Original article

Effects of coexisting polymorphisms of CYP2C19 and P2Y12 on clopidogrel responsiveness and clinical outcome in patients with acute coronary syndromes undergoing stent-based coronary intervention

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Keywords: clopidogrel; acute coronary syndromes; CYP2C19; P2Y12; platelet reactivity

Background The CYP2C19 G681A single polymorphism has been proven to affect clopidogrel responsiveness. However, the effect of coexisting polymorphisms of other genes has not yet been reported in the Chinese population. This study investigated the effect of coexisting polymorphisms of CYP2C19 and P2Y12 on clopidogrel responsiveness and adverse clinical events in Chinese patients.

Methods In 577 Han Chinese patients undergoing stent placement because of acute coronary syndrome had platelet reactivity assessed by thromboelastography, and the CYP2C19 G681A and P2Y12 C34T polymorphisms were detected by the ligase detection reaction. Primary clinical endpoints included cardiovascular death, nonfatal myocardial infarction, target vessel revascularization, and stent thrombosis. The secondary clinical endpoints were thrombolysis in myocardial infarction bleeding. The follow-up period was 12 months.

Results Genotyping revealed 194 carriers of the wild type GG genotype of CYP2C19 and the wild type CC genotype of P2Y12 (group 1), 102 carriers of the wild type GG genotype of CYP2C19 and the mutational T allele of P2Y12 (group 2), 163 carriers of the mutational A allele of CYP2C19 and the wild type CC genotype of P2Y12 (group 3), and 118 carriers of the mutational A allele of CYP2C19 and the mutational T allele of P2Y12 (group 4). Group 4 had the lowest ADP-inhibition (49.74±32.61) and the highest prevalence of clopidogrel low response (29.7%) of the four groups. The rate of the composite of primary clinical endpoints increased more in group 4 (8.5%) than in the other three groups; the rate of composite primary endpoints in group 2 (2.9%) and group 3 (3.7%) were not significantly different than that of group 1 (1.5%).

Conclusion Coexisting polymorphisms of different genes affected clopidogrel responsiveness and clinical outcome more than single polymorphism in Chinese patients with acute coronary syndrome undergoing percutaneous coronary intervention.

Current guidelines recommend that patients with acute coronary syndrome (ACS) or those undergoing percutaneous coronary intervention (PCI) and drug-eluting stent (DES) implantation be treated with a combination of aspirin and a P2Y12 antagonist for at least one year.1,2 Dual anti-platelet treatment with aspirin and clopidogrel is an established therapy for those patients to prevent thrombotic events and other adverse cardiovascular events.3 Clopidogrel is a pro-drug that requires biotransformation into an active metabolite in order to exert its inhibitory effect on platelet activation and aggregation.4 Platelet inhibitory responses to clopidogrel have shown wide inter-individual variability in some studies,5 and a poor clopidogrel responsiveness was linked to cardiovascular events after PCI, including stent thrombosis.6,7 The phenomenon of a poor response to clopidogrel may be related to genetic polymorphisms, alternative pathways of platelet activation, patients’ non-compliance, pretreatment platelet activation, or drug interactions.5 Genetic polymorphisms that influence the

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response to clopidogrel may encode the proteins that are responsible for clopidogrel absorption, its biotransformation to the active form, and the platelet drug receptor.

Cytochrome P450 2C19 (CYP2C19) is the gene that is responsible for the hepatic metabolism of the pro-drug of clopidogrel to its active form. The CYP2C19 G681A polymorphism was confirmed to be linked to clopidogrel responsiveness and ischemic events in ACS patients or PCI-treated subjects. Intriguingly, there are considerable ethnic differences in the distribution of CYP2C19 genetic variants. The carriage prevalence of the CYP2C19 G681A polymorphism is 25%–35% and 20%–30% among Caucasians and Africans, respectively, whereas it is 45%–70% among Asians. Prior studies revealed that only 3.7%–12% of the clopidogrel responsiveness variability is explained by CYP2C19 genetic variations. Hence, there are still many unknown factors that could explain the inter-individual variability of clopidogrel response.

P2Y12 is the platelet receptor for adenosine diphosphate (ADP) that is targeted by the active form of clopidogrel. Primary studies led to the discovery of several P2Y12 polymorphisms that formed two distinct haplotypes (H1 and H2) that influence ADP-induced platelet activation in healthy subjects. However, subsequent studies did not observe a correlation between the P2Y12 receptor haplotypes and increased ADP-induced platelet aggregation in either healthy volunteers or patients with coronary disease who were treated with clopidogrel. Another frequent polymorphism of the P2Y12 gene (34 C>T in exon 2) is not linked to the four polymorphisms that were found to be associated with a reduced clinical response to clopidogrel in patients with symptomatic peripheral artery disease (PAD). We hypothesized that coexisting polymorphisms have a greater effect than single gene polymorphisms in clopidogrel responsiveness and clinical outcome. Therefore, we evaluated the effect of coexisting CYP2C19 G681A and P2Y12 C34T polymorphisms on clopidogrel responsiveness and long-term clinical outcome in Chinese patients with ACS undergoing PCI.

**METHODS**

**Study population**

Patients with all types of ACS undergoing PCI were enrolled between October 1, 2010 and April 30, 2011 in Fu Wai Hospital of the Chinese Academy of Medical Sciences and Peking Union Medical College. Consecutive patients were assessed for eligibility for enrollment based on the following inclusion criteria; age >18 years, all types of ACS, had undergone coronary stent implantation and an uneventful PCI, and could be followed up for >1 year after PCI. The major exclusion criteria were concomitant glycoprotein IIb/IIIa inhibitor administration, hemodynamic instability, active bleeding and bleeding diatheses, contraindication to antiplatelet therapy, cerebrovascular event within the last three months, major surgical procedure within one week prior, non-cardiac disease with a life expectancy of <1 year, or inability to follow the study protocol.

All patients received a 300 mg loading dose (LD) of clopidogrel and aspirin at least 12 hours before PCI. Patients were given a 300 mg/d maintenance dose of aspirin for one month then a 100 mg/d maintenance dose of aspirin for life, and 75 mg/d of clopidogrel for one year. The decision for PCI was based on the coronary angiography results, and all interventions were conducted according to the current standard guidelines. The stent type was chosen by the operator. The study design was reviewed and approved by the local ethics committee, and all participants gave written informed consent before enrollment.

**Genotyping**

Blood samples from a peripheral vein were obtained from each patient and stored in 4 ml evacuated vacuum tubes containing ethylenediaminetetraacetic acid (EDTA). Genomic DNA was extracted from white blood cells according to the standard salting-out method and was stored in 200 μl of TE (10 mmol/L Tris/HCl and 1.0 mmol/L EDTA, pH 8.0). The selected single nucleotide polymorphisms (SNPs) rs4244285 and rs6785930 were genotyped using the ligase detection reaction (LDR) and a commercially available detection system (ABI 3730XL DNA Analyzer System; Applied Biosystems, Foster City, CA, USA). Repeat genotyping was performed on random duplicate samples (n=40), and sequencing techniques were used to ensure quality control.

**Thromboelastograph platelet-mapping assay**

Blood was collected in an evacuated vacuum tube containing 3.2% trisodium citrate and lithium heparin at least six hours after the patient had taken the clopidogrel dose. The vacuum tube was filled to capacity and inverted 3–5 times to ensure complete mixing of the anticoagulant. Modified thromboelastography (TEG) uses 4 channels to detect the effects of antiplatelet therapy action via the arachidonic acid (AA) and ADP pathways. A detailed description of this method has been outlined previously. The TEG Hemostasis Analyzer (Haemonetics Corp, Braintree, MA, USA) and automated analytical software were used to measure the physical properties.

The percentage of platelet inhibition by clopidogrel was computed as the contribution of ADP-stimulated platelets to the maximal clot strength (ADP-inhibition): 100–100× ([MAAA or ADP–MAfibrin]/[MAthrombin–MAfibrin]), where MA AA is the AA-induced clot strength (measurement of the aspirin effect), MA ADP is the ADP-induced clot strength (measurement of the ADP effect), MA fibrin is the activator-induced clot strength (measurement of the fibrin contribution), and MAthrombin is the thrombin-induced clot...
Kaplan-Meier analysis was performed. Statistical analysis event-free survival for the composite ischemic events, a composite ischemic events. To assess the cumulative proportional-hazards regression model with the were calculated using the univariate Cox 12-month follow-up period. Stent thrombosis was within and beyond the target lesion limits during the coronary vessel that was treated at the index procedure, luminal stenosis (>75% on angiography) in the same intervention required (surgical or percutaneous) to treat the TEG platelet-mapping assay. Previous studies, which employed modified TEG percent platelet inhibition to measure the response to clopidogrel, used cutoff values of <30% to define hypo-responsiveness to clopidogrel (clopidogrel low response) or high platelet reactivity.

Primary clinical endpoints included cardiovascular death, nonfatal myocardial infarction (MI), unplanned target vessel revascularization (TVR), and stent thrombosis during the 12-month follow-up period. All deaths were considered cardiovascular unless an unequivocal non-cardiovascular cause could be established. Nonfatal MI was defined as ischemic symptoms with electrocardiogram (ECG) abnormalities and upper normal limits of troponin. Unplanned TVR was defined as any intervention required (surgical or percutaneous) to treat luminal stenosis (>75% on angiography) in the same coronary vessel that was treated at the index procedure, within and beyond the target lesion limits during the 12-month follow-up period. Stent thrombosis was defined as definite or probable according to the Academic Research Consortium definitions. The composite ischemic events indicated the composite of primary clinical endpoints (cardiovascular death, nonfatal MI, unplanned TVR, and stent thrombosis). The secondary clinical endpoint was the composite of major and minor bleeding during the observation period. Bleeding was quantified according to the Thrombolysis in Myocardial Infarction (TIMI) criteria. Two independent physicians blinded to the laboratory data adjudicated events after reviewing the source documents.

**Endpoints and definitions**

Continuous variables were expressed as mean±standard deviation (SD) and compared using the Student’s t test, or one-way analysis of variance (ANOVA) test as appropriate. After demonstrating significant differences among variables by the ANOVA test, post hoc comparisons between the groups were performed with the Student-Newman-Keuls test for multiple comparisons. Categorical variables were expressed as frequencies and percentages and were compared with a chi-square test ($\chi^2$) or Fisher’s exact test. All SNPs evaluated in our study were tested for deviation from the Hardy-Weinberg equilibrium using the chi-square test. Clinical follow-up was censored at the day of the first cardiovascular event corresponding to the clinical endpoints. For subjects without a clinical event, clinical follow-up was censored either at the last clinic visit after 12 months of taking clopidogrel or at the day of clopidogrel discontinuation. The hazard ratio (HR) and 95% confidence interval (CI) were calculated using the univariate Cox proportional-hazards regression model with the composite ischemic events. To assess the cumulative event-free survival for the composite ischemic events, a Kaplan-Meier analysis was performed. Statistical analysis was performed with SPSS version 17.0 (SPSS Inc., Chicago, IL, USA), and a two-tailed probability value less than 0.05 was considered statistically significant.

**RESULTS**

**Genotype distributions and clinical characteristics**

Genetic samples for analysis were available from 577 patients with ACS who underwent PCI, received aspirin and clopidogrel, completed the one-year clinical follow-up, and had available platelet function measures (Figure 1). The P2Y12 C34T polymorphism had the following distribution in the 577 patients; 354 patients (61.4%) were homozygous for the major C allele, 193 (33.4%) were heterozygotes, and 30 (5.2%) were homozygous for the minor T allele. The CYP2C19 G681A polymorphism had the following distribution in the 577 patients; 296 patients (51.3%) were homozygous for the major G allele, 233 (40.4%) were heterozygotes, and 48 (8.3%) were homozygous for the minor A allele. No significant deviation from a Hardy-Weinberg equilibrium was observed for the distribution of the polymorphisms ($P > 0.05$). We divided the study population into four groups; 194 carriers of the wild type GG genotype of CYP2C19 and the wild type CC genotype of P2Y12 (group 1), 102 carriers of the wild type GG genotype of CYP2C19 and the mutant T allele of P2Y12 (group 2), 163 carriers of the mutant A allele of CYP2C19 and the wild type CC genotype of P2Y12 (group 3), and 118 carriers of the mutant A allele of CYP2C19 and the mutant T allele of P2Y12 (group 4).

**Statistical analysis**

The baseline characteristics of the study groups are presented in Table 1. The baseline demographics, clinical presentation, and treatments were not significantly different between the groups. The average age of these 577 patients was (59.0±11.4) years and >75% of the patients were men. PCIs were mostly performed with drug-eluting stents (96.2%).

**Clopidogrel responsiveness**

The median level of ADP inhibition was (61.35±30.80)%, and 18.8% of the patients met the predetermined criteria...
of clopidogrel low response (n=108). The platelet function detected by the TEG platelet mapping assay and the prevalence of clopidogrel low response were significantly different among groups (P<0.001). Carriers of the mutant A allele of CYP2C19 and the mutant T allele of P2Y12 (group 4) had the lowest ADP-inhibition and the highest prevalence of clopidogrel low response by the TEG platelet mapping assay. In contrast, carriers of the wild type GG genotype of CYP2C19 and the wild type CC genotype of P2Y12 (group 1) had the highest ADP-inhibition and the lowest prevalence of clopidogrel low response by the TEG platelet mapping assay (Table 2).

Clinical endpoints

Only five patients (0.9%) died of cardiovascular death, eight patients (1.4%) had nonfatal MI, and three patients had stent thrombosis (0.52%). Composite ischemic events during the follow-up period occurred in three patients in group 1 (1.5%), three patients in group 2 (2.9%), six patients of group 3 (3.7%) and 10 patients of group 4 (8.5%). The rate of the composite ischemic events (cardiovascular death, nonfatal MI, unplanned TVR, or stent thrombosis) was significantly higher in group 4 compared with the other three groups (P=0.028). Forty-six patients had TIMI bleeding (8.0%), which included nine (1.6%) cases of major bleeding and 37 (6.4%) cases of minor bleeding. The bleeding risk did not differ between groups (Table 2).

In univariate Cox proportional hazard model analysis, carriers of the mutant A allele of CYP2C19 and the mutant T allele of P2Y12 (group 4) were associated with a 5.1-fold (95% CI 1.4–18.4) higher relative risk of an ischemic event occurrence (P=0.014) compared with group 1 during the 12-month follow-up. The occurrence of composite ischemic events in group 2 (HR: 1.8, 95% CI: 0.4–9.0, P=0.467) and group 3 (HR: 2.3, 95% CI: 0.6–9.4, P=0.229) was not significantly different from that in group 1. The occurrence of cardiac events over time is depicted in Kaplan-Meier event-time curves, which demonstrate the cumulative risk of composite ischemic events according to coexisting polymorphisms of CYP2C19 and P2Y12 (Figure 2).

Figure 2. Cumulative risk of cardiovascular death, myocardial infarction, and unplanned-target vessel revascularization and stent thrombosis according to coexisting polymorphisms of CYP2C19 and P2Y12 (Figure 2).

Table 1. Baseline characteristics of the four study groups

<table>
<thead>
<tr>
<th>Variables</th>
<th>Total (n=577)</th>
<th>Group 1 (n=194)</th>
<th>Group 2 (n=102)</th>
<th>Group 3 (n=163)</th>
<th>Group 4 (n=118)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean±SD, year)</td>
<td>59.0±11.4</td>
<td>58.5±11.3</td>
<td>60.5±12.0</td>
<td>58.6±11.5</td>
<td>60.0±10.8</td>
</tr>
<tr>
<td>Male (n (%))</td>
<td>444 (76.9)</td>
<td>248 (75.8)</td>
<td>133 (71.6)</td>
<td>77 (47.1)</td>
<td>86 (72.9)</td>
</tr>
<tr>
<td>BMI (mean±SD, kg/m²)</td>
<td>32.2±9.9</td>
<td>33.8±10.4</td>
<td>33.8±10.9</td>
<td>30.7±9.5</td>
<td>31.2±10.1</td>
</tr>
<tr>
<td>LVEF (mean±SD)</td>
<td>56.2±8.8</td>
<td>55.6±9.0</td>
<td>56.0±8.5</td>
<td>56.7±8.2</td>
<td>56.5±9.7</td>
</tr>
<tr>
<td>WBC (mean±SD, ×10³/mm³)</td>
<td>9.0±1.3</td>
<td>9.0±3.4</td>
<td>9.2±3.6</td>
<td>9.1±4.0</td>
<td>8.9±3.5</td>
</tr>
<tr>
<td>Hemoglobin (mean±SD, g/dl)</td>
<td>13.6±1.7</td>
<td>13.6±1.7</td>
<td>13.6±1.5</td>
<td>13.5±1.5</td>
<td>13.5±1.7</td>
</tr>
</tbody>
</table>

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DISCUSSION

The results of this study indicated that the coexistence of the mutant A allele of the G681A polymorphism of
In conclusion, our results demonstrated that carriers of both the mutant A allele of the G681A polymorphism of CYP2C19 and the mutant T allele of the C34T polymorphism of P2Y12 had the poorest response to clopidogrel and the highest risk of composite ischemic events during long-term follow-up in Chinese patients with ACS undergoing PCI. In the future, multiple gene polymorphisms may be considered as a risk factor for persistent platelet activation with clopidogrel treatment. Meanwhile, additional studies should focus on altered treatment strategies based on genotypes in the Chinese population.
population.

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REFERENCES


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