Clopidogrel resistance in North Indian patients of coronary artery disease and lack of its association with platelet ADP receptors P2Y1 and P2Y12 gene polymorphisms

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Abstract
Aspirin and Clopidogrel are used in prophylaxis of patients undergoing percutaneous coronary intervention and long-term prevention of cardiovascular and cerebrovascular events. Clopidogrel resistance has been attributed to P2Y1 and P2Y12 adenosine diposphate (ADP) receptor polymorphisms. This study enrolled 100 patients of coronary artery disease (CAD) who were on the maintenance dose of clopidogrel (75 mg OD) with or without aspirin. In addition, 10 received loading dose (300 mg) prior to percutaneous coronary intervention. Relevant clinical and drug history were elicited. ADP-induced platelet aggregation study and PCR-RFLP for P2Y1 (1622A>G) and P2Y12 (i-T744C) polymorphisms were performed. Two groups of controls were used for defining cut-off for platelet aggregation response. Follow-up data, wherever available was recorded. The most common pattern of aggregation response was disaggregation, either complete (46.4%) or partial (53.6%). A frequency of 13% clopidogrel non-responders and 19% semi-responders was found. All the cases were H1/H1 haplotype for P2Y1 gene polymorphism and 28 (29.2%) patients carried P2Y12 1622A>G (21(21.9%) AG and 7(7.3%) GG) gene polymorphism, the frequency being greater in clopidogrel responders compared to semi/non-responders but difference was not statistically significant. There was no statistically significant difference between responders and semi/non-responders in terms of the history of risk factor for CAD, concurrent atorvastatin use or past history of an acute vascular event. On follow up, the two patients who developed myocardial infarction/acute coronary syndromes (MI/ACS) were clopidogrel semi- and non-responder, respectively. Variability in clopidogrel response with 13% non-responders and 19% semi-responders was seen in this study with adverse outcome (MI/ACS) on follow up seen in two patients. Hence, poor response to clopidogrel may be related to increased likelihood of adverse long-term coronary event that may benefit from additional or alternative anti-platelet therapy. Clopidogrel resistance was not associated with ADP receptor P2Y1 and P2Y12 gene polymorphisms. Hence, it is postulated that clopidogrel resistance in CAD patients is multifactorial and not caused by single-gene polymorphisms.

Keywords: Clopidogrel, clopidogrel resistance, aspirin, ADP receptor, P2Y1, P2Y12 gene polymorphisms

Introduction
Platelet aggregation is a key event in arterial thrombosis. It is also involved in the initiation and development of atherosclerotic lesions through platelet adhesion to dysfunctional endothelium and release of growth factors and cytokines [1]. Hence, anti-platelet agents like aspirin and thienopyridines (Clopidogrel and Ticlopidine) are used in prophylaxis of patients undergoing vascular grafting or percutaneous coronary intervention (PCI), management of acute coronary syndromes (ACS), and in the long-term prevention of cardiovascular and cerebrovascular events. However in recent years, concern over resistance to the effect of anti-platelet agents has emerged and clopidogrel resistance has been associated with an increase in cardiovascular events [2]. Several mechanisms have been proposed and studied including platelet receptor polymorphisms.

The thienopyridines irreversibly inhibit adenosine diposphate (ADP) binding to the P2Y12 receptor on the platelet surface. Two platelet ADP receptors, P2Y1 and P2Y12, have been shown to initiate platelet activation when stimulated in concert [3]. Fontana et al. identified two functional haplotypes of P2Y12 designated as H1 and H2, tagged by four single nucleotide polymorphisms (SNPs) in absolute linkage disequilibrium (i-C139T, i-T744C, i-ins801A, G52T) and the minor haplotype (H2) was found to be associated with enhanced platelet reactivity, suggesting a role for gene sequence variations of the P2Y12 receptor in atherothrombotic processes [4]. Similarly, a dimorphism, 1622A>G, in the P2Y1 gene was associated with a significant (p = 0.007) effect on the platelet ADP response, with a greater response in carriers of the G allele [5].

This study was undertaken primarily to evaluate the prevalence of clopidogrel resistance in a North Indian cohort of coronary artery disease (CAD) patients and its association with ADP receptors P2Y1 and P2Y12 polymorphisms. The secondary objectives were to evaluate the risk factor profile, atorvastatin coadministration, prior vascular event, and adverse vascular event on follow up in clopidogrel responders and resistant patients.

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(Received 13 September 2011; revised 11 May 2012; accepted 11 May 2012)
Material and methods

Patients and controls
This was a prospective study in which 100 patients of CAD presenting with ACS or chronic stable angina (CSA) or due for PCI attending the cardiology outpatient department were enrolled. Majority of the patients (99 cases) were on maintenance dose (75 mg OD) of clopidogrel for more than a week when they presented to the cardiology OPD started by the referring local physicians since this was a tertiary care centre. In addition, 10 patients who underwent PCI during the sampling period received a loading dose (300 mg) of clopidogrel. Drug history and other relevant clinical details were elicited. Patients were enrolled irrespective of the additional use of aspirin, but none were on platelet glycoprotein blockers.

Control subjects were enrolled for defining normal range for platelet aggregation study and to determine the cutoff values for categorization of patients. As control subjects, there were 20 patients of CAD who were only on aspirin without clopidogrel (aspirin-only controls) and 20 age- and sex-matched healthy subjects not on any anti-platelet drugs (normal controls). For all the cases, 5 ml of blood was collected by atraumatic venepuncture in citrate anticoagulant. For those patients who received a clopidogrel loading, an additional sample was taken 24 hours after clopidogrel loading. Informed consent was taken from all the subjects regarding their participation and the study was approved by the institute’s ethics committee. Follow-up duration was 1.5 years and the records were either from the patients’ case files or through telephonic interview.

Aggregation studies
Platelet-rich plasma (PRP) and platelet poor plasma (PPP) were prepared by the standard method. Aggregation studies were done in all the cases on platelet aggregometer (Chrono-Log 440 model, Chrono-Log Corp., Havertown Pa) between 30 minutes and 2 hours after sampling using 5 μl mol/l of ADP agonist. Nonresponders were defined as those with <10% reduction in platelet aggregation to ADP, semiresponders as those with 10–29% reduction and responders as those with >30% reduction in platelet aggregation compared to control values, as has been defined in an earlier study [6]. In random patients other agonists like adrenaline, ristocetin, and arachidonic acid were used to ensure that the dimunition in ADP-induced platelet aggregation was due to drug effect and not due to any intrinsic platelet function defect.

Genotyping
Genomic DNA was extracted from the samples as per standard protocol. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was performed for P2Y$_{12}$ receptor polymorphism and P2Y$_1$ receptor dimorphism. Genotyping of P2Y$_{12}$ H2 haplotype was performed by targeting the i-T744C single nucleotide polymorphism with 5'-TCACTTATCTCTGGTGAATAAATAAGATTTAGCTA-3' (sense primer) and 5'-GTCAGAAAATGGCCTGTGTATATATGGATGAGTGCGGCTAC-3' (antisense primer). The thermal cycling conditions comprised an initial denaturation step at 95°C for 5 minutes and 30 cycles at 95°C for 1 minute, 61°C for 1 minute, and 72°C for 1 minute. A final extension step was performed at 72°C for 10 minutes. The PCR product was then digested with Real restriction enzyme (RE) and analyzed by gel electrophoresis. The PCR product before the digest by the RE was 1200 bp and the SNP abolishes the restriction site. Hence the H1/H1 haplotype would be denoted by 750 and 450 bp bands, H1/H2 haplotype by 1200, 750, and 450 bp bands and H2/H2 haplotype by 1200 bp bands. Genotyping of the P2Y$_1$ gene 1622A>G polymorphism was done using the primers 5'-CGAGTACCTGCGAAGTTATT-3' (sense primer) and 5'-TTGCTGGGGTCTGAATAC-3' (antisense primer). The thermal cycling conditions comprised an initial denaturation step at 95°C for 6 minutes and 30 cycles at 95°C for 30 seconds, 53°C for 1 minute, and 72°C for 1 minute. A final extension step was performed at 72°C for 10 minutes. The PCR product was digested with the use of BclI restriction enzyme and visualized by agarose gel electrophoresis. The PCR product before the digest by the RE was of 260bp and the SNP creates a restriction site. Hence the normal AA haplotype would be denoted by 260 bp band, homozygous GG haplotype by 162 and 98 bp bands and heterozygous AG by 260,162 and 98 bp bands.

Statistical analysis
Statistical analysis was done using STATA 9.1 statistical software (Statacorp, College Station, Texas, USA). The groups were analyzed individually and by clubbing semi-responders and non-responders. Pearson χ² test and Fisher’s exact test was used to determine the statistical significance of the difference between these groups in terms of variables in the presenting symptoms, history, and drug usage.

Results
There were 90 male and 10 female patients and the age ranged from 31 to 85 years with a mean of 55 (SD 10.5) years. The relevant drug history, present and past history, is given in Table 1. The maximum % ADP-induced platelet aggregation for patients on the maintenance dose of clopidogrel (n = 99) ranged from 0% to 56% with a mean of 22.1% (SD = 12). Aggregation was absent in one case. The most common pattern of aggregation response seen was disagggregation seen in 84

Table 1. Relevant drug history, presenting and past history of 100 patients of CAD.

<table>
<thead>
<tr>
<th>Drug, present and past history</th>
<th>% of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clopidogrel (maintenance dose)</td>
<td>99</td>
</tr>
<tr>
<td>Clopidogrel (loading dose)</td>
<td>10</td>
</tr>
<tr>
<td>Antihypertensive drugs</td>
<td>95</td>
</tr>
<tr>
<td>Aspirin</td>
<td>90</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>74</td>
</tr>
<tr>
<td>Angina at rest</td>
<td>73</td>
</tr>
<tr>
<td>Unstable angina</td>
<td>16</td>
</tr>
<tr>
<td>Previous myocardial infarction</td>
<td>40</td>
</tr>
<tr>
<td>Stroke/TIA</td>
<td>11</td>
</tr>
<tr>
<td>Congestive cardiac failure</td>
<td>2</td>
</tr>
<tr>
<td>Hypertensive (long-standing)</td>
<td>41</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>23</td>
</tr>
</tbody>
</table>
cases (85.7%) (Figure 1). Dissaggregation was either complete (46.4%) with the aggregometer pen coming back to touch the baseline or partial (53.6%) with aggregometer chart tracing curving down towards the baseline. For normal controls and aspirin-only controls, the mean of maximum % aggregation was 47.4% and 35.4%, respectively (Table 2).

Based on the cutoff range, as stated earlier for aspirin-only controls and normal controls, the patients on clopidogrel maintenance with and without aspirin, respectively, were segregated into responders, semi-responders, and non-responders (Table 3) (Figure 2). As none of the control patients showed complete dissaggregation, hence regardless of maximum %aggregation, patients showing complete dissaggregation were taken as complete-responders. Taking the cutoff values of control as applicable to the patients, there were 19 semiresponders and 13 non-responders. One of the semi-responders on maintenance dose showed absent aggregation after a loading dose and had to be re-categorized as a complete-responder. All 13 non-responders were on both clopidogrel and aspirin and 17 semiresponders were on both drugs.

PCR-RFLP for the P2Y12 gene showed 750 and 450 bp bands in all cases denoting the H1/H1 haplotype (Figure 3). Based on polymorphism in the P2Y1 gene, our patients were divided into two major groups according to the presence or absence of the G allele. There were 28 (29.2%) patients who carried the P2Y1 1622A>G gene polymorphism (21 (21.9%) AG and 7 (7.3%) GG) while 68 (70.8%) patients carried the wild-type allele (AA). In four patients the PCR amplification was inadequate. Genotype frequencies of the polymorphism in the different subgroups of patients' were as follows: 34.3% (23/67) in complete-responders, 11.8% (2/17) in semi-responders, and 25% (3/12) in non-responders (Figure 4). The P2Y1 gene polymorphism (AG and GG) frequency was greater in clopidogrel responders compared to clopidogrel poor responders (semi and non-responders) singly or combined, but the difference was not statistically significant.

There was no statistically significant difference between the three groups individually and between responders and semi/non-responders in terms of history of known risk factor for CAD or concurrent atorvastatin use or past history of an acute vascular event. Eighty-one patients underwent PCI within few months of inclusion in the study and none had any significant in-lab vascular complications. Subsequent follow up available in 32 patients out of whom 30 were either normal or had exertional angina and 2 had an adverse coronary event of unstable angina or MI. The patient who died of MI was a clopidogrel semi-responder and the patient who had unstable and rest angina was a non-responder.

**Discussion**

Treatment with anti-platelet agents is an important part in the management and prevention of patients with arterial thrombosis. However variability in the response to these agents exists, which is multifactorial: the cause can be extrinsic related to compliance with the medication and possible drug

<table>
<thead>
<tr>
<th>Patient categories</th>
<th>CAD patients on clopidogrel (%)</th>
<th>CAD patients on clopidogrel + aspirin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-responders</td>
<td>&gt;42.7</td>
<td>&gt;31.9</td>
</tr>
<tr>
<td>Semi-responders</td>
<td>33.2–42.7</td>
<td>24.8–31.9</td>
</tr>
<tr>
<td>Complete-responders</td>
<td>&lt;33.2</td>
<td>&lt;24.8</td>
</tr>
</tbody>
</table>

**Table 3. Cutoff values of maximum % aggregation for determining categories of clopidogrel response.**

Figure 1. Types of platelet aggregation response in patients and normal controls.

Figure 2. Patient categorization as per platelet aggregation values.

### Table 2. Platelet aggregation response with 5μmol ADP.

<table>
<thead>
<tr>
<th>Platelet aggregation with 5μmol ADP</th>
<th>Patients on clopidogrel ± aspirin [n = 100]</th>
<th>Aspirin –only controls [n = 20]</th>
<th>Normal controls [n = 20]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean of max % aggregation (SD)</td>
<td>22.1(12)</td>
<td>35.4 (5.6)</td>
<td>47.4 (9.2)</td>
</tr>
<tr>
<td>Range of max % aggregation</td>
<td>0–56</td>
<td>30–45</td>
<td>35–70</td>
</tr>
<tr>
<td>Cases showing disaggregation (%)</td>
<td>84 (85.7)</td>
<td>5 (25)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Complete disaggregation (%)</td>
<td>39 (46.4)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Partial disaggregation (%)</td>
<td>45 (53.6)</td>
<td>5 (100)</td>
<td>–</td>
</tr>
</tbody>
</table>
interactions or it may be intrinsic related to difference in metabolic pathways or reside in the platelet receptors itself. This study was undertaken to study if variability in response to clopidogrel exists in terms of extent of inhibition of ADP-induced platelet aggregation and whether polymorphisms in the ADP receptor genes P2Y\textsubscript{1} and P2Y\textsubscript{12} contribute to clopidogrel resistance. This study is important in defining clopidogrel poor responders in those patients who are on a maintenance dose who may benefit from doubling the maintenance dose, as suggested by a recent study [7].

Since the arachidonic acid pathway is involved in the secondary wave or sustained platelet aggregation response to ADP, the use of aspirin along with clopidogrel would also affect the maximal aggregation (35.4% in aspirin only controls vs. 47.4% in normal controls). As most of the patients were on both clopidogrel and aspirin, it was decided to take patients on aspirin only as control to define the cut-off values of maximum aggregation to group these patients into various categories of clopidogrel response.

The most common pattern of ADP-induced platelet aggregation in the clopidogrel-treated patients was disaggregation which was either complete (46.4%) or partial (53.6%). ADP-induced platelet aggregation is influenced by the activity of both the P2Y\textsubscript{1} and P2Y\textsubscript{12} purinergic receptors, whereas clopidogrel blocks only the latter. The stabilization of aggregation, measured by disaggregation and late aggregation, is mainly driven by the activity of P2Y\textsubscript{12} receptor [8]. Hence, measures of disaggregation rather than just the measurement of peak aggregation may reflect better the interactions clopidogrel and the P2Y\textsubscript{12} receptor. As none of the control patients showed complete disaggregation in conjunction with a previous study that showed disaggregation at 6 minutes was 62% with clopidogrel, but was absent with placebo and aspirin [8], hence regardless of maximum %aggregation, patients showing complete disaggregation were taken as complete-responders.

Prior studies [9, 10] have shown an adverse drug interaction between atorvastatin, a commonly prescribed lipid lowering drug in CAD patients and clopidogrel use. The premise of this

![Figure 3](image3.jpg)

**Figure 3.** PCR-RFLP for P2Y\textsubscript{12} ADP receptor polymorphism i T744C: molecular base pair ladder in the extreme left. All the lanes show TT homozygous pattern (H1/H1) haplotype with 750 and 450 bp bands.

![Figure 4](image4.jpg)

**Figure 4.** PCR-RFLP for P2Y\textsubscript{1} ADP receptor polymorphism 1622A/G: molecular base pair ladder in the extreme left. Lanes 1,3,5,6,9,13, 16 show AG heterozygous pattern with 260, 162 and 98 bp bands, lane 12 is GG homozygous with 162 and 98 bp bands and the rest are normal/AA homozygous with 260 bp band.
was that clopidogrel is a pro-drug which is converted to an active, unstable drug by cytochrome P450 (CYP isoform, 3A4). Since certain lipophilic statins (i.e., simvastatin, atorvastatin, lovastatin) are substrates of CYP3A4, they competitively inhibit the metabolic activation of clopidogrel. As a result, the relative clopidogrel-induced platelet inhibition is diminished. However, we did not find any statistically significant association between atorvastatin use and clopidogrel resistance in conjunction with later studies refuting this finding [11–13].

No statistically significant difference was found between clopidogrel responders and non-responders in terms of prior adverse coronary event. But, on follow up, the two patients (6%) who developed MI/ACS were clopidogrel semi- and non-responder, respectively. A longer duration of follow up with more number of patients would be better to draw statistical conclusions. However, since both patients with an adverse coronary event showed poor response to clopidogrel in terms of inhibition of maximum aggregation, this simple lab test may be of use in identifying a subset of patients who will benefit from alternative anti-platelet therapy. Earlier studies [2] have also shown increased risk of recurrent atherothrombotic events in patients with acute myocardial infarction with clopidogrel resistance.

In our study, a frequency of 13% clopidogrel non-responders and 19% semi-responders was detected. The incidence of clopidogrel non-responders is widely variable with 24.2% incidence in a recent western study [14] and 14% incidence of clopidogrel non-responders and 19% semi-responders was detected. The P2Y12 gene polymorphism was neither detected in our cohort of patients nor in a previous study (unpublished) from our department in a different cohort of larger numbers of patients, indicating that the polymorphism does not exist in the North Indian population. The genotype frequency of the P2Y1 gene polymorphism was 29.2% in our study, similar to the 30.8% frequency reported in the West [14].

Although earlier studies [16, 17] have suggested H2 haplotype of the P2Y12 gene polymorphism to be associated with clopidogrel resistance, however, several recent studies [14, 18, 19] have not found any association between platelet receptor polymorphism and clopidogrel resistance. Similarly, a polymorphism 1622A>G in the P2Y1 gene was reported to be associated with greater platelet ADP response [5], and another study [20] showed that the residual platelet activation of 2MeS-ADP, a stable ADP analogue, after clopidogrel treatment is partly due to an inadequate antagonistic effect of clopidogrel on the P2Y12 receptor and partly due to activation of the P2Y1 receptor, which is unaffected by clopidogrel. It was postulated that mutations conferring increased function of the P2Y1 gene may allow an escape from P2Y12 blockade by clopidogrel, and therefore be associated with response to the drug. However this has been refuted by a later study [14] that did not find any difference between polymorphism frequencies and response to the drug similar to the findings in this study.

The lack of association of these gene polymorphisms with clopidogrel could be due to the fact that the drug response depends on multifactorial causes including factors affecting its bioavailability like variable absorption and metabolism. One such factor that is in recent focus is the difference in the metabolic activity of the P450 enzyme system. Recent studies [21, 22] have reported that carriers of a reduced-function CYP2C19 allele had significantly lower levels of the active metabolite of clopidogrel, diminished platelet inhibition, and a higher rate of major adverse cardiovascular events, including stent thrombosis, than did noncarriers.

One limitation of our study was that all patients enrolled were on maintenance dose of clopidogrel where compliance to therapy was based on history and not objectively assessed. Another limitation of this study was that since only 10 patients received loading dose whose repeat samples were assessed, the effect of increased dose on overcoming resistance could not be adequately assessed.

In conclusion, variability in response to clopidogrel exists with an incidence of 13% non-responders and 19% semi-responders in our population. No statistically significant difference was found between clopidogrel responders and poor responders in terms of prior adverse coronary event. But on follow up, the two patients who developed MI/ACS were clopidogrel semi- and non-responder, respectively. Hence poor response to clopidogrel may be related to increased likelihood of adverse long-term coronary event who may benefit from additional or alternative anti-platelet therapy. In the absence of the P2Y1 and P2Y12 gene polymorphisms contributing to its pathogenesis, it is postulated that clopidogrel resistance in CAD patients is due to multifactorial causes and not caused by single-gene polymorphisms.

Acknowledgments

This study was supported by a financial grant from the Indian Council of Medical Research (ICMR).

References


