A study into the genetic basis of aspirin resistance in Pakistani patients with coronary artery disease

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Abstract: Aspirin is a key player in the management and prevention of stroke and myocardial infarction in patients with atherothrombosis. About 12% of Pakistanis suffering from coronary artery disease are resistant to aspirin's effects. Clinical, biochemical and genetic factors are known to be responsible for this phenomenon. We conducted this study to investigate whether previously studied polymorphisms in COX-1, GPIIIa, GPIa and P2RYI genes could be the cause of aspirin resistance in our population. Blood samples were collected from 29 aspirin non-responders and 60 ethnically matched responders. Aspirin response assay was performed on IMPACT-R and DNA prepared from blood using the phenol: chloroform method. Genotyping was carried out for four SNPS including COX-1 C50T (rs3842787), GPIIIA PIA1/A2 polymorphism (rs5918), GPIA C807T (rs1126643) and p2RY1 C893T (rs1065776). No statistically significant differences were observed in the allele or genotype frequencies between the aspirin non responders and responders indicating the possible involvement of different genetic determinants of aspirin resistance in our population. This study paves the way for further research into the field of aspirin resistance in Pakistan.

Keywords: Aspirin resistance, Aspirin response assay, Pakistan, COX-1.

INTRODUCTION

Aspirin (Acetylsalicylic Acid, ASA) is one of the most effective and commonly prescribed antiplatelet drug worldwide for the management and prevention of stroke and myocardial infarction in patients with atherothrombosis (Antiplatelet Trialists' Collaboration 1994, 2002). However, despite the proven benefit of aspirin as an antiplatelet drug, a significant number of individuals on aspirin remain at risk of vascular events (Wang 2006). The failure of this drug to prevent clinical atherothromboembolic ischemic events in patients who are taking aspirin has been called "clinical aspirin resistance" (Sanderson 2005). The underlying cause of aspirin resistance has yet to be determined. However, clinical, biochemical and genetic factors are known to be involved. Studies in different populations have shown that 5-45% individuals may be aspirin resistant (Mason 2005). A study on aspirin resistance in Pakistani patients with coronary artery disease revealed that 12% of participants were aspirin resistant (Akhtar 2009).

Aspirin inhibits the cyclooxygenase (COX) enzyme irreversibly, by acetylating a serine residue at position 530 (Lecomte 1994). COX has two distinct isoforms COX-1, and COX-2. COX-1 is the constitutive isoform of cyclooxygenase and is expressed in most tissue types. It is the only form found in mature platelets. COX-1 converts arachidonic acid to prostaglandin H2, which in platelets is further converted to thromboxane A2. Thromboxane A2 is a potent vasoconstrictor and platelet agonist. Aspirin suppresses platelet function by blocking the access of arachidonic acid to the catalytic site of COX-1 so TXA-2 cannot be synthesized (Sanderson 2005). Single nucleotide polymorphism (SNPs) in the COX- 1 gene may therefore be expected to affect COX-1 enzymatic activity, as well as its interaction with aspirin.

Platelet aggregation is regulated through binding of cross-linking molecules and agonists to specific receptors present on the platelet cell surface. GPIIb/IIIa (integrin $\alpha_{IIb}\beta_3$), the receptor for von Willebrand factor and fibrinogen, is the most abundant integrin present on the surface of platelet cells. The binding of fibrinogen to this receptor leads to platelet aggregation and formation of thrombi. The genes for the GpIIb/IIIa receptor are polymorphic and have been studied in association with the risk of atherothrombosis and aspirin sensitivity (Goodman 2008).

The GPIa/IIa (integrin $\alpha_{II}\beta_1$) receptor, also present on the platelet cell surface, binds to collagen. Collagen is the major adhesive protein exposed to platelets after a blood vessel injury and acts as an activator of platelet aggregation leading to thrombus formation. Several SNPs have been described in the GPIa subunit of the receptor, of which the C807T variant is been the most commonly studied polymorphism (Kunicki 1997). Although it has been demonstrated through platelet aggregation assays that aspirin non-responders are more sensitive to low concentrations of collagen than aspirin responders, the

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role of any polymorphism of the GPIa gene in aspirin resistance remains unclear (Shanker 2011).

ADP is one of the key molecules in platelet aggregation mediating its action through the G-protein coupled receptors P2RY1 and P2RY12 on the platelet surface. The binding of P2RY1 to ADP results calcium release from internal stores, change in platelet shape and reversible platelet aggregation (Jin 1988). ADP signalling through the P2RY12 receptor results in the activation of GPIIb/IIIa and the stabilization of platelet aggregates (Kauffenstein 2001). During aspirin treatment, the TXA2 pathway of platelet aggregation is blocked. However, the ADP mediated pathway may remain unaffected. Thus polymorphisms in the ADP receptors, associated with an increase in platelet aggregation, may result in aspirin resistance. As high as a three-fold increase in platelet aggregation has been associated with a C to T substitution at position 893 of the P2RY1 gene (Jefferson 2005).

We conducted this study to investigate whether the polymorphisms in the above mentioned genes could prove to be useful markers in predicting aspirin resistance in Pakistani patients.

MATERIAL AND METHODS

Blood samples were collected from 29 aspirin non-responders and 60 ethnically matched responders who had been part of a study on the frequency of aspirin resistance in Pakistan carried out at the Departments of Cardiology and Hematology at the Shifa International Hospital, Islamabad, Pakistan. All participants of this study had been on aspirin (75-300mg/day) for more than seven days and had established coronary artery disease or coronary artery disease equivalent conditions (carotid artery disease, diabetes mellitus, peripheral vascular disease) (table 1). Aspirin response assay was performed on IMPACT-R (Dia Med AG 1785 Cressier Morat, Switzerland) as described previously (Akhtar 2009).

DNA was prepared from blood using a standard organic extraction method. Genotyping was carried out for four SNPS including COX-1 C50T (rs3842787), GPIIIA PIA1/A2 polymorphism (rs5918), GPIA C807T (rs1126643) and p2RY1 C893T (rs1065776). 6/29 DNAs could not be typed due limitation in quality or quantity of DNA and had to be dropped from the study. The GPIIIA polymorphism was typed as described previously (Undas 2001). A PCR-RFLP assay was designed for the p2RY1 C893T polymorphism. Allele-specific PCR (AS-PCR) assays were designed for COX C50T and GPIA C807T using the WASP website (Wangkumhang 2007). The details of the PCR assays are as given in table 2. PCR was performed in 15-25µl reaction volumes with 1µM primer, 2.0mM $MgCl_2$, 1 Unit Taq DNA polymerase, 200 µM dNTPs and 20-40ng DNA. The p2RY1 master mix also contained 8% DMSO. PCR cycling conditions consisted of a denaturation cycle of 4 minutes at 94^{o}C followed by 30 cycles of denaturation (94^{o}C) for 45 sec, annealing at the temperatures specified in table 2 for 45 sec and extension (72^{o}C) for 45 sec. Restriction digestion was carried out as per the manufacturer's instructions. PCR products were run on 2-3% agarose gels and visualized under UV trans illumination. The genotypes of COX-1 and GPIA were ascertained on the basis of absence/presence of allele specific bands. The genotypes of GPIIIA and P2RY1 were based on the presence of digested/undigested PCR product.

The allele and genotype frequencies were determined by direct counting. Hardy-Weinberg equilibrium (HWE) was calculated using the Koonec Hardy-Weinberg Equilibrium Calculator available at http://www.koonec.com/k-blog/2010/06/20/hardy-weinberg-equilibrium-calculator/. Fisher's exact test, required for comparison between the two study groups, Odds ratio (OR) and 95% confidence intervals were determined by the use of an online statistical software package for 2-way Contingency table Analysis (Pezzullo 2010). A p value <0.05 was considered significant.

RESULTS

Deviation from HWE was observed P2RY1 in the aspirin responders. The remaining loci showed HWE in both aspirin-resistant and control groups. The allele and genotype frequencies along with p values by Fisher's exact test, ORs and their 95% CI for GPIa, GPIIIa, P2RY1 and COX1 are given in table 3. The COX1, C and T alleles were present at a frequency of 0.98 and 0.02 respectively in both responders and non-responders. No significant difference was observed in the frequencies of the genotypes C/C and C/T between the two groups (p= 1.0; OR=1.16; CI=0.10-30.55 and p=1.0; OR=0.83; CI= 0.03-9.87 respectively). The homozygous T/T genotype for this polymorphism was not found in our population. At the GPIA locus, the C and T alleles were present at frequencies of 0.54 and 0.46 in the non-responders and 0.58 and 0.42 in the responders respectively. There was no statistically significant variation in the genotype frequencies between the aspirin non responders and responders (C/C: p=0.8, OR=0.8, CI=0.26-2.42; C/T: p=1.0, OR=1.11, CI 0.37-3.32; T/T: p=0.78, OR=1.16, CI=0.33-3.95). At the GPIIIA locus, T was the major allele present in both groups at a frequency of 0.89 while C was present at a frequency of 0.11. There difference observed in the genotype frequencies between the two groups at this locus was not significant. The CC genotype was absent in responders and present in non-responders at a frequency of 0.04. The C/T genotype was present at frequencies of 0.13 and 0.22 while TT was present in 0.83 and 0.78 of non responders and responders respectively. The P2RY1 C allele was present at frequencies of 0.83

Table 1: Characteristics of the study groups

		Responders (N=23)	Non-responders (N=60)
Gender	Male	20 (87)	37 (62)
	Female	3 (13)	23 (38)
Age		57.43 ± 10.41	59 ± 11.74
Diabetes Mellitus		11 (47.8)	29 (48.3)
Hypertension		5 (21.7)	28 (46.6)
Hyperlipidemia		8 (34.8)	29 (48.3)
Family history		2 (0.09)	6 (0.10)
Smokers		3 (13.0)	3 (0.05)

Table 2: Details of the PCR assays used for genotyping

Gene	Primer sequence	Assay	Tm	Enzyme	PCR product
COX-1	FN - GCTGTTCCTGCTCCTGCTCAC MN-GCTGTTCCTGCTCCTGCTCAT R - AGCCTCAGTCTTTCTCAGC	ARMS -PCR	59^{o}C	-	195 bp
GPIA	F- GACAGCCCATTAATAAATGTCTCCTCTG RN - CCTTGCATATTGAATTGCTACG RM - CCTTGCATATTGAATTGCTACA	ARMS -PCR	57^{o}C	-	188 bp
GPIIIA	F- GCTCCAATGTACGGGGTAAAC R -GGGGACTGACTTGAGTGACCT	PCR-RFLP	55^{o}C	MspI	undigested =282 bp digested =157+125 bp
P2RY1	F- AAAGCGCAGTCGGAAAGTTA R- TGCTGCCATAGAGGTTCACA	PCR-RFLP	56^{o}C	RsrII	undigested =735 bp digested =378+357 bp

and 0.84 and the T allele at frequencies of 0.17 and 0.16 in the non-responders and responders, respectively. No statistically significant difference was observed in the genotype frequencies between the aspirin non responders and responders (C/C: p=0.78, OR=0.86, CI=0.25-3.01; C/T: p=0.75, OR=1.19, CI=0.27-4.99; T/T: p=1.0, OR= 1.05, CI=0.13-6.87).

DISCUSSION

Oral antiplatelet agents, including aspirin are a fundamental and indispensible part in the secondary prevention and treatment of cardiovascular disease. However, it has been demonstrated in multiple studies that all patients prescribed aspirin do not gain a similar desired effect, resulting in a greater chances of further vascular events or even death. Krasopoulos (2008) showed in their study that patients who were aspirin resistant had a nearly four times greater risk of suffering further cardiovascular events and a six fold increase in mortality. It has been shown in different populations that up to 40% of patients may be aspirin resistant which may be due to a number of reasons including aspirin dose, non-compliance, co-morbid conditions, smoking, hyperlipidemia and genetic factors.

The heritable nature of platelet ASA responsiveness has been well documented (Faraday 2007) and many studies have been conducted to investigate the association of aspirin resistance with polymorphisms in genes involved in platelet activation and function. A recent study using mRNA profiling to identify the therapeutic response to aspirin identified a set of 60 co-expressed genes which could serve as novel biomarkers to identify patients unlikely to obtain therapeutic benefit from aspirin (Voora 2013).This study was carried out to study the genetic association of aspirin resistance by analysing four SNPs in different candidate genes in the Pakistani population.

Polymorphisms in the COX-1 gene (*PTGS1*), have been of special importance as COX-1 is the primary molecular target of aspirin and individual single nucleotide polymorphism (SNPs) and/or their haplotypes may affect COX-1 enzymatic activity, as well as its interaction with aspirin. The non-synonymous variant, C50T (rs3842787) has been widely studied in relation to aspirin resistance (Goodman 2008). This SNP is in linkage disequilibrium with the A-842G variant, present in the promoter region of the COX-1. According to Patinella (2009) the C50T polymorphism reduces the inhibition of aspirin on platelet aggregation *in vitro* and on TXA-2 synthesis *in vitro* and *in vivo* while others have found no relationship between the C50T variant and aspirin resistance. Clappers (2008) established that C50T polymorphism of COX-1 gene has no association with an increased risk of atherothrombotic events in patients on aspirin. Gonzalez (2005) showed that carriers of the 50T allele of COX-1 had persistent high levels of 11-dehydro thromboxane B_2 despite treatment with aspirin. Our results showed no significant association of the C50T variant of COX-1 gene with aspirin resistance in our population.

Table 3: Genotype and allele frequencies in aspirin non-responders (cases; n=23) and aspirin responders (controls; n=60) in the Pakistani population.

	Genotype Frequency			Allele Frequency	
GPIA	C/C	C/T	T/T	C	T
Cases	0.35	0.39	0.26	0.54	0.46
Controls	0.40	0.37	0.23	0.58	0.42
p-value	0.80	1.00	0.781	0.727	
Odds ratio	0.80	1.11	1.16	0.85	1.18
95% CI	0.26-2.42	0.37-3.32	0.33-3.95	0.41-1.78	0.56-2.5
GPIIIA					
Cases	0.04	0.13	0.83	0.11	0.89
Controls	0	0.22	0.78	0.11	0.89
p-value	0.277	0.53	0.769	1.00	
Odds ratio	inf	0.542	1.31	1.00	0.996
95% CI	0.149-inf	0.11-2.38	0.34-5.50	0.29-3.29	0.30-3.44
P2Y1					
Cases	0.74	0.17	0.09	0.83	0.17
Controls	0.77	0.15	0.08	0.84	0.16
p-value	0.78	0.75	1.00	0.817	
Odds ratio	0.86	1.19	1.05	0.89	1.12
95% CI	0.25-3.01	0.27-4.99	0.13-6.87	0.33-2.44	0.41-2.99
COX1					
Cases	0.96	0.04	0	0.98	0.02
Controls	0.95	0.05	0	0.98	0.02
p-value	1.0	1.0	1.0	1.0	
Odds ratio	1.16	0.83		1.15	0.87
95% CI	0.10-30.55	0.03-9.87		0.10-29.5	0.03-9.73

GPIIIa is characterized by several heritable dimorphisms according to Santoso (1998). The C>T polymorphism at position 1565 in the GPIIIa gene (*ITGB3*), commonly known as the PIA1/A2 polymorphism, leads to a leucine (PIA1) to proline (PIA2) amino acid substitution. The PIA1/A2 variant of this gene has shown an association with aspirin resistance in previous studies. Goodman (2008) in their systematic review of literature on candidate genes in association with aspirin resistance established that healthy subjects carrying the PIA2 allele were 2.36 times more likely to be resistance to aspirin. They failed to demonstrate a similar association in subjects with cardiovascular disease. The latter is in conjunction with our findings of the PIA1/A2 SNP of the GPIIIa receptor gene not having a significant impact on aspirin effect in our subjects.

Three different genotypes of the C807T polymorphism of the GPIa subunit have been associated with differing expression of GPIa/IIa on platelets. It has been demonstrated that individuals with the TT genotype have the highest level of expression of the receptor on the platelet cell surface while the CC individuals have the lowest level of expression (Kunicki 1997). The presence of the T allele results in increased platelet activation and aggregation and is a potential risk factor for thrombotic diseases according to Williams (2010). We however found that there was no association between the C807T and aspirin responsiveness in our study. Our finding is in accordance with those of Gonzalez (2005) and Fontana (2006) who demonstrated a similar trend in healthy individuals.

ADP mediates its action on P2RY1 through the Gq-phospholipase C–inositol 1,4,5-trisphosphate-Ca2+ pathway. This cascade ultimately results in the activation of the fibrinogen receptor (GPIIb/GPIIIa) present on platelets (Sangkuhl 2011). Antagonism of P2RY1 and P2RY12 receptors leads to reduced platelet aggregation, P-selectin expression and platelet-monocyte aggregates (Storey 2000). Li, 2007 found that after aspirin administration in healthy Chinese participants, the net reduction in arachidonic acid-induced platelet aggregation was significantly greater in the P2RY1 C893T genotype panel compared with C893C genotypic group. C893T polymorphism of P2RY1 is conservative and leads to no change in the overall amino acid sequence, hence the biochemical mechanism of the risk associated with this polymorphism remains unclear (Jefferson 2005).

This study, the first of its kind in Pakistan, paves way for further research into the field of aspirin resistance. There remains a need to define the term ‘aspirin resistance’, one which unifies clinical expression of failed treatment and

biochemical parameters. Further, Fontana (2006) highlighted how use of different techniques to measure aspirin resistance yields different results. Our efforts do elicit an eminent danger of 'failed treatment' in 12% of Pakistani population, but we could not associate one of the common culprit gene polymorphisms with this variation in aspirin response. This finding may be due to a different polymorphism that we did not include in our study or may be a limitation of the small study population. The question thus turns to how a clinician can effectively pre-empt and treat this phenomenon and perhaps pave way for personalised health care.

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