Allele frequency distribution of CYP2C19*2 allelic variants associated with clopidogrel resistance in cardiac patients

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Received May 24, 2014; Accepted February 18, 2015

DOI: 10.3892/etm.2015.2493

Abstract. Drug resistance is a phenomenon that has become a critical issue in medical practice. Such is the case in the response to clopidogrel treatment, which is variable inter-individually and inter-ethnically due to genetic polymorphisms in the cytochrome P40 (CYP) gene. Clopidogrel is an anti-platelet agent administered to cardiac patients in the form of a prodrug, which is further metabolized into an active form by CYP enzymes. There are many allelic variants of the CYP gene that are involved in clopidogrel resistance, of which CYP2C19*2 has been demonstrated to be one of the most significant loss-of-function alleles. In the present study, 100 cardiac patients with percutaneous coronary intervention (PCI) or acute coronary syndrome (ACS) who were undergoing treatment with clopidogrel were selected and the patients were analyzed for CYP2C19*2 allelic variants using an allele-specific primer extension polymerase chain reaction method. The variant amplicons were visualized on gel and validated by Sanger sequencing. The observed allelic frequency distribution of CYP2C19*2 variants was 18% heterozygous for CYP2C19*2 A/C/G variants, 35% heterozygous for A/G variants, 13% heterozygous for C/G variants, 6% heterozygous for A/C variants, 7% homozygous for A variant, 5% homozygous for C variant and 16% homozygous for G wild-type. Furthermore, tri-allelic single nucleotide polymorphisms (SNPs) were identified in the CYP2C19*2 allele in cardiac patients for the first time, to the best of our knowledge; these were CYP2C19*2 A/C/G SNPs (18%). The overall frequency observed for new allelic variant C of CYP2C19*2 was 42%. These results suggested that there are significant inter-ethnic variations in the allelic frequencies of CYP2C19*2, which may be responsible for the variable clopidogrel response in cardiac patients.

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Key words: clopidogrel resistance, single nucleotide polymorphisms, CYP2C19*2, percutaneous coronary interventions, acute coronary syndrome, pharmacogenetics

Introduction

Drug resistance is a phenomenon that has received serious attention in recent years in everyday medical practice, and this may also be described as responsiveness or non-responsiveness as patients respond partially to medical treatment or have no response at all (1). Cardiovascular diseases (CVDs) are the most common cause of mortality globally (2). An estimated 17.3 million people succumbed to CVDs in 2008, representing 30% of all global mortalities (2). It is estimated that 7.3 million of these mortalities were due to coronary heart disease and 6.2 million were due to stroke (3). Low and middle income countries are highly affected, as >80% of CVD mortalities occur in such countries, with almost equal incidence in men and women (2). Clopidogrel is an antiplatelet drug that is administered in the form of a prodrug to cardiac patients with acute coronary syndrome (ACS) and patients undergoing percutaneous coronary intervention (PCI). It has been reported that clopidogrel non-responsiveness varies between 5 and 44% among different populations; therefore, clopidogrel and aspirin are recommended in combination as the two therapies inhibit the aggregation of platelets via different mechanisms (4). As a monotherapy, clopidogrel does not effectively inhibit platelet aggregation; therefore, it is preferentially used with aspirin, which provides additive advantages in the reduction of atherothrombotic events as compared with either drug alone (5).

The non-responsiveness to clopidogrel in cardiac patients of different populations is due to genetic variations in the cytochrome P450 (CYP) gene (6,7). During its action, clopidogrel is converted into its active metabolite (via 2-oxo-clopidogrel) by hepatic CYP isoenzymes in the liver. Clopidogrel functions as an inhibitor of adenosine diphosphate-induced platelet activation and aggregation by blocking the P2Y12 receptor selectively and irreversibly (1). The pharmacokinetics and pharmacodynamics of clopidogrel are modulated by genetic polymorphism of CYP2C19 in healthy volunteers and in patients (6,8-11). In comparison with subjects without a CYP2C19 variant allele, subjects carrying one or two CYP2C19 loss-of-function alleles have been found to have lower plasma concentrations of the

active metabolite of clopidogrel and a reduction in the anti-platelet effect of clopidogrel as evaluated by *ex vivo* platelet aggregation tests (8). These CYP isoforms are involved in the inter-individual response variability (6,7). In the response to clopidogrel therapy, polymorphisms in CYP2C19 are considered to affect both steps of the hepatic metabolism of clopidogrel. Carriers of at least one 'poor metabolizer allele' of CYP2C19 (either *2 or *3) have lower levels of the active metabolite of clopidogrel and have reduced platelet inhibition (12). Furthermore, the significant inter-ethnic variability in the allelic frequencies of CYP2C19*2 has been associated with differential clopidogrel resistance (13). The significance of screening for the loss of function allelic variants of CYP2C19*2 and their association with clopidogrel resistance has been discussed in detail in a review (14). Such mutations in this variant allele are responsible for the inability of the CYP enzyme to convert clopidogrel into its active metabolite, which results in the increased risk of mortality, heart attack or stroke among patients who have undergone PCI. Thus, the genotyping of allelic variant CYP2C19*2 is recommended as a suitable and affordable strategy for the identification of patients at risk of thrombosis (15).

In the present study, the aim was to determine the association of genetic polymorphism of the CYP2C19 isoform CYP2C19*2 with clopidogrel resistance in Pakistani cardiac patients. A total of 100 cardiac patients with PCI or ACS who were on clopidogrel therapy were analyzed for the CYP2C19*2 variant using an allele-specific primer extension polymerase chain reaction (PCR) technique. The allele frequency distribution of CYP2C19*2 variants was determined. Furthermore, the gel-based single nucleotide polymorphism (SNP) identification technique was validated using Sanger sequencing of CYP2C19*2 variant amplicons.

Materials and methods

Methodology. Blood samples of 100 cardiac patients on clopidogrel therapy were collected in K3 ethylenediamine tetraacetic acid (EDTA) vials from the Punjab Institute of Cardiology and Mayo Hospital (both Lahore, Pakistan). The samples were processed for molecular analysis. The present study was approved by the ethical committee of the Department of Zoology, University of Punjab (Lahore, Pakistan). Informed consent was obtained from either the patients or the patients' families.

Molecular analysis

Extraction of DNA. The DNA was extracted using a Genomic DNA Extraction kit (Invitrogen Life Technologies, Carlsbad, CA, USA). The quality of DNA was determined by agarose gel electrophoresis. The DNA was quantified using a spectrophotometer (NanoDrop™ 2000; Thermo Scientific, Wilmington, DE, USA).

Design of primers. The allele-specific and amplification primers for allelic variants of CYP2C19*2 were designed using Primer 3 (http://frodo.wi.mit.edu) and their sequences are shown in Table I. Genomic DNA flanking the SNP was amplified with allele-specific primers. Three different pairs of primers were used for SNP amplification, one with wild-type allele-specific primer and the other two with mutant allele-specific primer; the reverse primer was non-allele-specific and identical in wild and mutant genotypes (Fig. 1A). The strategy for the design of allele-specific primers followed that of Hirotsu *et al* (16).

PCR amplification. PCR was performed in a 20-μl reaction volume containing 10 ng genomic DNA, 0.4 pM of each oligonucleotide primer, 1X PCR Buffer (Fermentas, Thermo Fisher Scientific, Waltham, MA, USA), 200 μM dNTPs (Fermentas), 2 mM $MgCl_2$ and 2 U *Taq* Polymerase (Fermentas). The following PCR cycling conditions were used: 5 min at 95˚C for 1 cycle, 32 cycles at 95˚C for 30 sec, with various annealing temperatures (as given in Table I) for 30 sec and 72˚C for 30 sec, followed by 1 cycle at 72˚C for 5 min. PCR was carried out using a 2720 Thermal Cycler (Applied Biosystems Life Technologies, Foster City, CA, USA).

Gel electrophoresis. Amplified SNP products were electrophoresed on 2% agarose gel stained with ethidium bromide (EtBr) and visualized with a UV transilluminator (Benchtop 3UV Transilluminator; UVP, LLC, Upland, CA, USA). The CYP2C19 variant CYP2C19*2 was genotyped by the gel-based genotyping method (Fig. 1B).

Sanger sequencing. In order to validate the gel-based method of SNP identification, Sanger sequencing of the purified PCR products of selected samples was performed to confirm the different allelic variants of CYP2C19*2. Sequencing of the purified products using reverse primers was conducted with the BigDye® Terminator v3.1 Cycle Sequencing kit according to the instructions of the manufacturer (Applied Biosystems, Foster City, CA, USA). The amplification consisted of pre-denaturation at 96˚C for 1 min, followed by 35 cycles of denaturing at 96˚C for 15 sec, annealing at 55˚C for 15 sec, and extension at 72˚C for 1 min, and a final extension at 72˚C for 5 min. Sequencing products were resuspended in 10 μl formamide and denatured at 95˚C for 5 min. The DNA sequencing was performed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The sequencing results were assembled using ABI PRISM sequencing analysis software version 3.7 (Applied Biosystems) and analyzed with Chromas software (http://www.technelysium.com.au/chromas.html). The chromatograms of different SNP variants using reverse primers for sequencing are presented in Fig. 2A (CYP2C19*2A), Fig. 2B (CYP2C19*2G) and Fig. 2C (CYP2C19*2C).

Results and Discussion

The present study screened Pakistani cardiac patients enrolled for clopidogrel therapy for loss-of-function alleles of CYP2C19*2, which have been observed to be involved in clopidogrel resistance in numerous ethnic groups (17-20). A total of 100 cardiac patients on clopidogrel therapy were analyzed by an allele-specific extension-based SNP identification method with gel electrophoresis in this study. The amplified PCR products for three different allelic variants were confirmed by the Sanger sequencing method. The results of sequencing chromatograms demonstrated 100% concordance with the results of the gel electrophoresis method in the selected samples. The allele frequency distribution of CYP2C19*2 variants was calculated in the Pakistani cardiac patients. For the first time, the present study screened out the C variant of the CYP2C19*2 allele in a

Table I. Allele specific primer sequences for CYP2C19*2.

CYP2C19 allele	Name of primer	Sequence of primer	Annealing temperature (˚C)
CYP2C19*2	A forward	5'-CCACTATCATTGATTATTTCCCA-3'	53
	C forward	5'-CCACTATCATTGATTATTTCCCC-3'	55
	G forward	5'-CCACTATCATTGATTATTTCCCG-3'	55
	Universal reverse	5'-TAAAGTCCCGAGGGTTGTTG-3'	58

CYP2C19*2, *2 allele of cytochrome P450 2C19.

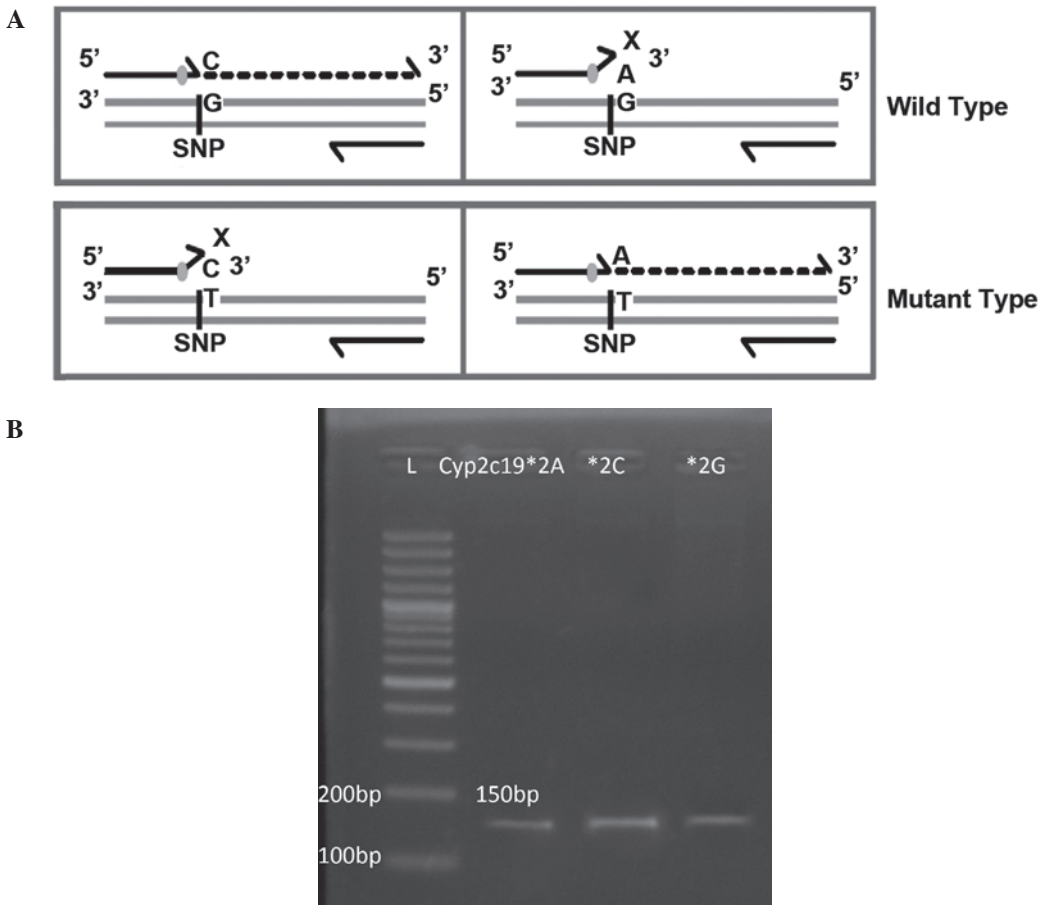

Figure 1. (A) Amplification strategy for the allele-specific extension of CYP2C19*2 (rs4244285). (B) Gel showing the allele-specific PCR product of CYP2C19*2 (SNPs A, C and G). CYP2C19*2, *2 allele of cytochrome P450 2C19; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism; bp, base pairs;L, GeneRuler 100 bp Plus DNA Ladder (Thermo Fisher Scientific, Waltham, MA, USA).

Pakistani population; this variant, to the best of our knowledge has not been reported before in any other population. Among the 100 cardiac patients analyzed in this study, 18% were heterozygous for CYP2C19*2 A/C/G variants, 35% were heterozygous for A/G variants, 13% were heterozygous for C/G variants, 6% were heterozygous for A/C variants, 7% were homozygous for A variant, 5% were homozygous for C variant and 16% were homozygous for G wild-type (Table II). The complete list of allelic variants of CYP2C19*2 found in the Pakistani cardiac patients is shown in Table III.

Tri-allelic SNPs in the CYP2C19*2 allele (CYP2C19*2 A/C/G SNPs) were identified for the first time, to the best of our knowledge, in Pakistani cardiac patients with an incidence of 18%. There is no evidence of tri-allelic variants in any other populations, including the Asian population, in the literature. The association of bi-allelic variants has been observed in many other populations (13,15,20,21). However, the present study observed a significant frequency (42%) of new allelic variant C, both in homozygous and heterozygous forms, in Pakistani cardiac patients. The allelic variant CYP2C19*2 is secondary to G<A and G<C nucleotide substitution at position 681 at the junction of intron 4 and exon 5 with loss of function as confirmed by the Sanger sequencing method (Fig. 2). The mechanisms responsible for

Table II. Comparison of the allele frequency distribution (%) of variants of CYP2C19*2 (SNP accession number, rs4244285) associated with clopidogrel resistance among different populations.

Allelic variant of CYP2C19*2	Population (ref.)						
	Pakistani	Tunisian (15)	Thai (21)	Malaysian (13)	Indian (13)	Chinese (13)	Caucasian (13)
A	7	-	7.32	9	13	6	2-3
A/G	35	11.5	5.61	-	-	-	-
C	5	-	-	-	-	-	-
A/C	6	-	-	-	-	-	-
C/G	13	-	-	-	-	-	-
A/C/G	18	-	-	-	-	-	-

CYP2C19*2, *2 allele of cytochrome P450 2C19; SNP, single nucleotide polymorphism.

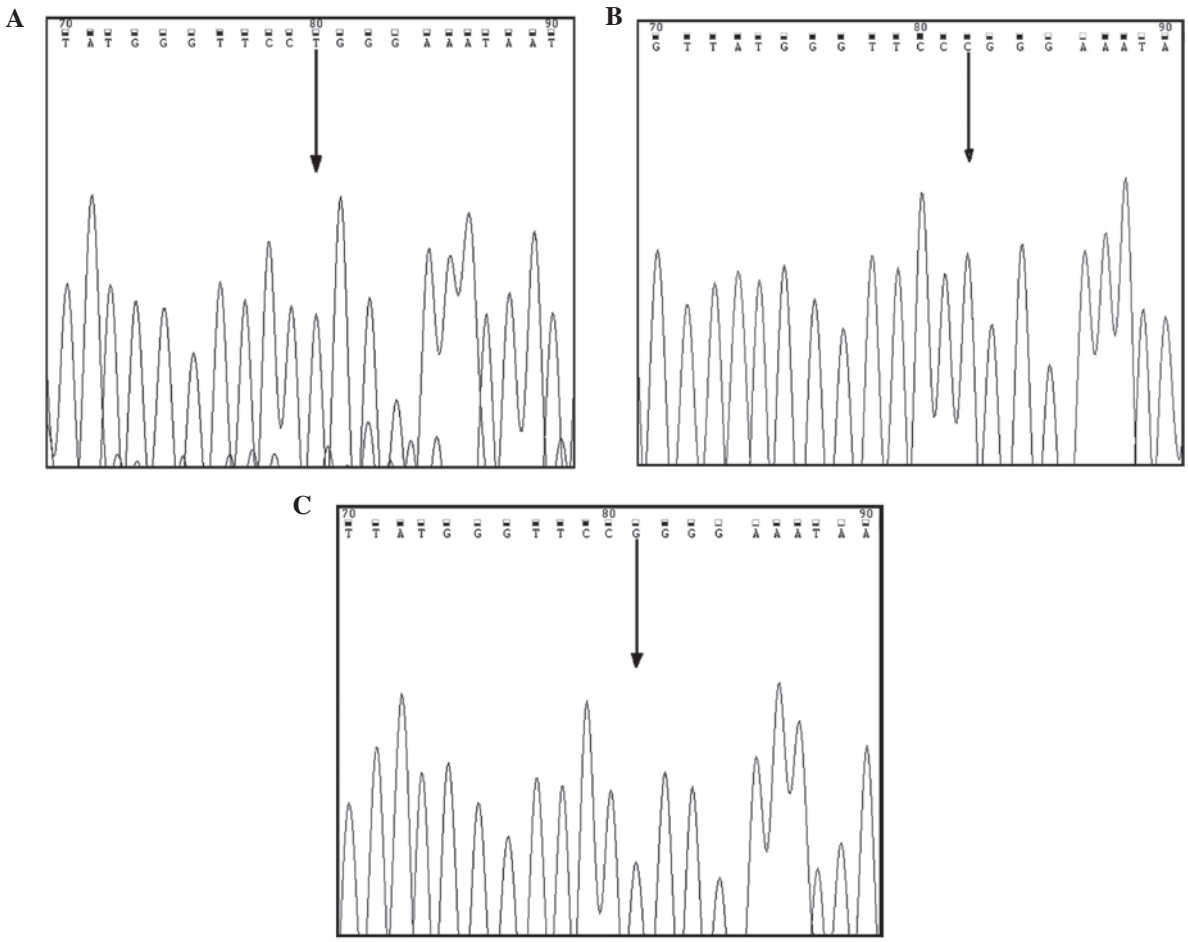

Figure 2. Sequencing chromatograms of (A) CYP2C19*2A, (B) CYP2C19*2G and (C) CYP2C19*2C (reverse primer used for sequencing). CYP2C19*2, *2 allele of cytochrome P450 2C19.

inducing mutations on the two strands of DNA duplex can be easily understood if it is considered that if a base mismatch is present, it may create instability. Thus, following the mutation of a G-C base pair to G-A, further mutation to C-A may occur; if DNA replication reads through this mismatch, the G allele will have mutated to both C and T. Alternatively, mutations may occur concurrently across the two strands of the duplex, due to a chemical or radiation event, for example. Although bi-allelic SNPs have a reasonably low density in the human genome, some tri-allelic and tetra-allelic sites are present in the human population (22). There are considered to be approximately twice as many tri-allelic sites than are expected by chance (23,24). Three different mutational mechanisms that are reported to be responsible for generating such an excess of tri-allelic sites are: i) Hyper-mutable regions in DNA; ii) the simultaneous generation of two of

Table III. Complete list of allelic variants of CYP2C19*2 (SNP accession number, rs4244285) in Pakistani cardiac patients.

Sample ID	Allele A	Allele C	Allele G	Genotype	Sample ID	Allele A	Allele C	Allele G	Genotype
1	A		G	A/G	51	A		G	A/G
2			G	C/G	52	A			A/A
3			G	G/G	53	A		G	A/G
4			G	G/G	54	A			A/A
5	A	C	G	A/C/G	55	A	C		A/C
6	A		G	A/G	56	A		G	A/G
7			G	G/G	57		C	G	C/G
8		C	G	C/G	58	A		G	A/G
9	A			A/A	59	A		G	A/G
10	A		G	A/G	60		C		C/C
11	A		G	A/G	61		C	G	C/G
12		C	G	C/G	62	A	C	G	A/C/G
13	A	C	G	A/C/G	63	A		G	A/G
14	A		G	A/G	64	A		G	A/G
15			G	G/G	65	A			A/A
16		C	G	C/G	66	A	C	G	A/C/G
17		C	G	C/G	67	A		G	A/G
18			G	G/G	68	A	C	G	A/C/G
19	A			A/A	69	A	C		A/C
20	A	C	G	A/C/G	70		C	G	C/G
21		C		C/C	71	A	C		A/C
22	A		G	A/G	72			G	G/G
23		C	G	C/G	73			G	G/G
24			G	G/G	74	A	C	G	A/C/G
25	A		G	A/G	75	A		G	A/G
26	A	C		A/C	76		C		C/C
27	A		G	A/G	77	A	C	G	A/C/G
28	A			A/A	78			G	G/G
29	A		G	A/G	79			G	G/G
30	A	C	G	A/C/G	80		C	G	C/G
31	A		G	A/G	81	A		G	A/G
32	A			A/A	82		C	G	C/G
33	A		G	A/G	83	A		G	A/G
34		C	G	C/G	84			G	G/G
35	A		G	A/G	85		C		C/C
36	A		G	A/G	86			G	G/G
37	A	C	G	A/C/G	87	A		G	A/G
38	A		G	A/G	88	A	C	G	A/C/G
39			G	G/G	89	A		G	A/G
40	A		G	A/G	90			G	G/G
41	A	C		A/C	91	A	C		A/C
42	A		G	A/G	92	A	C	G	A/C/G
43			G	G/G	93	A		G	A/G
44	A		G	A/G	94		C		C/C
45	A	C	G	A/C/G	95		C	G	C/G
46	A		G	A/G	96	A	C	G	A/C/G
47	A		G	A/G	97	A	C	G	A/C/G
48	A		G	A/G	98	A	C	G	A/C/G
49	A	C	G	A/C/G	99	A	C	G	A/C/G
50	A		G	A/G	100			G	G/G

CYP2C19*2, *2 allele of cytochrome P450 2C19; SNP, single nucleotide polymorphism.

the alleles at a tri-allelic site within a single individual; and iii) subsequent mutations induced by a single SNP by the process of base mismatching in heteroduplex DNA during recombination (23,24). Certain sites may be hypermutable, and an elevation of the mutation rate of at least two pathways at such sites will result in an excess of tri-allelic sites. The mutation rate of a site depends upon the nucleotides adjacent to it; the best known example is the CpG dinucleotide at which transition and transversion mutations occur at increased frequency (23,24). Other adjacent nucleotides are also known to affect the mutation rate (25-27). However, the role of this new allelic variant C of CYP2C19*2 in clopidogrel resistance remains to be determined.

There is significant inter-ethnic variability in the allelic frequencies of CYP2C19*2. As shown in the present study, the prevalence of bi-allelic heterozygous CYP2C19*2 variants was very high (54%) compared with that in other studied populations such as Tunisian (11.5%) (15) and Thai populations (5.61%) (21). The prevalence of the bi-allelic homozygous CYP2C19*2A variant was 7% in the population of the present study compared with 2-3% in Caucasian, 6% in Chinese, 7.32% in Thai, 9% in Malaysian and 13% in Indian populations (13). Furthermore, the prevalence of a bi-allelic homozygous CYP2C19*2C variant was observed for the first time with a frequency of 5% in the present study population; this has not been found in any other population. Such differences in the frequencies of CYP2C19*2 variants are responsible for the differential dose response in cardiac patients during treatment. Therefore, the present study is further supports that the genotyping of allelic variant CYP2C19*2 as a more suitable and affordable strategy for identifying patients at risk of thrombosis than repeatedly performing platelet monitoring (15).

Despite providing informative genotyping data of CYP2C19*2 variants in a Pakistani population, there were several limitations to the present study. Only patients admitted to hospital were enrolled in the study. Therefore, some selection bias is likely. The small sample size of patients is insufficient to provide significant allele frequency distribution of CYP2C19*2 variants. A large-scale study is required to strengthen the present findings regarding the allele frequency distribution of CYP2C19*2 variants that contribute as a risk factor for stent thrombosis. It has been demonstrated in previous studies that CYP2C19*2 polymorphisms inhibit the antiplatelet effect of clopidogrel (28,29). However, platelet aggregation was not assessed in the present study population. Therefore, it is suggested that it is necessary to investigate the role of the newly reported allelic variant CYP2C19*2C in the inhibition of the antiplatelet response to clopidogrel.

In conclusion, allelic variants of CYP2C19*2 have been screened out for the first time, to the best of our knowledge, and new variant CYP2C19*2C has been identified in Pakistani cardiac patients with ACS and PCI. Due to the new variant C, tri-allelic SNPs in CYP2C19*2 allele (CYP2C19*2 A/C/G SNPs) were observed in Pakistani cardiac patients, in contrast with observations in other studied populations. Significant inter-ethnic variability has been observed in the allelic frequencies of CYP2C19*2, providing evidence that genetic polymorphism of CYP2C19*2 is important in the response to clopidogrel. Hence, the genetic screening of CYP2C19*2 allele variants is emphasized as a more suitable tool for selecting the appropriate anti-platelet agent to treat a cardiac patient, and would be a step towards personalized medicine. However, further studies are required to investigate other likely factors involved in clopidogrel resistance, and there is also a requirement for a larger study to better assess the role of genotyping in the evaluation of the phenomenon of clopidogrel resistance.

Acknowledgements

The authors thank Punjab University Research Committee 2012/2013 (University of the Punjab, Lahore-Pakistan) for providing financial assistance. The laboratory facility provided by Vital Molecular Diagnostic Laboratory (Lahore, Pakistan) and the sequencing facility provided by the Center for Applied Molecular Biology (CAMB), Lahore are also highly acknowledged.

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