Impact of CYP2C19 Polymorphisms on the Antiplatelet Effect of Clopidogrel in an Actual Clinical Setting in Japan

Toshikazu Jinnai, MD; Hisanori Horiuchi, MD; Takeru Makiyama, MD; Junichi Tazaki, MD; Tomohisa Tada, MD; Masaharu Akao, MD; Koh Ono, MD; Kozo Hoshino, MD*; Yumiko Naruse, MT; Kanako Takahashi, MT; Haruyo Watanabe, MT; Toru Kita, MD**; Takeshi Kimura, MD

Background: The P2Y12 adenosine diphosphate (ADP) receptor blocker, clopidogrel, an essential drug for the prevention of stent thrombosis after percutaneous coronary intervention (PCI), is a prodrug that requires CYP2C19- and CYP3A4-mediating activation. CYP2C19*2 and *3 polymorphisms are known to lack enzymatic activity. CYP2C19 polymorphisms have been reported to exhibit weaker antiplatelet response to clopidogrel in healthy subjects. The effect of polymorphisms of CYP2C19, CYP3A4 and P2Y12 on the antiplatelet effect of clopidogrel in clinical patients was examined in the present study.

Methods and Results: Single nucleotide polymorphisms of CYP2C19*2, *3, CYP3A4 (IVS10 +12G>A) and P2Y12 (T744C) were determined in 25 PCI-scheduled patients who had been systematically analyzed for the antiplatelet effect of clopidogrel in a previous study. On the basis of CYP2C19 genotype, 11 patients (44%) were classified as extensive metabolizers (EMs), 8 (32%) as intermediate metabolizers (IMs) and 6 (24%) as poor metabolizers (PMs). The rates of inhibition of 5 μmol/L ADP-induced platelet aggregation by clopidogrel intake at 48 h were 31.6 ± 14.3% in EMs, 18.4 ± 10.0% in IMs (P=0.04 vs EMs) and 16.0 ± 13.0% in PMs (P=0.02 vs EMs).

Conclusions: CYP2C19 polymorphisms are frequent in Japanese, and the antiplatelet effect of clopidogrel is strongly affected by them in the real-world clinical setting. (Circ J 2009; 73: 1498–1503)

Key Words: Antiplatelet therapy; Clopidogrel; Polymorphisms; Stent; Thrombosis

In arterial thrombus formation, the interaction of adenosine diphosphate (ADP) with its receptor P2Y12 plays an important role in platelet activation and its maintenance.1–4 Dual antiplatelet therapy with aspirin and thienopyridine derivative is the standard therapy for the prevention of stent thrombosis in patients after percutaneous coronary intervention (PCI).5–8 Ticlopidine, a 1st-generation P2Y12 ADP receptor antagonist, has been reported to have a relative high rate of side-effects. Clopidogrel, a 2nd-generation P2Y12 ADP receptor antagonist, has been demonstrated to reduce the risk of cardiovascular events in patients with prior vascular disease.9–11 and is widely used for secondary prevention in patients with coronary artery disease. However, it is well known that clopidogrel exerts little antiplatelet effect in a certain proportion of patients, a phenomenon termed “clopidogrel resistance.”12–15 Importantly, cardiovascular risk is elevated after coronary stent implantation in patients with clopidogrel resistance.16

Clopidogrel is a prodrug that requires two-step biotransformation processes mediated mainly by 2 cytochrome p450 enzymes (CYP), CYP2C19 and CYP3A4.17,18 Two major single nucleotide polymorphisms (SNPs) of CYP2C19 are known to make the enzyme activity non-functional.19,20 One is CYP2C19*2, which is the mutation of guanine to adenine at position 681 in exon 5, causing a splicing defect and the other is CYP2C19*3, which is the mutation of guanine to adenine at position 636 in exon 4, forming a stop codon.20 The frequency of the CYP2C19*2 or *3 mutation varies among races. The frequency of CYP2C19*2 in Asians is higher (30%) than in Western people (~15%), whereas the CYP2C19*3 allele has been reported as frequent in Asians (~5%) and rare in Caucasians (0.04%).21

Results of a recent study of healthy volunteer subjects showed that CYP2C19 polymorphisms are associated with a weaker antiplatelet response to clopidogrel.22 Furthermore, the plasma concentration of the active metabolite of clopidogrel is affected by CYP2C19 genotype.23 However, to our knowledge no study has evaluated the effect of CYP2C19 polymorphisms on the antiplatelet effect of clopidogrel in actual clinical patients with cardiovascular risks on aspirin therapy. The SNP of CYP3A4 (IVS10 +12G>A) has been reported to enhance the antiplatelet effect of clopidogrel, and the SNP of P2Y12 (T744C) has been reported to reduce the antiplatelet effect of clopidogrel when it coexists with CYP2C19 SNPs.24,25

We recently systematically evaluated the antiplatelet effect of clopidogrel during low-dose aspirin therapy in 30 Japanese patients scheduled for PCI, and reported a wide inter-individual variation in the antiplatelet effect of clopidogrel, with 4 cases (14%) being classified as non-responders.26 Comparison of our data with results from a similar study performed in the US also showed that the effectiveness of

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Department of Cardiovascular Medicine, Graduate School of Medicine, Kyoto University, Kyoto, *Division of Cardiology, Nagaig Hospital, Tsu and **Division of Cardiology, Kobe City Medical Center General Hospital, Kobe, Japan
Mailing address: Hisanori Horiuchi, MD, Department of Cardiovascular Medicine, Graduate School of Medicine, Kyoto University, 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan. E-mail: horiuchi@kuhp.kyoto-u.ac.jp
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Clopidogrel and CYP2C19 Polymorphisms

They received a single 300 mg dose of clopidogrel on the 1st day, which has been reported necessary to achieve rapid adequate platelet inhibition in Japanese patients. They received a single 300 mg dose of clopidogrel on the 1st day, which has been reported necessary to achieve rapid adequate platelet inhibition in Japanese patients. Then, 75 mg/day maintenance dose of clopidogrel was given from the 2nd day. Platelet-rich plasma was obtained and 5 μL ADP-induced aggregation was evaluated by optical aggregometer at baseline, 4, 24 and 48 h, and on the 14th and 28th days. We evaluated the maximal aggregation rate (MAR), and the inhibition of platelet aggregation (IPA) value was calculated as the percent inhibition of baseline aggregation according to the following equation:

\[
IPA(\%) = \left( \frac{MAR_{baseline} - MAR_{time \ after \ treatment}}{MAR_{baseline}} \right) \times 100
\]

Using the IPA values at 48 h after clopidogrel loading, 28 patients (2 patients were excluded because of missing IPA data at 48 h) were classified into 3 groups: responders (IPA <10%) according to the definition proposed by direct sequencing using an ABI 3100 genetic analyzer. Responders (IPA <10%) according to the definition proposed by direct sequencing using an ABI 3100 genetic analyzer.

**Methods**

**Subjects**

In our previous study, 30 patients (22 males) on low-dose aspirin therapy (81–100 mg/day) scheduled for coronary intervention based on symptoms or results of noninvasive tests were enrolled in a study to evaluate the antiplatelet effect of clopidogrel. They received a single 300 mg loading dose of clopidogrel on the 1st day, which has been reported necessary to achieve rapid adequate platelet inhibition in Japanese patients, and then 75 mg/day maintenance dose of clopidogrel was given from the 2nd day. Platelet-rich plasma was obtained and 5 μL ADP-induced aggregation was evaluated by optical aggregometer at baseline, 4, 24 and 48 h, and on the 14th and 28th days. We evaluated the maximal aggregation rate (MAR), and the inhibition of platelet aggregation (IPA) value was calculated as the percent inhibition of baseline aggregation according to the following equation:

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\]

Using the IPA values at 48 h after clopidogrel loading, 28 patients (2 patients were excluded because of missing IPA data at 48 h) were classified into 3 groups: responders (IPA ≥30%), hypo-responders (10% ≤IPA <30%) and non-responders (IPA <10%) according to the definition proposed in the study performed by Angiolillo et al using a similar protocol. We aimed to determine the SNPs of CYP2C19, CYP3A4 and P2Y12 among the patients with IPA values at 48 h (n=28).

The study protocol was approved by the Ethics Committee of Kyoto University Hospital, and written informed consent was given by all participants.

**Genotyping of CYP2C19, CYP3A4, and P2Y12**

Genomic DNA was extracted from peripheral whole blood samples using a DNA Extractor WB-Rapid Kit (Wako Pure Chemical Industries, Ltd, Osaka, Japan). For each sample, the regions coding CYP2C19*2, CYP2C19*3, CYP3A4 (IVS10 +12G>A) and P2Y12 (T744C) were screened for mutations. The sense and antisense primers used for polymerase chain reaction (PCR) are described in Table 1. The regions were amplified, and mutant alleles were detected by direct sequencing using an ABI 3100 genetic analyzer.

**Statistical Analysis**

Continuous variables are expressed as means±SDs. Categorical variables are expressed as frequency (%). Student’s t-test was used to compare continuous variables. A P-value <0.05 was defined as indicating statistical significance. Statistical analysis was performed using StatView (SAS Institute, Cary, NC, USA).

**Results**

**Incidence of CYP2C19 SNPs**

Of the 28 candidates, 25 (21 males) were evaluated (3 patients were excluded: 1 had died of cancer, 1 refused genomic analysis, 1 was lost to follow-up). Baseline characteristics are presented in Table 2. All participants were free from stent thrombosis. Figure 1 shows the distribution.

![Figure 1](image-url)

**Table 1. Primers Used for Polymerase Chain Reaction**

<table>
<thead>
<tr>
<th>SNP</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2C19*2</td>
<td>Sense: 5’-CAACCAAGCCTTGCCATATT-3’</td>
</tr>
<tr>
<td></td>
<td>(681 G&gt;A) Anti-sense: 5’-TACGCAAGCAGTCACATAAC-3’</td>
</tr>
<tr>
<td>CYP2C19*3</td>
<td>Sense: 5’-CCCTGTGATCCCACTTTTAC-3’</td>
</tr>
<tr>
<td>(636 G&gt;A)</td>
<td>Anti-sense: 5’-ATGGCTGTCTAGGCAAGGACT-3’</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Sense: 5’-TGATATGAAGCAGGCACTCAC-3’</td>
</tr>
<tr>
<td>(IVS10+12G&gt;A)</td>
<td>Anti-sense: 5’-GAGCCTTCTCTACATAGAGTC-3’</td>
</tr>
<tr>
<td>P2Y12</td>
<td>Sense: 5’-TGATATGTCAGAGCCAGTCAG-3’</td>
</tr>
<tr>
<td>(IVS1+744T&gt;C)</td>
<td>Anti-sense: 5’-CCTGCTACTCCTACATAGGC-3’</td>
</tr>
</tbody>
</table>

**Table 2. Baseline Characteristics of the Patients**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>70.0±8.3</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>21 (84)</td>
</tr>
<tr>
<td>Platelet count (10^9/L)</td>
<td>21.4±6.0</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>17 (68)</td>
</tr>
<tr>
<td>Hyperlipidemia, n (%)</td>
<td>16 (64)</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>4 (16)</td>
</tr>
<tr>
<td>Current smoker, n (%)</td>
<td>4 (16)</td>
</tr>
<tr>
<td>Prior myocardial infarction, n (%) (3%)</td>
<td>12 (48)</td>
</tr>
<tr>
<td>Medication</td>
<td></td>
</tr>
<tr>
<td>β-blockers, n (%)</td>
<td>4 (16)</td>
</tr>
<tr>
<td>Nitrates, n (%)</td>
<td>4 (16)</td>
</tr>
<tr>
<td>ACEI/ARB, n (%)</td>
<td>11 (44)</td>
</tr>
<tr>
<td>Statins, n (%)</td>
<td>18 (72)</td>
</tr>
<tr>
<td>CCB, n (%)</td>
<td>12 (48)</td>
</tr>
<tr>
<td>PPI, n (%)</td>
<td>5 (20)</td>
</tr>
</tbody>
</table>

ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin-receptor blocker; CCB, calcium-channel blocker; PPI, proton-pump inhibitor.
of CYP2C19 polymorphisms: 11 of the 25 patients (44%) were CYP2C19 wild-type (*1/*1) homozygotes; 3 patients (12%) were CYP2C19*2 homozygotes (*2/*2) and 9 patients (36%) were CYP2C19*2 heterozygotes (*1/*2); 5 patients (20%) were CYP2C19*3 heterozygotes (*1/*3); 3 patients (12%) had both CYP2C19*2 and CYP2C19*3 mutant alleles (*2/*3). Because 1 case of both CYP2C19*2 and CYP2C19*3 on the same allele had been reported, we analyzed whether both SNPs in these 3 patients were on the same allele and we found that they were present on different alleles in each case. Thus, 25 patients were divided into 3 groups according to their CYP2C19 genotype: 11 extensive metabolizers (EMs: CYP2C19*1/*1), 8 intermediate metabolizers (IMs: CYP2C19*1/*2 (n=6) and CYP2C19*1/*3 (n=2)), and 6 poor metabolizers (PMs: CYP2C19*2/*2 (n=3) and CYP2C19*2/*3 (n=3)).

**Effect of CYP2C19 SNPs on the Antiplatelet Effect of Clopidogrel**

The IPA values at 48 h after clopidogrel administration of EMs, IMs and PMs were 31.6±14.3%, 18.4±10.0% and 16.0±13.0%, respectively (Figure 2A). The IPA values of the IMs and PMs were significantly lower than that of the EMs (P=0.04 and P=0.02, respectively). The IPA values for each patient at 48 h are shown in Figure 2B. In the EM group, the response to clopidogrel was sufficient in most patients, although it had little effect in 1 patient. In most patients in the PM group, the response to clopidogrel was weaker, although 1 patient responded sufficiently. In the IM group, the antiplatelet effects of clopidogrel were various and largely appeared intermediate between those of the EM and PM groups. According to the definition previously described, the EM group (n=11) included 7 responders...

![Figure 2](image-url)  
**Figure 2.** Values for IPA induced by 5μmol/L ADP at 48 h after clopidogrel intakeaccording to CYP2C19 genotype. (A) IPA values at 48 h for the EM, IM and PM groups. Data are mean±SD. IPA values for both the IM and PM groups are significantly lower than in the EM group. (B) IPA values for individual patients. IPA, inhibition of platelet aggregation; ADP, adenosine diphosphate; EM, extensive metabolizers (*1/*1); IM, intermediate metabolizers (*1/*2 and *1/*3); PM, poor metabolizers (*2/*2 and *2/*3).

![Figure 3](image-url)  
**Figure 3.** Time-dependent changes in values for inhibition of platelet aggregation (IPA) for individual patients according to CYP2C19 genotype. IPA value changes in EMs (A), IMs (B) and PMs (C). EM, extensive metabolizers (*1/*1); IM, intermediate metabolizers (*1/*2 and *1/*3); PM, poor metabolizers (*2/*2 and *2/*3).
(IPA ≥30%) (63.6%), 3 hypo-responders (10% ≥IPA <30%) (27.3%), and 1 non-responder (IPA <10%) (9.1%). The IM group (n=8) had 1 responder (12.5%), 6 hypo-responders (75.0%), and 1 non-responder (12.5%) and the PM group (n=6) comprised 1 responder (16.7%), 3 hypo-responders (50.0%), and 2 non-responders (33.3%).

Figure 3 shows the time-dependent changes in IPA values of the patients based on their CYP2C19 genotype. The effects of clopidogrel were rather steady for each patient over the observation period, indicating that it is individual-specific, as described in our previous study.

SNPs in CYP3A4 and P2Y12
We also analyzed a SNP of CYP3A4 (IVS10+12G>A), which was reported to enhance the antiplatelet effect of clopidogrel. Of the 25 subjects, 8 (32%) were CYP3A4 (IVS10+12G>A) heterozygotes, and the other 17 were wild-type homozygotes. The IPA values at 48 h of the patients with wild-type CYP3A4 and with this SNP were 21.4 ±10.8% and 28.4 ±19.8%, respectively (P=0.26) (Figure 4A). The IPA values for each patient at 48 h are shown in Figure 4B. Although the SNP of P2Y12 (T744C) has been reported to reduce the antiplatelet effect of clopidogrel when it coexists with CYP2C19 SNPs, none of the subjects in the present study possessed this P2Y12 SNP.

Discussion
In this study, we analyzed SNPs of CYP2C19, an enzyme required for clopidogrel activation, in 25 patients on low-dose aspirin therapy who had been enrolled in our previous study that evaluated the antiplatelet effect of clopidogrel. Of the 25 patients (56%) had some SNPs of CYP2C19, indicating that these polymorphisms were very frequent. The antiplatelet effect of clopidogrel was rather strong in the EM group, rather weak in the PM group, and intermediate in the IM group.

It has been demonstrated in healthy subjects that the antiplatelet effect of clopidogrel is strongly reduced in those with a CYP2C19*2 mutation, compared with wild-type homozygotes. Even though these healthy subjects were all CYP2C19*2 heterozygotes (ie, IMs), clopidogrel has been shown to exert reduced antiplatelet effect. It was recently reported that in healthy subjects receiving 75 mg clopidogrel the IPA values averaged approximately 60% in EMs, 40% in IMs, and 20% in PMs. Thus, CYP2C19 SNPs would contribute to a reduced clopidogrel-induced antiplatelet effect in healthy subjects. However, we are not aware of reports linking CYP2C19 SNPs to the antiplatelet effect of clopidogrel in actual clinical patients scheduled for coronary stent therapy, who are likely to be older, have multiple risk factors and take several medicines, such as aspirin, statins and proton-pump inhibitors.

In our study, the IPA values using 5 μmol/L ADP as a stimulus were 31.6 ±14.3% in the EM group, 18.4 ±10.0% in the IM group, and 16.0 ±13.0% in PM group at 48 h after clopidogrel administration (P=0.04; IMs vs EMs, and P=0.02; PMs vs EMs). Thus, the antiplatelet effect of clopidogrel was affected by CYP2C19 polymorphisms. It could be related to the effect of aspirin that the IPA values in our study were relatively lower than those in the previous study using healthy volunteers. The IPA value at 48 h in a US study with a protocol similar to ours was approximately 35%, which was much higher than those observed in our study. We reported previously that the effectiveness of clopidogrel in Japanese was weaker than in Western people, but the ratios of CYP2C19 PMs in the Western population are less (~1–7%) than in Asians (~12–23%). The mean IPA value at 48 h in the EMs among the present subjects was 31.6 ±14.3%, which could be comparable with the Western patients (~35%), most of whom should be EMs. This result suggests that the high frequency of CYP2C19 polymorphisms contributes greatly to the lower effectiveness of clopidogrel among Japanese.

Observations of individual subjects revealed that the IPA values at 48 h in PMs were less than 20% in 5 of 6 patients, whereas the IPA values of most EMs were more than 20% (9 of 11 patients). Importantly, however, there were some exceptions. One EM patient had a very low IPA at 48 h of 9.7%, and was categorized as a non-responder, indicating that clopidogrel resistance can be caused by factors other than CYP2C19 SNPs, such as drug interactions. It has been
demonstrated that CYP2C19-metabolized drugs, such as omeprazole and lansoprazole, reduce the antiplatelet effect of clopidogrel. On the other hand, 1 of the PM patients showed a good response to clopidogrel, with an IPA value at 48h of 40.6%. The reason remains unclear, but other enzymes such as CYP1A2, 2B6, 2C9 and 3A4/5 that are involved in clopidogrel activation might substitute for CYP2C19 in such cases.

CYP3A4 is also required for the activation of clopidogrel and because CYP3A4 (IVS10+1G>A) has been reported to enhance the antiplatelet effect of clopidogrel, we analyzed this mutation and detected it in 8 of the present subjects (32%). However, we did not find any differences in the IPA values (28.4±19.8% in heterozygous subjects vs 21.4±10.8% in the wild-type subjects, not significant). Further examination is required to draw any conclusion.

In conclusion, the antiplatelet effect of clopidogrel at 48h after intake is strongly affected by CYP2C19 polymorphisms in the real-world clinical setting. Because the incidence of CYP2C19 polymorphisms is much higher in Japanese and Asians than in Western people, we should pay special attention to treatment with clopidogrel in these groups. It is essential to investigate in future whether CYP2C19 mutations are associated with stent thrombosis.

Note Added in Proof
CYP2C19 polymorphisms have been recently reported to be associated with cardiovascular events in patients with ischemic heart disease on clopidogrel therapy.

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Disclosure
There are no conflicts of interest.

References