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Association of COX-2 rs20417 with aspirin resistance

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Abstract Aspirin is the most commonly used antiplatelet drug for treatment of a serious vascular event, most notably stroke and myocardial infarction. However, despite the demonstrated benefit of aspirin, significant fraction of aspirin-treated patients may be resistant to the antiplatelet effects of the drug. The possible mechanisms of aspirin resistance (AR) are multifactorial. A genetic basis for AR has been suggested to exist. Therefore, the present study was taken up to investigate the role of −765G/C polymorphism (rs20417) in the cyclooxygenase-2 (COX-2) gene with AR in stroke patients. Four hundred and fifty stroke patients and four hundred and forty age and sex matched healthy controls were involved in the study. Baseline clinical data were collected and follow-up telephone interviews were conducted with patients at 3 months post event to determine stroke outcome using Modified Rankin Scale. Blood samples were collected and genotypes determined by polymerase chain reaction-restriction digestion technique. The association between the genotypes and outcome was evaluated by stepwise multiple logistic regression analysis. The COX-2 CC and GC genotype showed a significant association with bad outcome. Therefore, the carriers of C allele of COX-2 −765G/C polymorphism are more prone to AR in comparison with non-carriers. These results support a potential role of −765G/C COX-2 gene polymorphism with AR in ischemic stroke patients.

Keywords Stroke · Aspirin resistance · Genetics · Cyclooxygenase-2 gene (COX-2) · Pharmacogenetics

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Introduction

Despite the development of new antiplatelet drugs, still many patients are treated with aspirin to prevent primary or secondary cardiovascular death and stroke, for acute management of arterial occlusion and other serious vascular events. In the largest meta-analysis to date, the 2002 antithrombotic trialist collaboration representing more than 200,000 subjects, reported that among high-risk vascular patients, aspirin therapy was associated with a 34 % reduction in non-fatal myocardial infarction, 25 % reduction in non-fatal stroke, and an 18 % reduction in all [1]. Aspirin is an O-acetyl derivative of salicylic acid and is obtained by acylating the hydroxyl group with acetic anhydride using sulphuric acid as catalyst [2]. After its

existence for more than 100 years, aspirin is still considered golden standard of antiplatelet therapy [3]. Aspirin is a non-steroidal anti-inflammatory drug (NSAID) and acts by inhibiting the platelet cyclooxygenase system involved in the formation of thromboxane A2, a potent aggregator of platelets and also a vasoconstrictor. Cyclooxygenase also known as prostaglandin endoperoxide synthase, is a rate limiting enzyme that converts free arachidonic acid into important prostaglands and eicosanoids such as prostaglandin H2 [4]. There are at least two isoforms of COX identified i.e. COX-1 and cyclooxygenase-2 (COX-2). Aspirin inactivates permanently the activity of COX-1 and COX-2 enzymes catalyzing the first step in prostanoid biosynthesis [5]. After a single dose of aspirin, platelet COX activity recovers by 10 % per day in parallel with the entry of new platelets in the circulation. The usual antiplatelet aggregator dose of aspirin ranges from 75 to 325 mg/day.

Despite aspirin intake several patients develop adverse vascular events, an observation that gave rise to the concept of aspirin resistance (AR) [1]. Studies have shown that AR occurs in 5–65 % of people with ischemic stroke [6]. AR is a poorly defined term. The term has been used to describe not only an absence of the expected pharmacological effects of aspirin on platelets but also poor clinical outcome such as recurrent vascular events in patients on aspirin treatment. The mechanisms of AR are multifactorial. It might be on account of inadequate dose of aspirin, reduced absorption, and/or increased metabolism of aspirin. Some drugs may compete with aspirin at the COX-1 receptor site, most commonly encountered are NSAIDs, like ibuprofen which can offset the clinical benefit of aspirin in number of ways [7]. Poor glucose control and body weight have also been proposed to contribute to AR [8]. In addition to this a number of other clinical and miscellaneous factors have been proposed which contribute to AR [9]. Apart from all these factors a genetic aetiology to AR has also been proposed. Genetic variants of COX-1 and COX-2 genes and several receptors on the surface of platelets have been examined for the association with AR [10, 11].

A large number of variants have been described within the COX-2 gene. Most of the polymorphisms are in the intronic region with unlikely impact on COX-2 enzymatic activity. Only a limited number of polymorphisms have been found in the promoter region of COX-2 gene with potential impact on COX-2 expression and activity. In 2002, a new variant in the COX-2 promoter $-765G > C$ was identified and it was shown that the C variant has significantly lower activity in comparison with the G allele in vitro [12]. In accord with this observation the present study was taken up with an aim to study whether this functional polymorphism located 765 bp (base pairs) upstream from the transcription start site, in the COX-2 gene is associated with AR in ischemic stroke patients.

Methods

Subjects

Four hundred and fifty ischemic stroke patients (Males: females = 315:135) presenting with new stroke, evaluated in the stroke clinic of Nizam's Institute of Medical Sciences, Hyderabad (AP, India) between January 2007 and December 2010, were included in the study. The study was approved by ethical committee of the study hospital and written informed consent was obtained from all the subjects included in the study. All patients were examined by a qualified stroke neurologist to confirm diagnosis. Patients were confirmed to have suffered an ischemic stroke by computed tomography scans and magnetic resonance imaging. Patients with major renal, hepatic, cardiac diseases and cancers were excluded from the study. Ischemic stroke was classified according to Trial of ORG 10172 in Acute Stroke Treatment (TOAST) classification [13].

Four hundred and forty healthy individuals matched for sex and age formed control group (Males: females = 313:127). The controls were healthy volunteers recruited simultaneously from the same demographic area. They were blood donors from the study hospital and staff members of the Institute of Genetics and Hospital for Genetic Diseases. They underwent a routine medical check up in the outpatient clinic of the Institute of Genetics. The controls had no clinical evidence of any cerebrovascular disease.

Information on demographic characteristics and risk factors were collected by using a structured questionnaire. Hypertension, alcohol use, diabetes and smoking were defined as reported previously [14].

Follow-up

All the patients were on antiplatelet therapy with aspirin. The recommended doses of aspirin were 75, 150 and 325 mg/day. The follow-up telephone interviews were conducted with patients at 3 months after hospital discharge. Bad outcome was defined as a score of more than 2 on modified rankin scale score (mRS) from stroke onset. The good clinical outcome at 90 days was defined as a score of <2 on mRS. The AR was defined as bad clinical outcome and recurrent stroke events and death in patients on aspirin treatment.

DNA isolation and genotyping

5 ml of blood was collected in EDTA tubes. Genomic DNA was extracted from Blood samples using standard phenol–chloroform method. The COX-2 G765C polymorphism was detected by PCR–RFLP technique [15]. The

amplified 157 bp PCR product was digested with *Bsh*1236I restriction enzyme (Fermentas Fast digest) by incubating at 37 °C for 5 min followed by separation of fragments on 3 % agarose gel. −765C allele was detected as fragment of 157 (bp) while as −765G allele was detected as fragments of 134 and 23 bp.

Statistical analysis

Hardy–Weinberg equilibrium was tested for COX 2 gene polymorphism. Association between genotypes and stroke was examined by odds ratio with 95 % confidence interval (CI) and χ^2 analysis using EPI 6 software (EPI info 6 CDC). Allelic frequencies were calculated according to the number of different alleles observed and the total number of alleles examined. Multiple logistic regression analysis with forward stepwise selection (Wald) was performed using SPSS18 software. The independent variables were decoded as the following dummy variables: genotype, 0 for normal homozygotes and 1 for heterozygotes homozygotes and 2 for homozygous mutant; sex, 1 for male and 2 for female; smoking, 1 for smokers and 2 for non-smokers; hypertension, 1 for hypertension and 2 for normotension; for diabetes mellitus, 1 for presence and 2 for absence, for outcome, 1 for good outcome and 2 for bad outcome and for dosage, 1 for 75 mg/day, 2 for 150 mg/day and 3 for 325 mg/day Statistical significance was defined as $p < 0.05$. The association between the genotypes and clinical outcome was examined by multiple logistic regression analysis.

Results

During the study period, 450 ischemic stroke patients and 440 controls were included in the study. All patients belonged to a South Indian population from A.P. The clinical characteristics of the stroke patients and controls have been presented in Table 1. Mean age was 49.3 years in the former group and 49.01 years in the later. Risk factor profile of the patients reveals hypertension in 56.6 %, diabetes in 44.2 %, smoking in 43.7 %, alcohol use in 31.7 % and family history of stroke in 26 % subjects. In the control group 29.6 % had hypertension, 28.2 % were diabetic, 31.9 % smokers, 25.5 % alcohol users and 12 % had family history of stroke. Stroke patients also had significantly higher levels of systolic and diastolic blood pressure, higher serum triglycerides and random glucose and a lower level of HDL ($p < 0.001$).

The genotypic distribution of COX-2, −765G/C polymorphism and allele frequency of G and C alleles in patients and controls have been given in Table 2. The frequency of GG, GC and CC genotypes in patients was 46.4, 43.3 and 10.2 %, respectively, while as in controls it was 55.6, 37.2 and 7.04 %, respectively. The frequency of C allele was 0.31 in patients and 0.25 in controls. Examining the association of COX-2 polymorphism with stroke subtypes classified according to TOAST classification, we found significant association with extracranial large artery atherosclerosis ($p = 0.038$) (Table S1).

During the follow-up period of 3 months 233 (51.7 %) patients had good outcome (score of <2 on mRS), 217 (48.2 %) had bad outcome (score of >2 on mRS) in Table 3. A stepwise multiple logistic regression analysis was carried out to evaluate the association of different genotypes with outcome after statistically adjusting for potential confounding effect of other covariates. The confounding factors were sex, hypertension, diabetes, smoking, alcoholism, aspirin dose, total cholesterol, HDL cholesterol and triglycerides. A significant association of

Table 1 Clinical characteristics of stroke patients and controls

	Patients ($n = 450$)	Controls ($n = 440$)	p value
Age	49.3 (17.34)	49.01 (16.78)	
Male:female	315:135	313:127	$p < 0.001$
Systolic BP (mmHg)	142 (17.2)	128 (16.2)	$p < 0.001$
Diastolic BP (mmHg)	88.4 (20.2)	79 (15.3)	$p < 0.001$
Total cholesterol (mg/dl)	197.45 (40.45)	195.36 (47.50)	$p > 0.05$
Triglycerides (mg/dl)	178.5 (40.02)	138.68 (43.3)	$p < 0.001$
Random glucose (mg/dl)	128.08 (7.2)	118.73 (23.28)	$p < 0.001$
HDL cholesterol (mg/dl)	53.25 (20.23)	59.56 (22.62)	$p < 0.001$
Hypertension	56.6 %	29.6 %	$p < 0.001$
Diabetes	44.2 %	28.2 %	$p < 0.001$
Smokers	43.7 %	31.9 %	$p = 0.001$
Alcohol use	31.7 %	25.5 %	$p < 0.001$
Family history of stroke	26 %	12 %	$p < 0.001$

Age, systolic BP, diastolic BP, total cholesterol, high density lipoprotein (*HDL*) cholesterol, random glucose and triglycerides are given as mean (SD).Stroke patients have significantly higher levels of systolic and diastolic blood pressure, higher serum triglycerides and random glucose and a lower level of HDL($p < 0.001$). p values were calculated using students' paired t test (SPSS 18)

Table 2 Distribution of COX 2 (−765G/C) genotypes frequencies of the study population

Study group	COX 2 genotypes (%)			
	GG	GC	CC	Total
Patients *n* (%)	209 (46.4)	195 (43.3)	46 (10.2)	450
Control *n* (%)	245 (55.6)	164 (37.2)	31 (7.04)	440

Table 3 Genotype wise distribution of good and bad outcome in study population

Genotypes	Disease outcome (%)	
	Good outcome ($n = 233$)	Bad outcome ($n = 217$)
GG	121 (51.9)	80 (36.8)
GC	102 (43.7)	101 (46.5)
CC	10 (4.2)	36 (16.5)

GC and CC genotypes with bad outcome [for GC genotype; $p = 0.02$; adjusted odds ratio = 1.745 (95 % CI; 1.059–2.875) and for CC genotype; $p = 0.016$; adjusted odds ratio = 3.157 (95 % CI; 1.241–8.033)] was observed. However, GG genotype did not show any significant association with bad outcome.

In subgroup analysis, in addition to genotype, hypertension [($p = 0.01$); adjusted odds ratio = 2.460 (95 % CI; 1.116–3.821) and total cholesterol [($p = 0.01$); adjusted odds ratio = 1.89 (95 % CI; 1.015–2.242)] also associated significantly with outcome. The patients were on three different doses (75, 150 or 325 mg/day) of aspirin. These doses were grouped into three dummy variables (1, 2 and 3) and the association of the dose with outcome was also evaluated by multiple logistic regression analysis after controlling all other confounding risk factors. However, we did not find a significant association of the aspirin dose with outcome. The death rate was high in stroke patients bearing CC genotype. Therefore, the carriers of −765C allele of COX-2 gene had a significantly bad outcome than non-carriers.

Discussion

The efficacy and safety of aspirin has been evaluated in patients covering the whole spectrum of atherothrombosis. These include apparently healthy low risk individuals to patients presenting with an acute myocardial infarction or an acute ischemic stroke. However, antiplatelet effects of aspirin may not be equal in all individuals. Previous studies have shown that adequate antiplatelet effects are not achieved in 5–45 % of patients [16] taking aspirin suggesting that many patients are resistant to aspirin. Since there is no single definition, or validated method to define AR, its reported prevalence varies greatly. In one study it has been reported as high as 60 % [17]. In the present study 48.2 % of the patients were found to be aspirin resistant or more appropriately presented "clinical treatment failure" [18]. A study investigating the AR in Indian patients with coronary artery disease reported 41.66 % patients showing inadequate response to aspirin [19]. AR is multifactorial in origin A genetic etiology to AR has also been suggested. A largest and most comprehensive review by Goodman et al. [20] analyzed thirty-one candidate gene studies of AR. 50 polymorphisms in 11 genes were identified. These included COX-1 and COX-2 genes. In COX-2 gene a functional G/C polymorphism 765 bp upstream from transcription site has been found to be related to AR. This polymorphism of COX-2 has also been associated with an increased risk of stroke in African-Americans [21]. Colaizzo et al. [22] also found an association between rs20417 of COX-2 gene and cerebrovascular ischemia. However, in contrast, Hegener et al. [23] found no evidence for association of the COX-2 genetic variants neither with risk of incident MI or ischemic stroke.

A study by Huuskonen et al. [24] examining the association of COX-2 765G/C polymorphism with the risk of severity of atherosclerosis at the coronary artery level revealed that Finnish men possessing C allele with sudden death had more often advanced coronary plaques, characterized by more extensive areas of complicated lesions. We examined the association of COX-2 gene polymorphism with AR by evaluating the post stroke functional outcome as measured by well-validated stroke scales by multiple logistic regression analysis by controlling all other confounding risk factors. The demographic features of the patients were compared with controls. The controls selected for the study were blood donors from the study hospital. The blood donors were healthy volunteers. They belonged to the same demographic area and it was easy to collect large number of samples from these volunteers. To the best of our knowledge this is the first study from India to examine pharmacogenetics of AR. We found that the carriers of −765C allele of COX-2 gene had a significantly bad outcome in stroke patients on aspirin treatment in comparison with the non-carriers. However, dose of aspirin did not show any significant association with outcome. In subgroup analysis we found a significant association of hypertension and total cholesterol with outcome controlling all other confounding risk factors. These results indicate that the outcome of CC genotype bearers having hypertension and high cholesterol levels is expected be very bad. The analysis of the small subgroups of study participants helps to extract as much information as possible. However, subgroup analysis can lead to overstated and misleading results because it introduces analytical challenges.

A recent study has found a significant association between COX-2 variants (rs20417 and rs5275) and functional outcome at 90 days [25]. They reported these COX-2 variants contributing to a more favourable stroke outcome. On the contrary we found the COX-2 rs20417 contributing to an unfavourable stroke outcome in patients on antiplatelet treatment. However, Maguire et al. [25], have not

discussed about aspirin treatment in these patients. Larger and more robust studies are needed to truly understand whether a genetic aetiology can partly explain the phenomenon of AR. As suggested by Goodman et al. [20] that in order to truly understand the genetic contribution to AR the researchers need to agree on a standard technique to measure and define AR.

In conclusion our results suggest C allele of COX-2 gene associated significantly with AR in stroke patients.

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