

Genetic etiology of coronary artery disease considering NOS 3 gene variant rs1799983

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Abstract

Reduced production of nitric oxide due to rs1799983 single nucleotide polymorphism in nitric oxide synthase 3 gene (NOS3) may enhance the risk of coronary artery disease. The association of rs1799983 polymorphism with coronary artery disease was investigated in the local population of Pakistan. Study consisted of 376 individuals, out of which 198 were coronary artery disease patients and 178 were normal healthy individuals. Allele-specific polymerase chain reaction (PCR) based strategy was used for the detection of different genotypes of rs1799983 polymorphism. PCR amplification results were obtained for 354 samples. Frequency of T allele was higher as compared to G allele in our population. Strong association between rs1799983 and coronary artery disease was observed ($p < 0.01$). TT genotype was found to enhance 5.717 times the risk of coronary artery disease (odds ratio (OR): 5.717; 95% confidence interval (95% CI) 3.586–9.115). On the basis of present results, it can be concluded that rs1799983 is strongly associated with coronary artery disease in our population and TT genotype of this polymorphism enhanced the risk of coronary artery disease in Pakistani population.

Keywords

Coronary artery disease, Pakistan, rs1799983, smoking, nitric oxide synthase 3, single-nucleotide polymorphism

Introduction

The most common form of cardiovascular disorders is coronary artery disease (CAD). It is considered as serious trouble resulting in high morbidity and mortality rate. According to World Health Organization (WHO, 2011), casualties as a result of CAD reached 196,258 (15.36% of all casualties in Pakistan).¹ Pakistan is at 17th number among the world on the basis of death rate due to CAD. Classical risk factors of CAD include hyperglycemia, high blood pressure, smoking, and alcohol consumption. Mental stress and sedentary life style, which account for more than 90% of the cardiovascular risk, are also considered as classical risk factors.² The formation of atherosclerotic plaques is the major reason of CAD. Several molecular pathways are involved in their formation. Genetic polymorphisms affecting these pathways may predispose some individuals toward the CAD.

Considerable efforts have been made for the understanding of genetic etiology of CAD. Numerous genetic and environmental factors and their interactions have been reported to be involved in its causation. For the

identification of susceptibility genes related to CAD, case-control association studies were used frequently.³ This method involves the selection of candidate gene on the basis of its possible contribution in disease development. Single-nucleotide polymorphisms (SNPs) are identified in the selected gene. These SNPs are then genotyped in patients and healthy controls. Allele frequencies of SNPs are evaluated. Association with the disease is prescribed if allele frequencies were significantly different among cases and controls.³ The results should be interpreted carefully because most of the studies are based on biased selection of controls and cases.⁴ Factors like admixtures of population, mismatched cases and controls, errors in phenotyping, and small sample size also affect the validity of findings.⁴ Many case-control base association studies have

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been reported in literature and provide evidence for genetic variance as a cause for CAD susceptibility.^{5,6} True association of genetic polymorphisms with CAD in different populations is debatable due to inconsistent results.^{5,6}

Nitric oxide synthase (NOS) enzyme produces nitric oxide (NO) from L-arginine. There are two functional classes of NOS, inducible class of NOS and constitutive class of NOS. Constitutive class of NOS includes endothelial-NOS (eNOS—also known as NOS3) and neuronal-NOS. The eNOS protein is involved in the synthesis of NO by converting L-arginine to L-citrulline.⁷ NO is involved in vasodilation and inhibiting the adhesion of leukocyte and platelets with vascular endothelium. It also decreases the proliferation of smooth cells and their migration. In this way, it imparts vasoprotective effect by scavenging superoxide radicals. These functions indicate the role of NO in reducing the chances of atherosclerosis.⁸ Gene for NOS3 or eNOS is located at locus 7q35–36. It spans 4.4 kb of the genomic DNA, comprising 26 exons and encoding a 135-kD protein which contains 1203 amino acids.⁹ NOS3 polymorphism rs1799983 at exon 7 involves G→T substitution at nucleotide position 894 (G894T). This conversion causes an amino acid substitution of glutamic acid for aspartic acid in amino acid residue 298 (Glu298Asp). The alteration in structure of this variant affects the NOS3 susceptibility to cleavage and lowers the activity of this enzyme. Polymorphisms in NOS3 affect the levels of NO. This change is conservative as these two amino acids are similar. Decline in NO as a result of Glu298Asp polymorphism can accelerate atherosclerosis.¹⁰

SNP rs1799983 in NOS3 gene plays pivotal role in the development of CAD in various populations.^{11–14} Meager information exists about the role of this polymorphism in modulating the chances of CAD in Pakistani population. Present study targets to find the allele frequency in rs1799983 polymorphism in NOS3 in the local population of Pakistan and investigates the association between rs1799983 and CAD. Controversial reports are available regarding association of rs1799983 polymorphism with CAD in different populations and no such information is available about local population, therefore the study is focused on this association.

Methods

Population studied

All procedures were in compliance with the declaration of Helsinki. The Advance Research and Study Board, University of Sargodha approved the protocol of present study. Approval of Ethical Committee, University

of Sargodha was also obtained before the start of research work. The subjects were divided into two groups on the basis of disease presence. One group was consisted of CAD patients (CAD group) and the other was comprised of age-matched healthy controls (normal). For each sample, data for age, smoking habit, diabetes, and hypertension were collected. Study consisted of 376 subjects out of which 198 samples were CAD patients and 178 were healthy persons.

Sample collection

Samples were collected from July 2012 to December 2012. After taking proper consent and completion of ethical criteria, CAD patients (confirmed angiographically) and controls (healthy individuals) were selected for the present study. All blood samples were taken between 8 and 11 am. To draw blood sample cubital vein was punctured by using sterilized syringe (BD, USA). Collected blood samples were stored in ethylenediaminetetraacetic acid (EDTA) coated vials (BD, USA) as whole blood for genetic analysis. For long-term storage, samples were kept stored at -20°C until further analysis. Information of diabetes and hypertension were obtained from the hospitals from where samples were collected. Subjects were considered as smoker if they were smoking at the time of sample collection with smoking history of 5 years. Hypertensive individuals were defined on the basis of systolic and diastolic blood pressure. Hypertensive individuals were having systolic blood pressure values of more than 140 mmHg and their diastolic blood pressure values were more than 90 mmHg. Persons were considered as diabetic on the basis of their blood sugar levels. This information was also provided by the hospitals. If blood sugar level was higher than 120 mg/dl (fasting), individuals were considered as diabetic.

Genetic analysis

Genetic study was based on the SNP (rs1799983) detection in NOS3 gene using allele-specific PCR-based strategy. Genomic DNA was isolated by using GF-1 blood DNA extraction kit (Vivantis Cat. No. GF-BD-100 USA) as per manufacturer's protocol. Genomic DNA and PCR products were detected by using agarose gel electrophoresis. Allele-specific PCR reactions were carried out in XP Thermal cycler (BIOER TECHNOLOGY CO., LTD., TC-XP-G, China). Primers used in the study were synthesized by Invitrogen, USA through local representative. Two forward primers, Forward1 (F1) 5'CTGCTGCA GGCCCCAGATGAG3 and Forward2 (F2) 5'CTG CTGCAGGCCCCAGATGAT3 and one reverse primer 5'CGCACCTCAC CACCAGGATGT3, were used. For each sample, two PCR reactions (one for

each allele) were performed. PCR Master Mix (Invitrogen #12532-016) was used for amplification. Annealing temperature was 56.5°C for both alleles. PCR product of 372 bp was detected using UV transilluminator. In the presence of GG homozygotes, bands appeared with F1 primer; in TT homozygotes bands appeared with F2 primer; and in case of GT heterozygotes, bands appeared with both F1 and F2 primers.

Statistical analysis

For the analysis of Hardy–Weinberg equilibrium (HWE), chi square test was used. Genetic frequencies, allelic frequencies, and difference in genotypic and allelic frequencies among different groups were also examined by chi square analysis. Chi square test and other nonparametric tests were applied by SPSS® Software version 12 for windows (SPSS Inc., Chicago Illinois, USA 1989–2003). Odds ratio was calculated using an online calculator.¹⁵

Results

Table 1 describes the baseline characteristics of samples of the study along with analysis of the groups for age, gender, smoking habit, presence of diabetes, and hypertension. It was noticed that the two groups were significantly different on the basis of hypertension ($p < 0.01$), diabetes ($p < 0.05$), and smoking habit ($p < 0.01$).

Table 2 describes the genotype and allele frequencies and results for HWE estimation. The present data show that G allele frequency was higher in controls as compared to that of T allele. When the same data were analyzed without stratification on the basis of disease presence, frequency of T allele was found to be higher than that of G allele. The HWE estimation indicates that allele frequencies were deviant from HWE in normal individuals and in CAD patients.

Table 3 presents the association of smoking habit with CAD without adjustment of data (for age and gender of the participants). The data suggested the

strong association of CAD with the smoking behavior ($p < 0.01$). Analysis with odds ratio revealed that smoking habit increased 1.867 times the risk of CAD development (OR: 1.863; 95% confidence interval (95% CI), 1.220–2.846). After the adjustment of data (for age and gender of the participants of the study), the results remained unchanged. A strong association was present between the smoking habit and CAD ($p < 0.05$). Odds ratio estimation presents an increase of 1.782 times in the risk of CAD with the smoking habit (OR: 1.782; 95% CI, 1.129–2.813). Table 3 further presents the significant association of diabetes with CAD ($p < 0.05$) without adjusted data (for age and gender of the participants). Results suggested 1.675 times the increase in risk of CAD development due to diabetes (OR: 1.675; 95% CI, 1.083–2.590) whereas the adjusted data did not show any association between CAD and diabetes ($p < 0.05$). Results for odds ratio estimation were slightly changed. It was found that risk of CAD was at margin in diabetic patients (OR: 1.564; 95% CI, 0.970–2.522). Table further indicates the strong association of hypertension with CAD ($p < 0.01$), using both adjusted and unadjusted data (for age and gender of the participants). Presence of hypertension increased 2.733 times the risk of CAD development (OR: 2.738; 95% CI, 1.793–4.183). Hypertension was found to have an

Table 2. Genotype and allele frequencies.

Allele	Normal (N = 172)	CAD (N = 182)	Total (N = 354)
GG	109 (63.37%)	32 (17.58%)	141 (39.83%)
TT	39 (22.68%)	114 (62.64%)	153 (43.23%)
GT	24 (13.95%)	36 (19.78%)	60 (16.94%)
G	0.7	0.27	0.48
T	0.3	0.73	0.52
HWE (p)	76.18 (0.000)	46.16 (0.000)	154 (0.000)

HWE: Hardy–Weinberg equilibrium; p: statistical p-value; <: value less than.

Table 1. Baseline characteristics.

Characteristics	CAD patients (N = 198)	Normal (N = 178)	Total (N = 376)	p-value
Age (years) ^a	54.47 ± 10.343	55.25 ± 10.521	54.84 ± 10.421	0.957
Gender, male (%age) ^b	140(70.70%)	121(67.97%)	261(69.41%)	0.566
Smokers, yes (%age) ^b	90(45.45%)	55(30.89%)	145(38.56%)	0.003*
Diabetic, yes (%age) ^b	77(38.88%)	49(27.52%)	126(33.51%)	0.019 ^ψ
Hypertensive, yes (%age) ^b	109(55.05%)	55(30.89%)	164(43.61%)	0.000*

^aData are shown as mean ± standard deviation. Student's t-test was used for comparison of groups of CAD and normal.

^bData are shown as percentage. Chi square test of the difference between the two groups (CAD and normal) defined in terms of disease presence.

* $p < 0.01$, ^ψ $p < 0.05$.

Table 3. Effect of different factors on CAD occurrence.

Factors	Analysis without adjustment of data for age and gender				Analysis with adjustment of data for age and gender				
	CAD (N = 198)	Control (N = 178)	OR (95% CI)	Chi square (p-value)	CAD (N = 171)	Control (N = 150)	OR (95% CI)	Chi square (p-value)	
Smoking	Yes	90	55	1.863 (1.220–2.846)	8.382 (0.000)	78	48	1.782 (1.129–2.813)	6.211 (0.012)
	No	108	123			93	102		
Diabetes	Yes	77	49	1.675 (1.083–2.590)	5.430 (0.019)	62	40	1.564 (0.970–2.522)	3.39 (0.065)
	No	121	129			109	110		
Hypertension	Yes	109	55	2.738 (1.793–4.183)	22.232 (0.000)	91	46	2.572 (1.625–4.070)	16.609 (0.000)
	No	89	123			80	104		

OR: odds ratio; 95% CI: 95% confidence interval.

Table 4. Association of genotype with CAD.

Groups	GG (N = 141)	TT (N = 153)	GT (N = 60)
CAD	32	114	36
Control	109	39	24
OR	0.1233	5.7172	1.5205
(95% CI)	(0.075–0.201)	(3.586–9.115)	(0.864–2.675)
Chi square (p-value)	80.996 (0.000)		

OR: odds ratio; 95% CI: 95% confidence interval.

increased risk of 2.572 times for CAD (OR: 2.572; 95% CI, 1.625–4.070).

Table 4 describes the results of association between the polymorphism and the CAD. Association is estimated in terms of Chi square test and odds ratio with 95% CI. The analysis indicates a strong association between the rs1799983 polymorphism and CAD ($p < 0.01$). GG genotype was found to show the protective effects against the disease development. It lowers the risk of CAD for 0.123 times (OR: 0.123; 95% CI, 0.075–0.201). TT genotype shows 5.717 times the increased risk of CAD development (OR: 5.717; 95% CI, 3.586–9.115). GT has mild association with the CAD (OR: 1.520; 95% CI, 0.864–2.674). In conclusion, GT was found to have no association with CAD.

Discussion

CAD being a multifactorial disease is affected by various risk factors including traditional and genetic factors. Individuals exposed to traditional risk factors always do not develop CAD. There may be some other potential factors playing their role. Genetics may help in unveiling the mystery of the reasons attributed in modulation of risks of CAD.¹⁶

NO derived from endothelium acts as a relaxing factor for endothelial walls. Its production is regulated by the enzyme NOS3. Different variants have been identified in the NOS3 gene. rs1799983 polymorphism in exon 7 results in the substitution of guanine by thymine at 894 position. This substitution results in the change of amino acid glutamic acid to aspartic acid at position 298.¹⁷ rs1799983 polymorphism of NOS3 gene has extensively been studied as a risk factor for CAD. In the presence of T allele, reduced amount of NO is produced. NO being vasoprotective maintains the vascular tone. In the presence of reduced NO, endothelium gets damaged which enhances the risk of development of atherosclerosis and ultimately results in CAD.^{7,8}

CAD results due to the increased buildup of atherosclerotic plaques. CAD found to appear in the late 50s of men and late 60s of women.¹⁸ In the present study, the mean age with standard deviation of CAD subjects was 54.47 ± 10.343 . In old age, endothelial functions get disturbed and vascular tone is not maintained. Present study also considers old age as a risk for CAD.

Smoking as key factor of CAD imparts serious damage to the endothelium. Smoking results in vasoconstriction, aggregation of platelets, and impaired cholesterol metabolism. Endothelium dysfunction has been reported in both active and passive smokers. Smoking enhances the oxidative stress and spoils the functional endothelium.¹⁹ Yathish et al. reported the injurious effects of smoking on the development of CAD.²⁰ Smokers have more than 80% chances of developing CAD. Result of present study depicts strong association of smoking with CAD. Results considering smoking as a risk factor were in accordance with several studies.^{21–23} However, smoking is not significantly associated with CAD in North Indians.¹⁶

Among the traditional risk factors for CAD, diabetes shows characteristic association with CAD. Diabetic individuals are at three- to four fold increased

risk of CAD as compared to nondiabetic individuals.^{24–26} Incidence of death due to presence of CAD in diabetic individuals was found to be equal when compared with nondiabetic individuals having myocardial infarction.^{24–26} The present study suggests that diabetic individuals were at marginal risk of CAD. These findings did not show clear view of the facts and suggest a detailed investigation with larger sample size. Diabetes being a risk factor does not impart strong association with CAD in our population. However, diabetes is considered as risk factor in the Egyptian, Turkish, Indian, and Taiwanese population.^{22,23,27,28}

Hypertension has been reported to have association with CAD. Increased blood pressure stimulates the impairments in functions of endothelium. Hypertension along the endothelial dysfunction worsens the process of atherosclerosis.²⁹ Present study noticed the hypertension to have strong association with the CAD. Cam et al. observed hypertension as an independent risk of CAD in Turkish population.²⁷ Similar results were observed in Egyptians.²² Tripathi et al. suggest hypertension as an important predictor of CAD in Indian population.²³ Cai et al. found the association of hypertension and CAD in Taiwanese population.²⁸ Leili et al. also found hypertension as an independent risk factor for CAD in Iranian population.¹²

Several studies have investigated the role of this SNP in etiology of cardiovascular diseases in different populations. Present study focused on this SNP for analysis of its association with CAD in local population of Pakistan. The results for allele frequency suggest that T allele frequency was higher in the population under observation. A strong association was observed between CAD and rs1799983. Present data is in accordance with previous finding from different populations.^{12–14,21,22,27,30–36} Inharmonious results were also reported in different populations.^{11,23,24,37–44}

Results in contradiction can be explained in terms of ethnic variations among different populations. Our results match with East Asian and non-East Asian population.¹⁴ Results are also in accordance with findings of Tian et al. in Asians.¹³ However, finding of Bhanushali and Das in Asian Indians does not consider rs1799983 as a risk for CAD.³⁹ Pakistanis being part of Indo-Pak subcontinent were thought to have strong genetic association with Indians. In Indian region, two contradictory results have been reported in two different local populations. In North Indians, rs1799983 does not depict any association with the CAD. While in Tamilian Indian population (South India) strong association of this polymorphism and CAD was observed. rs1799983 TT genotype is considered as a risk factor for CAD in South Indians.

These findings further strengthen the fact that geo-ethnic variations exist among different populations. Results of rs1799983 polymorphism were in accordance with two different studies 2010 and 2012 in North Indians.^{23,24} Both studies do not consider this polymorphism as a risk of CAD. Similarly three different studies in Turkish population also does not consider this SNP as risk of CAD.^{38,39} Two different studies in Iranian population consider rs1799983 as a risk factor of CAD. Similarly two meta-analysis studies in Asian populations also consider it as a risk of CAD. Up to our best knowledge this is the first study in Pakistani population which considers rs1799983 as a risk for CAD. Further studies considering large population size are thought to be useful in considering rs1799983 as a risk of CAD.

The limitations of the present study may be described in terms of small sample size. Lack of detailed information of smoking habits, use of single technique for SNP detection, and consideration of only one SNP also make the study restricted. Nevertheless, the present findings shall be useful for future investigations considering these weak points.

Conclusion

Frequencies of GG, TT, and GT genotypes differ between CAD and normal individuals. TT genotype of rs1799983 may enhance the risk of CAD development. On basis of these results, it can be concluded that rs1799983 polymorphism of NOS3 gene is associated with CAD in population under observation. Smoking and hypertension also increase the chances of CAD in local population of Pakistan. Generally speaking, the smoking habit, hypertension, and genotype of individuals decide the onset of CAD in local population of Pakistan.

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Conflict of interest

None declared.

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