Pharmacogenomics Study of Clopidogrel by RFLP based Genotyping of CYP2C19 in Cardiovascular Disease Patients in North-East Population of India

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Abstract

Introduction and Objective: Pharmacogenetics is a genetically determined variability in drug responses. The genes and their allelic variants which affect our response to drugs are the main routes in development of pharmacogenetics. Clopidogrel is an antiplatelet drug, used against athero-thrombotic events in cardiovascular patients. The objective of our study was to identify the CYP2C19 Single Nucleotide Polymorphisms, responsible for altering the metabolism of clopidogrel, at gene level. And to document the prevalence of CYP2C19 gene mutations in clopidogrel treated cardiovascular disease patients in Assam population, Guwahati Medical College & Hospital, in North-East India.

Patients and Methods: We have studied 60 patients who received clopidogrel from Gauhati medical college and hospital Assam. Genomic DNA was extracted by using Hipura blood genomic DNA extracting mini preparation kit by following the manufacturer’s instructions. RFLP analysis was done by DNA amplification which was carried out by using set of primers and resulting ampicons of CYP2C19*2; CYP2C19*3 and CYP2C19*17 were subjected for Restriction digestion with Smal, BamHI and Lwe01 respectively.

Results: We found that CYP2C19*2 had allelic frequency of ~40% in Gauhati Medical College and Hospital, Assam, North East India. None of the samples were mutated with CYP2C19*3 and CYP2C19*17 allele. Other CYP2C19 variant alleles with reduced or absent enzymatic activity have been identified.

Conclusion: We found that loss of functional allele CYP2C19*2 had higher carriage frequency; whereas, CYP2C19*3 and *17 alleles were not found in cardiovascular patients who were taking clopidogrel. Personalized therapy targeting patients who carry these genetic variants might help to improve the clinical outcome.

Keywords: Clopidogrel; Pharmacogenomics; CYP2C19; RFLP analysis

Introduction

Variation of individual persons in response to drug is crucial clinical problem. Optimal drugs and doses regimen depends on various factors such as gender, age, body weight, organ function, co-morbidity, drug-drug interactions, disease states, culture, lifestyle, race/ethnicity, genetics, smoking, diet [1]. However, genetic variation can make up as much as 95% of variability in drug disposition and its effect [2-6]. Clopidogrel is an antiplatelet drug, used against athero-thrombotic events in patients undergoing percutaneous coronary interventions (PCIs) with stent implantation [7-11]. Clopidogrel after biotransformation by Cytochrome 2C19 inhibits platelet activation through an irreversible blockage of adenosine diphosphate (ADP) P2Y12 receptor [12-22]. CYP2C19 gene, member of CYP450 family is highly polymorphic, as it is occurring in 25 variant alleles. The aims of this study were to determine the prevalence of clinically relevant CYP2C19*2, CYP2C19*3 and CYP2C19*17 genes in North East India, study population and finally determine whether the allele distributes and predicts metabolic phenotype in clopedogrel treatment patients. Study population include 90% male with an average of about 50.66 years. All other statistical analysis were carried out with the level of significance at P<0.05. The most common CYP2C19 loss-of-function allele is *2 (c.636G>A; rs4986893) in Asians (2-9%) [24-27-32]. Based on identified CYP2C19 genotypes, individuals can be categorized as extensive metabolizers (e.g., *1/*1), intermediate metabolizers (e.g., *1/*2), or poor metabolizers (e.g., *2/*2) (Table 1). The frequencies of CYP2C19 poor metabolizers are ~2–5% among Caucasians and Africans and ~15% in Asians. In contrast, the CYP2C19*17 allele (~0.86C-T; rs12248560) results in increased activity, with normal multi-ethnic allele frequencies of ~3-21% [33-35]. Individuals who possess this allele may be categorized as ultra-rapid metabolizers (e.g., *17/*17). Some studies reveals that this allele results in enhanced platelet inhibition and clopidogrel response [30,33,34], and perhaps an increased risk of bleeding complications [28]. However, other studies have not documented an effect of CYP2C19*17, and satisfactory evidence for an independent effect of this allele on clinical outcomes is missing [31,35,36]. In this study, typically <1%, with the exception of CYP2C19*3 (c.636G>A; rs4986893) in Asians (2-9%) [24,27-32]. Based on identified CYP2C19 genotypes, individuals can be categorized as extensive metabolizers (e.g., *1/*1), intermediate metabolizers (e.g., *1/*2), or poor metabolizers (e.g., *2/*2) (Table 1). The frequencies of CYP2C19 poor metabolizers are ~2–5% among Caucasians and Africans and ~15% in Asians. In contrast, the CYP2C19*17 allele (~0.86C-T; rs12248560) results in increased activity, with normal multi-ethnic allele frequencies of ~3-21% [33-35]. Individuals who possess this allele may be categorized as ultra-rapid metabolizers (e.g., *17/*17). Some studies reveals that this allele results in enhanced platelet inhibition and clopidogrel response [30,33,34], and perhaps an increased risk of bleeding complications [28]. However, other studies have not documented an effect of CYP2C19*17, and satisfactory evidence for an independent effect of this allele on clinical outcomes is missing [31,35,36]. In this study,
we shows that the frequency of allelic variation of CYP2C19*2 allele is ~40% in comparison with allele frequencies of ~15% in Caucasians and Africans, and 29-35% in Asians.

Materials and Methods

Sample size
We have studied 60 patients who received clopidogrel from Gauhati medical college and hospital Assam, for period of six month from September 2012 to February 2013. 2 ml of whole blood for DNA extraction was collected using 20-gauge needle and syringe in EDTA tubes which were obtained after informed consent according to declaration of Gauhati medical college and Hospital, Guwahati.

DNA extraction
Genomic DNA was extracted by using Hipura blood genomic extracting mini preparation kit by following the manufacturer’s instructions. The presence of DNA was confirmed by running DNA in 0.8% agarose gel.

Genotyping procedure PCR

CYP2C19*1*2*3 and *17 genotyping: DNA amplification was carried out by using set of primers which were selected from published literature and confirmed by using BLAST analysis. Two PCR reactions specific for *2 and *3 were conducted in parallel for each specimen in a final volume of 20 μl. CYP2C19*2 amplification was done with 5'-CAGAGCTTTGGCATATTGATC-3'; 5'-GAAACACACAAAAACTATGCATATG-3'sense and antisense primers respectively at 94°C as initial denaturation for 5min with 35cycles of 94°C for 20 sec as a denaturation, annealing at 53°C for 10sec, with polymerization at72°Cfor 10sec and final extension of72°Cfor 5min. For amplification ofCYP2C19*3gene, 5'ACATCGAGGATTGAAACGG-3' and 5'-TCAGGGCTTGGTCAATATAG-3' primers with same PCR conditions as above for CYP2C19*2 were used. However, CYP2C19*17 were carried out by set of 5'-GCCCTTAGCACAAAACTTCTC-3'; 5'TTTAACCCCTTAAAAAACAGG-3' primers. CYP2C19*17 allele PCR conditions were used as, 94°C initial denaturation for ten minutes; 35 cycles of 94°C for 30 seconds (denaturation), 58°C for 30 seconds (annealing), 72°C for 45 seconds (extension); and final extension of 72°C for 10 minutes.

RFLP analysis

Resulting amplicons of CYP2C19*2; CYP2C19*3 and CYP2C19*17 were subjected for restriction digestion with SmaI, BamHI and LweI (New England Biolabs) respectively. CYP2C19*2; CYP2C19*3 PCR products were digested at 37°C for 1 hour and digestion of CYP2C19*17 amplicon was carried out with LweI for 3 hour at same temperature. Enzyme deactivation was done at 65°C for 20 min and the resulted RFLP products were analysed by 2.5% (w/v) agarose gel electrophoresis.

Result

Genomic DNA of sufficient quality and quantity was extracted from 50 blood samples from Gauhati Medical College and Hospital, in Assam. Amplification of the CYP2C19*2, CYP2C19*3 and CYP2C19*17 target sequence from these archival samples resulted in 321, 119 and 438 bp products, respectively. Digestion of the CYP2C19*2 amplicon with SmaI resulted in products of 212 and 109 bp (homozygous wild type; c.681 G/G); 321, 212 and 109 bp (heterozygote; G/A); and a single undigested product of 321bp (homozygous*2; A/A) (Figure 1). Digestion of the CYP2C19*3 amplicon with BamHI resulted in products of 93 and 26 bp (homozygous wild-type; c.636 G/G); products of 119, 93 and 26 bp (heterozygote *3; G/A); and a single undigested product of 119 bp (homozygous*3; A/A) (Figure 2). Digestion of CYP2C19*17 (wild type; -806 C) amplicon with LweI resulted in products of 183, 142, 113 bp (Tables 2 and 3).

Allelic frequencies were calculated for CYP2C19 gene with *2, *3 and *17 alleles by using the Hardy-Weinberg equation ($p^2 + 2pq + q^2 = 1$). Where q is the number of the variant alleles (CYP2C19*2,
CYP2C19 at the corresponding CYP2C19*2, CYP2C19*3

χ² test for equilibrium (HWE). Data were recorded on a predesigned proforma

Weinberg equation (p² + 2pq + q²)

Expected genotype numbers were calculated using the Hardy-Weinberg equilibrium (HWE).

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Table 4: Allelic frequencies of CYP2C19 in Cardiovascular Disease Patients in North-East Population of India. J Pharmacogenomics Pharmacoproteomics 5: 132.

Table 5: Baseline Demographics and Past Medical History.

Table 2: Allelic variants tested for CYP2C19 gene.

Table 3: Polymorphisms observed in CYP2C19 gene with *2, *3 alleles and their Genotype frequencies.

Table 4: Allelic frequencies of CYP2C19*2, *3 and *17 alleles.

Table 5: Polymeric frequencies of CYP2C19*2, *3 and *17 alleles.

CYP2C19*3 and CYP2C19*17 and p is the number of wild-type alleles at the corresponding CYP2C19*2, CYP2C19*3 and CYP2C19*17 loci (Table 4).

Statistical analysis

Genotype and allele frequencies were calculated from the counts. Expected genotype numbers were calculated using the Hardy-Weinberg equation (p² + 2pq + q² = 1) using the allele numbers, where q is the number of the variant alleles (CYP2C19*2, CYP2C19*3 and CYP2C19*17) and p is the number of wild-type alleles at the corresponding CYP2C19*2, CYP2C19*3 and CYP2C19*17 loci. The χ² test P value (P=0.05) was consistent with the Hardy-Weinberg equilibrium (HWE). Data were recorded on a predesigned proforma and managed on an MS Office Excel Spread sheet. The descriptive statistics were represented by mean ± standard deviation and percentage (Table 5).

Discussion

This study aimed to determine the influence of genetic variations related to the cardiovascular disease patients who were on clopidogrel treatment at the time of the event. Clopidogrel is a thiopyridine prodrug that requires hepatic biotransformation to form an active metabolite that selectively and irreversibly inhibits the purinergic P2RY12 receptor, and thereby platelet aggregation, for the platelet's life span (~10 days) [37-39]. Only 15% of the prodrug is available for transformation to the active agent; other 85% is hydrolyzed by esterases to inactive forms. Conversion of clopidogrel to its active metabolite requires two sequential oxidative steps involving several CYP450 enzymes (e.g., CYP1A2, CYP2B6, CYP2C9, CYP2C19, and CYP3A4/5) [40]. The hepatic CYP2C19 enzyme contributes to the metabolism of many clinically relevant drugs such as antidepressants, benzodiazepines, mephenytoin, some proton pump inhibitors, and clopidogrel. Like many other CYP450 super-family members, the CYP2C19 gene is highly polymorphic, having more than 25 known variant alleles (http://www.cypalleles.ki.se/cyp2c19.htm). The CYP2C19*1 allele is associated with functional CYP2C19-mediated metabolism. The most common CYP2C19 loss-of-function allele is *2 (c.681G>A; rs4244285), with allele frequencies of ~15% in Caucasians and Africans, and 29–35% in Asians [38,39,41,42].

We found that CYP2C19*2 had allelic frequency of ~40% in Gauhati Medical College and Hospital, Assam, North East India. None of the samples were mutated with CYP2C19*3 and CYP2C19*17 allele. Other CYP2C19 variant alleles with reduced or absent enzymatic activity have been identified (e.g., *3–*8); however, their allele frequencies are typically <1%, with the exception of CYP2C19*3 (c.636G>A; rs4986893) in Asians (2-9%) (Table 4). CYP2C19*2 is inherited as an autosomal codominant trait; platelet responsiveness to clopidogrel in heterozygotes (*1/*2) lies somewhere between the responsiveness in individuals with the *1/*1 genotype and that in those with the *2/*2 genotype.
Therefore, based on identified CYP2C19 genotypes, individuals can be categorized as extensive metabolizers (e.g., *1/*1), intermediate metabolizers (e.g., *1/*2), or poor metabolizers (e.g., *2/*2). In contrast, the CYP2C19*17 allele (c.-806C>T; rs12248560) results in increased activity as a consequence of enhanced transcription, with average multi-ethnic allele frequencies of ~3–21% [33,34]. Individuals who carry this allele may be categorized as ultra-rapid metabolizers (e.g., *17/*17). Some studies indicate that this allele results in enhanced platelet inhibition and clopidogrel response, and possibly an increased risk of bleeding complications [28]. However, other studies have not identified an effect of CYP2C19*17 [31,35,36], and adequate evidence for an independent effect of this allele on clinical outcomes is lacking. Probably this was the first study carried out in North East Indian population which had a higher prevalence rate of CYP2C19*2 ~40% when compared with allele frequencies of ~15% in Caucasians and Africans, and 29–35% in Asians. This study will help to make best use of benefit path and reducing harm appears to lie in stratifying patient individuality in response to the treatment. This genetic testing aims to match treatment to an individual genetic profile. It will be helpful in designing clinical trials particularly at initial phases of drug development. It helps to reduce the number of patients needed, prove efficiency, and identify subgroups in trial design, also alternative treatment can be targeted. Over recent years, genetic testing has been increasingly used in clinical practice. Newer antiplatelet agents are failed to demonstrate superiority to clopidogrel without trade off of more bleeding [42-47]. The functional information on variants is important for justifying its clinical use. Understanding the functional meanings of CYP2C19 variants is an essential step toward shifting the current medical paradigm to highly personalised therapeutic regimen. The clinical decision strategies following CYP2C19 genotyping suggest two regimens: 1) an adjustment of drug dose according to genotype 2) an alternative drug choice [48].

To date the pharmacogenomic data on CYP2C19 clearly supports the genetic variants alter the drug response of its substrate drugs. However, clinical application of CYP2C19 pharmacogenetics is limited to certain genotypes [49]. The number of study population that would benefit from pharmacogenomics research would be greatly reduced if such studies focused on common variants for strong statistically evidence. One goal of pharmacogenomics is to provide personalised medicine and to provide an appropriate dose of the most appropriate drug to him or her. Therefore, more diversified investigations.

**Conclusion**

In conclusion, we have shown that loss of functional allele CYP2C19*2 had higher carriage frequency; whereas, CYP2C19*3 and *17 alleles were not found in cardiovascular patients who were taking clopidogrel, in Gauhati Medical College and Hospital, Assam, North East India. Personalized therapy to target patient's carrying these genetic variants might help to improve the clinical outcome. Our findings suggest that CYP2C19 loss of functional allele had higher carriage frequency in northeast Indian population, furthermore studies on large sample size are needed in this population so that before prescribing the medication through the detection of these alleles in a patient may help reduce the harmful adverse events and so that necessary steps should be taken like changing the dose or prescribing alternative drugs. More comprehensive and diverse research wrapping a large number of CYP2C19 variants will lay the foundation for better adapted medicine for future.

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**Conflict of Interest**

The authors declare that they have no competing interests or conflict of interests.

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