fortune Journals

Research Article

FORTUNE JOURNAL OF HEALTH SCIENCES

ISSN: 2644-2906

Inducible Nitric Oxide Synthase Gene Polymorphism and Markers of Pro-inflammatory Endothelial Dysfunction in Coronary Artery Disease: Case Control Study in North Indian Population

Shilpa Bhardwaj[1], Richa Dixit[2*], Jayashree Bhattacharjee[3], MK Bhatnagar[4], Sanjay Tyagi[5]

Abstract

Coronary Artery Disease (CAD) is a major public health issue globally and is the leading cause of death worldwide (1). According to the World Health Organization (WHO), cardiovascular diseases (CVDs), particularly CAD, are responsible for an estimated 17.9 million deaths each year, which accounts for 31% of all global deaths. The WHO projects that by 2030, nearly 23.6 million people will die annually from cardiovascular diseases. The WHO reports that India is experiencing a surge in the number of CAD cases, making it a significant public health concern (2). The age-adjusted mortality rate from CAD in India is among the highest in the world (3). According to recent statistics, the prevalence of CAD in urban India is estimated to be between 7-13%, while in rural areas, it is between 2-7%. [4].

The etiology of CAD is multifactorial and the various environmental, lifestyle, and genetic factors interact to affect the evolution and progression of the disease. Apart from traditional risk factors, chronic "low grade" inflammation mediated by macrophage and lymphocytes, has been implicated in the pathogenesis of atherosclerosis and cardiovascular events [5]. Inflammation has become one of the central themes in the pathogenesis of heart disease, over the past decade along with endothelial dysfunction, representing a key early step in the development of atherosclerosis and is also involved in plaque progression and the occurrence of atherosclerotic complications.

Keywords: Coronary Artery Disease (CAD), World Health Organization (WHO), cardiovascular diseases (CVDs)

Introduction

Coronary Artery Disease (CAD) is a major public health issue globally and is the leading cause of death worldwide (1). According to the World Health Organization (WHO), cardiovascular diseases (CVDs), particularly CAD, are responsible for an estimated 17.9 million deaths each year, which accounts for 31% of all global deaths. The WHO projects that by 2030, nearly 23.6 million people will die annually from cardiovascular diseases. The WHO reports that India is experiencing a surge in the number of CAD cases, making it a significant public health concern (2). The age-adjusted mortality rate from CAD in India is among the highest in the world (3). According to recent statistics, the prevalence of CAD in urban India is estimated to be between 7-13%, while in rural areas, it is between 2-7%. [4].

The etiology of CAD is multifactorial and the various environmental,

Affiliation:

[1]Associate Professor, Department of Neurochemistry, Institute of Human Behaviour & Allied Sciences Hospital, Delhi, India-110093

[2]Tutor, Department of Biochemistry, Army College of Medical Sciences, Delhi, India- 110010

[3](ex-HOD), Department of Biochemistry, LHMC), Delhi, India- 110001

[4](ex-HOD), Department of Medicine, LHMC), Delhi, India- 110001

[5](ex-HOD), Department of Cardiology, GB Pant Hospital), Delhi, India-110002

***Corresponding author:**
Richa Dixit, Tutor, Department of Biochemistry, Army College of Medical Sciences, Delhi, India-110010.

Citation: Shilpa Bhardwaj, Richa Dixit, Jayashree Bhattacharjee, MK Bhatnagar, Sanjay Tyagi. Inducible Nitric Oxide Synthase Gene Polymorphism and Markers of Pro-inflammatory Endothelial Dysfunction in Coronary Artery Disease: Case Control Study in North Indian Population. Fortune Journal of Health Sciences. 7 (2024): 307-312.

Received: May 28, 2024
Accepted: June 04, 2024
Published: June 11, 2024

Bhardwaj S, et al., Fortune J Health Sci 2024
DOI:10.26502/fjhs.190

lifestyle, and genetic factors interact to affect the evolution and progression of the disease. Apart from traditional risk factors, chronic "low grade" inflammation mediated by macrophage and lymphocytes, has been implicated in the pathogenesis of atherosclerosis and cardiovascular events [5]. Inflammation has become one of the central themes in the pathogenesis of heart disease, over the past decade along with endothelial dysfunction, representing a key early step in the development of atherosclerosis and is also involved in plaque progression and the occurrence of atherosclerotic complications.

Angiographic evidence of coronary atherosclerosis proves the presence of Endothelial dysfunction and it appears to precede manifest atherosclerotic lesion development. Endothelial dysfunction is characterized by a reduction of the bioavailability of vasodilators, in particular, Nitric oxide (NO), whereas endothelium-derived contracting factors like Endothelin-1(ET-1) are increased. The endothelium has an important role in vascular homeostasis. The development of an imbalance in the release of vasoconstrictor and vasodilator agents from the endothelium results in impaired endothelium-dependent vasodilatation, the hallmark of endothelial dysfunction. NO is produced in the cells from the amino acid L-arginine by the enzymatic action of nitric oxide synthase (NOS). Emerging evidence suggests that coronary artery disease (CAD) is related to defects in the generation or action of NO. It inhibits both platelet adhesions to endothelial cells and platelet aggregation along with reduction of monocyte adhesion to the endothelium and expression of monocyte chemo-attractant protein-1 (MCP-1), which has been implicated in early recruitment during atherogenesis [6, 7]. NO reduces the activation of NF-kB and the induction of the leukocyte (vascular cell) adhesion molecule (VCAM) by cytokines and by oxidized LDL in endothelial cells [8]. The inflammatory etiology of atherosclerosis has led to research into more pro-inflammatory cytokines like IL-2 and IL-6. Cytokines have been studied as potential markers for the prediction of future cardiovascular events. Another widely studied marker of cardiovascular risk is highly sensitive C-reactive protein (hsCRP), which is the only inflammatory marker recommended for clinical application [9-12]. The pro-inflammatory cytokine, interleukin-6 (IL-6), secreted by macrophages, is largely responsible for hsCRP production by the liver and also is an independent risk factor for future myocardial infarction [13,14] and is elevated in patients with CAD [15,16]. Additional investigators have reported the role of IL-2 as a potential marker for predicting cardiovascular events [17, 18].

Genetic polymorphisms are being widely implicated in predisposition to CAD. One of the polymorphisms in exonic region C150T of the iNOS gene is being investigated for its role in NO production. The inducible (or inflammatory) NOS (iNOS) is involved in the inflammatory reactions following infection, disease, or tissue damage. The iNOS gene expression is under the transcriptional control of several inflammatory mediators including nuclear factor (NF) kB, IFN-g, NF-1, and IL-6 [19]. Out of the several polymorphisms studied in the iNOS gene, C150T in exon 16 (E16) was found to be the most frequent. Studies have indicated that even a single amino acid change may have dramatic effects on enzymatic activity [20]. With this background, a preliminary pilot study was conducted to explore the effect of C150T polymorphism in exon 16 of the iNOS gene on the levels of nitric oxide in CAD. After analyzing the results of our pilot work, we designed a larger study considering iNOS gene exonic region (C150T) polymorphism about inflammatory (IL-2, IL-6, and hs-CRP) and endothelial dysfunction markers (Nitric oxide and Endothelin-1) in CAD. We hypothesize the role of iNOS gene polymorphism along with endothelial dysfunction and inflammation in the pathogenesis of CAD in the Indian population.

Materials and Methods

A preliminary cross-sectional hospital-based case-control study was carried out in tertiary care & super speciality hospital in Delhi. We enrolled 120 subjects to study iNOS gene (C150T) polymorphism in exon 16 using PCR-RFLP, along with endothelial dysfunction markers in our pilot study [21]. Based on our findings from the previous study, we designed this project recruiting 300 subjects for studying markers of endothelial dysfunction, pro-inflammatory markers, and genetic study for the C150T polymorphism at exon 16 of the iNOS gene in CAD. Cases (n=150) were selected from angiographically proven patients of CAD attending the cardiac clinic of and 150 age and sex-matched healthy volunteers without clinical or ECG evidence of CAD and without any family history of CAD formed the control group All subjects were enrolled after taking informed written consent. The study protocol was in agreement with the guidelines of the institutional ethics committee. None of the subjects had signs of acute or chronic inflammation and did not undergo any recent surgery. A detail of clinical and anthropometric characteristics was recorded in a performance for all the participants. Ten ml of fasting venous sample was collected into plastic tubes containing EDTA under sterile conditions and processed immediately for separation of plasma. For ET-1, IL-6, IL-2, and hs-CRP estimation, the samples were aliquot and stored at -40ºC. NO levels were estimated on the same day. The remaining cell aggregate (Buffy-coat) was stored at -70ºC till analyzed for PCR-RFLP. Repeated freeze-thaw cycles were avoided. Samples were processed without knowledge of their case-control status.

Analytical Procedure

Estimation of nitric oxide in plasma was done indirectly by measurement of stable decomposition products nitrate and nitrite by employing the modified Griess reaction [22].

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Cytokines (IL-2, IL-6, and hs-CRP) and endothelin (ET-1) levels were estimated by Enzyme-Linked Immuno-Sorbent Assay (ELISA) using standard kits (DIACLONE Research, France) according to manufacturer's instructions.

iNOS gene C150T polymorphism on exon 16 was studied by extracting DNA from whole blood and performing PCR-RFLP. The details of the procedure have been published in our previous work [21].

Statistical Analysis

Statistical analysis was done using the SPSS statistical package, version 18.0 (SPSS Inc., Chicago, IL, USA). Data are expressed as mean ± SEM (standard error of the mean). The difference between groups was compared by student t-test. One-way ANOVA was used to compare mean values between the study groups. The frequency of alleles and genotypes were compared between the study and the control group by Chi-square test with values predicted by Hardy-Weinberg equilibrium model. Pearson's correlation was used to analyze the relationship between NO and inflammatory cytokines in the study and control group. Two-tailed p-value <0.05 was considered statistically significant.

Results

In the present study, 150 diagnosed patients of CAD were included in the case group with a mean age of 54 years (67% males and 33% females). An equal number of age & sex-matched control subjects were selected.

Endothelial Dysfunction & Inflammatory markers

The endothelin-1 levels in plasma were significantly higher in the case group whereas nitric oxide levels were found to be significantly lower in the case group as compared to the control group ($p<0.001$). A statistically significant increase in the levels of pro-inflammatory cytokines (IL-2, IL-6, and hsCRP) was found in the case group as compared to the control group ($p<0.001$) (Table 1).

Genotypes and Alleles frequencies of C150T iNOS gene (PCR-RFLP). (table 2)

CC genotype was found in 70 % of cases and 75.3% of controls, whereas CT genotype was found in 30% of cases and 25% of controls. No TT was found in any group (figure 1). The genotype distribution was in Hardy Weinberg equilibrium ($c2=0.467$, $df=1$, $p=0.652$). The frequency of the C allele was 76.7% in the case group and 92.7% in the control group while the frequency of the T allele was 23.3% in the case group and 7.3% in the control groups. Significant differences were found between allelic distributions among the two groups.

Inter-genotypic levels of plasma Nitric oxide among study groups

A significant inter-genotypic variation of NO among and between both cases as well as the control group was found. The mean plasma NO levels in CC and CT genotypes were found to be higher in controls as compared to cases. Within the case group, NO levels were found to be significantly elevated in CT genotypes as compared to their CC genotype group (Table 3).

Table 1: Biochemical profile in study population

PARAMETERS	CASES	CONTROL	p-VALUE
ENDOTHELIN (PG/ML)	29.1 ± 2.08	12.5 ± 1.21	< 0.001
NITRIC OXIDE (MM/L)	13.5 ± 1.67	24.8 ± 1.45	< 0.001
il-2 (pg/ml)	541.5 ± 5.1	125.4 ± 4.01	<0.001
il-6 (pg/ml)	42.2 ± 4.3	4.1 ± 0.6	<0.001
hsCRP (mg/l)	11.4 ± 0.24	0.6 ± 0.5	<0.001

p-value <0.05 is statistically significant, Data is expressed as Mean ±S.E.M.

Table 2: Distribution of Genotype and Allele frequencies of iNOS gene C150T (PCR-RFLP)

Genotypes and Alleles	Case Group (N=150)	Control Group (N=150)
Genotypes		
CC	105 (70%)	113 (75.3%)
CT	45 (30%)	37 (24.6%)
TT	0	0
Alleles		
C	-76.70%	-92.70%
T	-23.30%	-7.30%

$\chi^2=0.487$, df =1 p=0.652

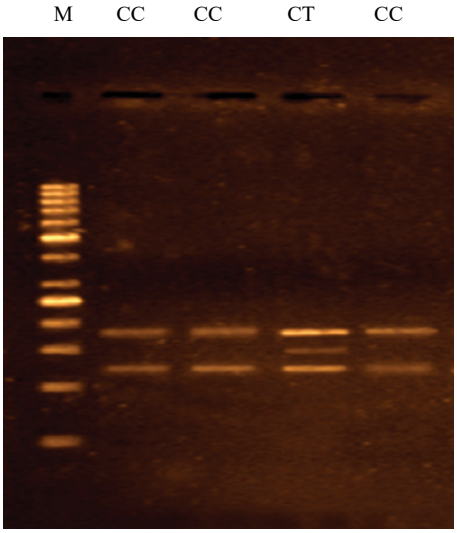

Figure 1: Electrophoretic separation of the iNOS (exonic region, C150T) gene detected by Tsp509I restriction endonuclease digestion of the 288 bp PCR product. Lane 1 shows ladder (M) and Lane 2, 3, 5 shows the CC, (wild type) genotype, and Lane- 4 -shows the CT (heterozygous mutated type) genotype in subjects. The CC and CT were represented by 175, 113 & 288, 175, and 113 bp respectively.

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Table 3: Intergenotypic variation of plasma nitric oxide level in study groups

Genotype	Nitric Oxide (Mm/L)		P-Value
	Study Group	Control Group	
CC	9.21 ± 0.12	25.7 ± 4.1	<0.001
CT	13.4 ± 0.02	21.5 ± 2.2	<0.001
CC+CT	11.30 ± 0.07	23.7 ± 3.12	<0.001

p-value <0.05 is statistically significant, Data is expressed as Mean ±S.E.

Discussion

Coronary artery disease has a multifactorial etiology. The mean plasma levels of IL-2 in our study were significantly different between the two groups (cases and controls). IL-2 acts as the primary growth factor for Treg cells. Even though IL-2 is expressed in atherosclerotic plaques, its direct role in atherogenesis has not been studied. In our study, the levels were found to be higher in the case group than controls.

In our study, the mean plasma levels of IL-6 were significantly higher in the case group as compared to controls. IL-6 is a key pro-inflammatory and immune-stimulatory cytokine that activates acute-phase proteins. It enhances atherosclerotic lesion development and induces endothelial dysfunction. Gotsman et al. reported that more overt coronary occlusion correlates with increased production of IL-6 in patients with coronary artery disease [23]. IL-6 is independently predictive of the cardiovascular risk [24]. In patients with CAD, the coexistence of peripheral artery disease was associated with a greater inflammatory status evident by increased levels of IL-6 and hsCRP.

It has been consistently demonstrated that hs-CRP is a marker of adverse events in patients with stable CAD. However, there is disagreement concerning hsCRP levels and severity of CAD as assessed by invasive or computed tomography angiography, with some studies supporting [25-27] and others denying an association [28-30]. In our study, the mean plasma hs-CRP levels were significantly higher in cases as compared to controls. ET-1, the most potent vasoconstrictor known, was found to be significantly elevated in patients of IHD as compared to controls suggestive of enhanced vascular tone in CAD. The results were similar to the observations of other investigators [31, 32]. Moreover, it has been demonstrated that circulating and tissue endothelin immunoreactivity correlates with the severity of human atherosclerotic disease [33]. Taken together, these findings strongly suggest a role for ET-1 in the evolution and progression of coronary atherosclerosis in humans.

The iNOS gene is located at chromosome 17. In contrast to the constitutively active isoforms, iNOS exerts its functions independent of Ca2+. The C150T (Ser608Leu) polymorphism is associated with diabetes mellitus and its complications [34]

Considering endothelial dysfunction to be a common feature with CAD, we studied C150T polymorphism leading to amino acid change ser608leu, in CAD. In our study, the CC genotype was found in 105 subjects (70%) of the study group (case) and 113 subjects (75.3%) of the control group. CT genotype was found in 45 subjects (30%) of the study group and 37 subjects (24.6%) of the control group. No TT was found in any group, probably because of the smaller sample size. The difference was not found to be significant (p=0.65). The genotypic distribution was in Hardy Weinberg equilibrium (c2= 0.487, df =1, p=0.652).

The iNOS genotype frequency is coincident with the results presented by Shen et al [35] which represents the genotype frequency in Asians. Not much study has been done regarding iNOS C/T genotype and allelic distribution in India. Bhatnagar et al. studied this polymorphism in preeclampsia in Indians [36] and allelic frequency in controls was found to be 78% for the C allele and 22% for the T allele. In our study, the frequency of genotypes and alleles in the study and control groups showed no significant difference, although the frequency of the T allele was higher in the study group as compared to the control group (23.3% versus 7.3%), showing that T allele might be the susceptibility allele for CAD in Indian population. In this study, we demonstrated significantly decreased NO levels in the (CC+CT) genotype in the study group as compared to controls, suggesting that iNOS gene expression is tightly regulated and tissue-specific and is induced only when eNOS expression is blunted. It also suggests a complex milieu of various factors which ultimately aim at maintaining NO levels. Studies have suggested that iNOS microsatellite polymorphism may contribute to atrial fibrillation [37]. Since NO alone has been considered to be an important anti-atherogenic factor with antioxidant, anti-inflammatory, antiproliferative, and vasodilatory effects on vasculature, the lower NO levels in coronary artery disease patients may be considered to be one of the contributing factors to atherosclerotic process. The decreased level of nitric oxide is influenced by the presence of inflammatory cytokines. An IL-6 mediated endothelium-dependent nitric oxide-cyclic guanosine monophosphate (cGMP) mediated relaxation pathway is inhibited in systemic vessels in CAD. Also, the presence of inflammatory cytokines might affect the expression of the iNOS gene thereby decreasing the amount of NO in CAD. In our study, CAD is associated with the iNOS gene (C150T) polymorphism with altered levels of nitric oxide pointing toward the role of endothelial dysfunction in CAD. The presence of the T allele may be considered a susceptibility allele for CAD.

Conclusions

The data suggested the hypothesis that persistent inflammatory instability is present among stable CAD patients. Decreased levels of nitric oxide and increased

levels of endothelin-1 and pro-inflammatory cytokines in (CT) heterozygous mutants of cases do suggest that the mutation may alter the formation of NO. Therefore, these findings suggest that endothelial status is not only determined by an individual risk factor but also the collective effect of all atherogenic and atheroprotective factors present in an individual, including known as well as unknown genetic predisposition. Novel therapies designed to attenuate inflammation and control endothelial dysfunction might be of some advantage in CAD. However, before endothelial markers and inflammatory markers can be recommended as a primary endpoint to guide the use of appropriate therapeutic strategies to reduce cardiovascular risk, it is important to have appropriate diagnostic tools that are accurate, reproducible, expensive, and with high sensitivity and specificity. More studies focusing on the molecular and cellular aspects of endothelium dysfunction need to be initiated for both research and clinical interests.

Acknowledgments

This work has been funded by a grant from the Indian Council of Medical Research (ICMR).

Conflicts of Interest: None declared.

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