.926
Rates of dyslipidemia (<i>n</i> , %)	75 (55.15)	80 (50.63)	0.473
TC (mM)	5.76 ± 1.56	5.62 ± 1.32	0.786
LDL-C (mM)	3.15 ± 1.27	2.99 ± 1.19	0.475
HDL-C (mM)	1.23 ± 0.51	1.21 ± 0.45	0.722
TG (mM)	1.96 ± 1.12	1.83 ± 1.14	0.628
Previous treatments (<i>n</i> , %)			
Antihypertensive drugs	61 (55.45)	70 (55.12)	0.998
Hypoglycemic drugs	19 (40.43)	22 (42.31)	0.998
Statins	29 (21.32)	33 (20.89)	0.998
Antiplatelet drugs	41 (30.15)	48 (30.38)	0.998

MI, myocardial infarction; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides

lowing values were used to evaluate the degree of stenosis: 50%–69% with peak systolic velocities of 120–220 cm/s and internal carotid artery/common carotid artery ratio of 1.5–3.7; and 70%–99% with peak systolic velocities of >220 cm/s and internal carotid artery/common carotid artery ratio of >3.7²⁰). To assess the measurement reproducibility of the degree of stenosis in the current study, for 28 randomly selected patients, the analyses of the degree of stenosis were performed twice using an investigator (Jie Li), and subsequently using another sonographer (Hong Chen), after which the consistency was determined. The coefficients of intra-observer and inter-observer variations for the degree of stenosis were 7.4% and 7.8%, respectively, suggesting relatively reliable measurements in the current study. One of the investigators (Jie Li) then assessed all patients. These duplex-based stenosis groups were also compared with 80 carotid arteries blindly measured on conventional angiographies according to North American Symptomatic Carotid Endarterectomy Trial (NASCET) criteria²¹). Exact agreement was found in 85% of the cases, and there were no major (>1 stenosis group up or down) disagreements.

Genotyping

Eleven SNPs of seven CYP genes (**Supplementary**

Table 1), including *CYP2J2* (rs10889160), *CYP2C8* (rs17110453, rs1934980), *CYP2C9* (rs1799853, rs1057910), *CYP3A5* (rs776746), *EPHX2* (rs751141), *CYP4F2* (rs3093135, rs2108622), and *CYP4A11* (rs2269231, rs9333025), were selected according to the following criteria: (i) Minor allele frequency >0.05 (<http://www.ncbi.nlm.nih.gov/SNP>); (ii) nonsynonymous variants; (iii) SNPs that have been implicated in previous studies^{13–16}).

Whole blood (3 ml) collection, genomic DNA extraction, and genotyping were performed as previously described¹³. Each assessed SNP was designed with two amplification primers and one extension primer (**Supplementary Table 1**).

Measurement of Plasma 20-HETE, EETs, and DiHETEs Levels

A blood sample (4 ml) was collected into EDTA/BHT (butylated hydroxytoluene)/glutathione at the second day after admission, and plasma was isolated following centrifugation and samples were stored at –80°C until analysis. CYP450 plasma metabolite levels were measured in 186 consecutive IS patients (90 patients with carotid stenosis and 96 patients without carotid stenosis) between August 2011 and March 2012. Plasma 20-HETE level was analyzed using a stable isotope dilution gas chromatography/mass spectrometry

Table 2. Genotype distribution of patients with or without carotid stenosis

	carotid stenosis (<i>n</i> = 136)	Non-carotid stenosis (<i>n</i> = 158)	<i>P</i> value	OR [†] (95% CI)
rs10889160				
AA, n (%)	103 (75.74)	121 (76.58)	0.826	0.96 (0.56-1.75)
AG, n (%)	32 (23.52)	35 (22.15)		
GG, n (%)	1 (0.74)	2 (1.27)		
rs17110453				
AA, n (%)	52 (38.23)	76 (48.10)	<0.001	2.28 (1.31-5.42)
AC, n (%)	49 (36.03)	69 (43.67)		
CC, n (%)	35 (25.74)	13 (8.23)		
rs1934980				
CC, n (%)	21 (15.44)	23 (14.56)	0.894	1.03 (0.66-2.01)
CT, n (%)	61 (44.85)	71 (44.93)		
TT, n (%)	54 (39.71)	64 (40.51)		
rs1799853				
CC, n (%)	136 (100)	158 (100)	–	–
rs1057910				
AA, n (%)	122 (89.71)	143 (90.51)	0.768	1.06 (0.52-2.23)
AC, n (%)	12 (8.82)	12 (7.59)		
CC, n (%)	2 (1.47)	3 (1.90)		
rs776746				
AA, n (%)	11 (8.09)	24 (15.19)	0.042	1.36 (0.98-3.75)
AG, n (%)	47 (34.56)	65 (41.14)		
GG, n (%)	78 (57.35)	69 (43.67)		
rs751141				
GG, n (%)	99 (72.79)	86 (54.43)	0.007	3.01 (1.18-7.06)
AG, n (%)	34 (25.00)	65 (41.14)		
AA, n (%)	3 (2.21)	7 (4.43)		
rs2269231				
AA, n (%)	28 (20.59)	28 (17.72)	0.658	0.95 (0.52-1.89)
AT, n (%)	73 (53.68)	86 (54.43)		
TT, n (%)	35 (25.73)	44 (27.85)		
rs9333025				
AA, n (%)	2 (1.47)	4 (2.53)	0.006	1.97 (1.22-6.16)
AG, n (%)	22 (16.18)	51 (32.28)		
GG, n (%)	112 (82.35)	103 (65.19)		
rs3093135				
TT, n (%)	115 (84.56)	129 (81.65)	0.766	1.01 (0.51-2.12)
AT, n (%)	19 (13.97)	25 (15.82)		
AA, n (%)	2 (1.47)	4 (2.53)		
rs2108622				
GG, n (%)	74 (54.41)	78 (49.37)	0.392	0.98 (0.48-1.96)
GA, n (%)	51 (37.50)	66 (41.77)		
AA, n (%)	11 (8.09)	14 (8.86)		

OR, odds ratio; CI, confidence interval.

[†]OR adjusted for age, gender, hypertension, diabetes, smoking and hypercholesterolemia.

Table 3. Comparison of the best models, prediction accuracies, cross-validation consistencies, and *P* values identified by GMDR

Best model*	Training Balanced Accuracy	Testing Balanced Accuracy	Cross-validation consistency	Sign test (P)
1	0.5124	0.5115	9/10	7 (0.1726)
1, 2	0.5685	0.5418	10/10	9 (0.0116)
1, 2, 3	0.5422	0.5211	7/10	8 (0.2566)
1, 2, 3, 4	0.5691	0.6188	5/10	7 (0.5264)
1, 2, 3, 4, 5	0.6236	0.5217	10/10	9 (0.6326)
1, 2, 3, 4, 5, 6	0.6124	0.4988	8/10	5 (0.7235)
1, 2, 3, 4, 5, 6, 7	0.6328	0.4946	6/10	3 (0.8233)
1, 2, 3, 4, 5, 6, 7, 8	0.6422	0.5123	8/10	5 (0.2367)
1, 2, 3, 4, 5, 6, 7, 8, 9	0.6527	0.5011	7/10	6 (0.9112)
1, 2, 3, 4, 5, 6, 7, 8, 9, 10	0.6625	0.5133	6/10	5 (0.6357)
1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11	0.6472	0.5311	7/10	4 (0.4336)

*rs17110453, rs9333025, rs751141, rs1934980, rs10889160, rs1057910, rs776746, rs1799853, rs3093135, rs2269231, rs2108622 are symbolized as 1–11, respectively.

GMDR, generalized multifactor dimensionality reduction.

ter (GC/MS). Total plasma EETs and DiHETEs levels were measured using a stable isotope dilution GC/MS following base hydrolysis and separation on high performance liquid chromatography (HPLC), as previously described¹²⁾.

Statistical Analysis

For quantitative and qualitative demographic and clinical variables, the Student's *t*-test and χ^2 -test were performed, respectively. Hardy–Weinberg equilibrium was evaluated using the χ^2 -test. Differences of genotype frequencies were determined using the χ^2 -test, and levels of plasma 20-HETE, EETs, and DiHETEs were compared using the Student's *t*-test. Analysis of variance (ANOVA) followed by the Student–Newman–Keuls test was employed to evaluate differences of plasma 20-HETE, EETs, and DiHETEs levels between patients with carotid stenosis and patients without carotid stenosis stratified by genotypes. Multivariate logistic regression analyses accounting for potential confounders of age, gender, hypertension, diabetes, smoking, and hypercholesterolemia were conducted to estimate the associations between assessed variants and carotid stenosis risk. For associations reaching *P*=0.05 threshold, a Bonferroni correction was conducted to account for multiple comparison issues^{22, 23)}. Specifically, a threshold of 0.05/7=0.0071 was used because 11 variants from 7 genes were tested. All statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA).

Gene–gene interactions were investigated using the statistical software program generalized multifactor dimensionality reduction (GMDR) Beta, version

0.7 (www.healthsystem.virginia.edu/internet/addiction-genomics/Software)^{13, 24)}.

Plasma 20-HETE, EETs, and DiHETEs levels identified significantly associated variants and variant interactions, as well as hypertension and diabetes mellitus were jointly incorporated in a multi-variable regression model to assess whether the associations with carotid stenosis remain after controlling for other factors.

Results

Patient Characteristics

There were no significant differences between patients with or without carotid stenosis in terms of demographic and clinical characteristics (all *P*>0.05; **Table 1**).

Genotype Distributions in Patients with or without Carotid Stenosis

The genotype distributions of the 11 variants in patients without carotid stenosis were consistent with the Hardy–Weinberg Equilibrium (all *P*>0.05). Frequencies of rs17110453CC, rs776746GG, rs751141GG, and rs9333025GG genotypes were significantly higher in patients with carotid stenosis than patients without carotid stenosis (**Table 2**). After adjusting for potential confounding variables of age, gender, hypertension, diabetes, smoking, and hypercholesterolemia, CC, GG, and GG genotypes of rs17110453, rs751141, and rs9333025 were significantly associated with the risk of carotid stenosis (OR=2.28, 95% CI: 1.31–5.42, *P*<0.001; OR=3.01, 95% CI: 1.18–7.06, *P*=0.007; and

Table 4. CYP plasma metabolite levels of patients with or without carotid stenosis

Characteristics	carotid stenosis (<i>n</i> =90)	Non-carotid stenosis (<i>n</i> =96)	<i>P</i> value
20-HETE (pmol/L)	1712 ± 176	1612 ± 157	<0.001
DiHETEs (nmol/l)	84.32 ± 5.61	76.66 ± 5.22	<0.001
EETs (nmol/l)	60.41 ± 4.43	68.66 ± 4.56	<0.001

HETE, hydroxyecosatetraenoic acid; EET, epoxyecosatrienoic acids; DiHETE, dihydroxyecosatrienoic acid

OR=1.97, 95% CI: 1.22–6.16, *P*=0.006; respectively, **Table 2**), after correcting for multiple comparisons.

Gene–Gene Interactions in Mediating the Risk of Carotid Stenosis

Significant high-order interactions were detected for the association with carotid stenosis risk (**Table 3**). With covariates adjustment, the best model indicated an interaction between rs17110453 and rs9333025, which scored 10/10 for cross-validation consistency and 9/10 for the sign test (*P*=0.0116), suggesting a synergistic effect of the two variants in mediating the risk of carotid stenosis.

Different Genotype Combinations of rs17110453 and rs9333025 in Affecting the Risk of Carotid Stenosis

Supplementary Table 2 shows associations between carotid stenosis risk and the nine different genotype combinations of rs17110453 and rs9333025 compared with the reference genotype combination of rs17110453AA and rs9333025AA. Two interactions conferring increased phenotype risks were rs17110453CC, rs9333025GG and rs17110453CC, rs9333025AG. The estimated risk of carotid stenosis was significantly higher in individuals with rs17110453CC, rs9333025GG or rs17110453CC, rs9333025AG than in patients harboring rs17110453AA, rs9333025AA (OR=2.06, 95% CI: 1.11–7.25, *P*=0.014 and OR=1.93, 95% CI: 1.00–5.42, *P*=0.041, respectively, **Supplementary Table 2**). The results indicate that the interaction of specific CYP genetic variants confers a higher risk of carotid stenosis.

CYP Plasma Metabolite Levels with Carotid Stenosis and Their Relationship with CYP Genetic Variants

Plasma levels of 20-HETE and DiHETEs were significantly higher in patients with carotid stenosis compared with those in patients without carotid stenosis; and EETs levels were significantly lower in patients with carotid stenosis (all *P*<0.001, **Table 4**). Stratified analyses based on different genotypes of the three identified variants revealed that the identified

significant differences existed in the strata of specific genotypes. Particularly, carotid stenosis patients carrying the rs9333025GG genotype had higher 20-HETE levels than patients without carotid stenosis who carried the rs9333025GG genotype; carotid stenosis patients carrying the rs17110453CC genotype had lower plasma EETs levels than patients without carotid stenosis who carried the same genotype; and carotid stenosis patients carrying the rs751141GG genotype had lower plasma levels of EETs and higher plasma levels of DiHETEs compared with those in patients without carotid stenosis who carried the rs751141GG genotype (all *P*<0.01, **Table 5**). When focusing on patients with carotid stenosis, individuals with the genotype of GG for rs9333025 tended to have higher 20-HETE levels than individuals with other two genotypes for this variant (1833 ± 187 vs 1623 ± 171; *P*<0.001); individuals with the genotype of CC for rs17110453 tended to have lower EETs levels than individuals with other two genotypes for this variant (58.72 ± 5.63 vs 63.74 ± 5.54; *P*<0.001); individuals with genotype of GG for rs751141 tended to have lower EETs levels and higher DiHETEs levels than individuals with other two genotypes for this variant (58.24 ± 4.67 vs 63.14 ± 5.62; *P*<0.001; and 106.24 ± 11.22 vs 74.58 ± 5.96; *P*<0.001, respectively).

Logistic regression analysis indicated that rs751141GG was independently associated with low EETs (OR=1.72, 95% CI 1.04–3.82, *P*=0.021), also the genotype combination of rs17110453CC and rs9333025GG was independently associated with high 20-HETE (OR=1.96, 95% CI 1.17–5.56, *P*=0.004), high DiHETEs (OR=1.87, 95% CI 1.21–5.42, *P*=0.005) and low EETs (OR=2.11, 95% CI 1.34–6.29, *P*=0.001), after adjusting for diabetes mellitus and hypertension. It was also shown that high 20-HETE, high DiHETEs, and low EETs were independent risk factors for carotid stenosis, after adjusting for hypertension, diabetes mellitus, rs751141GG, and the genotype combination of rs17110453CC and rs9333025GG (**Table 6**).

Table 5. Association of 20-HETE and DiHETE levels with genotype distribution in patients

	20-HETE (pmol/L)			DiHETE (nmol/l)			EET (nmol/l)		
	Carotid stenosis (n=90)	Non-carotid stenosis (n=96)	P*	carotid stenosis (n=90)	Non-carotid stenosis (n=96)	P*	Carotid stenosis (n=90)	Non-carotid stenosis (n=96)	P*
rs17110453									
AA	1682 ± 186	1668 ± 180		83.13 ± 6.15	79.18 ± 7.14		63.80 ± 6.11	64.66 ± 5.53	
AC	1656 ± 181	1698 ± 182		83.35 ± 10.72	81.68 ± 7.22	0.14	63.67 ± 5.14	63.11 ± 6.21	
CC	1748 ± 193	1699 ± 191	0.55	83.12 ± 10.24	81.87 ± 6.13		58.72 ± 5.63	70.23 ± 6.55	<0.01
rs751141									
GG	1702 ± 192	1679 ± 181		106.24 ± 11.22	78.24 ± 6.37	<0.01	58.24 ± 4.67	73.28 ± 6.36	<0.01
AG	1623 ± 167	1612 ± 169		76.36 ± 6.15	74.16 ± 6.13		61.46 ± 6.58	62.42 ± 6.35	
AA	1635 ± 184	1641 ± 176	0.42	71.88 ± 4.66	70.78 ± 5.22		63.68 ± 5.66	64.77 ± 6.44	
rs9333025									
AA	1602 ± 177	1567 ± 179		82.12 ± 8.51	79.36 ± 9.28	0.32	61.11 ± 5.67	62.68 ± 6.82	
AG	1646 ± 161	1620 ± 171		85.89 ± 9.12	83.98 ± 8.76		61.89 ± 6.17	63.04 ± 6.35	
GG	1833 ± 187	1635 ± 168	<0.01	83.87 ± 8.02	81.88 ± 7.01		60.78 ± 5.66	61.92 ± 6.91	0.78

*Statistical significance was based on analysis of variance.

HETE, hydroxyeicosatetraenoic acid; EET, epoxyeicosatrienoic acids; DiHETE, dihydroxyeicosatrienoic acid.

Discussion

In the present study we investigated the relationship between CYP450 genetic polymorphisms, CYP plasma metabolite levels, and the risk of symptomatic carotid stenosis in IS patients. We detected that 20-HETE and DiHETEs were significantly higher and EETs were significantly lower in patients with symptomatic carotid stenosis. Research has shown that 20-HETE can constrict cerebral arteries²⁵⁾ and is involved in endothelial dysfunction²⁶⁾. EETs have vascular relaxation effects and play protective roles in the cardiovascular system²⁷⁾. EETs can be metabolized by sEH to yield less biologically active DiHETEs¹¹⁾. The pharmacological inhibition or genetic deletion of sEH has been shown to increase EETs levels and protect from stroke-induced brain injury²⁸⁾. This protective effect of EETs has also been demonstrated in an animal model²⁹⁾. Overall, it is plausible that altered 20-HETE, EETs, and DiHETEs levels could contribute to carotid stenosis or IS onset¹²⁾.

Our data revealed that genotypes of rs17110453CC, rs751141GG, and rs9333025GG were significantly associated with the risk of carotid stenosis in IS patients. Additionally, these genetic variants might affect CYP450 metabolite levels in IS patients with carotid stenosis versus those without. A number of studies have explored the association of CYP450 genes with blood pressure and IS¹³⁻¹⁶⁾. Some studies have

also shown that CYP450 plasma metabolite levels are associated with IS risk^{10, 12)}. To the best of our knowledge, this is the first study to investigate the association between the genetic variations of CYP pathway genes and carotid stenosis, as well as the potential effects of these variants on mediating the differences of CYP450 metabolite levels in patients with carotid stenosis versus those without. A previous study has shown that a functional variant in *CYP4A11* is associated with 20-HETE levels and essential hypertension³⁰⁾. Our data indicated that the rs9333025GG genotype might be associated with a higher 20-HETE level, which might partially explain its association with a higher risk of carotid stenosis. Additionally, the rs17110453CC genotype is associated with a lower EETs level, and the rs751141GG genotype is associated with a lower level of EETs and a higher level of DiHETEs. These results indicate that CYP450 polymorphisms can influence AA metabolism enzymes and further alter 20-HETE, EETs, and DiHETEs levels, which eventually affect the risk of carotid stenosis.

Besides these important findings, we detected gene-gene interaction in mediating metabolite levels and disease risk. Variants rs17110453 and rs9333025 were identified to interact together to influence the risk of carotid stenosis. Compared with the reference genotype combination of rs17110453AA and rs9333025AA, there was a 2.06-fold increased risk for carotid stenosis in individuals with a combined geno-

Table 6. Multiple regression analysis of the major risk factors for carotid stenosis

Risk factor	OR	95% CI	P value
Hypertension	2.23	1.24–5.86	0.012
Diabetes mellitus	1.85	1.08–3.89	0.027
High 20-HETE	2.32	1.27–6.25	0.006
High DiHETEs	1.86	1.04–4.12	0.031
Low EETs	1.98	1.18–5.32	0.004
rs751141GG	2.08	1.51–6.36	0.005
combination of rs17110453CC and rs9333025GG	2.86	1.64–8.65	<0.001

HETE, hydroxyecosatetraenoic acid; EET, epoxyecosatrienoic acids; DiHETE, dihydroxyecosatrienoic acid.

type of rs17110453CC and rs9333025GG. One possible explanation for the two-factor interaction is that the two relevant genes encode enzymes that participate in AA metabolism. Therefore, the interactions of rs17110453 and rs9333025 can predispose these individuals to have lower circulating EET levels and higher 20-HETE levels than those without the particular genotype combination. In addition, a recent study suggested that CYP2C8 overexpression coupled with EPHX2 downregulation significantly attenuates NF- κ B dependent vascular inflammatory response³¹. Aligning with previous findings, our results provide empirical evidence for supporting the interactive role of these two genetic variants in influencing the risk of carotid stenosis.

Several potential limitations need to be considered when interpreting our findings. First, our study was focused on Han Chinese population; hence, replication of this study in other populations will be needed before generalizing our findings to other ethnicities. Second, our study used a case-control study design; therefore, relevant biases may play roles in affecting the validity of our results. Third, this was a single hospital based study with limited sample size. Our findings thus need to be confirmed in multi-center studies with larger sample sizes. We also acknowledge that although the current study examined the roles of several important CYP450 genes, other relevant genes were not examined. Additionally, further studies are needed to clarify how the identified SNPs affect CYP450 expression and their role in carotid stenosis formation. Fourth, CYP450 plasma metabolite levels may be strongly affected by acute cerebral ischemia itself³². In the present study, CYP450 plasma metabolite levels were measured in the acute phase, and thus, may be influenced by acute cerebral ischemia. Further studies are needed to evaluate CYP450 plasma metabolite levels in the chronic phase to validate our findings. Fifth, some previous studies indi-

cate that patients with carotid stenosis usually show higher frequencies of elderly population, hypertension, cigarette smoking, dyslipidemia, diabetes, and ischemic heart disease than those seen in patients with no carotid stenosis^{2, 3}. However, there were no significant differences of these relevant factors between patients with and without carotid stenosis in our study sample. There may be certain biases involved in sample selection in our study. Furthermore, the carotid plaque morphological characters such as echolucent plaque and ulcerative plaque were strong risk factors for IS³³ and it would be interesting to explore their association with CYP genetic variants and AA metabolites levels. This research question, however, is beyond the scope and focus of the current study, and we will work on this research question in our future study.

Conclusion

In conclusion, we identified that variants rs17110453, rs751141, and rs9333025 were significantly associated with carotid stenosis risk. Furthermore, these polymorphisms were associated with CYP plasma metabolite levels in patients with carotid stenosis. There was a significant gene–gene interaction between rs17110453 and rs9333025 in affecting the risk of carotid stenosis. Overall, specific CYP450 gene SNPs and their interactions are associated with CYP450 plasma metabolite levels, which may partially explain their associations with carotid stenosis risk. Further studies are needed to validate our findings.

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Conflicts of Interest

None.

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Supplementary Table 1. Amplification and extension primers

SNPs	Forward primer and Reverse primer (5'→3')	Extension primer (5'→3')
<i>CYP2J2</i> (rs10889160)	F: CGTTGGATGGGATAATGAGAGGAAGTTGC R: ACGTTGGATGGTAGATTACATCAGATTCC	AAGGGAGAAAATCGAACTTTGTG
<i>CYP2C8</i> (rs17110453)	F: ACGTTGGATGACACTGATTTCCCTCAAGGT R: ACGTTGGATGCTGAAGTAAATGATTCTATG	CATTTCCCTCAAGGTCATAAA
<i>CYP2C8</i> (rs1934980)	F: ACGTTGGATGAACTGATGCTTTTGCTTGGG R: ACGTTGGATGTACAAATGGGAGAGTGGAGC	CCTAATCAATGAAGCATTCAATTTC
<i>CYP2C9*2</i> (rs1799853)	F: ACGTTGGATGCTGCGGAATTTTGGGATGGG R: ACGTTGGATGACCCACCCCTTGGTTTTTCTC	GAGGAGCATTGAGGAC
<i>CYP2C9*3</i> (rs1057910)	F: ACGTTGGATGCTACACAGATGCTGTGGTGC R: ACGTTGGATGTGTACAGGTCACTGCATGG	CACGAGGTCCAGAGATAC
<i>CYP3A5</i> (rs776746)	F: ACGTTGGATGGATGAAGGGTAATGTGGTCC R: ACGTTGGATGCCATAATCTCTTTAAAGAGC	CCAAACAGGGAAGAGATA
<i>EPHX2</i> (rs751141)	F: ACGTTGGATGTTTTCTAGATCCCTGCTCTG R: ACGTTGGATGAGCAGATGACTCTCCATAGC	CCCAGGCAGGTTACC
<i>CYP4F2</i> (rs3093135)	F: ACGTTGGATGAGACCCTGGCCATATAACAGC R: ACGTTGGATGTTCCACCTGTCTAACCTCTG	CATATAACAGCCATTGTAGAG
<i>CYP4F2</i> (rs2108622)	F: ATCAACCCGTTCCACCT R: ACATTGTGCTCCCAGACG	CCTAATCAATGAAGCA
<i>CYP4A11</i> (rs2269231)	F: ACGTTGGATGGCCTGGCTTTTTTCATGTACC R: ACGTTGGATGCCAACACTGGCTTTACAGTC	TCCTGTTTTTTCCTGTAGC
<i>CYP4A11</i> (rs9333025)	F: ACGTTGGATGCCACATCCTCAAGGCTAAAG R: ACGTTGGATGAATCGTGAATGCTACCTGGG	GCTCAAGGCTAAAGTCAGAT

SNPs, single nucleotide polymorphisms; F, forward primer; R, reverse primer.

Supplementary Table 2. Associations between carotid stenosis and different combinations of genotypes

rs17110453	AA	CC	AC	CC	AC	CC	AA	AA	AC
rs9333025	AA	GG	GG	AG	AG	AA	AG	GG	AA
OR	1*	2.06	1.21	1.93	1.06	1.02	0.95	1.01	0.98
95% CI	–	1.11–7.25	0.62–2.24	1.00–5.42	0.72–2.11	0.60–1.64	0.48–1.52	0.73–1.81	0.57–1.59
P value	–	0.014	0.286	0.041	0.524	0.718	0.821	0.682	0.792

* Non-risk genotype for each genetic factor was used as the reference OR. OR, odds ratios.