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Original Article

Association of *Cyclooxygenase 2 (COX2)* Polymorphism at SNP RS20417 with Aspirin Resistance in Pakistani Patients with Ischemic Heart Disease

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ABSTRACT

Objective: to find a link between a single nucleotide polymorphism (SNP) called rs20417 in the cyclooxygenase-2 gene and Aspirin resistance in Pakistani patients with heart disease.

***Study Design*:** Cross-sectional study.

***Place and Duration of Study*:** Pharmacology Department, National University of Medical Sciences, in collaboration with the National Institute of Heart Diseases and the Institute of Biomedical and Genetic Engineering in Islamabad Pakistan, from Oct 2018 to Dec 2021.

Methodology: The study was carried out on 384 patients (272 males and 112 females) with ischemic heart disease. Patients who had been on Aspirin for at least seven days were selected using non-probability convenience sampling. Platelet aggregation was performed using a Transmission Aggregometer, with arachidonic acid as an agonist. DNA extraction was done using the kit method (Invitrogen, Thermofisher). Then, Polymerase chain Reaction, Restriction Fragment Length Polymorphism, and gel electrophoresis were used to identify SNP rs20417.

Results: In this study, 14.0% (n=54) of ischemic heart disease patients were found to be Aspirin resistant, while 86.0% (n=330) were Aspirin responders. The genotyping of COX2 SNP rs20417 showed that 55.46% (n=213) of patients were homozygous with the GG genotype, 34.11%(n=131) had CG (heterozygous), and 10.41% (n=40) of patients were carriers of homogeneous (CC). Statistical analysis did not demonstrate a significant association of any of the alleles of the evaluated SNP with Aspirin resistance (p>0.05).

***Conclusion*:** In the Pakistani population, Aspirin resistance is not associated with any specific polymorphic form of Cyclooxygenase-2 at SNP rs20417.

Keywords: Aspirin resistance, *Cyclooxygenase 2* polymorphism, Ischemic heart disease, Platelet aggregation, Restriction fragment length (RFLP), Single nucleotide (SNP) rs20417

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INTRODUCTION

Nearly 1 million people die globally just because of ischemic heart disease (IHD), making it the number one cause of death worldwide[1]. The prevalence of cardiovascular diseases in Punjab, Pakistan, remained at 17.5% during 2017[2]. Platelet aggregation inhibitors are the most important pillars of IHD prevention and treatment. Platelets play a role in the pathogenesis of thromboembolic diseases. Platelets are synthesized in the bone marrow and circulate as enucleate cells for 7 to 10 days until they are eliminated by phagocytosis in Kupffer cells.[3] These vascular guards rigorously monitor the vascular epithelium's integrity. As soon as the vascular epithelium is damaged, these sentries become activated through various receptors present on their surface, and a series of events take place, ultimately resulting in clot formation. The cells in this layer talk to platelets through integrin a2b1, glycoprotein (GP)VI, and GPIb3 to fix any damage to the epithelial layer.4 Cyclooxygenase 2 is activated in platelets, leading to prostaglandin formation and platelet adhesion. Overactivity in these processes causes thromboembolic disorders. Activation, adhesion, release of chemical mediators, and aggregation of platelets are all natural responses of the body to unexpected damage to the vessel wall. If these responses are stimulated too much, they can lead to the formation of an intraluminal clot and vascular obstruction, followed by cellular ischemia or infarction.[5] Antiplatelet drugs, particularly Aspirin, are most frequently used by IHD patients. This crucial role of Aspirin in prevention has drawn attention to a

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clinical issue commonly known as Aspirin resistance.[5]. A significant number of patients worldwide have developed Aspirin resistance, i.e., 24.7% (pool prevalence). [6] The cause of this resistance is being investigated so that the dose can be adjusted in advance to avoid serious consequences.[7] Cyclo-oxygenase 2 *(*COX-2) is one of the major enzymes involved in platelet aggregation. [8] spirin irreversibly acetylates and thus permanently inactivates COX-2 in platelets. Platelets are non-nucleated particles and thus are deprived of their aggregation ability due to a lack of active COX-2 in them for the rest of their lives, which is 7–10 days in humans.[9] Hence, low-dose Aspirin (75–150 mg per day) is used worldwide to prevent and treat cardiac thrombotic events.10. The cause of Aspirin resistance is unclear. A COX-2 enzyme variant may be involved, or its genetic variation may be implicated. In this study, a single nucleotide variation (rs20417) is selected to study whether it is associated with Aspirin resistance in the Pakistani population.

METHODOLOGY

The cross-sectional study was carried out at the Pharmacology Department of the Constituent Medical College of the National University of Medical Sciences, Rawalpindi Pakistan, in collaboration with the National Institute of Heart Diseases, Rawalpindi Pakistan, and the Institute of Biomedical and Genetic Engineering (IBGE), Islamabad Pakistan, from October 2018 to December 2021. The study commenced after ethical approval by the Institutional Ethical Review Board (ERC/SA-15, dated December 1, 2015). The National Institute of Heart Diseases (NIHD) is a tertiary care hospital with patients coming from all over Pakistan. The sample size was calculated by the WHO calculator using a 95% confidence interval and 80% power of study with an anticipated minor allele frequency (MAF) of 0.6 in patients with Aspirin resistance.[11]

Inclusion Criteria: Ischemic heart disease patients of either gender, aged 18 to 70 years, taking Aspirin 75 mg or more once daily for at least 8 days, were enrolled in the out-patient and in-patient departments were included.

Exclusion Criteria: Individuals taking other anti-platelet agents or having any bleeding disorder were excluded. Patients with other comorbidities and those taking drugs that interact with Aspirin were excluded.

We used consecutive sampling to select patients from every province of the country, and informed written consent was obtained before sample collection. We collected 8 ml of blood after 2.5 to 10 hours of the last dose of Aspirin, carried out platelet aggregation studies using 4 ml of sample within 3 hours, and saved the remaining blood for genetic studies.

Before aggregation studies, platelet counts were assessed using a hematology analyzer (Erba H360). The blood was given a spin at 800 rpm to get platelet-rich Rich Plasma (PRP). Using a micropipette, the PRP-containing upper layer was taken out, and the rest was centrifuged at 4000 rpm for 5 minutes to get Platelet Poor Plasma (PPP). This was then used as a control in light transmission aggregometry (Chrono-Log, Havertown, Pa., USA). Arachidonic acid (0.5 mM) was used as a stimulator, and platelet aggregation > 20% was considered Aspirin resistance [12,13].

DNA was extracted from 384 samples of whole blood taken in EDTA vacutainers using the kit method (Invitrogen, Thermofischer). DNA quality and quantity were analyzed using an ultraviolet (UV) spectrophotometer (U-3210, Hitachi) at 260/280 nanometers (nm) and a 1:50 dilution in distilled water. To copy the important part of the Cyclo-oxygenase-2 (COX-2) gene from samples with an OD range of 1.7 to 1.9, PCR was used. Primers for PCR were designed using Primer 3 software and procured by Inqaba Biotech.

Restriction enzyme BspACI (recognizes sequence C^CGC prepared by New England Biolabs, UK) was used at 37 °C for 1 hour. To do restriction enzyme digestion, 10U/µL of enzyme and 10µl of reaction buffer were added to the PCR product. Distilled water (dH2O) was then added, and the mixture was left to sit at 37°C overnight. This led to the production of fragments of different lengths. The restriction enzyme produced 3 fragments (237bp+131bp+106bp) in heterozygotes, 2 fragments (131bp+106bp) in wild-type, and 1 fragment (237bp) in homozygotes. The temperature was kept at 65°C for 25 minutes to inactivate residual enzymes. Agarose gel electro-phoresis was carried out to separate the fragments. A 100-bp gene ladder (GeneRuler from ThermoScientific) helped identify fragment length.

Statistical Package for Social Sciences (SPSS) version 23 was used for data analysis. Allele frequencies were calculated. Chi-square test was applied to explore the inferential statistics. The p value of ≤0.05 was considered statistically significant.

RESULTS

In this study, 54 (14%) cardiovascular disease patients were Aspirin-resistant, while 330 (86%) Individuals were responsive to Aspirin.

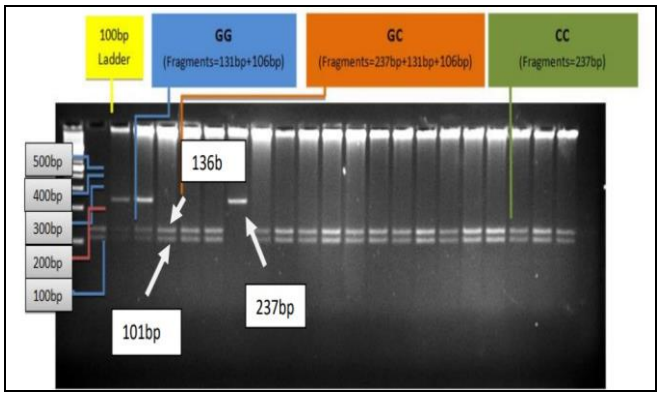

Figure-1: RFLP Analyses for Genotyping of SNP rs20417 (n=384)

Figure-1 shows that the GG, GC, and CC genotypes each made 2, 3, and 1 fragments. To tell them apart, a 100-bp ladder was used in 2% gel electrophoresis.

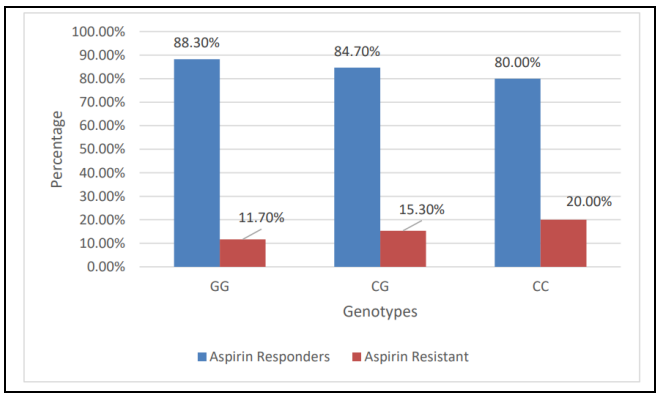

Figure-2: Aspirin Response in Different Genotypes of SNP RS20417 (n=384)

The GG genotype has the highest prevalence in our population, followed by the CG genotype, and the least prevalent is the CC genotype, as shown in Figure-2. None of these genotypes is significantly associated with the risk of Aspirin resistance.

Based on rs20417 analysis, 55.46%(n=213) had the wild-type allele (GG), 34.11%(n=131) had the homozygous (CC) genotype, and 10.41%(n=40) had the heterozygous (CG) genotype. This is shown in Table.

Table-: Genotype Distribution in Aspirin Resistance (n=384)

SNP rs20417	Aspirin Responders	Aspirin Resistant	Total	*p*-value
GG	188(88.3%)	25(11.7%)	213(55.5%)	0.145
CG	111(84.7%)	20(15.3%)	131(34.1%)	
CC	32(80.0%)	8(20.0%)	40(10.4%)	
Total	331(86.2%)	53(13.8%)	384(100%)	

DISCUSSION

Different results have been reported about the possible role of COX2 SNP (rs20417) in the development of Aspirin resistance.[14-15] A lack of response to Aspirin was seen in 25(11.7%), 20(15.3%), and 8(20%) cardiovascular patients who were wild-type, heterozygous, and homozygous carriers of the rs20417 alleles, respectively. This is likely not due to rs20417. Yi and colleagues' study on 634 Aspirin-taking ischemic stroke patients reinforces our findings. They looked at rs20417 (G765C) as part of their genotypic study, but they couldn't find a meaningful statistical difference between Aspirin responders and non-responders in the frequencies of rs20417 (COX2) [16]. However, the permutation test indicated that the presence of variants at rs20417 significantly increased the risk of Aspirin resistance. On the other hand, a meta-analysis looked at and summed up six double-blinded trials, including ACTIVE-A, CURE, epi DREAM/DREAM, ONTARGET, RE-LY, and WGHS. The goal of the authors was to find out how the rs20417 variant affected the risks of bad cardiovascular events in six different groups [17]. It was proven that people who carried rs20417 had a much lower likelihood of having a heart attack, stroke, or vascular death (OR=0.78, 95% CI: 0.70–0.87, p=1.20 × 10^{-3})[17]. It was also observed that the use of Aspirin by the CC carriers at rs20417 reduces the risk of major cardiovascular problems.[17]Our results are not supported by a meta-analysis that compiled data from 26 different case-control studies, following the guide-lines set by the Preferred Reporting Items for Sys-tematic Reviews and Meta-analysis (PRISMA). The authors demonstrated that the presence of COX2 gene variation in rs20417 may contribute to a higher risk of Aspirin resistance. In patients, the risk-allele fre-quency of rs20417 was 20.36%, compared to 12.6% in healthy individuals.[18] Another study involved 450 stroke patients and an equal number of healthy individuals of similar age and sex to investigate the role of rs20417 in Aspirin resistance. The antiplatelet efficacy of Aspirin was evaluated by follow-up interviews with

stroke patients up to ninety days after enrollment. The genotyping results showed that people with GC (p=0.02) and CC (p=0.016) genotypes were more likely to have bad ischemic events within the given timeframe, even if they regularly took Aspirin to lower their blood pressure, compared to people with GG variants [12]. In this study, they finally concluded that rs20417 is significantly associated with poor Aspirin efficacy, and carriers of this SNP are at greater risk of encountering repeated ischemic cardio-vascular events.

In other studies, there was a strong link between this SNP and inability to respond to Aspirin, but this one could not find a link between the COX2-rs20417 variant and IHD. It is concluded that even larger sample sizes and Sanger sequencing of COX2 can be a good choice to get a clear picture or significance of this SNP.

CONCLUSION

Aspirin resistance in Pakistani patients with ischemic heart disease is not related to a polymorphism in the SNP (rs20417) of the COX2 gene.

Conflict of Interest: None.

Authors Contribution

Following authors have made substantial contributions to the manuscript as under:

MN: Data acquisition, data analysis, drafting the manuscript, critical review, approval of the final version to be published.

SZHS: Study design, data interpretation, drafting the manuscript, critical review, approval of the final version to be published.

UN & QM: Conception, data acquisition, drafting the manuscript, approval of the final version to be published.

Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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