Original Article

Associations of *CYP3A4*, *NR1I2*, *CYP2C19* and *P2RY12* polymorphisms with clopidogrel resistance in Chinese patients with ischemic stroke

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Aim: There is a high incidence of the antiplatelet drug clopidogrel resistance (CR) in Asian populations. Because clopidogrel is a prodrug, polymorphisms of genes encoding the enzymes involved in its biotransformation may be the primary influential factors. The goal of this study was to investigate the associations of polymorphisms of *CYP3A4*, *NR1I2*, *CYP2C19* and *P2RY12* genes with CR in Chinese patients with ischemic stroke.

Methods: A total of 191 patients with ischemic stroke were enrolled. The patients were treated with clopidogrel for at least 5 days. Platelet function was measured by light transmission aggregometry. The SNPs *NR1I2* (rs13059232), *CYP3A4**1G (rs2242480), *CYP2C19**2 (rs4244285) and *P2RY12* (rs2046934) were genotyped.

Results: The CR rate in this population was 36%. The *CYP2C19**2 variant was a risk factor for CR (*2/*2+wt/*2 vs wt/wt, OR: 2.366, 95% CI: 1.180–4.741, $P=0.014$), whereas the *CYP3A4**1G variant had a protective effect on CR (*1/*1 vs *1G/*1G+*1/*1G, OR: 2.360, 95% CI: 1.247–4.468, $P=0.008$). The *NR1I2* (rs13059232) polymorphism was moderately associated with CR (CC vs TT+TC, OR: 0.533, 95% CI: 0.286–0.991, $P=0.046$). The C allele in *P2RY12* (rs2046934) was predicted to be a protective factor for CR (CC+TC vs TT, OR: 0.407, 95% CI: 0.191–0.867, $P=0.018$). In addition, an association was found between hypertension and CR ($P=0.022$).

Conclusion: The individuals with both the *CYP2C19**2 allele and hypertension are at high risk of CR during anti-thrombosis therapy. The *CYP3A4**1G allele, *P2RY12* (rs2046934) C allele and *NR1I2* (rs13059232) CC genotype may be protective factors for CR. The associated SNPs studied may be useful to predict clopidogrel resistance in Chinese patients with ischemic stroke.

Keywords: clopidogrel resistance; ischemic stroke; genotype analysis; SNPs; *CYP3A4**1G; *NR1I2*; *CYP2C19**2; *P2RY12*; pharmacogenomics

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Introduction

Clopidogrel (Plavix®) is an irreversible P2Y₁₂ receptor antagonist prescribed for the treatment of arteriosclerotic events in patients with recent stroke, myocardial infarction (MI) or established peripheral arterial disease^[1, 2]. Although clopidogrel is safe and effective in most patients, there is an inevitable variability in responsiveness among individuals^[3]. Current investigations indicate that 4% to 30% of patients fail to attain

platelet inhibition after clopidogrel therapy^[4–6], and this phenomenon is called clopidogrel resistance (CR). In fact, several clinical studies conducted in Chinese, Japanese and Korean patients reveal that the frequencies of clopidogrel resistance in Asians may vary from 20% to 65%, which highly exceeds the incidence reported in other ethnic groups^[7, 8]. The underlying mechanisms of clopidogrel resistance remain unknown. Previous studies indicate that genetic factors may greatly contribute to the variability of platelet activity^[8].

Because clopidogrel is a prodrug that requires a two-step oxidation process for activation in the liver, polymorphisms of genes encoding the metabolic enzymes involved in clopidogrel biotransformation may be the primary influential factors of clopidogrel responsiveness. *CYP2C19* plays a substantial role in both of these steps by producing nearly 50% of the

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active CYP2C19 metabolites^[9]. CYP3A4 also participates in hepatic clopidogrel metabolism, producing 39.8% of the active metabolites during the second oxidation step^[9]. Pregnane X receptor (PXR), a member of the nuclear receptor subfamily 1 (NR1I2), is a transcriptional regulator of several metabolic enzymes, including CYP3A4 and CYP2C19^[10]. It has been previously reported that the genetic polymorphisms in *NR1I2* may account for the interindividual variation in drug metabolism and disposition in certain diseases^[11, 12]. In addition, the *P2RY12* gene encodes the P2Y12 ADP receptor, and genetic variants of *P2RY12* have been associated with alterations in the platelet inhibition response in patients^[13]. Many studies have focused on the association between genetic polymorphisms and clopidogrel resistance^[14–16].

According to the clopidogrel black box warning released by the US FDA, CYP2C19 genotyping now allows clinicians to adjust the treatment therapy for an individual patient. The different CYP2C19 genotypes have a significant impact on the response of clopidogrel and the prognosis of patients with ischemic stroke^[17]. Although the hepatic *CYP2C19* LOF genotypes are associated with a lower clopidogrel responsiveness^[18, 19], only 12% of the platelet response to clopidogrel can be explained by the presence of the *CYP2C19**2 polymorphism, as suggested by an intervention (PAPI) study^[20, 21]. Thus, other potential genetic factors and mechanisms remain to be discovered, which together may contribute to the individual variability of clopidogrel efficacy^[22]. Moreover, limited documents have been published concerning the relationship between genetic polymorphisms and clopidogrel resistance in Chinese ischemic stroke patients. The aim of this study was to investigate the association between the *CYP2C19**2 (rs4244285), *CYP3A4**1G (rs2242480), *NR1I2* (rs13059232) and *P2RY12* (rs2046934) polymorphisms and clopidogrel resistance in Chinese ischemic stroke patients.

Material and methods

Study population

This study was approved by the Ethics Committee of Guangdong Provincial Hospital of Traditional Chinese Medicine, China. It was conducted in accordance with the Declaration of Helsinki and was consistent with applicable guidelines for good clinical practice. In total, 191 ischemic stroke patients between 40 and 80 years of age were enrolled in the study from January 2014 to January 2015. The inclusion criteria were as follows: (1) clinical diagnosis of ischemic stroke according to the revised guideline of the 4th Cerebrovascular Disease Forum of China, as shown by CT or MRI; (2) treatment with clopidogrel for at least 5 d with complete laboratory examinations and clinical records; and (3) signed informed consent prior to the study. The exclusion criteria were as follows: (1) patients who were allergic to clopidogrel; (2) a platelet count greater than $450 \times 10^9/\text{L}$ or less than $150 \times 10^9/\text{L}$; (3) anti-coagulation therapy with warfarin or heparin within 1 month of the study; (4) recent history of active bleeding; (5) ALT, AST or TB values 3-fold higher than the normal range; (6) serum creatinine 50% above the normal upper limit; and

(7) major surgery within 1 month of the study. Depending on their condition, enrolled patients were treated with either a 300 mg loading dose of clopidogrel followed by a 75 mg maintenance dose per day or a 75 mg maintenance dose daily. Demographic characteristics, clinical information and medications were collected from patient electronic medical records at the Guangdong Provincial Hospital of Traditional Chinese Medicine.

Extraction of peripheral blood DNA

Whole blood samples were obtained from participants and collected in EDTA tubes and stored at -80°C . The extraction method was performed based on a previous study with some modifications^[23]. The concentration and purity of extracted DNA samples were calculated using a Nanodrop 2000 Spectrophotometer (ThermoFisher, USA).

Genotype analysis

Four single nucleotide polymorphisms were selected for the study, including *NR1I2* (rs13059232), *CYP3A4**1G (rs2242480), *CYP2C19**2 (rs4244285) and *P2RY12* (rs2046934), and they were genotyped using Sequenom MassARRAY iPLEX technology (Sequenom, San Diego, CA, USA). Operations were based on the instructions for multiplexed genotyping analysis using iPLEX gold in a 96-well format for MassARRAY. DNA (10 ng) samples were amplified by multiplex PCRs, and the amplification products were subsequently used for iPLEX extension reactions. The final products were desalted and dispensed into a 96-pad SpectroCHIP. Allele detection was performed using MALDI-TOF mass spectrometry (Sequenom). MassARRAY Typer software was utilized to analyze the mass spectrums.

Platelet function assay

Platelet function was measured by the light transmission aggregometry method to evaluate antiplatelet responses. Before the next dose of clopidogrel was administered, peripheral venous blood samples were collected from patients. The platelet aggregation rate was measured within 3 h. The blood was centrifuged to separate the platelet-rich plasma (PRP) and the platelet-poor plasma (PPP), and the PRP sample was diluted to 2×10^9 to $3 \times 10^9/\text{L}$ using the PPP sample. Next, both the PPP and PRP samples were heated to 37°C for 3 min. To measure platelet aggregation, $10 \mu\text{mol}/\text{L}$ of ADP was added to the PRP sample to induce platelet aggregation, and the change in light transmission in the PRP sample was recorded until the response reached a plateau. The maximal platelet aggregation rate (MPAR) was calculated and recorded. After the baseline platelet aggregation measurements were obtained, follow-up platelet aggregation studies were repeated 5 d after the last dose of clopidogrel.

Definition of clopidogrel resistance

Clopidogrel resistance was defined as either less than a 10% change in MPAR on d 5 or the MPAR (d 5) was greater than 50% of the baseline magnitude (d 0)^[24, 25]. The platelet

inhibition rate was quantified as follows:

$$\text{Platelet inhibition rate (PIR)} = \text{PAR (d 0)} - \text{PAR (d 5)}.$$

PIR < 10% × PAR (d 0) or PAR (d 5) > 50% PAR (d 0) was defined as clopidogrel resistance. According to this standard, participants were divided into clopidogrel resistance (CR) or non-resistance (NCR) groups.

Statistical analysis

Data are presented as the mean ± SD for continuous variables and as frequencies for categorical variables. The significant differences between groups were assessed using the Chi-square test (when variables were categorical) or the independent-samples *t*-test (when variables were continuous). Hardy-Weinberg equilibrium and allele frequency comparisons were predicted using the Chi-square test. A significance level of *P* < 0.05 was based on the two-sided probability test. All statistical analysis was performed using SPSS version 21.0.

Results

Baseline characteristics of study participants

In total, 191 ischemic stroke patients with different disease statuses were enrolled. There were 110 males (58%) and 81 females (42%) included in our study. The average age of the participants was 67 years old. All of the patients were divided into the NCR or CR group according to the definitions specified in the methods section. The CR rate was 36% of the research population. In our study population, 131 subjects were in an acute phase, 11 subjects were in a recovery phase, 32 subjects were in a sequela phase and 17 subjects were suffering from transient ischemia. No discrepancies in the CR rate were found among these four categories of stroke patients. Other characteristics of the study population are presented in Table 1. No significant differences in the distribution of age, sex, diabetes, smoking, alcohol, medications, previous history, NIHSS score or biochemical indexes were found between the CR and NCR groups. Patients with hypertension exhibited a higher prevalence of CR (81% with hypertension in 69 patients).

Genotype distributions and allele frequencies of CYP2C19*2, CYP3A4*1G, NR1I2 and P2RY12

Table 2 shows the genotype distributions and allele frequencies

Table 1. Baseline characteristics of the patients.

	NCR	CR	P-value
Total cases, <i>n</i>	122	69 (36%)	
Age (year)	67 ± 10	67 ± 10	1.000
Sex			
Male, <i>n</i> (%)	72 (59%)	38 (55%)	0.595
Female, <i>n</i> (%)	50 (41%)	31 (45%)	
Acute phase	82 (67%)	49 (71%)	0.633
Categories of stroke			
Recovery phase	6 (5%)	5 (7%)	
Sequela	21 (17%)	11 (16%)	
TIA	13 (11%)	4 (6%)	
Diabetes, <i>n</i> (%)	36 (30%)	22 (32%)	0.493
Hypertension, <i>n</i> (%)	80 (66%)	56 (81%)	0.022
Smoke, <i>n</i> (%)	31 (25%)	17 (25%)	0.906
Drink, <i>n</i> (%)	8 (7%)	8 (12%)	0.227
Statins, <i>n</i> (%)	107 (88%)	65 (94%)	0.209
Antihypertensive drugs, <i>n</i> (%)	47 (39%)	34 (49%)	0.149
Previous stroke or TIA, <i>n</i> (%)	49 (40%)	36 (52%)	0.304
NIHSS score	3.93 ± 4.26	3.93 ± 4.26	1.000
PLT count (×10 ⁹ /L)	246 ± 85	245 ± 85	0.938
TC (mmol/L)	7.50 ± 3.67	7.45 ± 3.75	0.929
TG (mmol/L)	1.67 ± 1.09	1.66 ± 1.09	0.952
LDL-C (mmol/L)	2.94 ± 1.05	2.93 ± 1.05	0.950
HDL-C (mmol/L)	1.14 ± 0.35	1.14 ± 0.35	1.000

of the four selected SNPs. Allele frequencies of all SNPs were in Hardy-Weinberg equilibrium (*P* > 0.05). The frequencies of the mutant alleles and genotypes of CYP3A4*1G, CYP2C19*2 and P2RY12 (rs2046934) were similar to previously reported findings^[13, 15, 26]. The genotype distribution and allele frequency of NR1I2 (rs13059232) was not documented well in research articles; therefore, we consulted the NCBI database and found that there was no significant difference between the results in the database and our data.

Association of CYP2C19, CYP3A4, NR1I2 and P2RY12 gene polymorphisms with clopidogrel resistance

The association of CYP2C19, CYP3A4, NR1I2 and P2RY12 gene polymorphisms with clopidogrel resistance are shown in Table 3. CYP3A4*1G was significantly associated with a lower

Table 2. Alleles and genotypes frequencies for the four SNPs in the research population.

Gene	SNP	Allele frequency	Genotypes frequency	HWE <i>P</i> -value
CYP3A4*1G	rs2242480	*1 (0.30) *1G (0.70)	*1G/*1G (0.12); *1/*1G (0.37); *1/*1 (0.51)	0.11
CYP2C19*2	rs4244285	*2 (0.29) Wt (0.71)	*2/*2 (0.11); wt/*2 (0.36); wt/wt (0.53)	0.11
P2RY12	rs2046934	T (0.85) C (0.15)	TT (0.72); TC (0.25); CC (0.03)	0.68
NR1I2	rs13059232	T (0.43) C (0.57)	TT (0.19); TC (0.47); CC (0.34)	0.57

Table 3. Association of four selected SNPs with clopidogrel resistance (CR) in ischemic stroke patients.

Gene	SNP	Genotype	NCR	CR	Chi square	P-value	OR	95% CI
CYP3A4*1G	rs2242480	*1/*1	43	42	7.085	0.008	2.360	(1.247, 4.468)
		*1G/*1G+*1/*1G	58	24				
CYP2C19*2	rs4244285	*2/*2+wt/*2	38	31	6.011	0.014	2.366	(1.180, 4.741)
		wt/wt	58	20				
P2RY12	rs2046934	CC+TC	39	11	5.617	0.018	0.407	(0.191, 0.867)
		TT	75	52				
NR1I2	rs13059232	CC	34	30	3.998	0.046	0.533	(0.286, 0.991)
		TT+TC	83	39				

rate of clopidogrel resistance (*1/*1 *vs* *1G/*1G+*1/*1G, OR: 2.360, 95% CI: 1.247–4.468, $P=0.008$), and the CR rate was higher in the *1/*1 genotype than in those carrying the variant genotypes *1G/*1G and *1/*1G (25.1% *vs* 14.3%). *NR1I2* rs13059232 was moderately associated with CR (CC *vs* TT+TC, OR: 0.533, 95% CI: 0.286–0.991, $P=0.046$). Subjects with mutant homozygotes (CC) of *NR1I2* rs13059232 had a lower rate of CR than those carrying the T allele (16.1% *vs* 21.0%). *CYP2C19**2 allele carriers had a higher risk of CR compared with wt/wt carriers (21.1% *vs* 13.6%). *P2RY12* rs2046934 was positively associated with CR (CC+TC *vs* TT, OR: 0.407, 95% CI: 0.191–0.867, $P=0.018$), and mutant allele (C) carriers exhibited a lower rate of CR compared with wild-type homozygotes (TT) (6.2% *vs* 29.4%).

Discussion

The prevalence of clopidogrel resistance in our study population was approximately 36% ($n=69$), which is within the previously reported range^[7, 8]. Previous studies have shown that the antiplatelet effect of clopidogrel is achieved after taking a daily dose of 75 mg for 3 to 7 d^[4, 27]. Michelson *et al* reported that clopidogrel resistance may be explained by a pre-existing difference in the platelet response to ADP, indicating that there may be interindividual variation in ADP-induced platelet aggregation at baseline before clopidogrel therapy^[28]. Therefore, we compared the platelet aggregation rate on d 0 with the follow-up rate on d 5 (defined as the steady state of clopidogrel treatment) in each patient. No variation in MPAR (d 0) was detected between the CR and NCR groups, and we observed significantly decreased platelet activity on d 5.

*CYP2C19**2 was an independent predictor of clopidogrel resistance in patients with cardiovascular and cerebrovascular diseases. In previous studies^[15, 17], the *CYP2C19**2 variant was widely accepted as a risk factor for clopidogrel resistance, and this was confirmed in our study (*2/*2+wt/*2 *vs* wt/wt, OR: 2.366, 95% CI: 1.180–4.741, $P=0.014$). The CR rate of subjects with the *2/*2 and wt/*2 genotypes was 7.6% higher than those with the wt/wt genotype, and the estimated CR risk of the *CYP2C19**2 allele carriers was 2.366-fold higher than in

the wt/wt carriers. Therefore, these results confirm that the *CYP2C19**2 allele reduced the activity of *CYP2C19*, decreasing the production of active metabolites and thereby impairing clopidogrel-induced platelet inhibition.

*CYP3A4**1G, a SNP in intron 10 of *CYP3A4* and characterized by a G to A substitution at position 82266, was the most frequent mutant allele of *CYP3A4* in Asians. The allele frequency of *CYP3A4**1G was 0.22–0.37 in the Chinese population^[26, 29]. This mutant genotype was correlated with a higher *CYP3A* metabolic activity, thus increasing the formation of metabolites, particularly for prodrugs, which are activated through biotransformation via *CYP3A*^[30]. In addition, there is evidence of an association between the presence of *CYP3A4**1G and clopidogrel response variability in Spanish patients with stable coronary artery disease^[31]. However, the relationship between *CYP3A4**1G and clopidogrel resistance in Chinese patients with ischemic stroke had not been investigated previously. In this study, we found that the *CYP3A4**1G variant had a protective effect on the clopidogrel resistance of ischemic stroke patients in China. The *CYP3A4* *1/*1 genotype added a 10.8% increased risk of CR, which resulted in a 2.360-fold higher estimated risk for *1/*1 carriers of CR compared with *1G allele carriers. The variant allele increased the activity of *CYP3A4*, leading to higher production of active metabolites and thereby increasing the antiplatelet efficacy of clopidogrel. Therefore, the *CYP3A4**1G variant was determined to be a protective factor in clopidogrel resistance.

It is well known that the Pregnane X Receptor (*NR1I2*), an upstream regulator of *CYP450*s, is closely associated with the metabolism of xenobiotics and endogenous substances. Recently, many SNPs in the *NR1I2* promoter and intron regions were studied in different races^[32, 33]. *NR1I2* (rs13059232), a SNP in intron 1 of *NR1I2*, was consistently associated with *CYP3A4* phenotypic measures. In our investigation, *NR1I2* (rs13059232) was moderately associated with clopidogrel resistance ($P=0.046$, OR: 0.533). The estimated CR risk of subjects carrying the T allele was 1.876-fold higher than those carrying the mutant genotype (CC). Because *NR1I2* (rs13059232) may influence the phenotype of *CYP3A4*^[34, 35]

and the *CYP3A4*1G* polymorphism would affect the enzyme activity of *CYP3A4*, there is potential interaction between *CYP3A4*1G* and *NR1I2* (rs13059232). In this study, we found a moderate association between *NR1I2* (rs13059232) and CR in Chinese ischemic stroke patients, which had not been previously reported.

The *P2RY12* gene encodes the P2Y₁₂ receptor protein, which is the pharmacological target of clopidogrel. Genetic polymorphisms of the P2Y₁₂ ADP receptor might alter the receptor activation induced by ADP or the response to platelet inhibitors in patients^[36]. Goran *et al* reported that *P2RY12* (rs2046934) was consistently associated with a higher platelet reactivity in patients on clopidogrel in the ADP-induced LTA and the VerifyNow P2Y₁₂ assay; this may provide protection against atherothrombotic events^[16]. Robert *et al* provided evidence for an association between the *P2RY12* haplotype H2 (composed of dbSNP rs10935838, rs2046934, rs5853517 and rs6809699) and a lower risk of DVT/PE^[37]. We found that the *P2RY12* polymorphism was significantly associated with individual variation of clopidogrel sensitivity in ischemic stroke patients. In the current study, *P2RY12* (rs2046934) mutant allele carriers (CC+TC) exhibited a lower risk of CR than TT carriers, with an OR of 0.407. Therefore, *P2RY12* (rs2046934) was included as one of the variations in the *P2RY12* gene that was examined and was found to contribute to interindividual variability during clopidogrel therapy. The *P2RY12* (rs2046934) C allele also exhibited a protective effect on CR.

In the current study, hypertension was found as a non-genetic factor significantly associated with the antiplatelet effects of clopidogrel. A correlation between CR and hypertension was also documented in a recent study conducted on 303 ischemic cerebral infarction patients in China^[38]. However, the relationship between hypertension and the incidence of clopidogrel resistance remains unclear. Although diabetes mellitus was previously suggested to influence the antiplatelet effects of clopidogrel^[39, 40], we did not observe any correlation between CR and diabetes mellitus in our study population. In addition, sex, age, smoking, drinking or other biomedical indexes were not associated with CR in ischemic stroke patients. Concomitant use of lipophilic statins (atorvastatin, simvastatin) and calcium-channel blockers were suspected to be associated with a diminished antiplatelet response, which is likely due to a shared metabolic pathway via the *CYP2C19* or *CYP3A4* isoenzymes^[41, 42]. Therefore, we evaluated the association between CR and the co-administration of statins, anti-hypertensive drugs and other co-administered drugs that might affect the efficacy of clopidogrel according to previous reports^[43, 44]. Our results did not demonstrate a significant association of co-medication with CR in our research population. Several previous investigations similar to ours also did not find any obvious association between CR and potential drug-drug interactions^[45–47]. Therefore, combination therapy might have an inconsequential impact and likely does not contribute to the influence of genetic factors on CR. Discrepancies in non-genetic factors influencing CR occurrence may vary among population and races. In addition, the impact of non-

genetic factors may be masked by the effect of genetic factors.

In summary, our findings indicate that the *CYP2C19*2* allele and hypertension are risk factors for CR, whereas the *CYP3A4*1G* allele, *P2RY12* (rs2046934) C allele and *NR1I2* (rs13059232) CC genotype are protective against CR in Chinese ischemic stroke patients. Because our study was limited by the sample size, further investigations should be conducted to verify the associated genetic and non-genetic factors with CR. The current results may be useful for predicting the development of clopidogrel resistance in different patient populations.

Abbreviations

MI, myocardial infarction; PAPI, pharmacogenomics of antiplatelet intervention; CT, computed tomography; MRI, magnetic resonance imaging; TB, total bilirubin; CR, clopidogrel resistance; NCR, non-clopidogrel resistance; OR, odds ratio; CI, confidence interval; MPAR, max platelet aggregation rate; EDTA, ethylenediaminetetraacetic acid; PD, pharmacodynamics; PK, pharmacokinetics; SNP, single nucleotide polymorphism; NIHSS, National Institutes of Health stroke scale; ADP, adenosine diphosphate; ALT, alanine transaminase; AST, aspartate aminotransferase; PLT, platelet; TC, total cholesterol; TG, triglyceride; LTA, light transmission aggregation; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; HWE, Hardy-Weinberg equilibrium; TIA, transient ischemic attack.

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Author contribution

Jin JING, Min HUANG, and Rui LIU designed the study; Rui LIU, Zi-yi ZHOU, and Ye-feng CAI performed the research; Min ZHAO, Yuan-qi ZHAO, Wei-bang YU, and Yi-bei CHEN assisted with the research; Xin-meng CHEN contributed reagents; Rui LIU analyzed data; Rui LIU, Jia-li LI, and Jin JING wrote the paper.

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