A Rapid Real-Time PCR Assay for CYP2C19 Gene Variants to Optimize the Use of Clopidogrel and Other Anti-Platelet Drugs for PCI Stent Patients

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Abstract

Introduction: Clopidogrel is the most commonly prescribed anti-platelet medication in combination with aspirin, post percutaneous cardiac intervention (PCI). However, the response to clopidogrel in patients is known to be highly variable as the bioavailability is dependent upon the conversion of the prodrug into the pharmacologically active form of clopidogrel. This conversion is dependent upon the activity of several liver enzymes of the Cytochrome P450 family, of which CYP2C19 plays a central role. Key mutations in this gene elicit either a loss-of-function or a gain-of-function in CYP2C19, ultimately leading to a change in the platelet reactivity in an individual. The risk was considered to be high enough that FDA added a 'boxed warning' to the medication cautioning healthcare professionals to use the best information available to treat patients better. Therefore, identifying the genotype of an individual before taking clopidogrel is necessary to achieve its optimal anti-platelet activity.

Methods: Blood samples were collected from consenting patients after they were on the maintenance dose of the anti-platelet therapy. A novel allele-specific real-time PCR assay was developed for the identification of the specific mutations in the CYP2C19 gene. The real-time PCR method using SYBR green was validated against the conventional RFLP method described previously and used to determine the frequency and type of mutations in a population of post-PCI patients.

Results: The real-time PCR method was faster and more cost-effective in identifying the variants of CYP2C19 gene. The new method was comparable to the conventional RFLP method. Results from the study indicate high prevalence of mutations that would alter the normal function of the CYP2C19 gene in majority of the Indian population.

Conclusions: Based on the high frequency of mutations present in Indian PCI patients we conclude that a genotyping variant of CYP2C19 provides an excellent opportunity for optimizing the anti-platelet regimen post-PCI.

Keywords: Percutaneous cardiac intervention (PCI); Clopidogrel; Cytochrome p450 (CYP450)

Introduction

The pharmacological effect of certain drugs in the body is dependent on many factors including mutations in drug target, drug transporters, and the metabolism of the drug in the body [1-3]. The study of pharmacogenomics has vastly improved with research in the area of drug metabolizing enzymes closely followed by that in drug transporters and drug receptors. Such studies are important as it is thought that nearly 50% of the patients may not respond to the given drug due to polymorphisms in any of the above proteins [4,5].

Providing the correct dose of any particular drug to a patient may depend on many factors that are both environmental as well as genetic. However, polymorphisms in the Cytochrome P450 family of enzymes (CYP2C9, CYP2C19 and CYP2D6 to name a few), cause changes in the metabolism of nearly 40% drugs [4]. If the mutation causes a reduction in the enzyme activity, based on the genotype, the individuals are categorized as poor metabolizers (bearing mutations in both the alleles), intermediate (mutation of one allele), and extensive or good metabolizers (no mutation, also known as wild type genome). If the mutation causes an increase in activity, again the individuals may be classified as rapid metabolizers (mutation in one allele) or ultra-rapid (both alleles mutated).

Post percutaneous cardiac intervention (PCI) procedure, patients are given a maintenance dose of one of the three Thioenopyridine classes of anti-platelet medications in combination with Aspirin. Of these, clopidogrel is the most economical and commonly prescribed medication. However, the pharmacologically active form of clopidogrel has to be made available by metabolism in the liver. During the process of metabolism, the inactive form of clopidogrel is converted to its active form and the CYP2C19 gene plays a prominent part in this conversion [6].

CYP2C19 gene activity is affected by many mutations. However, of these, two loss-of-function mutations in relation to clopidogrel metabolism, named variants 2 (CYP2C19*2; 681 G>A) and 3 (CYP2C19*3; 636 G>A) and a gain-of-function mutation variant 17 (CYP2C19*17; -806 C>T) have been identified to be of higher occurrence in population studies [7]. Homozygous mutants of variant 2 or 3 (poor metabolizers) have a near total lack of active clopidogrel while the heterozygous mutants (intermediate metabolizers) have a reduced bioavailability.

Patients whose genotype is that of a poor metabolizer do not benefit from clopidogrel and therefore need to be on alternative medication while the intermediate metabolizers may benefit from dose escalation. If this adjustment is not done, the risk of cardiovascular diseases or cardiovascular death, myocardial infarction, or stroke (MACE) may increase as much as 60%-75% in severe cases or the risk of stent thrombosis in mild cases [8].

Mutations in specific genes can be determined either by sequencing or more cost effectively by PCR-based methods. In a previous study we reported RFLP-based PCR determination of the genotype of patients

who underwent PCI procedure [2]. In this study, we describe a SYBR green based real-time PCR method for identification of specific alleles of interest. This allele-specific (AS) PCR method is meant to target the specific SNP polymorphisms using primers specific to the SNP site [9]. Two specific forward primers are used in this PCR; one of the primers matches with the wild type sequence while the other one matches with the mutant allele sequence. This method would specifically amplify the SNP polymorphism using a primer that has the 3' end that matches only one of the SNPs. Thus in a heterozygotic genome, both the wild type and the allele specific primers would result in product amplification while in either the null mutant or the wild type genome, only one of the primers would result in an amplified product.

Methods

Sample collection

Patients undergoing PCI procedure with coronary artery disease were offered the genetic test by the treating cardiologist for identifying the genotype of CYP2C19 gene. All patients were duly informed of the benefit of such a test and were asked to sign an informed consent form before blood collection. Ethical clearance was obtained from the Apollo Hospitals institutional ethics committee

SYBR green-based real-time allele-specific PCR (AS-PCR)

The detailed methodology and the sequence of the primers used for the genotyping of CYP2C19 gene using the RFLP method was described in a previous report [2]. Allele-specific primers were used to differentiate b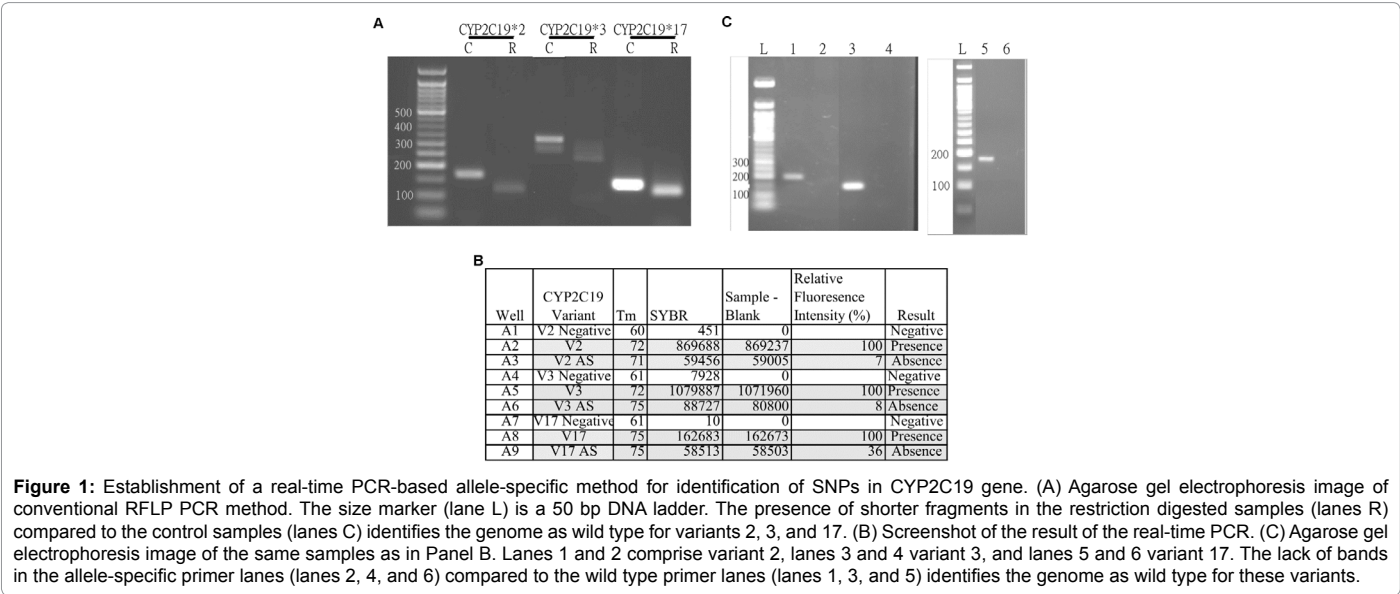etween the wild type and the mutant base at the site of the mutation (Table 1). In the first step, the touch down PCR method was used to amplify the fragments of interest. Briefly, the annealing temperature was reduced by 0.5°C for every cycle for 15 cycles and the final annealing temperature of 60°C was used for the next 20 cycles. To this reaction, 4 μl of SYBR green dye (diluted to 10X from a 1000X stock in DMSO, Lonza, Basel, Switzerland) was added and the PCR amplification reaction was carried out for one further cycle in a real-time machine (7500 Real Time PCR System, ABI, Foster City, CA, USA). The SYBR green fluorescence intensity for each sample was calculated by subtracting the fluorescence intensity of the negative control, where template DNA was not added. The percentage fluorescence intensity of the allele-specific primer reaction was calculated and the genotype was determined based on the percentage values obtained.
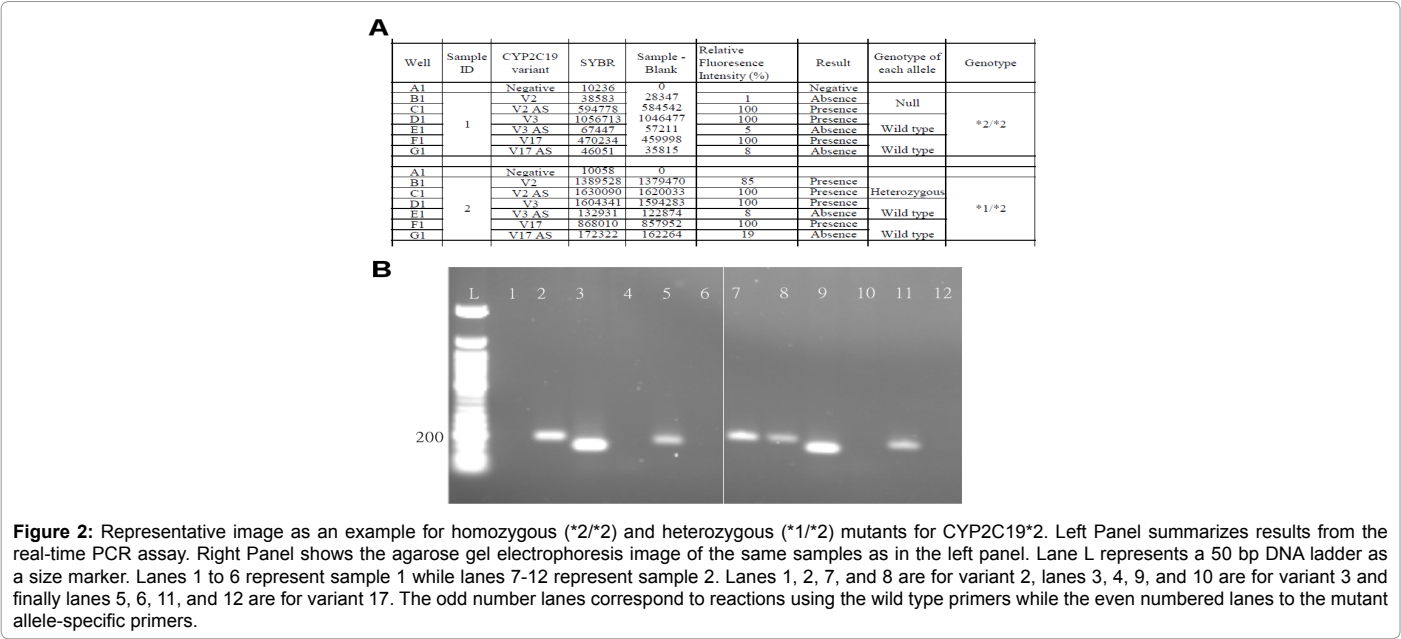
Results

Real-time PCR analysis using the allele-specific PCR compared well with the traditional RFLP method (Panel A) (Figure 1). The presence of a single band of lower molecular weight (lanes R) compared to the control (C lanes, undigested) band showed that the genomic DNA is wild type for all the three variants. The details of the RFLP method have been described previously in detail in Rath et al. but the presence of a CYP2C19*2 allele renders the restriction site inactive and the 169 bp PCR fragment remains intact. However, in the case of the CYP2C19*1 wild type, two bands of 120 and 49 bp are observed. In the case of CYP2C19*3, a single band of 329 bp is amplified which is cut into two bands of 233 and 96 bp in case of the wild type allele of Variant 3. Finally, the wild type allele for CYP2C19*17 produces two bands of 116 and 27 bp while a single band of 143 bp is amplified in the case of the mutant allele.

S. No.	Variants	Primer	Oligo sequence (5' to 3')	PCR product size (bp)
1	Variant-2; G>A change at nucleotide 681 in Exon 5	2-FP	CCCACTATCATTGATTATTTCTCG	203
2		2-ASP	CCCACTATCATTGATTATTTCTCA	
3		2-RP	ACGCAAGCAGTCACATAACTA	
4	Variant-3; G>A change at nucleotide 636 in Exon 4	3-FP	GGATTGTAAGCACCCCCAGG	159
5		3-ASP	GGATTGTAAGCACCCCCAGA	
6		3-RP	TCACCCCATGGCTGTCTAGG	
7	Variant 17; C>T change at nucleotide -806 in promoter	17-FP-4	TTGTGTCTTCTGTTCTCAACGC	188
8		17-ASP-2	TTGTGTCTTCTGTTCTCAACGT	
9		17-RP-4	CACGTGAAGGCAGGAATTGT	

Table 1: The sequence of the PCR primers used in the AS-PCR analysis of the CYP2C19 gene variants used in the study. The expected PCR products size is mentioned.

Well	CYP2C19 Variant	Tm	SYBR	Sample - Blank	Relative Fluoresence Intensity (%)	Result
A1	V2 Negative	60	451	0		Negative
A2	V2	72	869688	869237	100	Presence
A3	V2 AS	71	59456	59005	7	Absence
A4	V3 Negative	61	7928	0		Negative
A5	V3	72	1079887	1071960	100	Presence
A6	V3 AS	75	88727	80800	8	Absence
A7	V17 Negative	61	10	0		Negative
A8	V17	75	162683	162673	100	Presence
A9	V17 AS	75	58513	58503	36	Absence

Figure 1: Establishment of a real-time PCR-based allele-specific method for identification of SNPs in CYP2C19 gene. (A) Agarose gel electrophoresis image of conventional RFLP PCR method. The size marker (lane L) is a 50 bp DNA ladder. The presence of shorter fragments in the restriction digested samples (lanes R) compared to the control samples (lanes C) identifies the genome as wild type for variants 2, 3, and 17. (B) Screenshot of the result of the real-time PCR. (C) Agarose gel electrophoresis image of the same samples as in Panel B. Lanes 1 and 2 comprise variant 2, lanes 3 and 4 variant 3, and lanes 5 and 6 variant 17. The lack of bands in the allele-specific primer lanes (lanes 2, 4, and 6) compared to the wild type primer lanes (lanes 1, 3, and 5) identifies the genome as wild type for these variants.

A

Well	Sample ID	CYP2C19 variant	SYBR	Sample - Blank	Relative Fluorescence Intensity (%)	Result	Genotype of each allele	Genotype
A1		Negative	10236	0		Negative		
B1		V2	38583	28347	1	Absence	Null	
C1		V2 AS	594778	584542	100	Presence		
D1	1	V3	1056713	1046477	100	Presence		*2/*2
E1		V3 AS	67447	57211	5	Absence	Wild type	
F1		V17	470234	459998	100	Presence		
G1		V17 AS	46051	35815	8	Absence	Wild type	
A1		Negative	10058	0				
B1		V2	1389528	1379470	85	Presence		
C1		V2 AS	1630090	1620033	100	Presence	Heterozygous	
D1	2	V3	1604341	1594283	100	Presence		*1/*2
E1		V3 AS	132931	122874	8	Absence	Wild type	
F1		V17	868010	857952	100	Presence		
G1		V17 AS	172322	162264	19	Absence	Wild type	

B

Figure 2: Representative image as an example for homozygous (*2/*2) and heterozygous (*1/*2) mutants for CYP2C19*2. Left Panel summarizes results from the real-time PCR assay. Right Panel shows the agarose gel electrophoresis image of the same samples as in the left panel. Lane L represents a 50 bp DNA ladder as a size marker. Lanes 1 to 6 represent sample 1 while lanes 7-12 represent sample 2. Lanes 1, 2, 7, and 8 are for variant 2, lanes 3, 4, 9, and 10 are for variant 3 and finally lanes 5, 6, 11, and 12 are for variant 17. The odd number lanes correspond to reactions using the wild type primers while the even numbered lanes to the mutant allele-specific primers.
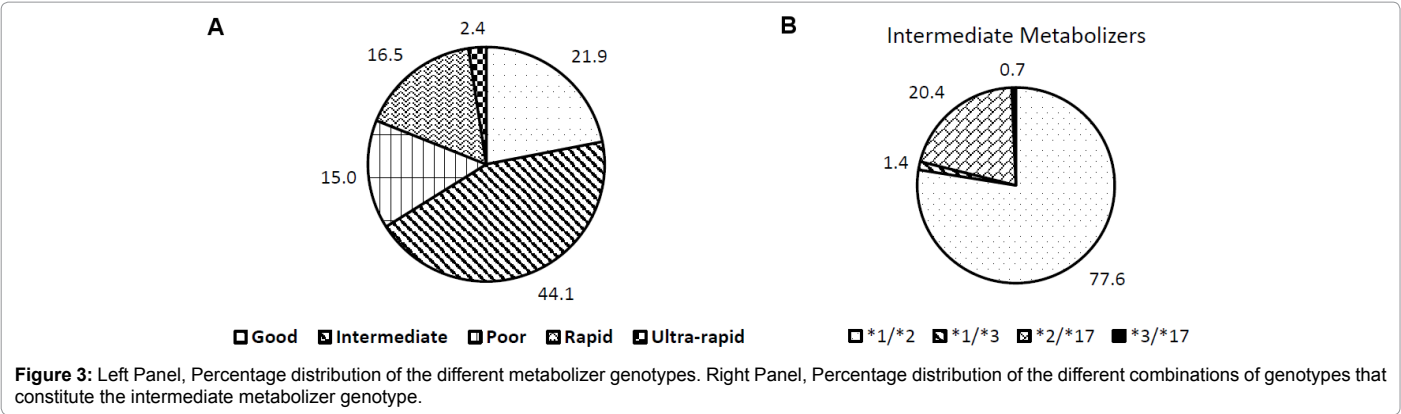
Figure 3: Left Panel, Percentage distribution of the different metabolizer genotypes. Right Panel, Percentage distribution of the different combinations of genotypes that constitute the intermediate metabolizer genotype.

This result was also seen in the allele-specific PCR method as shown by the fluorescence intensity, tabulated in panel B. The V2 negative denotes standard negative control where the genomic DNA was not added to the reaction. The relative fluorescence intensities (%) of the wild type and the Allele Specific (AS) primer were calculated for each variant. To confirm the absence of bands in the reactions with the allele-specific primers in Panel B, same samples were also analyzed by a 2% agarose gel (Panel C); the bands in the gel confirmed that the genomic DNA was indeed wild type for variants 2, 3, and 17 in the CYP2C19 gene. The PCR-amplified fragments obtained from the RFLP method were sequenced to further confirm the absence of the specific mutations in the gene.

A total of 30 patient samples, 10 each for each variant, were used in the validation of the allele-specific PCR which was followed by 10 blinded samples that were analyzed by both the methods (Figure 2). The left panel shows the relative fluorescence intensities obtained in the real-time PCR reaction, which were further confirmed by resolving the samples on a 2% agarose gel as shown in the right panel.

The genotypes were distributed as good or normal metabolizers (CYP2C19*1/*1; also called extensive metabolizers in literature), intermediate (*1/*2; *1/*3, *2/*17, and *3/*17), poor (*2/*2 and *3/*3), rapid (*1/*17), and ultra-rapid metabolizers (*17/*17). Results showed that more than 75% of the patients had one or the other mutation in any of the three variants with the intermediate metabolizers being the highest population (44%, Left Panel). Poor metabolizers, with both alleles being mutated and hence unable to metabolize clopidogrel, formed 15% of the population while about 19% of the patients had rapid or ultra-rapid genotype variants that is expected to lead to a higher than normal rate of clopidogrel metabolism. Of the intermediate metabolizers, about 77% were heterozygous for CYP2C19*2, followed by about 20% carrying heterozygous mutations in both variant 2 and variant 17 (Right Panel). Mutations in variant 3 were rare, comprising a very small percentage of the population (about 2%) (Figure 3).

Discussion

We have successfully established a real-time method of allele-specific PCR for the identification of common variants in the CYP2C19 gene. The conventional RFLP method while used more frequently takes a long time. The allele-specific PCR method does not require a restriction digestion step and is therefore faster as well as more sensitive due to the addition of the real-time detection step. Furthermore, much lower amounts of genomic DNA are needed due to the higher sensitivity of the SYBR green dye. This may be advantageous where small quantities of blood are available. Using a combination of the two genotyping methods, a total of 333 patients who underwent PCI

procedure for vessel occlusion were genotyped for the CYP2C19 gene. Of these only 22% of the patients were normal or wild type for the three variants and hence candidates for the normal dose of clopidogrel. Majority of the patients had genetic variants that have been shown to cause changes in the bioavailability of clopidogrel, hence these patients would benefit from reliable genotyping followed by the adjustment of their medication and/or dosage as per the recommendations of FDA.

Mutations in variant 2 were the highest (58%), followed by variant 17 (28.2%). The most common genotype was heterozygous mutation for CYP2C19*2 (*1/*2) which was found in 34.2% of the patient population. Variant 3 was the most uncommon with barely 2% of the patients being intermediate metabolizers due to variant 3 (either *1/*3 or *3/*17 genotype). Overall, more than 44% of the patients were in the intermediate metabolizer category and may benefit from a higher than normal dose of clopidogrel. Poor metabolizers were 15% and would benefit from a change in their medication, while about 19% of the patients were in the rapid or ultra-rapid metabolizer group and may need either a lower dose of clopidogrel or increased monitoring to reduce bleeding complications. Though the *2/*17 and *3/*17 genotype have also been considered as intermediate metabolizers, not much research has been done to firmly establish the dosage regimen for these cases. The same is true in the case of rapid and ultra-rapid metabolizers.

Conclusion

In conclusion, we have developed a rapid, economical, more sensitive method for the genotyping of variants of the CYP2C19 gene. Using this method, the common mutations that cause changes in the bioavailability of clopidogrel may be reliably identified and the medication and dosage adjusted accordingly.

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