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. Clinically, stroke can be classified into ischemic and hemorrhagic categories because their management significantly differs. For example, antiplatelet drugs are recommended for the treatment and prevention of noncardioembolic ischemic stroke (IS). Depending on the risk of stroke, antithrombotic drugs such as aspirin or warfarin can effectively reduce IS in certain populations. Previous studies have shown that clopidogrel is superior to aspirin for preventing IS in high-risk individuals. However, antiplatelet drugs such as clopidogrel may not completely prevent ischemic events, causing a number of subsequent IS events to occur. Such a situation is referred to as drug or clopidogrel resistance, which is due to an individual’s variability in response to drug or clopidogrel therapy. A study suggested that hyporesponsiveness to antiplatelet drugs is associated with poor clinical outcome after an acute coronary syndrome. Thus, further research on this individual variability could help us develop more effective drugs to control or prevent IS in clinical settings.
To date, the molecular and clinical mechanisms underlying clopidogrel resistance remain to be determined\(^\text{10}\). Clopidogrel is an oral thienopyridine-class antiplatelet drug used for the treatment and prevention of coronary artery disease, myocardial infarction (MI), and cerebrovascular disease\(^\text{5,7}\). However, it is a prodrug that requires biotransformation to an active metabolite by cytochrome P450 (CYP) enzymes\(^\text{11}\). 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\(^\text{12}\). Previous in \textit{vitro} metabolic and clinical studies have demonstrated that certain \textit{CYP} gene single nucleotide polymorphisms (SNPs) reduce enzymatic activities \textit{in vitro} and affect the conversion of clopidogrel into its active metabolite and that this is associated with the degree of platelet coagulation capacity\(^\text{13,14}\). To date, gene polymorphism-related clopidogrel resistance has been widely studied in coronary heart disease patients\(^\text{15-17}\). 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, \textit{ABCB1}, \textit{CYP3A5}, \textit{P2Y12}, and \textit{PLA1/A2} polymorphisms have not been associated with clopidogrel resistance\(^\text{15-17}\), 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 losocalon 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\(^\text{18,19}\). We therefore hypothesized that relevant \textit{CYP} genetic variants and interaction among these variants contribute to clopidogrel resistance and the outcome of acute IS patients treated with clopidogrel.

\textbf{Materials and Methods}

\textbf{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 \(\geq 40\) 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\(^\text{20}\), (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, hemotological 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\(\times 10^9\)/L or >450\(\times 10^9\)/L.

All patients received standard therapies on the basis of guidelines\(^\text{3}\) 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.

\textbf{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\(^\text{21}\). 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 treatment22, 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 studies15-17, (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 study19. 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 nomenclature12, 23. 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 χ² 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 χ² test or Fisher’s exact test when 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
An 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. 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 for 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 the first stroke, and values of the hazard ratio (HR) with 95% CI were reported. The $\chi^2$ 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 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/m²)  | 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      |
| HDL-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.
Table 2. Allelic frequencies of CYP genes between patients with or without clopidogrel resistance (%)

| Genotype                  | 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)                    |         |
| CYP3A45 (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 CYP3A45 (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 (rs4244285) AA.
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%, \( P<0.001 \)).
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, four died, 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 and the primary outcome was observed.

With regard to the second outcome, a better outcome was defined as mRS ≤2 points, whereas mRS >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 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/m²)  | 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, %) | 32 (86.49) | 279 (85.58) | 0.71    |
| Antihypertensive drugs   | 21 (56.76)                          | 175 (53.68)                            | 0.23    |
| Hypoglycemic drugs       | 36 (97.29)                          | 318 (97.55)                            | 0.91    |
| Statins                  | 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.
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: 2.08–3.00, P = 0.003) 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. 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. 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.

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 was a 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*5 polymorphism contributed significantly to clopidogrel resistance with a higher OR. Suh et al. 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 reveal any association between CYP3A5
SNP interactions may be the three and pharmacodynamics. Previous studies have demonstrated that carriers with CYP2C19 of major adverse cardiovascular events. Patients had a diminished platelet inhibitory rate after the administration of clopidogrel, resulting in a higher rate of major adverse cardiovascular events. Specifically, the reduction in platelet aggregation response, which is in triple of the USFDA recommended dose of 75 mg. 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. 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. A number studies showed that genetic determined the response to clopidogrel and cardiovascular events. 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.

### 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.

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. 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. 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. 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. A number studies showed that genetic determined the response to clopidogrel and cardiovascular events. 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.
There are several potential limitations in the present study. 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\(^4\). 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

| Gene   | Genotypes 1 | Genotypes 2 | Genotypes 3 | Genotypes 4 | Genotypes 5 | Genotypes 6 |
|--------|-------------|-------------|-------------|-------------|-------------|-------------|
| rs4244285 | GG          | AA          | AA, AG      | AA          | AG          | AA, AG      |
| rs776746  | AA          | GG          | GG          | AA, AG      | AG          | AA, 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.