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Diversity of platelet function and genetic polymorphism in clopidogrel-treated Chinese patients

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ABSTRACT. We investigated the correlation between genetic polymorphisms of cytochrome P450 enzyme genes and the outcome of clopidogrel treatment in 118 coronary disease patients after percutaneous coronary intervention at the Chinese PLA General Hospital. Patients were divided into an ischemia event relapse group (IERG) and a non-IERG group (NIERG) based on relapse of ischemia events within 6 months after percutaneous coronary intervention. Ischemia occurred in 26.27% of patients. Thromboelastogram platelet mapping results showed that compared with the NIERG, the ADP-induced platelet inhibition ratio in the IERG was significantly lower (31.33 ± 24.91% *vs* 54.68 ± 26.63%, $P < 0.05$). The platelet inhibition ratio of patients carrying mutant alleles *CYP3A5*3* (41.98 ± 29.33% *vs* 52.89 ± 26.49%), *CYP2C19*2* (43.15 ± 27.97% *vs* 55.89 ± 26.71%), and *P2Y12*1* (38.74 ± 24.36% *vs* 52.19 ± 28.58%) was lower than patients with the wild-type alleles. The frequency of ischemia event relapse in patients

with the mutant alleles *CYP3A5*3* and *CYP2C19*2* was significantly higher than patients carrying the G/G genotype; however, there was no significant difference between patients carrying the T/T genotype and C allele of *P2Y12*1*. Thus, coexisting polymorphisms of *CYP3A5*3* and *2C19*2*, but not *P2Y12*1*, play an important role in the variability of clopidogrel's curative effect.

Key words: Coronary heart disease; Genetic polymorphisms; Clopidogrel; Percutaneous coronary intervention; Platelet mapping

INTRODUCTION

Clopidogrel, a second-generation thienopyridine that inhibits platelet aggregation, is a first-line treatment along with aspirin in the management of patients with coronary artery disease, acute coronary syndromes, and/or after percutaneous coronary intervention (PCI). However, a significant proportion of patients remain at risk for subsequent death, myocardial infarction, stent thrombosis, and stroke because of insufficient clopidogrel-induced platelet inhibition (Gutliikonda et al., 2005; Wang et al., 2006; Angiolillo et al., 2007; Sugunaraj et al., 2010). It has been estimated that in 4-30% of patients, expected antiplatelet effects are absent after clopidogrel administration (Nguyen et al., 2005; Barsky and Arora, 2006). A multitude of patient-related (obesity, diabetes mellitus, and smoking) and drug-related (pharmacodynamic and pharmacokinetic) factors have been implicated for the variant response observed with clopidogrel treatment (Angiolillo et al., 2007; Camilleri et al., 2011).

Clopidogrel is an inactive prodrug that requires hepatic bioactivation via several cytochrome P450 enzymes, including CYP2C19, CYP1A2, CYP2B6, CYP2C19, CYP2C9, and CYP3A4/5 (Fitzgerald and Maree, 2007). The active metabolite irreversibly inhibits the platelet ADP receptor, P2Y12 (Fitzgerald and Maree, 2007). *P2Y12* is polymorphic; the *T744C* variant is in linkage disequilibrium (Angiolillo et al., 2005). It exists in 2 different functional haplotypes, H1 and H2, and the latter is the rarer version of the 2 and shows more potent functional aggregation in response to ADP (Fontana et al., 2003).

CYP2C19 is polymorphic, in which the loss-of-function allele, *CYP2C19*2* in exon 5, is the most commonly observed variant. In comparison, the loss of 1 allele results in partially compromised platelet aggregation post-clopidogrel treatment (Mega et al., 2009; Chen et al., 2010; Jeong et al., 2010; Pettersen et al., 2011). Another form, *CYP2C19*17*, has been implicated in bleeding following clopidogrel treatment (Sibbing et al., 2010). *CYP3A5* is also expressed polymorphically, among which only *CYP3A5*3* is in linkage disequilibrium (Kuehl et al., 2001; Balram et al., 2003; Evans and McLeod, 2003; Xie et al., 2004). Notably, the wild-type *CYP3A5*1* allele dictates enzyme activity and is present under either homo- or heterozygous conditions.

Previous studies have indicated that polymorphisms at either of the *CYP3A4/5*, *CYP2C10*, or *P2Y12* loci impact and influence clopidogrel therapy efficacy in Caucasians and Asians (Fontana et al., 2003; Pettersen et al., 2011). Therefore, in the current study, we examined the singular and multivariate effects of the *CYP3A5* (22893G>A), *CYP2C19* (681G>A), and *P2Y12* (744T>C) polymorphisms on clopidogrel treatment outcome.

MATERIAL AND METHODS

Patients

All study subjects gave written informed consent for participation in the study, which was approved by the Ethics Committee of People's Liberation Army General Hospital, Beijing, China. Between November 2010 and March 2011, 118 coronary artery disease patients at the Heart Institute, Department of Cardiology Care Unit, People's Liberation Army General Hospital, Beijing, China were enrolled in the study.

The 2007 American College of Cardiology/American Heart Association chronic stable angina pectoris guidelines (Tantry et al., 2005), 2008 American College of Cardiology and American Heart Association acute myocardial infarction treatment guidelines (Bliden et al., 2005), and 2011 American College of Cardiology and American Heart Association unstable angina and non-ST-segment elevation myocardial infarction treatment guidelines (Gurbel et al., 2005a) were used for diagnosis. Inclusion criteria were the need to administer clopidogrel after PCI in patients with coronary artery disease. Patients with co-morbidities such as hypertension and diabetes mellitus were not excluded.

Exclusion criteria were: 1) allergic to clopidogrel; 2) platelet count < 100 x 10^9/L or platelet count > 450 x 10^9/L and hemoglobin < 80 g/L; 3) severely abnormal liver and kidney function (alanine aminotransferase/aspartate aminotransferase 3 times higher than the upper limit of normal; blood urea nitrogen ≥ 20 mM or creatinine ≥ 445 μM); 4) severe uncontrolled hypertension (blood pressure ≥ 180/110 mmHg); 5) exposed to infections in the past week; and 6) trauma, surgery, and bleeding after January 2010.

Medications

Patients admitted were prescribed a 75 mg/day clopidogrel dose, while PCI patients were given a 300 mg clopidogrel loading dose after a 75 mg/day initial maintenance dose over the same duration along with 100 mg/day aspirin.

Blood sampling

Blood samples were collected in sodium citrate anticoagulant tubes (0.109 M, 0.3 mL) (BD Biosciences, Franklin Lakes, NJ, USA) 1-3 days after PCI and were immediately processed for platelet aggregation and hematology assays.

Platelet aggregation

Thromboelastogram platelet mapping was performed using the TEG 5000 Thrombelastograph Hemostasis Analyzer System (Haemonetics, Braintree, MA, USA) according to manufacturer instructions. The rate of inhibition of platelet aggregation was determined based on the ability of a blood clots agglutination force-generated curve to determine the function of platelet aggregation, which was the aggregation force of the maximum amplitude (MA) of the agglutination curve.

Definition of clopidogrel responsiveness

Arachidonic acid (AA) or ADP-induced platelet inhibition was calculated using the following formula: platelet inhibition rate (%) = [1 - ($MA_{prothrombin}$ - $MA_{fibrinogen}$)/($MA_{thrombin}$ - $MA_{fibrinogen}$)] x 100%. Responses included either poor aspirin responders induced by 1 mM AA, showing a platelet aggregation inhibition rate of ≤50%, and a low clopidogrel response induced by 2 μM ADP, showing platelet aggregation inhibition of <30%.

Experimental grouping

Samples were grouped according to the thromboelastography (TEG) inhibition of platelet aggregation rate, divided into low anti-platelet drug reactions (including low clopidogrel response and low aspirin reaction groups) and anti-platelet drug reactions in the normal group. All patients were followed up for 6 months to determine the occurrence of adverse ischemic events (in-stent restenosis, unstable angina requiring re-hospitalization, stroke, fatal or non-fatal myocardial infarction, acute or sub-acute thrombosis). All 118 patients were placed into the ischemic event recurrence group (IERG) or non-ischemic event recurrence group (NIERG).

Genotyping

Genomic DNA was extracted from blood samples post-TEG testing using a DNA extraction kit as per manufacturer recommendation (Shanghai Biological Engineering Technology Service, Shanghai, China). Polymerase chain reaction was performed using reagents obtained from Beijing Sport Century Biotech (Beijing, China). The following primers were used for genotyping: *CYP3A5*3* (22893G>A): FP: 5'-GGCATAGGAGATACCCA-3'; RP: 5'-GGT TCTAGTTCATTAGGGTG-3'; GTTCTTTTACTTTCTCCAA-3'; *P2Y12*1* (744T>C): FP: 5'-ATATCTTTTACACGAAAG-3'; RP: 5'-ATTACCACAATAGGCAG-3'.

Polymerase chain reaction products were resolved using agarose gel electrophoresis and analyzed using the AlphalmagerTM 2000 gel imaging analysis system (San Leandro, CA, USA). Amplification products were sequence-verified at the Beijing Sino-US Taihe Biotechnology Company (Beijing, China).

Statistical analysis

Continuous variables are reported as means ± standard deviation, whereas categorical variables are represented as percentages. Normally distributed measurement data between the 2 groups were compared using one way analysis of variance, independent samples, Student *t*-test, skewed distribution of measurement data to the median (P25 of P75) (expressed by the conversion to approximate a normal distribution using the Student *t*-test or analysis of variance, other non-parametric methods of analysis). Count data between the 2 groups were compared using the chi-squared test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Patient group comparisons

Of the 118 cases of coronary heart disease, 42 cases (35.59%) showed a low antiplatelet

drug response (low antiplatelet drug reactions group). Among them, the ADP-induced platelet aggregation inhibition rate was less than 30% (48.55 ± 28.05%) in 35 cases, accounting for 29.66% of all patients in the low clopidogrel response group. The AA-induced platelet aggregation inhibition rate was less than 50% (75.98 ± 27.54%) (aspirin reaction low group), accounting for 16.1% of all enrolled patients. The response to both clopidogrel and aspirin was lower in 12 cases compared to the response in 23 and 7 cases for clopidogrel and aspirin alone, respectively. There was no significant difference in patient characteristics or response to antiplatelet treatment regimens ($P > 0.05$), except that older women had a higher chance of performing as low responders following clopidogrel treatment (Table 1). Red blood corpuscles and hemoglobin levels were significantly lower in the low responder group ($P = 0.0473$ and 0.0110, respectively). Other hematologic and biochemical parameters showed no significant differences ($P > 0.05$) (Table 1).

Table 1. Demographic, clinical, procedural and laboratory information of the enrolled coronary heart disease patients (N = 118).

	Low responders	Normal responders	P value
General			
Gender (female)	12 (28.57%)	10 (13.16%)	0.0396
Age (years)	61.83 ± 12.75	56.13 ± 11.27	0.0178
BMI (kg/m^2)	25.48 ± 3.28	25.95 ± 3.69	0.4804
Systolic blood pressure at admission	129.86 ± 21.40	133.43 ± 19.44	0.3270
Diastolic blood pressure at admission	73.45 ± 11.60	75.50 ± 10.92	0.3513
Pulse pressure (mM/Hg)	56.95 ± 17.94	57.80 ± 17.08	0.8027
Pressure index	0.43 ± 0.08	0.43 ± 0.09	0.8034
Pulse at admission (beats/min)	76.00 ± 9.11	77.04 ± 8.92	0.5515
History			
Hypertension	20 (47.62%)	51 (67.11%)	0.0747
Diabetes	8 (19.05%)	22 (28.95%)	0.1086
Smoking	24 (57.14%)	39 (51.32%)	0.5435
Hyperlipidemia	17 (40.48%)	25 (32.89%)	0.2024
Drug use			
Clopidogrel use	31 (100%)	87 (100%)	-
Aspirin use	31 (100%)	87 (100%)	-
Statins	31 (100%)	87 (100%)	-
Heparin	34 (80.95%)	61 (80.26%)	0.9279
B blockers	30 (71.43%)	63 (82.89%)	0.1445
Angiotensin converting enzyme inhibitors	9 (21.43%)	24 (31.58%)	0.2395
Hematology parameters			
White blood corpuscles (x10^9/L)	7.61 ± 2.71	7.89 ± 3.39	0.6212
Red blood corpuscles (x10^{12}/L)	4.36 ± 0.58	4.57 ± 0.46	0.0473
Platelet (x10^9/L)	217.41 ± 81.76	211.86 ± 54.22	0.6946
Hemoglobin (g/L)	128.92 ± 14.93	138.75 ± 14.61	0.0110
Biochemical parameters			
Sodium (mM)	141.10 ± 5.36	142.11 ± 2.53	0.3780
Potassium (mM)	4.05 ± 0.36	3.96 ± 0.27	0.2705
Chlorine (mM)	105.40 ± 5.00	105.53 ± 2.61	0.9035
Creatine kinase (U/L)	61.25 (39.83, 152.55)	74.4 (58.1, 107.6)	0.8985
Creatine kinase-MB (U/L)	8.31 (2.84, 14.25)	7.9 (2.87, 12.93)	0.5093
Glucose (mM)	6.00 ± 1.87	6.34 ± 2.08	0.3651
High Density Lipoprotein-C (mM)	1.09 ± 0.43	0.99 ± 0.26	0.1817
Low Density Lipoprotein-C (mM)	2.70 ± 1.25	2.52 ± 1.12	0.4315
Total cholesterol (mM)	4.13 ± 1.34	4.06 ± 1.32	0.7835
Triglycerides (mM)	1.42 ± 0.58	1.52 ± 0.78	0.4334

AA or ADP-induced platelet inhibition rate is indicated by the MA decrease percentage in the curve. Specific equation use to calculate the inhibition rate was as follows: Platelet inhibition rate (%) = [1-(MA_p-MA_{Fib}) / ($MA_{Thrombin}$-MA_{Fib})] x 100%, where, Map is MA_{ADP} or MA_{AA}. Criteria used for classification was (a) low aspirin reaction, where in case of administration of aspirin at the rate of 1mM AA induced platelet aggregation inhibition rate ≤ 50%; and (b) low clopidogrel response, where in case of administration of Clopidogrel, 2μM ADP induced platelet aggregation inhibition rate <30%.

Coagulation indicators and TEG results

As expected, the low responder group had a significantly lower ADP (23.93 ± 21.93 *vs* 62.15 ± 20.92) and AA (51.92 ± 30.17 *vs* 89.27 ± 13.50) platelet aggregation inhibition rate (P = 0.000 in each case). However, there was no significant difference in other routine coagulation indices between the normal and low responder groups (data not shown). There was no statistical difference in the coronary stenosis distribution (P = 0.4080) and severity (Gensini scores: low responders, 58.83 ± 41.10 and normal responder, 62.62 ± 45.46, P = 0.6733). There was no observed difference in the 2 responder groups regarding the number, length, or diameter of the stent implantation in PCI (data not shown). No significant correlation was found between the responder groups and acute myocardial infarction or unstable angina pectoris (P > 0.05 in each case); however, low responders appeared to be negatively correlated with stable angina pectoris classification (P < 0.05).

Multifactor logistic regression analysis

We examined whether a combination of gender, age, red blood corpuscles, hemoglobin, and clinical classification (each was a dependent variable in univariate analysis) would be a better outcome predictor. As shown in Table 2, age [odds ratio (OR): 6.30; 95% confidence interval (95%CI): 1.541-25.762; P = 0.0104] and clinical classification (OR: 4.52; 95%CI: 1.279-15.962; P = 0.0192) were the only independent risk factors in the low responder group.

Table 2. Multifactor logistic regression analysis for predictor determination for clopidogrel response outcome.

Variable	OR values	95%CI	P
RBC	1.11	0.244-5.060	0.8917
Hb	0.96	0.914-1.015	0.1571
Age	6.30	1.541-25.762	0.0104
Gender	0.23	0.038-1.405	0.1119
Clinical classification	4.52	1.279-15.962	0.0192

Ischemic events during follow-up after PCI

The occurrence of recurrent ischemia during the follow-up occurred in 31 cases (26.27%) (in-stent restenosis: 13 cases; unstable angina pectoris requiring hospitalization: 10 cases; acute myocardial infarction: 2 cases; stroke: 4 cases; death: 2 cases). These 31 patients were grouped into the IERG, while the other 87 patients formed the NIERG. IERG occurred significantly more frequently in low responders (45.24%) than in normal responders (15.79%) (P = 0.005). In comparison, NIERG was more prominent in normal responders (84.21%) than in low responders (54.76%) (P < 0.05). Inhibition of platelet aggregation rate (ADP and AA) was significantly lower in the IERG (31.33 ± 24.91% and 66.84 ± 29.35%, respectively) than in NIERG (54.68 ± 26.63% and 79.23 ± 26.28%, respectively) (P = 0.000 for ADP and 0.0435 for AA, respectively).

Genetic polymorphism of *CYP3A5*3* (22893G>A), *CYP2C19*2* (681G>A), and *P2Y12*1* (744T>C) and modulation of clopidogrel response

Three polymorphic forms of each allele were identified (Figure 1 and Table 3). In

each case, the mutant allele was positively correlated with a significantly lower ADP-induced platelet inhibition rate (*CYP2C19*2*: wild-type: 55.89 ± 26.71%; mutant: 43.15 ± 27.97%, P = 0.0141; *CYP3A5*3*: wild-type: 52.89 ± 26.49%; mutant: 41.98 ± 29.33%, P = 0.038; and *P2Y12*1*: wild-type: 52.19 ± 28.58%; mutant: 38.74 ± 24.36%; P = 0.0199).

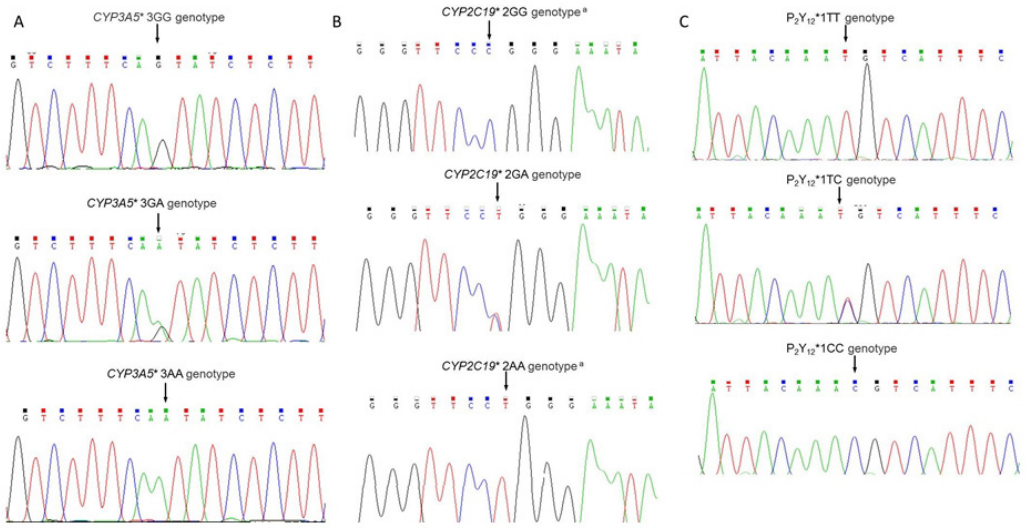

Figure 1. Representative chromatograms of the different polymorphic alleles for *CYP3A5*3* (**A**), *CYP2C19*2* (**B**), and *P2Y12*1* (**C**).

Table 3. Genotype distribution of *CYP3A5*3*, *CYP2C19*2*, *P2Y12*1* and comparison of detection results between wild type and mutant TEG. PCR product was sequenced and analyzed. Representative expression of the different polymorphic alleles for each of three aforementioned genes is shown on Table.

Loci	Wild-type (homozygous)	Mutant (heterozygous)	Mutant (homozygous)	ADP-induced platelet inhibition rate (%)		P
				Wild-type	Mutant	
*CYP3A5*3*	GG 71 (0.602)	GA 39 (0.331)	AA 8 (0.607)	52.89 ± 26.49	41.98 ± 29.33	0.038
*CYP2C19*2*	GG 50 (0.424)	GA 60 (50.9)	AA 8 (0.067)	55.89 ± 26.71	43.15 ± 27.97	0.0141
*P2Y12*1*	TT 86 (0.729)	TC (0.229)	CC (0.042)	52.19 ± 28.58	38.74 ± 24.36	0.0199

The *CYP3A5*3* (22893G> A) frequency of the wild-type GG genotype in NIERG was 70.12%, which was significantly higher than the 32.26% in the IERG. In comparison, the GA genotype in patients with IERG was significantly lower than those with the GG genotype (P < 0.05); the frequency of the A allele in the IERG was 38.71%, which was significantly higher than blood transfusion recurrence of 17.82% in this group. Patients carrying the A allele showed an incidence rate of ischemic events of 43.64%, which was much higher than in those with the G allele at 20.99% (P < 0.05).

The *CYP2C19*2* (681G> A) frequency of the GG genotype in NIERG was 51.72%, which was significantly higher than the 16.13% in the IERG; in patients with GA and AA genotypes, the risks of ischemic events were 35 and 62.5%, which were higher than those for

patients with the GG genotype (10%) ($P < 0.05$); the frequency of the A allele in the IERG was 50%, which was significantly higher than that in the NIERG.

Finally, for *P2Y12*1* (744T>C), the frequencies of the TT genotype in the IERG and NIERG were 72.41 and 74.19%, respectively, indicating that the chance of recurrence of ischemic events for patients with the TT, TC, and CC genotypes were 26.74, 25.93, and 20%, respectively ($P > 0.05$); the frequencies of the C allele in the IERG and NIERG were 14.52 and 16.09%, and the T allele and C allele in patients with ischemic events showed frequencies of 26.63 and 24.32%, respectively ($P > 0.05$).

DISCUSSION

In this study, the TEG test results showed that patients with poor response to antiplatelet drugs had a significantly higher risk of ischemic events occurring during follow-up compared to normal responders. Our results agree with those of previous studies regarding the benefit of measuring outcome criterion after clopidogrel treatment (Nguyen et al., 2005). However, our logistic regression analysis showed that age and severity of coronary heart disease were independent risk factors for the impact of platelet inhibition, which was not statistically significant between the NIERG and IERG groups, suggesting that other factors affect the rate of platelet inhibition. Thus, TEG platelet mapping can only partially predict the efficacy of antiplatelet drugs.

Clopidogrel differences can be multifaceted and are mainly related to gene polymorphisms (Gutliikonda et al., 2005), the degree of disease risk (Soffer et al., 2003), hyperinsulinism or insulin resistance (Kernan and Inzucchi, 2004), patients with poor compliance, drug dose (Montalescot et al., 2006), individual differences in drug absorption and clearing the active metabolite, drug interactions (Gurbel et al., 2005b), and platelet bypass via activation.

Some limitations of our study design were that the sample size was small and that we only screened polymorphisms of 3 genes. Future studies will focus on incorporating *ABCB-1* and *CYP3A4*. The current study showed that the presence of the *CYP3A5*3* (22893G> A) or *CYP2C19*2* (681G> A) mutant allele in patients after PCI was more likely to be associated with re-ischemic events, while the impact of the *P2Y12*1* (744T> C) polymorphism on the recurrence of ischemic events was not significant. The risk of postoperative recurrence of ischemic events was significantly higher when multi-locus mutations were present.

With the advent of personalized genomic medicine, coronary heart disease patients undergoing PCI or long-term use of clopidogrel should be screened for genetic polymorphisms. For cases in which polymorphisms are present, individualized treatment of patients such as increasing clopidogrel dose or selecting other ADP receptor antagonists (e.g., prasugrel, Kangeleiluo) in conjunction with GP IIb/IIIa receptor antagonists and other antiplatelet drugs should be considered.

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