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Original Article

Association of Cytochrome P450 Genetic Variants with Clopidogrel Resistance and Outcomes in Acute Ischemic Stroke

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***Aims*: Clopidogrel is an antiplatelet drug primarily used to treat or prevent acute ischemic stroke (IS) or myocardial infarction (MI). This prodrug requires biotransformation to an active metabolite by cytochrome P450 (CYP) enzymes, and *CYP* single nucleotide polymorphisms (SNPs) could affect the efficiency of such biotransformation.**

***Methods*: A total of 375 consecutive IS patients were genotyped for eight *CYP* SNPs using mass spectrometry. Platelet aggregation activity was measured before and after the 7−10 day treatment. Gene−gene interactions were analyzed using generalized multifactor dimensionality reduction (GMDR) analysis. All patients received clopidogrel therapy and were followed up for six months. Primary outcomes were evaluated as a composite of recurrent ischemic stroke (RIS), MI, and death. The secondary outcome was the modified Rankin Scale (mRS).**

***Results*: Clopidogrel resistance occurred in 153 patients (40.8%). The frequency of *CYP3A5* (rs776746) GG/AG and *CYP2C19*2* (rs4244285) AA/AG genotypes was significantly higher in clopidogrel-resistant patients than in sensitive patients. There was a significant gene−gene interaction between *CYP3A5* (rs776746) and *CYP2C19*2* (rs4244285). *CYP2C19*2* AA and its interaction with *CYP3A5* GG were independent predictors of clopidogrel resistance and affected the activity of platelet aggregation. Diabetes mellitus, *CYP2C19*2* (rs4244285), clopidogrel resistance, and the interaction of *CYP2C19*2* with *CYP3A5* were all independent risk factors for the primary outcomes of clopidogrel treatment. Clopidogrel-resistant patients were more likely to have poor outcomes (mRS >2 points) compared with clopidogrel-sensitive patients.**

***Conclusion*: *CYP* SNPs and their interactions are associated with drug resistance and outcomes in acute IS patients.**

Key words: Ischemic stroke, Clopidogrel resistance, Cytochrome P450 SNPs, Treatment outcome

Introduction

Stroke is a significant global health problem and is a common cause of death among the elderly[1]. Clinically, stroke can be classified into ischemic and hemorrhagic categories because their management significantly differs[2, 3]. For example, antiplatelet drugs are recommended for the treatment and prevention of noncardioembolic ischemic stroke (IS)[3]. Depending on the risk of stroke, antithrombotic drugs such as aspirin or warfarin can effectively reduce IS in certain populations[4]. Previous studies have shown that clopidogrel is superior to aspirin for preventing IS in high-risk individuals[5]. However, antiplatelet drugs such as clopidogrel may not completely prevent ischemic events, causing a number of subsequent IS events to occur[6]. Such a situation is referred to as drug or clopidogrel resistance[7], which is due to an individual's variability in response to drug or clopidogrel therapy[8]. A study suggested that hyporesponsiveness to antiplatelet drugs isassociated with poor clinical outcome after an acute coronary syndrome[9]. Thus, further research on this individual variability could help us develop more effective drugs to control or prevent IS in clinical settings.

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To date, the molecular and clinical mechanisms underlying clopidogrel resistance remain to be determined[10]. Clopidogrel is an oral thienopyridineclass antiplatelet drug used for the treatment and prevention of coronary artery disease, myocardial infarction (MI), and cerebrovascular disease[5, 7]. However, it is a prodrug that requires biotransformation to an active metabolite by cytochrome P450 (CYP) enzymes[11]. Thus, the expression and function of these CYP enzymes definitely have a pivotal role and are associated with the efficiency of clopidogrel in clinical settings. Previous studies have shown that genes that encode CYP enzymes are polymorphic and that certain alleles reduce enzymatic activities[12]. Previous *in vitro* metabolic and clinical studies have demonstrated that certain *CYP* gene single nucleotide polymorphisms (SNPs) reduce enzymatic activities *in vitro* and affect the conversion of clopidogrel into its active metabolite and that this is associated with the degree of platelet coagulation capacity[13, 14]. To date, gene polymorphism-related clopidogrel resistance has been widely studied in coronary heart disease patients[15-17]. However, there is no data on such an association in IS patients. Furthermore, previous studies have reported inconsistent results regarding the association between these factors and clopidogrel sensitivity, for example, *ABCB1*, *CYP3A5*, *P2Y12*, and *PlA1/A2* polymorphisms have not been associated with clopidogrel resistance[15-17], indicating that the genetic factors for clopidogrel resistance may be very complex. It is possible that genetic variants at individual loci only contribute to clopidogrel resistance by interacting with other gene variants. Moreover, the effects of individual locialone on clopidogrel resistance may be too small to be observed. Thus, the investigation of multiple gene−gene interactions is necessary to be able to understand the genetic basis of clopidogrel resistance risk in IS patients using alternative analytical methods, such as the generalized multifactor dimensionality reduction (GMDR) approach[18, 19]. We therefore hypothesized that relevant *CYP* genetic variants and interaction among these variants contribute to clopidogrel resistance and the outcome of acute IS patients treated with clopidogrel.

Materials and Methods

Study Population

This prospective study was reviewed and approved by the Ethics Committees of The People's Hospital of Deyang City and the Third Affiliated Hospital of Wenzhou Medical College. Written informed consent was obtained from each participant before enrollment.

In this study, we consecutively enrolled 375 patients diagnosed with their first IS. Patients were admitted to either the People's Hospital of Deyang City or the Third Affiliated Hospital of Wenzhou Medical College within 72 h of their index stroke onset between June 2014 and January 2015. Inclusion criteria were as follows: (i) Age ≥40 years, (ii) Patients diagnosed with IS on the basis of both clinical findings and results of a neurological examination using computerized tomography or magnetic resonance imaging, (iii) Patients with IS-related atherothrombotic or small-artery disease according to the Trial of Org 10172 in Acute Stroke Treatment classification system[20], (iv) Patients who did not take clopidogrel at least seven days before admission, (v) Patients with a National Institutes of Health Stroke Scale (NIHSS) score of <15 (mild or moderate IS), (vi) Patients who underwent clopidogrel treatment for at least six months, and (vii) Patients who provided consent to participate in this study. Exclusion criteria were as follows: (i) Patients who are allergic to clopidogrel; (ii) Those who underwent thrombolytic treatment; (iii) Those who used a protonpump inhibitor before or during hospital admission; (iv) Those with hemorrhagic stroke, hematological diseases, autoimmune diseases, or other severe concomitant diseases; (v) Those who underwent anticoagulation therapy with warfarin or heparin within seven days; (vi) Those with cerebral embolism and other determined or undetermined etiologies of IS; (vii) Patients withfever, hypoxia, alterations in consciousness, or any relevant hemodynamic compromise on admission; (viii) Patients who underwent any major surgical procedure within one week prior to enrollment in this study; and (ix) Patients with platelet count $<100\times10^9$/L or $>450\times10^9$/L.

All patients received standard therapies on the basis of guidelines[3] including 75 mg of clopidogrel (Sanofi Company Ltd., Shanghai, China) once daily. Whole blood (5 mL) was obtained for genotyping and adenosine diphosphate (ADP) -induced platelet aggregation test.

Data on vascular risk factors including body mass index, body weight, tobacco smoking, diabetes mellitus, and hypertension were collected, and fasting blood samples were analyzed for blood sugar, plasma total cholesterol, triglycerides, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and homocysteine levels.

ADP-Induced Platelet Aggregation Test

Platelet aggregation activity was measured using light transmittance aggregometry before clopidogrel treatment and within 7−10 days after clopidogrel treatment according to a previous study[21]. In brief, 5

mL of fasting venous blood was collected in 660 μL of 3.8% sodium citrate and centrifuged at 200×g for 10 min to obtain platelet-rich plasma (PRP). Platelet-poor plasma (PPP) was obtained from the remaining specimens by centrifugation at 4000×g for 10 min. The platelet aggregation test was performed using ADP (Helena Laboratories, Beaumont, TX, USA) at 10.0 μM and measured using light transmittance aggregometry with a BioData PAPS-4 platelet aggregometer (Helena Laboratories). Platelet aggregation rate was recorded as the change in the light transmission. The recorder was adjusted to make sure that the difference in the light transmission between PRP and PPP was 100%. The results were presented as the amplitude of light transmittance at 5 min after adding 10 μM of ADP agonist. Changes in values obtained from ADP-induced platelet aggregation tests before and 7−10 days after clopidogrel treatment reflected the sensitivity of the patients to clopidogrel treatment. Clopidogrel resistance was defined as a reduction of <10% in ADP-induced platelet aggregation values after 7−10 days of clopidogrel treatment[22], otherwise, it was considered as clopidogrel sensitivity (CS).

Assessment of Clinical Outcomes

All patients were followed up at our outpatient clinic starting one month after discharge and every two or three months thereafter. Clinical data were collected from hospital records by the referring physicians and data of some patients were collected via phone interview by our investigators. The investigators who evaluated the clinical endpoints were blinded to the results of other data such as DNA genotyping data.

The primary outcome of clopidogrel treatment was a composite of recurrent IS (RIS), MI, or death during six months after the first admission. RIS was defined as a new focal neurologic deficit of vascular origin that lasts for at least 24 h, which has been proven to be nonhemorrhagic by either CT or MRI. Death was defined as vascular mortality due to MI, IS, and other vascular causes. The secondary outcome was the modified Rankin Scale (mRS) at six months. Side effects (safety outcomes) included hemorrhagic episodes that occurred within six months. Hemorrhagic episodes were defined as the presence of any of the following: (i) Symptomatic or asymptomatic hemorrhagic transformation, symptomatic or asymptomatic intracerebral hemorrhage and (ii) Extracranial hemorrhages (e.g., gastrointestinal bleeding, hematoma, hematuria, and skin or mucosal bleeding). Serious hemorrhage was considered as any symptomatic intracranial hemorrhage or any hemorrhage requiring blood transfusion or prolonged hospitalization.

Selection of *CYP* SNPs and Genotyping

In this study, eight SNPs of the *CYP* gene were selected using tools listed in http://www.ncbi.nlm.nih.gov/SNP on the basis of the following criteria: (i) SNPs that have been assessed in previous studies[15-17], (ii) SNPs with minor allele frequency of >0.05, (iii) SNPs leading to amino acid changes, and (iv) Tagging SNPs across different human populations (http://pga.gs.washington.edu).

Genotyping was performed on genomic DNA extracted from periphery blood using the matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) method according to our previous study[19]. In brief, each SNP was amplified using two specific PCR primers and one extension primer. The reaction mixture was desalted by adding 6 mg of cation exchange resin (Sequenom Inc., San Diego, CA, USA), mixed, and resuspended in 25 μL of double-distilled water. Once the primer extension reaction was completed, samples were spotted onto a 384-well spectroCHIP (Sequenom) using a MassARRAY Nanodispenser (Sequenom) and genotyped using MALDI-TOF MS. Genotype call was performed in real-time with MassARRAY RT software version 3.0.0.4 and analyzed using a MassARRAY Typer software version 3.4 (Sequenom).

Each allele of these *CYP* SNPs was classified a priori by its known effect on enzymatic function according to literature and with the use of established common-consensus star allele nomenclature[12, 23, 24]. For each *CYP* gene, subjects were dichotomized a priori into two groups on the basis of whether they possessed at least one allele with significantly reduced function.

Statistical Analysis

All statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). The χ^2 test was used to analyze the deviation of Hardy−Weinberg equilibrium for genotype frequencies and compare genotype frequencies. Continuous variables were compared using Student's t-test. Discrete variables were compared using χ^2 test or Fisher's exact testwhen expected cell frequencies were small.

Gene−gene interaction was assessed using ageneralized multifactor dimensionality reduction (GMDR) program (beta version 0.7, www.healthsystem.virginia.edu/internet/addiction-genomics/Software)[18, 19]. GMDR computed the maximum likelihood estimates and the scores of all individuals under the null hypothesis. A cumulative score was then calculated within each multifactor cell, which was labeled either as highrisk if the average score met or exceeded a preassigned threshold of 0 or as lowrisk if the score was less than 0. An

Table 1. Baseline characteristics of patients

	Clopidogrel resistant ($n=153$)	Clopidogrel sensitive ($n=222$)	*P* value
Age (years)	69.97 ± 11.23	67.04 ± 12.16	0.013
Men (n, %)	99 (64.86)	143 (64.42)	0.98
Diabetes mellitus (n, %)	80 (52.29)	77 (34.68)	<0.001
Hypertension (n, %)	121 (79.08)	172 (77.48)	0.67
Body mass index (kg/m^2)	23.99 ± 3.11	23.96 ± 3.18	0.98
Current smoker (n, %)	64 (41.83)	94 (42.34)	0.86
Alcohol intake (n, %)	67 (43.79)	94 (42.34)	0.83
Previous MI (n, %)	8 (5.23)	10 (4.50)	0.78
TG (mmol/L)	1.54 ± 0.53	1.56 ± 0.64	0.96
TC (mmol/L)	5.52 ± 1.35	5.48 ± 1.38	0.87
LDL-C (mmol/L)	2.98 ± 0.96	2.94 ± 0.92	0.95
H DL-C (mmol/L)	1.23 ± 0.36	1.25 ± 0.44	0.93
Hcy (mmol/L)	14.52 ± 4.27	14.58 ± 4.45	0.89
HbA1c (%)	6.27 ± 1.58	6.19 ± 1.61	0.76
Admission NIHSS	5.93 ± 1.82	5.86 ± 1.91	0.45
Stroke subtype			
Atherothrombotic (n, %)	94 (61.44)	137 (61.71)	0.88
Small artery disease (n, %)	57 (37.25)	87 (39.19)	0.88
Previous treatment (n, %)			
Antihypertensive drugs	66 (43.14)	97 (43.69)	0.92
Hypoglycemic drugs	57 (37.25)	83 (37.39)	0.94
Statins	26 (16.99)	38 (17.12)	0.89
Aspirin	42 (27.45)	63 (28.39)	0.77
In-hospital treatment (n, %)			
Antihypertensive drugs	132 (86.27)	190 (85.59)	0.86
Hypoglycemic drugs	21 (56.76)	175 (53.68)	0.28
Statins	150 (98.04)	219 (98.65)	0.79
Aspirin	95 (62.09)	136 (61.26)	0.65

MI, myocardial infarction; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides; HbA1c, Hemoglobin A1C; Hcy, Homocysteine; NIHSS, National Institutes of Health Stroke Scale.

exhaustive search of all possible one- to eight-loci models was performed for all variants. The *P* value was determined using the sign test, a robust nonparametric test implemented in the GMDR software. A permutation test was applied for multiple testing corrections. The statistical significance was determined by comparing the average prediction error from the observed data with the distribution of average prediction errors under the null hypothesis of no associations empirically derived from 10,000 permutations. A permutation test (combined with cross-validation) can minimize false-positive results because of multiple tests[25]. This model with the minimum prediction error, the maximum cross-validation consistency score, and a *P* value of 0.05 or less (derived automatically from the sign test in the GMDR software) was considered as the best model. Furthermore, multivariate logistic regression analysis was performed to adjust covariate risk factors to assess the independent contribution of gene−gene interactions on clopidogrel resistance risk.

Significant independent predictors of clopidogrel resistance were detected by logistic regression analysis. The relative risk of a genotype with CR was expressed with an odds ratio (OR) and its 95% confidence interval (CI). Cox proportional-hazards model was used to describe risks for primary efficacy outcome during the six-month period after thefirst stroke, and values of the hazard ratio (HR) with 95% CI were reported. The χ^2 test was also used to compare changes in mRS scores at six months. All tests were two-sided, and a *P*-value of 0.05 was considered statistically significant.

Table 2. Allelic frequencies of *CYP* genes between patients with or without clopidogrel resistance (%)

	Clopidogrel resistant (n=153)	Clopidogrel sensitive (n=222)	P value
CYP3A4 (rs2242480)			
CC	83 (54.2)	123 (55.4)	0.91
TT + CT	70 (45.8)	99 (44.6)	
*CYP2C8*2* (rs17110453)			
AA	70 (45.8)	105 (47.3)	0.68
AC + CC	83 (54.2)	117 (52.7)	
*CYP2C8*3* (rs1934980)			
CC	24 (15.7)	31 (14.0)	0.69
CT + TT	129 (84.3)	191 (86.0)	
*CYP2C9*2* (rs1799853)			
CC	153 (100)	222 (100)	–
*CYP2C9*3* (rs1057910)			
AA	136 (88.9)	194 (87.4)	0.37
AC + CC	17 (11.1)	28 (12.6)	
CYP3A5 (rs776746)			
AA	12 (7.8)	36 (16.2)	0.016
GG + AG	141 (92.2)	186 (83.8)	
*CYP2C19*2* (rs4244285)			
GG	46 (30.1)	107 (48.2)	$<$0.001
AG + AA	107 (69.9)	115 (51.8)	
*CYP2C19*3* (rs4986893)			
GG	139 (90.8)	209 (94.1)	0.24
AG	14 (9.2)	13 (5.9)	

Results

Patient Characteristics

The mean platelet aggregation activity was 72.36 ± 17.23% before clopidogrel treatment and 33.92 ± 12.13 after clopidogrel treatment, leading to the mean inhibition of platelet aggregation of 40.78 ± 13.15%. Among the 375 IS patients, 153 (40.8%) patients were clopidogrel resistant, whereas 222 patients (59.2%) were clopidogrel sensitive. Clopidogrel-resistant patients were older (P=0.013) and were more frequently found in patients with diabetes mellitus (P<0.001), compared with clopidogrel-sensitive patients. In contrast, there was no significant difference in other risk factors between these two groups of patients (all P>0.05, **Table 1**).

CYP SNPs and its Association with Clopidogrel Response

Genotype distributions of *CYP* among the 375 IS patients are shown in **Table 2**. Specifically, frequency of the *CYP3A5* (rs776746) GG + AG and *CYP2C19*2* (rs4244285) AA + AG genotypes was significantly higher in clopidogrel-resistant patients than in clopidogrel-sensitive patients (P=0.016 and P< 0.001, respectively). However, genotype distributions of other variants did not differ significantly between these two groups of patients (all P>0.05).

The association of the high-order interactions of *CYP* SNPs with clopidogrel resistance was then assessed using the GMDR method (**Table 3**). After covariate adjustments, the best model for clopidogrel resistance including *CYP3A5* (rs776746) and *CYP2C19*2* (rs4244285) scored 10/10 for cross-validation consistency and nine for the sign test (P=0.011). The significance of this interaction was further confirmed by a permutation test (P=0.021). The prediction accuracies of these one-locus model was also computed for each variant, yielding a minimum P value of 0.9424, suggesting that the two variants together significantly contributed to clopidogrel resistance. Compared to patients harboring *CYP3A5* (rs776746) AA and *CYP2C19*2* (rs4244285) GG (wild-type genotypes), the relative risk of the nine combinations of genotypes of *CYP3A5* (rs776746) and *CYP2C19*2* (rs4244285) was analyzed. Two interactions made large contributions to this model: *CYP3A5* (rs776746) GG and *CYP2C19*2* (rs4244285) AA. The estimated risk of clopidogrel resistance was significantly higher in patients with *CYP3A5* (rs776746) GG and *CYP2C19*2*

Table 3. Comparison of the best models for predicting accuracy, cross-validation consistency, and *P* values assed by generalized multifactor dimensionality reduction analysis of clopidogrel resistance

Best model*	Training balanced accuracy	Testing balanced accuracy	Cross-validation consistency	Sign test (*P* value)
1	0.5268	0.4785	6/10	4 (0.92)
1, 2	0.7688	0.5721	10/10	9 (0.011)
1, 2, 3	0.5664	0.4852	7/10	2 (0.94)
1, 2, 4, 5	0.6523	0.4231	4/10	2 (0.96)
1, 2, 3, 4, 5	0.7325	0.4956	9/10	5 (0.26)
1, 2, 3, 4, 5, 6	0.7129	0.4785	8/10	5 (0.81)
1, 2, 3, 4, 5, 6, 7	0.6353	0.4874	8/10	3 (0.76)
1, 2, 3, 4, 5, 6, 7, 8	0.7218	0.4982	10/10	4 (0.88)

*Numbers 1-8 represent rs776746, rs4244285, rs2242480, rs17110453, rs1934980, rs1799853, rs1057910 and rs4986893, respectively.

Table 4. Logistic regression analysis of significant independent predictors of clopidogrel resistance

Risk factor	OR	95% CI	*P* value
Age (>68 years old)	0.68	0.58-1.48	0.12
Hypertension	1.01	0.87-2.86	0.11
Diabetes mellitus	1.66	1.06-3.15	0.032
CYP2C19 (rs4244285)	1.98	1.23-4.17	0.013
CYP3A5 (rs776746)	1.11	0.94-3.36	0.085
Interaction of *CYP2C19*2* AA and *CYP3A5* GG	2.46	1.97-6.54	0.001

OR, odds ratio; CI, confidence interval.
Note: Input variables include age, gender, tobacco smoking, hypertension, diabetes mellitus, stroke subtype, body mass index, previous myocardial infarction, total cholesterol, low-density lipoprotein cholesterol, and high-density lipoprotein cholesterol; triglycerides; hemoglobin A1C; homocysteine, *CYP3A4* (rs2242480), *CYP2C8*2* (rs17110453), *CYP2C8*3* (rs1934980), *CYP2C9*2* (rs1799853), *CYP2C9*3* (rs1057910), *CYP2C19*3* (rs4986893), *CYP3A5* (rs776746), *CYP2C19*2* (rs4244285), and the interaction of *CYP2C19*2* AA with *CYP3A5* GG.

(rs4244285) AA, as compared to patients harboring *CYP3A5* (rs776746) AA and *CYP2C19*2* (rs4244285) GG (OR=2.23, 95% CI: 1.08−5.87, *P*=0.025; **Supplementary Table 1**). These data suggest that these two *CYP* genetic variants together significantly contributed to clopidogrel resistance.

The relative risk conferred by the combinations of *CYP3A5* GG and *CYP2C19*2* AA was considered as a high-risk variable, with assigned as one, and other combinations of *CYP3A5* and *CYP2C19*2* as a low-risk variable, with assigned as zero. Logistic regression analysis revealed that the interaction of *CYP2C19*2* (rs4244285) AA with *CYP3A5* GG (OR=2.46, 95%CI: 1.97−6.54, *P*=0.001), *CYP2C19*2* (rs4244285) AA/AG (OR=1.98, 95%CI: 1.23−4.17, *P*=0.013), or diabetes mellitus (OR=1.66, 95%CI: 1.06−3.15, *P*=0.032) are significant independent predictors of clopidogrel resistance (**Table 4**).

Association of *CYP* SNPs with Platelet Aggregation Activity

There were no significant differences in the activity of pre-treatment platelet aggregation among these eight *CYP* SNPs. However, after 7−10 days of clopidogrel treatment, the percentage of reduced platelet aggregation activity was significantly lower in patients with the *CYP2C19*2* GA/AA genotype than in patients with the GG genotype (39.56±11.16% vs. 54.64±18.26%, *P*<0.001) or in patients with the *CYP3A5* GG/AG genotype than in patients with the AA genotype (42.56±12.38% vs. 54.97±16.38%, *P*<0.001). However, there were no significant differences among the genotypes of the other six variants. Furthermore, the percentage of reduced platelet aggregation activity was significantly lower in patients who carry interactive genotype *CYP2C19*2* AA and *CYP3A5* GG than in patients without interactive genotypes (32.58±10.23% vs. 53.84±17.25%, *P*<0.001).

Table 5. Baseline characteristics of patients with or without primary outcome

	Patients with primary outcome (n=37)	Patients without primary outcome (n=326)	P value
Age (year)	69.96 ± 10.98	67.03 ± 12.26	0.01
Men (n, %)	24 (64.86)	210 (64.42)	0.99
Diabetes mellitus (n, %)	20 (54.05)	133 (40.79)	0.01
Hypertension (n, %)	31 (83.78)	257 (78.83)	0.74
Body mass index (kg/m^2)	24.05 ± 3.12	23.92 ± 3.29	0.88
Current smoker (n, %)	16 (43.24)	136 (41.72)	0.76
Alcohol intake (n, %)	16 (43.24)	140 (42.94)	0.82
Previous MI (n, %)	2 (5.41)	15 (4.60)	0.72
TG (mmol/L)	1.53 ± 0.56	1.57 ± 0.67	0.58
TC (mmol/L)	5.53 ± 1.33	5.39 ± 1.28	0.25
LDL-C (mmol/L)	3.01 ± 0.97	2.97 ± 0.99	0.31
HDL-C (mmol/L)	1.22 ± 0.43	1.24 ± 0.48	0.97
Hcy (mmol/L)	14.46 ± 3.98	14.59 ± 4.68	0.22
Fasting blood glucose (mmol/L)	7.11 ± 2.68	6.89 ± 2.42	0.28
HbA1c (%)	6.36 ± 1.39	6.17 ± 1.56	0.15
Admission NIHSS	5.96 ± 1.87	5.85 ± 1.84	0.16
Stroke subtype			
Atherothrombotic (n, %)	23 (62.16)	200 (61.35)	0.72
Small artery disease (n, %)	14 (37.84)	126 (38.65)	0.72
Previous treatment (n, %)			
Antihypertensive drugs	16 (43.24)	143 (43.87)	0.92
Hypoglycemic drugs	14 (37.84)	119 (36.50)	0.78
Statins	6 (16.22)	56 (17.18)	0.89
Aspirin	10 (27.03)	92 (28.22)	0.64
In-hospital treatment (n, %)			
Antihypertensive drugs	32 (86.49)	279 (85.58)	0.71
Hypoglycemic drugs	21 (56.76)	175 (53.68)	0.23
Statins	36 (97.29)	318 (97.55)	0.91
Aspirin	23 (62.16)	199 (61.04)	0.58

MI, myocardial infarction; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides; HbA1c, Hemoglobin A1C; Hcy, Homocysteine.

Association of *CYP* SNPs with Outcome of Clopidogrel Treatment

Among these 375 patients, 363 patients (96.8%) completed the follow-up: 97.4% in clopidogrel-resistant patients (149 of 153) and 96.4% in clopidogrel-sensitive patients (214 of 222). Primary outcome of clopidogrel occurred in 37 patients (31 had RIS, fourdied, and two had MI) during the first six months of treatment. Baseline characteristics of these patients with or without primary outcome are summarized in **Table 5**. In brief, patients with primary outcome were older and had diabetes mellitus. In contrast, there was no statistical difference in other factors associated with primary outcome after clopidogrel treatment.

Furthermore, the frequency of primary outcome was higher in patients who carry the *CYP2C19*2* GA/AA genotype than in patients who carry the GG genotype, or in patients who carry the *CYP3A5* GG/AG genotype than in patients who carry the AA genotype, and in patients who carry interactive genotypes *CYP2C19*2* AA and *CYP3A5* GG than in patients without interactive genotypes (**Table 6**). However, there was no statistical association between any of the other six *CYP* genotypes andthe primary outcome was observed.

With regard to the second outcome, a better outcome was defined as mRS ≤ 2 points, whereasmRS > 2 points was considered as a poor outcome at six months after stroke. Patients with clopidogrel resistance and carrying the *CYP2C19*2* AG/AA genotype or interaction genotypes *CYP2C19*2* AA and *CYP3A5* GG were more likely to have a poor outcome (**Table 6**). However, there was no association of other *CYP* genotypes with poor outcome. Moreover, these results also showed that there was an association of clopidogrel

Table 6. Association of CR, *CYP2C19* (rs4244285) and *CYP3A5* (rs776746) with outcomes (%)

	Patients with Primary outcome (n=37)	Patients without primary outcome (n=326)	P value	mRS >2 (n=79)	mRS ≤2 (n=284)	P value
CR (n=149)	23 (15.44)	126 (84.56)	0.006	42 (28.19)	107 (71.81)	0.016
CS (n=214)	14 (6.54)	200 (93.46)	0.006	37 (17.29)	177 (82.71)	0.016
CYP3A5 (rs776746)						
AA (n=45)	0 (0.0)	45 (100)	0.018	5 (11.11)	40 (88.89)	0.071
GG + AG (n=318)	37 (11.64)	281 (88.36)	0.018	74 (23.27)	244 (76.73)	0.071
*CYP2C19*2* (rs4244285)						
GG (n=148)	7 (4.73)	141 (95.27)	0.003	19 (12.84)	129 (87.16)	<0.001
AG + AA (n=215)	30 (13.95)	185 (86.05)	0.003	60 (27.91)	155 (72.09)	<0.001
Interaction of *CYP2C19*2* AA with *CYP3A5* GG						
Yes (n=63)	15 (23.81)	48 (76.19)	<0.001	21 (33.33)	42 (66.67)	0.014
No (n=300)	22 (7.33)	278 (92.67)	<0.001	58 (19.33)	242 (80.67)	0.014

CR, clopidogrel resistance; CS, clopidogrel sensitivity.

responsive (platelet aggregation test) and *CYP* SNPs with primary and secondary clinical outcome. With regard to the association of poor outcome with RIS, the patients with poor outcome were associated with RIS, as compared with good outcome patients [12/79 (15.2%) vs. 19/284 (6.7%), P=0.018].

Cox regression analysis revealed that diabetes mellitus (HR=1.72, 95%CI: 1.21−3.96, P=0.017), *CYP2C19*2* (HR=2.01, 95%CI: 1.46−5.66, P=0.006), clopidogrel resistance (HR=1.98, 95%CI: 1.46−5.63, P=0.006), and interaction of *CYP2C19*2* AA with *CYP3A5* GG (HR=2.51, 95%CI: 2.08−8.72, P<0.001) were independent risk factors for primary adverse events (**Table 7**).

However, there was no significant difference in the rate of extracranial hemorrhage, asymptomatic intracerebral hemorrhage or hemorrhagic transformation between the clopidogrel resistance and clopidogrel sensitivity groups (all P>0.05). There were no association between hemorrhagic episodes and any of the eight *CYP* genotypes. Overall, there were no serious hemorrhage, symptomatic intracerebral hemorrhage, or hemorrhagic transformation events observed in this study.

Discussion

In the current study, we consecutively enrolled 375 IS patients who received clopidogrel, and associated genotyped *CYP* SNPs with clopidogrel resistance and adverse events in these patients. Our data revealed that 40.8% of IS patients were clopidogrel resistant, and these data were in accordance with previous studies[26-28]. Age and diabetes mellitus had a significant impact on clopidogrel response among IS patients, although the mechanisms of the hyporesponsiveness of clopidogrel associated with diabetes mellitus remains unclear. As patients age, the overall metabolic level in the body reduces, which could lead to clopidogrel resistance, whereas patients with diabetes mellitus may decrease periphery blood circulation and in turn affect the level of the active metabolite of clopidogrel available in the blood system. Thus, although there were normal *ex vivo* clopidogrel responses observed in patients who were unresponsive to clopidogrel, patients with diabetes mellitus would resist to clopidogrel[29]. Additional mechanisms for suboptimal clopidogrel-induced anti-platelet effects in diabetic patients include increased circulating ADP, calcium, or esterase levels, as well as platelet turnover, the expression of P2Y12 receptors, or the upregulation of other platelet activation pathways[30, 31].

Furthermore, our current data also revealed that IS patients who carry the *CYP3A5* (rs776746) GG + AG or *CYP2C19*2* (rs4244285) AA + AG genotype had a significantly higher prevalence of clopidogrel resistance; and that the *CYP2C19*2* genotype wasa significant independent predictor for clopidogrel response and adverse events. SNPs may affect clopidogrel activation, which could also contribute to the adverse events of this prodrug. Variant alleles and genotypes of *CYP3A5*3* polymorphism contributed significantly to clopidogrel resistance with a higher OR[32]. Suh *et al.*[33] also revealed an increased frequency of atherothrombotic events within six months after coronary angioplasty in patients with the *CYP3A5* nonexpression genotype (*CYP3A5*3*). However, other studies did not revealany association between *CYP3A5*

Table 7. Multivariate Cox regression analysis of independent predictors for adverse events

Factor	HR	95% CI	*P* value
Age (>68 years)	0.76	0.83-1.65	0.12
Hypertension	1.21	0.98-3.12	0.069
Diabetes mellitus	1.72	1.21-3.96	0.017
Smoking	0.92	0.86-1.48	0.27
High LDL-C	1.01	0.92-2.45	0.089
High TC	0.87	0.74-1.55	0.28
CYP2C19 (rs4244285)	2.01	1.46-5.66	0.006
CYP3A5 (rs776746)	1.18	0.95-3.43	0.097
Interaction of *CYP2C19*2* AA with *CYP3A5* GG	2.51	2.08-8.72	<0.001
Clopidogrel resistance	1.98	1.46-5.63	0.006

HR, hazard ratio; CI, confidence interval; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol.
Note: Input variables include: age, gender, tobacco smoking, hypertension, diabetes mellitus, stroke subtype, body mass index, previous myocardial infarction, total cholesterol, low-density lipoprotein cholesterol, and high-density lipoprotein cholesterol; triglycerides; hemoglobin A1C; homocysteine, *CYP3A4* (rs2242480), *CYP2C8*2* (rs17110453), *CYP2C8*3* (rs1934980), *CYP2C9*2* (rs1799853), *CYP2C9*3* (rs1057910), *CYP2C19*3* (rs4986893), *CYP3A5* (rs776746), *CYP2C19*2* (rs4244285), Interaction of *CYP2C19*2* AA with *CYP3A5* GG and clopidogrel resistance.

genetic variants and the antiplatelet effect of clopidogrelin acute MI or percutaneous coronary intervention patients[15, 34], although the genetic polymorphisms of *CYP2C19* can modulate clopidogrel pharmacokinetics and pharmacodynamics[35]. Previous studies have demonstrated that carriers with ≥1 variant of *CYP2C19* alleles (2* or 3*) had significantly lower levels of the active metabolite of clopidogrel; thus, these individuals hada diminished platelet inhibitory rate after the administration of clopidogrel, resultingina higher rate of major adverse cardiovascular events[35, 36]. Patients without these *CYP2C19* variant alleles had a better response rate, as demonstrated by better mRS scores at sixmonths after treatment[28]. Our current data are consistent with these previous studies[28, 35, 36].

In addition, our current study demonstrated that the interaction of *CYP3A5* (rs776746) GG with *CYP2C19*2* (rs4244285) AA had a significantly independent impact on clopidogrel response and adverse events. Specifically, the reduction in platelet aggregation activity in patients who carry the *CYP2C19*2* AA and *CYP3A5* GG genotypes was significantly lower than patients without such genotypes. However, the nature of these interactions remains unclear. One possible explanation for this may be because the two genes encode enzymes that participate in the modulation of clopidogrel pharmacokinetics and pharmacodynamics, which is one of the principal pathogenic factors of clopidogrel response. Our previous studies have shown that the interaction of *CYP* rs17110453, rs751141, and rs9333025 may confer a higher risk for IS[19, 37]. A possible explanation for these *CYP* gene SNP interactions may be the three *CYP* gene encode enzymes that participate in arachidonic acid metabolism, which is one of the principal pathogenic factors of IS. Using this combinatorial analysis with our current data may be helpful to elucidate complex genetic risk factors for clopidogrel resistance and IS. Our current study indicates that patients with these SNPs may need higher doses of clopidogrel or alternative drugs. Currently, there are no standardized treatment recommendations for these patients. The dosing regimen show that the response to clopidogrel is beyond pharmacokinetics and *CYP2C19*2* heterozygotes require a daily dose of 225 mg to maintain "normal" platelet inhibition response, which is in triple of the USFDA recommended dose of 75 mg[38]. Substitution of clopidogrel with another antiplatelet drug (like ticagrelor or prasugrel) is thought to another regime, and may help prevent the occurrence of vascular events[39]. The *ex vivo* platelet aggregation assay is commonly used to evaluate action of clopidogrel, but there were inconsistent results due to both technical and biological issues. To date, it is unclear whether there is a sufficiently validated *ex vivo* platelet aggregation assay to predict clinical response to clopidogrel, thus, these assays are not practical in the clinical setting[40]. A number studies showed that genetic determined the response to clopidogrel and cardiovascular events[14-16]. Among patients for whom clopidogrel therapy is indicated, genotyping rather than repeated platelet monitoring could be an affordable and suitable strategy to identify patients at high risk for atherothrombotic events. Thus, the USFDA suggested physicians avoid prescribing clopidogrel to patients with known *CYP2C19* polymorphisms[41].

There are several potential limitations in the presentstudy. For example, the current study did not include the assessment of plasma clopidogrel levels and its active metabolite. The current study may also have possible bias due to the relatively small sample size and short follow-up period. Moreover, although we genotyped multiple known functional variants in relevant *CYP* genes in this cohort of patients, some rare functional variants may have been left undetected in this population; thus, we could not exclude their role in regulation of the clopidogrel resistance. Furthermore, platelet aggregation activity was only measured using the light transmittance aggregometry in the current study. Other studies showed that vasodilator-stimulated phosphoprotein and VerifyNow P2Y12 assay could be a better choice for assessment of clopidogrel resistance[42]. Future studies with a larger set of genetic variants, a multi-center, large sample size, a longer follow-up period, vasodilator-stimulated phosphoprotein, and VerifyNow P2Y12 assay are necessary to confirm our current data.

Clinical Trial Registration Information

http://www.chictr.org/. Unique Identifier: ChiCTR-OCH-14004724.

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Conflict of Interest Statement

The authors declare no conflict of interest in this work.

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Supplementary Table 1. Associations of cerebral infarction with combined genotypes

rs4244285	GG	AA	AA, AG	AA	AG	AA, AG
rs776746	AA	GG	GG	GG, AG	AG	GG, AG
OR	1*	2.23	1.24	1.86	1.15	1.06
95% CI	–	1.08-5.87	0.63-2.02	0.98-3.67	0.58-1.96	0.61-1.82
P value	–	0.025	0.238	0.076	0.532	0.653

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