GENETIC TESTING AND MOLECULAR BIOMARKERS
Volume 19, Number 5, 2015

Pp. 235–241
DOI: 10.1089/gtmb.2014.0305

Cytochrome *4A11* Genetic Polymorphisms Increase Susceptibility to Ischemic Stroke and Associate with Atherothrombotic Events After Stroke in Chinese

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To evaluate the associations between four single-nucleotide polymorphisms (SNPs) in *CYP4A11* and *CYP4F2* and ischemic stroke (IS), and between these variants and atherothrombotic events after stroke. IS patients ($n=396$) and controls ($n=378$) were genotyped for two *CYP4A11* SNPs (rs2269231 and rs9333025) and two *CYP4F2* SNPs (rs2108622 and rs3093135). Patients were followed up for 12 months after the stroke for the atherothrombotic events. The frequency of the rs9333025 GG genotype was significantly higher in IS patients than in controls. Logistic regression analysis showed that the presence of rs9333025 GG in patients was associated with significantly higher risk of IS. Cox regression analysis revealed that the rs9333025 GG genotype was an independent risk factor for atherothrombotic events after stroke. The rs9333025 GG genotype increases patients' susceptibility to IS and is associated with high frequencies of atherothrombotic events in stroke patients.

Introduction

Stroke has emerged as a worldwide leading cause of mortality and is a major public health problem (Feigin, 2005; Domingues-Montanari *et al.*, 2008). In China, about 2.6 million new strokes have been estimated to occur each year, with ischemic stroke (IS) accounting for 43.7–78.9% of all strokes (Liu *et al.*, 2007). Stroke is a multifactorial, polygenic, complex disease resulting from the combination of vascular, environmental, and genetic factors (Della-Morte *et al.*, 2012).

The *CYP4A11* and *CYP4F2* genes encode cytochrome P450 (CYP) ω-hydroxylases, which are primarily responsible for metabolizing arachidonic acid (AA) into 20-hydroxyeicosatetraenoic acid (20-HETE), a potent vasoconstrictor (Powell *et al.*, 1998; Lasker *et al.*, 2000). 20-HETE constricts cerebral arteries by activating protein kinase C, depolarizing vascular smooth muscle cells through the inhibition of the large-conductance Ca^{2+}-sensitive K^+ channel, and increasing Ca^{2+} influx via L-type Ca^{2+} channels (Ma *et al.*, 1993; Alonso-Galicia *et al.*, 1997; Gladden *et al.*, 1998). Previous studies have indicated that nitric oxide (NO) inhibits the formation of 20-HETE, and a fall in 20-HETE levels appears to contribute to the vasodilator response to NO in cerebral arteries (Alonso-Galicia *et al.*, 1997). 20-HETE has been shown to play an important role in the autoregulation of cerebral blood flow (CBF) and systemic blood pressure (BP) (Gebremedhin *et al.*, 2000). In addition, blockade of the synthesis or vasoconstrictor actions of 20-HETE reduced infarct size in a middle cerebral artery occlusion model of IS (Omura *et al.*, 2006).

New single-nucleotide polymorphisms (SNPs) in genes that encode 20-HETE-synthesizing enzymes have recently been discovered, including two functional variants, F434S in *CYP4A11* and V433M in *CYP4F2*. These two variants result in enzymes that show significantly reduced ability to metabolize AA into 20-HETE *in vitro* (Gainer *et al.*, 2005; Stec *et al.*, 2007). Moreover, several variants and haplotypes of 20-HETE-synthesizing enzyme genes have been shown to be associated with hypertension (Liu *et al.*, 2008; Ward *et al.*, 2008). A large urban-based population study conducted in middle-aged Swedish patients suggested that the presence of the *CYP4F2* V433M SNP may increase the risk of IS in male subjects only, partially through the elevation of BP (Fava *et al.*, 2008). A study in Japanese men found that the *CYP4F2* rs2108622 G allele was associated with cerebral infarction (Fu *et al.*, 2008b), and yet another study from South India reported the association of the *CYP4F2* 1347 G/A polymorphism with stroke (Munshi *et al.*, 2012). In the Han Chinese population, the *CYP4A11* C-296T and *CYP4F2* V433M SNPs were shown to alter the susceptibility to stroke (Deng *et al.*, 2010; Ding *et al.*, 2010). However, all association studies between *CYP4A11* and *CYP4F2* genetic variations and stroke have been case–control studies, and none have included prospective follow-up data.

We hypothesized that polymorphisms in genes encoding 20-HETE-synthesizing enzymes might confer susceptibility

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to stroke and be associated with atherothrombotic (AT) events after stroke. To test this hypothesis, we evaluated four SNPs of *CYP4A11* and *CYP4F2* in IS patients and controls, these four SNPs were chosen from the NCBI database (www.ncbi.nlm.nih.gov/SNP) and according to the following criteria: (1) SNPs with the minor allele frequency (MAF) >0.05; (2) SNPs leading to amino acid changes; (3) SNPs have been examined in previous studies; (4) functional SNP. In addition, all stroke patients were followed up for 12 months after the stroke for the appearance of atherothrombotic events.

Materials and Methods

Study populations

The Ethics Committee of the People's Hospital of Deyang City and the Third Affiliated Hospital of Wenzhou Medical College reviewed and approved this study. Each patient or a legally responsible family member provided written informed consent before study enrollment.

The study population included 396 IS patients and 378 controls. Patients who had suffered their first IS and were admitted into the above two hospitals were consecutively recruited between August 1, 2010 and March 31, 2013. Inclusion criteria for patients were as follows: (1) age ≥ 18 years; (2) diagnosis of IS as defined by the World Health Organization (WHO) criteria; (3) IS related to AT ($n=260$) or small artery disease (SAD; $n=136$), according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification system (Han *et al.*, 2007). Exclusion criteria were as follows: (1) cardiogenic cerebral embolisms or cerebral infarction not related to AT or SAD; (2) family history of apoplexy or previous history of strokes; (3) cerebral hemorrhage; (4) the patients were treated with thrombolysis or carotid stenting; (5) unwillingness to participate in this study. Control subjects were selected from outpatients with no history of stroke as confirmed by medical history and physical and laboratory examinations at our center. Control subjects were not genetically related to the enrolled cerebral-infarction patients.

Demographic and clinical characteristics and presence of vascular risk factors were recorded for each individual and included age, gender, hypertension, diabetes mellitus, history of cigarette smoking and alcohol intake, and levels of total plasma cholesterol (TC), triglycerides (TG), and low-density lipoprotein cholesterol (LDL-C). Hypertension was defined as the mean of three independent measurements of BP $\geq 140/90$ mmHg or by the prescription of antihypertensive drugs from a medical doctor. Diabetes mellitus was defined as a fasting blood glucose level of >7.8 mM or of >11.1 mM at 2 h after an oral glucose challenge, or by the prescription of hypoglycemic drugs from a medical doctor.

Treatment regimens of IS patients

According to the Chinese acute ischemic stroke management guidelines, all the recruited patients were received aspirin 200 mg daily during the acute stroke period (i.e., within 2 weeks of the index stroke onset) and then 100 mg daily thereafter. In addition, the other stroke treatments were administered according to the Chinese ischemic stroke management guidelines, including a similar BP goal after stroke, deep venous thrombosis (DVT) prophylaxis, statin use, and rehabilitation. After discharge, the patients' adherence to taking aspirin and other drugs was checked in follow-up phone calls.

Genotyping

Genotyping markers for *CYP4A11* and *CYP4F2* were selected from the NCBI database (www.ncbi.nlm.nih.gov/SNP) based on previous studies showing significant associations between specific SNPs and stroke (Fava *et al.*, 2008; Fu *et al.*, 2008b; Liu *et al.*, 2008; Ward *et al.*, 2008; Deng *et al.*, 2010; Ding *et al.*, 2010; Munshi *et al.*, 2012) and considering each SNP MAF. Four tag SNPs were identified in the human HapMap project database (www.hapmap.org) with MAF ≥ 0.05 (rs226923 and rs9333025 for *CYP4A1l* and rs2108622 and rs3093135 for *CYP4F2*). Blood was drawn (3 mL) from an arm vein into a sterile tube containing ethylenediaminetetraacetic acid, and stored at -80°C until use for genotype analysis. Genomic DNA was extracted from peripheral blood using a modified phenol/chloroform method and purified using the UNIQ-10 kit (Sangon Biotech Co., Ltd., Shanghai, China).

Genotypes of the four variants were examined using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Aggarwal *et al.*, 2011; Chi *et al.*, 2014). In brief, the specific genotype of each SNP was determined using two amplification primers and one extension primer (Table 1). The reaction mix was desalted by mixing with 6 mg of cation exchange resin (Sequenom, Inc., San Diego, CA) and resuspended in 25 μl of water. Once the primer extension reaction was completed, the samples were spotted onto a 384-well SpectroCHIP (Sequenom, Inc.) using the MassARRAY Nanodispenser (Sequenom, Inc.) and genotyped using MALDI-TOF MS. Genotype calling was

Table 1. Amplification and Extension Primers Used to Genotype Each Single-Nucleotide Polymorphism

SNP	*Primers, 5′-3′*	*Extension primer, 5′-3′*
CYP4A1l (rs2269231)	F: CGTTGGATGGGATAATGAGAGGAAGTTGC R: ACGTTGGATGGTAGATTACATCAGATTCC	AAGGGAGAAAATCGAACTTTGTG
CYP4A1l (rs9333025)	F: ACGTTGGATGACACTGATTTCCCTCAAGGT R: ACGTTGGATGCTGAAGTAAATGATTCTATG	CATTTCCCTCAAGGTCATAAA
CYP4F2 (rs2108622)	F: ATCAACCCGTTCCCACCT R: ACATTGTGCTCCCAGACG	CCTAATCAATGAAGCA
CYP4F2 (rs3093135)	F: ACGTTGGATGCTGCGGAATTTTGGGATGGG R: ACGTTGGATGACCCACCCTTGGTTTTTCTC	GAGGAGCATTGAGGAC

F, forward primer; R, reverse primer; SNP, single-nucleotide polymorphism.

Table 2. Demographic and Clinical Characteristics of Stroke Patients and Controls

Characteristic	*Stroke patients,* n = *396*	*Controls,* n = *378*	p-*Value*
Age, years	68.79 ± 11.11	64.98 ± 10.29	< 0.001
Males	235 (59.34)	222 (58.73)	0.924
Diabetes mellitus	138 (34.85)	97 (25.66)	0.032
Hypertension	302 (76.26)	99 (26.19)	< 0.001
Body mass index, kg/m^2	24.10 ± 2.33	23.90 ± 2.62	0.221
Cigarette smoking	165 (41.67)	159 (42.06)	0.942
Alcohol intake	184 (46.46)	170 (44.97)	0.694
Triglycerides, mM	1.96 ± 1.12	1.83 ± 1.02	0.182
Total cholesterol, mM	5.54 ± 1.36	5.36 ± 1.21	0.061
Low-density lipoprotein cholesterol, mM	3.15 ± 1.27	2.99 ± 1.19	0.376

performed in real time using the MassARRAY RT software version 3.0.0.4 and analyzed using the MassARRAY Typer software version 3.4 (Sequenom, Inc.).

Study end points

The follow-up protocol for stroke patients included a medical visit to the outpatient clinic 1 month after discharge and every 2 or 3 months thereafter. Clinical events were assessed on the basis of the information provided by hospital readmission records, the referring physician, or a phone interview with the patient. The investigators who evaluated the clinical end points were blinded to the results of the DNA analysis. The end point was a composite of atherothrombotic events, including recurrent ischemic stroke (RIS), DVT, myocardial infarction (MI), and death, occurring in the 12 months after the first stroke. RIS was defined as a new focal neurologic deficit of vascular origin lasting at least 24 h that was proven to be nonhemorrhagic by either computer tomography or magnetic resonance imaging scanning. MI was defined by the presence of at least two of the following: ischemic symptoms, elevated cardiac enzyme (creatine kinase MB) concentration (2 × the upper limit of normal), and electrocardiographic changes compatible with MI. Death was defined as vascular mortality due to MI, IS, or other vascular causes.

Table 3. Genotype Frequencies in Stroke Patients and Controls

Genotype	*Stroke patients,* n = *396*	*Controls,* n = *378*	p-*Value*
rs2269231			
AA	78 (19.7)	66 (17.5)	
AT	217 (54.8)	206 (54.5)	
TT	101 (25.5)	106 (28.0)	0.362
rs9333025			
AA	7 (1.8)	17 (4.5)	
AG	90 (22.7)	113 (29.9)	
GG	299 (75.5)	248 (65.6)	< 0.001
rs2108622			
GG	209 (52.8)	186 (49.2)	
GA	155 (39.1)	155 (41.0)	
AA	32 (8.1)	37 (9.8)	0.293
rs3093135			
TT	331 (83.6)	314 (83.1)	
AT	58 (14.6)	58 (15.3)	
AA	7 (1.8)	6 (1.6)	0.999

Table 4. Genotype Frequencies in Atherothrombosis and Small Artery Disease Stroke Patients

Genotype	*AT stroke,* n = *260*	*SAD stroke,* n = *136*	p-*Value*
rs2269231			
AA	49 (18.8)	29 (21.3)	
AT	143 (55.0)	74 (54.4)	
TT	68 (26.2)	33 (24.3)	0.362
rs9333025			
AA	5 (1.9)	2 (1.5)	
AG	57 (21.9)	33 (24.3)	
GG	198 (76.2)	101 (74.3)	0.691
rs2108622			
GG	138 (53.1)	71 (52.2)	
GA	100 (38.5)	55 (40.4)	
AA	22 (8.5)	10 (7.4)	0.274
rs3093135			
TT	218 (83.8)	113 (83.1)	
AT	38 (14.6)	20 (14.7)	
AA	4 (1.5)	3 (2.2)	0.616

AT, atherothrombotic; SAD, small artery disease.

Statistical analysis

Based on a suggested sample size requirement (Wang and Zhao, 2003), we expected that our sample size of 360 patients and 360 controls would sufficiently provide 80% power at the 5% significance level calculated.

All statistical analyses were performed using the SPSS 16.0 software (SPSS, Inc., Chicago, IL). The χ^2 test was used to analyze the deviation from Hardy–Weinberg equilibrium

Table 5. Association of the Major Risk Factors for Ischemic Stroke with rs9333025 GG by Logistic Regression Analysis

Factor	*Adjusted odds ratio*	*95% Confidence interval*	p-*Value*
Females	0.98	0.72–1.83	0.482
Age			
> 68 years	1.01	0.86–2.99	0.042
Hypertension	1.65	1.12–4.86	0.022
Diabetes	1.46	1.02–3.46	0.031
Smoking	0.56	0.35–1.07	0.646
High triglyceride	0.68	0.53–1.26	0.423
High low-density lipoprotein cholesterol	1.03	0.89–2.91	0.076
High total cholesterol	0.97	0.86–1.97	0.092
rs9333025			
GG	1.82	1.24–5.04	0.016

for genotype frequencies. Continuous variables were compared between IS patients and controls using the Student's *t*-test. Discrete variables were compared using χ^2 tests or, if expected frequencies were small, Fisher's exact tests. Multiple logistic regression analysis was used to estimate adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for each SNP after adjustment for gender, age, body mass index, smoking status, and presence of hypertension, diabetes, and hyperlipidemia. The χ^2 test was also used to compare the incidence of clinical end points among the genotypes. The Cox proportional hazards model was used to calculate the risks for composite end points (RIS, DVT, MI, and death) during the 12 months after the first stroke. Relative risk (RR) with 95% CIs are reported. All tests were two-sided. Statistical significance was set at $p < 0.05$.

Table 6. Atherothrombotic Events During 12-Month Follow-Up in Stroke Patients ($N = 396$)

	Recurrent ischemic stroke	*Death*	*Myocardial infarction*	*Deep venous thrombosis*	*Total*
rs2269231					
AA, $n = 78$	8 (10.3)	0 (0.0)	1 (1.3)	1 (1.3)	10 (12.8)
AT, $n = 217$	20 (9.2)	3 (1.4)	3 (1.4)	4 (1.8)	30 (13.8)
TT, $n = 101$	9 (8.9)	2 (2.0)	2 (2.0)	1 (1.0)	14 (13.9)
rs9333025					
AA, $n = 7$	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
AG, $n = 90$	2 (2.2)	1 (1.1)	1 (1.1)	2 (2.2)	6 (6.7)
GG, $n = 299$	35 (11.7)[a]	4 (1.3)	5 (1.7)	4 (1.3)	48 (16.1)[a]
rs2108622					
GG, $n = 209$	20 (9.6)	4 (1.9)	3 (1.4)	4 (1.9)	31 (14.8)
GA, $n = 155$	14 (9.0)	1 (0.6)	2 (1.3)	2 (1.3)	19 (12.3)
AA, $n = 32$	3 (9.4)	0 (0.0)	1 (3.1)	0 (0.0)	4 (12.5)
rs3093135					
TT, $n = 331$	31 (9.4)	4 (1.2)	5 (1.5)	5 (1.5)	45 (13.6)
AT, $n = 58$	6 (10.3)	1 (1.7)	1 (1.7)	1 (1.7)	9 (15.5)
AA, $n = 7$	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Age					
>68 years, $n = 246$	29 (12.4)[a]	4 (1.6)	4 (1.6)	4 (1.6)	41 (16.7)[a]
≤ 68 years, $n = 150$	8 (6.0)	1 (0.7)	2 (1.3)	2 (1.3)	13 (8.7)
Gender					
Male, $n = 235$	22 (9.4)	3 (1.3)	4 (1.2)	3 (1.3)	32 (13.6)
Female, $n = 161$	15 (9.3)	2 (1.2)	2 (1.2)	3 (1.9)	22 (13.7)
Stroke subtype					
AT, $n = 260$	30 (11.5)[a]	4 (1.5)	4 (1.5)	4 (1.5)	42 (16.2)[a]
SAD, $n = 136$	7 (5.1)	1 (0.7)	2 (1.5)	2 (1.5)	12 (8.8)
Hypertension					
Yes, $n = 302$	34 (11.3)[a]	4 (1.3)	5 (1.7)	5 (1.7)	48 (15.9)[a]
No, $n = 94$	3 (3.2)	1 (1.1)	1 (1.1)	1 (1.1)	6 (6.4)
Diabetes					
Yes, $n = 138$	19 (13.8)[a]	3 (2.2)	3 (2.2)	2 (1.4)	27 (19.6)[a]
No, $n = 258$	18 (7.0)	2 (0.8)	3 (1.2)	4 (1.6)	27 (10.5)
High low-density lipoprotein cholesterol					
Yes, $n = 213$	27 (12.7)[a]	3 (1.4)	5 (2.2)	3 (1.4)	38 (17.8)[b]
No, $n = 183$	10 (5.5)	2 (1.1)	1 (0.5)	3 (1.6)	16 (8.7)
High total cholesterol					
Yes, $n = 200$	20 (10.0)	3 (1.5)	4 (2.0)	3 (1.5)	30 (15.0)
No, $n = 196$	17 (8.7)	2 (1.0)	2 (1.0)	3 (1.5)	24 (12.2)
Smoking					
Yes, $n = 165$	16 (9.7)	2 (1.2)	4 (2.4)	2 (1.2)	24 (14.5)
No, $n = 231$	21 (9.1)	3 (1.3)	2 (0.9)	4 (1.7)	30 (13.0)
Alcohol intake					
Yes, $n = 184$	17 (9.2)	3 (1.6)	4 (2.2)	2 (1.1)	26 (14.1)
No, $n = 212$	20 (9.4)	2 (0.9)	2 (0.9)	4 (1.9)	28 (13.2)
NIHSS score					
≤ 9, $n = 246$	21 (8.5)	3 (1.2)	4 (1.6)	3 (1.2)	31 (12.6)
>9, $n = 150$	16 (10.7)	2 (1.3)	2 (1.3)	3 (2.0)	23 (15.3)

[a] $p < 0.05$.
[b] $p < 0.01$.

Results

Patients' characteristics

Demographic and clinical characteristics of patients and controls are presented in Table 2. Stroke patients presented significantly higher prevalence of risk factors for stroke, including history of hypertension ($p < 0.001$), diabetes ($p = 0.032$), and older age ($p < 0.001$), than controls. However, patients and controls presented no statistically significant differences in other conventional risk factors, including smoking, alcohol intake, levels of LDL-C, TC, and TG, and body mass index.

Distribution of genotypic variants between stroke patients and controls

The distribution of genotypes analyzed in this study was consistent with the Hardy–Weinberg equilibrium model ($p > 0.05$). Genotype distributions for stroke patients and controls are shown in Table 3. The frequency of the rs9333025 GG genotype was significantly higher in stroke patients than in controls (75.5% vs. 65.6%; $p < 0.001$). However, no significant differences were observed in genotype distributions for rs2269231, rs2108622, and rs3093135 between the two groups ($p > 0.05$). Moreover, there were no significant difference in genotype frequencies between AT and SAD patients ($p > 0.05$; Table 4). Multiple logistic regression analysis showed that the rs9333025 GG genotype was associated with significantly higher risk of IS (adjusted for age, hypertension, and diabetes; OR = 1.82, 95% CI = 1.24–5.04; $p = 0.016$; Table 5).

Outcomes after stroke

Among the 396 stroke patients, 4 (1.01%) were lost during the follow-up period, resulting in a complete rate of follow-up of 98.99% (392/396). During the 12 months following the stroke, atherothrombotic events occurred in 54 patients (37 RIS, 5 death, 6 MI, and 6 DVT). Atherothrombotic effects that occurred during the 12 months following the stroke are shown in Table 6. The rs9333025 GG genotype was associated with significantly higher number of atherothrombotic events than the rs9333025 AG genotype ($p = 0.040$). However, there were no significant differences in the frequencies of atherothrombotic events among the three other genotypic variants ($p > 0.05$). Old age (>68 years), AT stroke, hypertension, diabetes, and high LDL-C levels were associated with significantly higher numbers of atherothrombotic events after stroke ($p < 0.05$). Multiple Cox regression analyses are shown in Table 7. Hypertension (RR = 1.46, 95% CI = 1.06–3.64; $p = 0.018$), diabetes (RR = 1.34, 95% CI = 1.01–3.02; $p = 0.036$), and presence of the rs9333025 GG genotype (RR = 1.87, 95% CI = 1.16–5.36; $p = 0.003$) were shown to be independent risk factors for atherothrombotic events (Table 7).

Table 7. Risk Factors for Atherothrombotic Events Assessed by Cox Regression Analysis

Factor	*Relative risk*	*95% Confidence interval*	p-*Value*
Females	0.92	0.86–1.86	0.258
Age >68 years	1.01	0.91–2.27	0.076
Hypertension	1.46	1.06–3.64	0.018
Diabetes	1.34	1.01–3.02	0.036
Smoking	0.95	0.83–1.76	0.862
NIHSS score >9	0.98	0.81–1.98	0.326
High low-density lipoprotein cholesterol	1.11	0.92–2.84	0.082
High total cholesterol	0.94	0.82–1.56	0.163
rs9333025 GG	1.87	1.16–5.36	0.003
AT stroke	1.06	0.93–2.86	0.079

Discussion

The main purpose of this study was to examine potential associations between polymorphisms in the *CYP4A11* and *CYP4F2* genes, which encode 20-HETE-synthesizing enzymes, and IS. We showed that the *CYP4A11* rs9333025 variation, but not *CYP4A11* rs2269231, *CYP4F2* rs2108622, or *CYP4F2* rs3093135, was independently associated with IS. These results are consistent with those of previous studies (Fu *et al.*, 2008a, 2008c, 2012; Liang *et al.*, 2014) and suggest that mutations in *CYP4A11* may contribute to altered 20-HETE production, which could ultimately lead to increased risk for stroke.

Kinetic analysis showed that the ability of CYP4F2 to convert AA to 20-HETE in the human kidney is nearly 10-fold greater than that of CYP4A11 (Lasker *et al.*, 2000). Immunoprecipitation studies revealed that treatment with anti-CYP4F2 antibodies inhibited 20-HETE synthesis in renal microsomes nearly 2×than treatment with anti-CYP4A11 antibodies (Lasker *et al.*, 2000). Ward *et al.* (2008) showed that SNPs in the *CYP4F2* gene, but not in the *CYP4A11* gene, were associated with increased 20-HETE secretion and BP. Some studies also indicated that the *CYP4F2* rs2108622 variant was independently associated with IS (Fava *et al.*, 2008; Fu *et al.*, 2008b; Deng *et al.*, 2010; Ding *et al.*, 2010; Munshi *et al.*, 2012). However, we found no association between the *CYP4F2* rs2108622 or rs3093135 variant and IS, which is not consistent with previously reported observations. The reasons for these differences are not clear, as many different factors contribute to the development of cerebral infarction at the molecular level. Differences in the patients' demographics, such as race, may have contributed to this discrepancy. In addition, stroke is a multifactorial, polygenic, complex disease and involves gene–gene and gene–environment interactions (Schork *et al.*, 2009). However, gene–gene interactions or environmental influences were not analyzed in this study.

To date, studies of associations between genetic variations and stroke have often employed case–control approaches, with few including a prospective follow-up component. To the best of our knowledge, our study is the first one to reveal the association between the *CYP4A11* rs9333025 GG variation and increased atherothrombotic events after stroke. In addition, multiple Cox regression analysis showed that the *CYP4A11* rs9333025 GG variant was an independent indicator of higher risk of atherothrombotic events after stroke; therefore, this variant may be useful as a marker for risk assessment of atherothrombotic events after stroke.

Our results indicate that mutations in *CYP4A11* may increase the susceptibility to IS and associate with increased

risk of atherothrombotic events after stroke. The underlying causes for these observations remain unknown. One possible cause is the involvement of the *CYP4A11* gene in the metabolism of AA into 20-HETE, which plays an important role in the regulation of cerebral vascular tone (Lange *et al.*, 1997; Gebremedhin *et al.*, 2000). 20-HETE can promote the formation of oxygen radicals (Guo *et al.*, 2007), contributes to endothelial dysfunction (Singh *et al.*, 2007), and is a potent constrictor of cerebral arteries, inhibiting Na^+, K^+-ATPase activity (Cheng *et al.*, 2008). In addition, 20-HETE activates a number of intracellular signaling pathways involved in apoptosis and cell death (Sun *et al.*, 1999; Randriamboavonjy *et al.*, 2003). Inhibitors of the synthesis of 20-HETE have been reported to reverse the decrease in CBF following subarachnoid hemorrhage and reduce infarct size following transient cerebral ischemia (Takeuchi *et al.*, 2005; Tanaka *et al.*, 2007).

Our study has a number of limitations. First, 20-HETE levels were not measured and, therefore, correlations between *CYP4A11* and *CYP4F2* polymorphisms, potential alterations in 20-HETE levels, and stroke risk could not be established. Second, due to the limited sample size and the use of patients from only two centers, our results may not be representative of the disease status among the entire Chinese population. Our results should be validated in larger, multicenter studies. Third, many genes in the CYP pathways may associate with stroke; however, our study exclusively focused on CYP genes encoding CYP ω-hydroxylases. Future studies should focus on additional candidate genes.

Acknowledgment

This study was supported by the Scientific Research Foundation of Sichuan Provincial Health Department (Grant No. 140025).

Author Disclosure Statement

No competing financial interests exist.

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