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Association of Cyclooxygenase 1 (*COX1)* Polymorphism at SNP *RS1330344* with Aspirin Resistance in Pakistani Patients of Ischemic Heart Disease

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ABSTRACT

Objective: To find an association between a single nucleotide polymorphism (SNP) rs1330344 in *cyclooxygenase-1* gene with Aspirin resistance in patients with cardiovascular disease in the Pakistani population.

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

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

Methodology: The study was conducted on 384 patients (272 males and 112 females) with ischemic heart disease. Patients on Aspirin for at least seven days were selected. Platelet aggregation was performed using a Light Transmission Aggregatometer and 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 rs1330344.

Results: In this study, 54 (14.0%) and 330 (86.0%) of ischemic heart disease patients were resistant to Aspirin and Aspirin, respectively. The genotyping of Cyclooxygenase 1, SNP rs1330344 showed 94 (24.41%), 67 (17.56%), and 223 (57.98%) patients had wild type allele (CC), homozygous (TT) and heterozygous (CT) genotype respectively. Gel electrophoresis results for allelic identification were correlated with the anti-platelet efficacy of Aspirin among ischemic heart disease patients. No association was found between a polymorphic form in SNP and the development of aspirin resistance.

Conclusion: Aspirin resistance is not associated with any specific polymorphic form of Cyclooxygenase-1 at SNP rs1330344 in the Pakistani population.

Keywords: Aspirin Resistance, Cyclooxygenase 1 Polymorphism, Ischemic Heart Disease, Platelet aggregation, Restriction Fragment Length (RFLP), Single Nucleotide (SNP) rs1330344.

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INTRODUCTION

Cardiovascular morbidity and mortality are on the rise globally.[1] After an acute attack of ischemia or in high-risk patients, low-dose Aspirin and clopidogrel are advised to prevent further serious coronary events. [2] Aspirin in anti-platelet dose is time tested, relatively safe, and efficacious compared to other anti-platelet drugs.[3] Low-dose Aspirin is being prescribed as prophylactic to coronary events the world over, especially in high-risk, elderly, and after cardiac intervention.[4]

Aspirin resistance has emerged in a significant number of patients globally, i.e., 24.7% pool prevalence of Aspirin resistance was noted in a recent study that collected data from different nations.[5] There have been efforts by researchers to find the cause of Aspirin resistance and any association with genetic variations so that the Aspirin responsiveness in a particular individual can be predicted and the dose can be adjusted in advance to avoid serious consequences.[6] Cyclooxygenase 1*(COX-1*) is one of the major enzymes involved in platelet aggregation.[7] Aspirin irreversibly acetylates and thus permanently inactivates COX-1 in platelets. Platelets are non-nucleated particles and thus are deprived of their aggregation ability due to their lack of active COX-1 for the rest of their life, which is 7-10 days in humans.[8] Hence, low-dose Aspirin (75-150mg daily) is used worldwide to prevent and treat cardiac thrombotic events.[9]

Aspirin resistance in the Pakistani population is around 13.8%, slightly more in males than in females.[10] Many inquiries have been into the cause/association of Aspirin resistance to different factors. If this phenomenon is not taken into account and the dose of

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Aspirin is not adjusted accordingly, the outcome may be fatal for cardiac patients. The mechanism behind Aspirin resistance needs to be clarified. However, SNPs in related genes (especially *COX-1*) have been suspected of being risk factors for Aspirin resistance. This study selected clinically important single nucleotide variation (rs1330344) 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, 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, after approval by the Institutional Ethical Review Board (ltr no. ERC/SA-15, dated 01 December 2015). The WHO calculator calculated the sample size using 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 taking Aspirin 75mg or more once daily for at least eight days were enrolled from the Out-patient and In-Patient Departments.

Exclusion criteria: Individuals taking other anti-platelet agents or having any bleeding disorder were excluded. Patients with other comorbidities and taking drugs with Aspirin interactions were excluded.

Informed written consent was obtained before sample collection. Drug interactions were checked using an interaction checker at drugs.com online. We carried out consecutive sampling and selected patients from every province of the country. Eight ml blood was collected 2.5 to 10 hours after the last Aspirin was taken, and platelet aggregation studies were carried out using 4 ml of sample within 3 hours after blood collection. The remaining blood was saved for genetic studies.

Before aggregation studies, the haematology analyser assessed platelet count (Erba H360). The blood was given a spin at 800rpm to get Platelet Rich Plasma (PRP). The upper layer containing PRP was collected by using a micropipette, and the rest was again centrifuged for 5 minutes at 4000rpm to obtain platelet-poor plasma (PPP), which was used as a control in light transmission aggregometry (Chronolog, Havertown). Arachidonic acid 0.5mM was used as a stimulator, and platelet aggregation >20% was considered Aspirin resistance.[12]

DNA was extracted from 384 samples of whole blood taken in EDTA vacutainers using the kit method (Invitrogen, Thermofischer). The quality and quantity of DNA were analysed using an ultraviolet (UV) spectrophotometer (U-3210, Hitachi) at 260/280 nanometers (nm) and 1:50 dilution of DNA to distilled water. The samples having optical density (OD) of 1.7 to 1.9 were subjected to PCR amplification of the area of interest in *Cyclo-oxygenase-1*(*COX-1*), also called *PTGS* (*Prostaglandin-Endoperoxide synthase-1*) gene.[13] Primers for PCR were designed using Primer 3 software and procured by Inqaba Biotech.

Restriction enzyme BccI (Recognises sequence CCATCNNNN^N_ prepared by New England Biolabs, UK) was used at 37°C for one hour.[14] Restriction enzyme digestion was carried out by the addition of 10U/µL of enzyme and 10µl reaction buffer to PCR product, and distilled water (dH2O) was added and incubated at 37°C overnight. This leads to the production of fragments of different lengths. Restriction enzyme produced three fragments (300bp + 164bp + 136bp) in heterozygotes, two fragments (164bp + 136bp) in wild type and one fragment (300bp) in homozygotes. Temperature was kept at 65°C for 25 minutes to inactivate residual enzymes. Agarose gel electrophoresis was carried out to separate the fragments. 100bp gene ladder (GeneRuler from Thermo-Scientific) helped identify fragment length.

Statistical Package for Social Sciences (SPSS) version 23.0 was used for the data analysis. Quantitative variables were expressed as Mean±SD and qualitative variables were expressed as frequency and percentages. Chi-square test, was applied to explore the inferential statistics. The *p*-value of 0.05 or less was taken as significant.

RESULTS

Out of the 384 cardiovascular disease patients, 54 (14%) showed more than twenty per cent platelet aggregation, making them aspirin-resistant cases. The remaining 330 patients (86%) had less than twenty per cent platelet aggregation, making them responsive to Aspirin. As shown in Figure-1, TT, CT, and CC genotypes produced 1, 3, and 2 fragments, respectively, and could be identified using a 100bp ladder in 2% gel electrophoresis.

Figure-2 elaborates that the CT genotype is prevalent in our population, almost 58%; CC is the next common genotype with almost 24% prevalence, while TT is the least prevalent at only 17.6%.

Analysis of rs1330344 showed that 94 (24.41%), 67 (17.56%), and 223 (57.98%) of patients had the wild-type allele (CC), homozygous (TT), and heterozygous (CT) genotypes, respectively, as shown in Table.

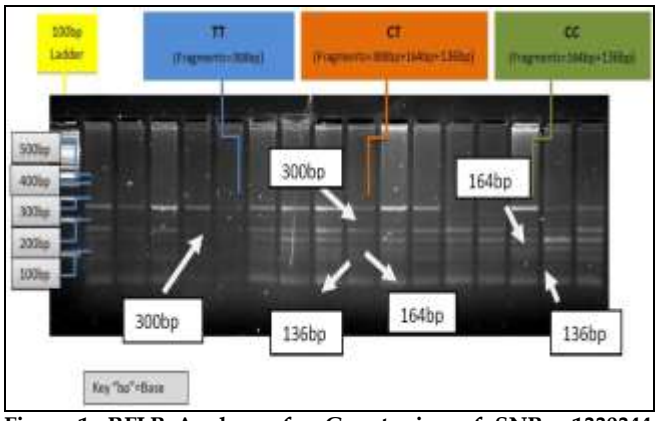

Figure-1: RFLP Analyses for Genotyping of SNP rs1330344 (n=384)

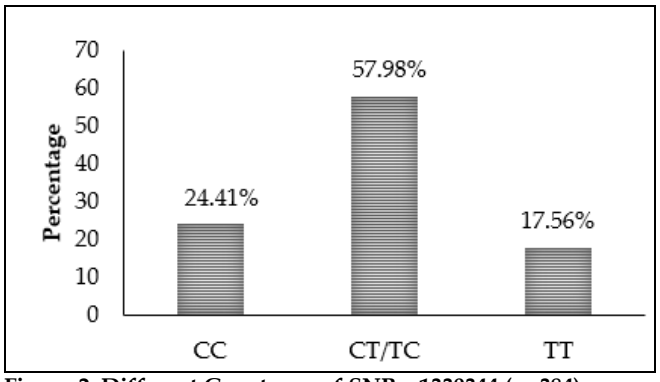

Figure-2: Different Genotypes of SNP rs1330344 (n=384)

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

SNP rs1330344	Aspirin Responders	Aspirin Resistant	*p*-value
CC	80(86.0%)	13(14.0%)	0.837
CT	190(85.6%)	32(14.4%)	
TT	61(88.4%)	8(11.6%)	
Total	331(86.2%)	53(13.8%)	

None of these genotypes was significantly associated with Aspirin resistance in our population.

DISCUSSION

In this study, 54 (14%) out of 384 cardiovascular patients showed Aspirin resistance. Poor response to Aspirin is linked with a four-fold risk of cardiovascular morbidity and mortality.[14] In Bacha Khan Medical College, an elaborate work on cardiovascular patients and Aspirin resistance demonstrated that Aspirin resistance is a growing concern in cardiovascular patients and can lead to treatment failure.[15] In this study, the prevalence of Aspirin resistance was around 14.5%, which is similar to our results.[16] Another study conducted at the University of Health Sciences Lahore in the pathology department of tertiary care cardiac hospitals of Lahore and Multan found around 11% Aspirin resistance in cardiac patients. The female gender and diabetes were reported as significant risk factors.[17] This study used an Aspirin Response Assay with DiaChidon (Arachidonic Acid 16mmol/L) to assess the Aspirin response rate in 71 patients. Our study used LTA with the arachidonic acid method (gold standard), and the number of cases enrolled was much greater than those used in similar studies in Pakistan. One thing ascertained is that around 14% of cardiovascular patients show Aspirin resistance in general in Pakistan, which can have fatal outcomes in these patients.

A few SNPs in *COX1* are proposed to be associated with certain cardiovascular diseases.[18] It is suspected that 33% of the varied responses to Aspirin are linked to genetic polymorphisms.[19] High throughput genomic technology, like genome-wide association studies and others, have pointed towards an association between Aspirin resistance and SNP rs1330344.[20] Another study in the Chinese population suggested a correlation between the SNP rs1330344 of *the COX-1* gene and Aspirin resistance.[21] Nevertheless, our results have been in contradiction to the findings of these studies. The presence of one of the CC, CT and TT variants revealed no relation with the anti-platelet response of Aspirin. The genetic makeup of our population may be different, or the sample size may be small. These varying results from different studies and opposing each other may be due to deficiencies in these studies. This led to confusion, and whether the C or T allele in *the COX-1* gene at position rs1330344 is associated with Aspirin resistance could not be identified. It is concluded that an even larger sample size and Sanger sequencing of COX1-rs1330344 can be a good choice to have a clear picture of the significance of this SNP. Despite a strong link reported between this particular SNP and Aspirin resistance in studies on other populations, it could not be established in this study in our cohort of Pakistani patients.

LIMITATIONS OF STUDY

The platelet function tests were not performed, the sampling was not appropriate, or the conclusions were clinical outcomes where the follow-up was not properly and timely done. In this study, the whole inquiry was done systematically, and platelet function tests were also performed according to the standard protocol, making the results more reliable. Coherent to already reported local studies, the current study failed to establish an association between *COX1*–rs1330344 variant and IHD.

CONCLUSION

Almost 14% of Pakistani cardiac patients show Aspirin resistance, which is not related to polymorphism in SNP (rs1330344) of *the COX1* gene.

Conflict of Interest: None.

Authors Contribution

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

MN & SZHS: Data acquisition, data analysis, data interpretation, critical review, approval of the final version to be published.

UN & QM: Study design, data interpretation, drafting the manuscript, critical review, 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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