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Research Brief

Endothelial nitric oxide synthase (eNOS) gene polymorphism (Glu298asp) and nitric oxide (NO) levels in patients with ST-segment elevation myocardial infarction (STEMI)

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ABSTRACT

Background: Genetic polymorphism in endothelial Nitric Oxide Synthase (eNOS) are associated with occurrence of multiple cardiovascular diseases (CVDs).

Methods: This study included 300 young ST-segment elevation myocardial infarction (STEMI) patients and 300 healthy controls. STEMI patients were divided into two groups: premature coronary artery disease [CAD] (STEMI<40 years of age) and older STEMI (>40 years of age). Genetic polymorphisms in the eNOS gene (894G/T) was evaluated in both subjects and controls. Plasma levels of nitric oxide (NO) were estimated for both patients as well as controls.

Results: Mean age of the study population was 49.7 ± 9.2 years with premature CAD being present in 58 (19.3 %) patients. No significant difference at genotypic ($P = 0.589$, odds ratio (OR) = 0.9, 95 % CI = 0.6–1.6) and allelic level ($P = 0.173$, OR = 1.2, 95 % CI = 0.9–1.4) was observed between STEMI patients and healthy controls. Genotype 894 TT had significantly higher frequency in STEMI patients >40 years ($P = 0.047$, OR: 2.5; 95 % CI = 1.0–6.0). No significant difference at genotypic ($P = 0.279$) and allelic level ($P = 0.493$) was observed between premature CAD (STEMI age <40 years) and healthy controls. NO levels (131 ± 59.6 μM vs 118.11 ± 49.96 μM; $P = 0.001$) was significantly higher in healthy controls as compared to STEMI patients >40 years of age (P= 0.001).

Conclusion: There was significant association of eNOS gene polymorphism Glu298Asp with STEMI patients > 40 years. However, this association was not observed in premature CAD patients. Lower levels of NO in STEMI patients >40 years suggests its potential role as a marker of CVD.

1. Introduction

There has been a growing burden of coronary artery disease (CAD) among the resource limited developing countries.[1] CAD is often multifactorial involving both the environmental and genetic components. Cardiovascular disease (CVD) is amongst the leading causes of death accounting for more than one-thirds of the deaths worldwide.[2] India of late has been experiencing an alarming increase in the incidence and prevalence of CVDs. A number of genetic factors have been evaluated in patients with CAD however, amongst them nitric oxide (NO) and endothelial nitric oxide synthase (eNOS) gene polymorphism has garnered much attention. eNOS gene polymorphism Glu298Asp, is a single nucleotide polymorphism (SNP) wherein there occurs a G to T conversion at nucleotide 894 located in exon 7 of the eNOS gene which results in the amino acid substitution of glutamate by aspartate at position 298 of the enzyme.[3] This polymorphism is thought to reduce the activity of eNOS gene, thereby resulting in decreased production of nitric oxide (NO) which may predispose the patient to hypertension and CAD.[4] NO plays an important role in vascular function during inflammatory responses. It is a potent vasodilator and is a key regulator of

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vascular endothelial cell activity. Unlike prostacyclin, it reduces both the platelet aggregation and adhesion. NO tends to inhibit platelet aggregation through cyclic guanosine monophosphate (cGMP) pathway and synergizes with prostacyclin, which inhibits platelet aggregation by increasing their concentration of cyclic adenosine monophosphate (cAMP). NO also tends to regulate leukocyte recruitment during inflammation by inhibiting leukocyte activation.[5]

The role of eNOS gene polymorphism Glu298Asp in CAD and acute coronary syndrome (ACS) has been evaluated in multiple studies with conflicting results. Initial studies identified it to be a risk factor for CAD among the Japanese and Caucasian population[6,7] while later studies from France[8] and Canada[9] reported a lack of association of this polymorphism and occurrence of CAD. Data regarding eNOS gene polymorphism and CAD from Indian population is very limited. Additionally, there is little evidence regarding the role of NO and eNOS gene polymorphism in the occurrence of STEMI in young (<40 years) individuals. This study aimed to determine the association between eNOS gene polymorphism Glu298Asp and NO levels in patients with STEMI in both >40 years of age and in those with premature CAD (<40 years of age) and to correlate it with disease severity and NO levels.

2. Methods

This was a single centre prospective observational study conducted in the department of Cardiology at a tertiary care medical centre in Delhi, India. Patients with STEMI presenting within 72 hours and consenting to be a part of the study were enrolled. A diagnosis of STEMI was based on the European Society of Cardiology/American College of Cardiology Foundation/American Heart Association/World Heart Federation Task Force for the Fourth Universal Definition of Myocardial Infarction.[10] Patients with previous myocardial infarction or previous revascularization, end stage renal/liver disease, malignancy and acute as well as chronic inflammatory condition and pregnancy were excluded. In this subset of patients with STEMI, premature CAD was defined as occurrence of CAD in those aged less than 40 years. In addition, age and sex matched healthy controls with no clinical evidence of CAD were also enrolled. Complete demographic profile of all the participants were noted. All patients underwent a detailed clinical evaluation, routine blood investigations, electrocardiography and 2D echocardiography following a written informed consent. In all the enrolled subjects, coronary angiogram was performed. Coronary artery stenosis was defined as significant when the luminal diameter was >50 % narrowed after nitroglycerin administration. The severity of coronary artery disease was expressed by the number of affected vessels (single vessel (SVD), double vessel (DVD) or three vessel disease [TVD]).

3. Analysis of eNOS gene polymorphism GLU298ASP and NO level estimation

Ten millilitres of peripheral venous blood were collected from all the subjects of which one ml in EDTA vacutainer for DNA isolation while the remaining blood volume in plain vacutainer was used for serum separation for analyses of routine and specific biochemical parameters. Peripheral blood leucocytes were used for DNA extraction and genetic studies. Plasma NO levels were estimated by an enzymatic Griess' method on a high throughput SpectraMax 190 Spectrophotometer (Molecular Devices, Sunnyvale CA, USA). Genomic DNA was extracted from peripheral blood leucocytes by using commercially available nucleic isolation kit QIA amp DNA Mini and Blood Kit (Qiagen, Chatsworth, CA, USA) and stored at −20 °C. The quality of DNA was checked by gel electrophoresis using 0.8 % agarose gel. Amplifications of the candidate gene loci were done through Polymerase Chain Reaction (PCR) method using specific forward (F:5′TCCCTGAGGAGGG CATGAGGCT 3′) and reverse primers (R:5′ TGAGGGTCACACAGGTTCCT 3′) in presence of restriction enzyme Ban II. Sense and anti-sense primers were designed using DNASTAR software to detect eNOS3 894G/T polymorphism The PCR product having genotype 894 TT was undigested by restriction enzyme Ban II, whereas, genotypes 894 GT and 894 GG were cut into different band sizes as shown in Fig. 1. A 100bp marker was used to confirm the size of the respective bands.

4. Consent and ethical issues

A written informed consent was obtained from all patients and controls. The present study was approved by the institutional ethics committee and was conducted in accordance with the ethical principles that are consistent with Good Clinical Practice and all local regulations.

5. Biostatistical analysis

Continuous data was expressed as mean ± standard deviation (SD) and categorical data was represented as proportions. Normality of distribution of continuous variables were assessed using the Kolmogorov–Smirnov test. A goodness of fit test was used for testing the Hardy–Weinberg equilibrium and a $\chi 2$ test compared the genotype and allele frequencies of NOS3 polymorphism between the two groups. Comparison of means of continuous variables was done using Student's *t*-test or Mann–Whitney *U* test as appropriate while Fisher exact test or $\chi 2$ test was used for categorical variables. A two-sided *P* value of <0.05 was considered to be statistically significant. SPSS version 24.0 (IBM Corp, Armonk, NY) software was used for statistical analysis. The power of the sample size to detect the association at $\alpha = 0.05$ was calculated using EPIINFO ver.6 (Centers for Disease Control, Atlanta, Georgia, USA) software.

6. Results

A total of 300 patients with STEMI and 300 age and gender matched controls were enrolled in the study. The mean age of the study population was 49.7 ± 9.2 years while that of the control group was 50.3 ± 9.3 years ($P = 0.42$). Premature CAD (STEMI<40 years) was present in 58 (19.3 %) patients. A majority of the enrolled subjects were males (90 %) with co-morbidities such as hypertension (32 %) and diabetes (18 %). Patients with STEMI had significantly higher levels of total cholesterol, LDL-C and triglycerides as compared to the control group. Additionally, STEMI had significantly lower levels of HDL-C as compared to healthy controls. The demographic, clinical and biochemical characteristics of the participants are presented in Table 1.

7. Genotype and allele distribution of NOS3 polymorphism

(a) STEMI vs controls:

The frequencies for the three genotypes in our study subjects were homozygous wild-type GG [180/300 (60 %)], heterozygous genotype

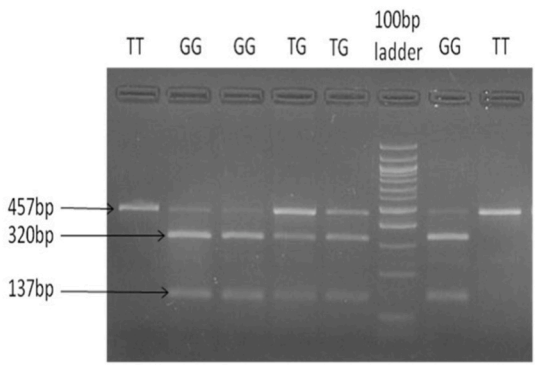

Fig. 1. Genotyping of NOS3 polymorphism, 3 % agarose gel showing allelic variants of the 894G/T polymorphism.

Table 1
Demographic and clinical characteristics of the study group.

Parameters	STEMI Patients (n = 300)	Controls (n = 300)	P-value
Age (years)	49.7 (9.2)	50.3 (9.3)	0.42
Male	270 (90 %)	269 (89.7 %)	0.89
Female	30 (10 %)	31 (10.3 %)	0.89
BMI (kg/m^2)	24 (3.7)	23.5 (4.1)	0.11
Hypertension	103 (34.3 %)	0 (0 %)	–
Diabetes	59 (19.7 %)	0 (0 %)	–
Family history	53 (17.7 %)	0 (0 %)	–
Alcohol History	110 (36.7 %)	88 (29 %)	**0.05**
Smoking History	191 (63.7 %)	108 (36 %)	**<0.0001**
Cholesterol (mg/dL)	184 (26.5)	174 (26.4)	**<0.0001**
HDL (mg/dL)	43 (6.8)	45.9 (5.3)	**<0.0001**
LDL (mg/dL)	117 (25.1)	109.6 (22.9)	**0.0002**
Triglycerides (mg/dL)	136 (34.8)	106 (34.9)	**<0.0001**

BMI: Body mass index; LDL: Low density lipoprotein; HDL: High density lipoprotein; SD: standard deviation; mg/dL: milligram per decilitre.
*Continuous variables represented as mean(SD) and categorical data represented as N(%).

GT [89/300 (29.7 %)] and homozygous mutant TT [31/300 (10.3 %)]. The observed genotype and allele frequencies were in agreement with the frequencies predicted by the Hardy–Weinberg equilibrium The multivariate logistic regression analysis after adjustment with confounding factors such as age, gender, BMI, smoking, alcohol, hypertension, family history and diabetes revealed no significant difference at genotypic (P = 0.589, $\chi2$ = 0.3, odds ratio (OR) = 0.9, 95 % CI = 0.6–1.6) and allelic level (P = 0.173, $\chi2$ = 1.8, OR = 1.2, 95 % CI = 0.9–1.4) between STEMI patients and healthy controls (Table 2).

(b) STEMI patients aged >40 years versus controls:

The multivariate regression model after adjustment with eight covariates revealed that genotype 894 TT had significantly higher frequency in STEMI patients >40 years as compared to age matched healthy controls ($P = 0.047, \chi2 = 4.0$). The OR for STEMI in the subjects with TT genotype was 2.5 (95 % CI = 1.0–6.0) as compared to GG genotype. The 894 T allele was significantly overrepresented (P = 0.036, $\chi2 = 4.4$) in STEMI patients >40 years of age with OR of 1.4 as compared to healthy controls (Supplementary Table 1).

(c) Premature CAD (STEMI age <40 years) versus controls:

Table 2
Genotype and allele distribution of the NOS3 polymorphism in STEMI patients and controls.

Gene/ SNP	Genotype/ allele	Patients (n = 300)	Controls (n = 300)	χ^2	P	OR (95 % CI)
eNOS3 G894T	GG	180 (60 %)	191 (63.7 %)			Reference
	GT	89 (29.7 %)	87 (29 %)	1.3	0.256	0.8 (0.5–1.2)
	TT	31 (10.3 %)	22 (7.3 %)	1.0	0.324	1.5 (0.7–3.1)
	GT + TT	120 (40 %)	109 (36.3 %)	0.3	0.589	0.9 (0.6–1.6)
	G$^{\#}$	449 (74.8 %)	469 (78.1 %)			Reference
	T$^{\#}$	151 (25.1 %)	131 (21.8 %)	1.8	0.173	1.2 (0.9–1.4)

Abbreviations: OR: odds ratio; SNP: single nucleotide polymorphism.
*P-value and OR were calculated after adjustment for age, gender, BMI, alcohol, smoking, family history, hypertensive and diabetic conditions using multivariate logistic regression analysis.
(n = 600).

Multivariate regression model after adjustment with eight covariates did not show any significant difference at genotypic and allelic level (P = 0.279, $\chi2$ = 1.2; P = 0.493, $\chi2$ = 0.5, respectively) between age matched premature CAD (STEMI age <40 years) and healthy controls (Supplementary Table 2).

STEMI patients were differentiated on the basis of single, double and triple vessel disease (Supplementary Table 3). The prevalence of risk genotypes 894 TT was higher in case of DVD (11 %) and TVD (18 %) compared to SVD (8 %). There was a significant difference (P = 0.033, $\chi2$ = 6.81) between three genotypes of 894G/T polymorphism between SVD and TVD. The risk allele 894 T was significantly overrepresented in TVD compared to SVD ($P = 0.011, \chi2 = 6.40$) and DVD ($P = 0.023, \chi2 = 5.19$).

8. NO levels in STEMI patients and healthy controls

The NO levels in premature CAD (STEMI age <40 years), STEMI>40 years of age, and healthy controls were 131.23 ± 50.20 μM, 118.11 ± 49.96 μM, and 131 ± 59.6 μM, respectively. NO levels was significantly higher in healthy controls as compared to STEMI patients >40 years of age (P = 0.001) and was comparable to the level in premature CAD (STEMI age <40 years) patients. There was a uniform trend among various genotypes wherein NO levels were highest in patients with GG followed by GT followed by TT genotype however, there was no statistically significant difference (Supplementary Figs. 1 and 2).

9. Discussion

The present study found a significant association of eNOS gene polymorphism Glu298Asp (mutant TT genotype) with STEMI in patients>40 years of age. However, this association was not reported in individuals with premature CAD. Findings of our study also revealed that a significantly higher frequency of homozygote TT in patients with severe CAD (TVD). Additionally, in our study NO levels were significantly higher in the control population as compared to STEMI patients >40 years of age. The role of eNOS gene polymorphism in patients with CAD is still unclear with most of the published studies reporting conflicting evidence. Previous studies conducted among the French[8] and the Canadian[9] population reported no significant association of eNOS gene polymorphism Glu298Asp with CAD. In one of the earliest meta-analysis (HuGE review) involving 42 studies and comprising 13,876 CHD cases and 13,042 controls, Casas JP et al.[11]reported a per-allele odds ratios of 1.17 (95 % CI: 1.07, 1.28) for CAD for the Glu298Asp gene polymorphism. However, later two larger meta-analysis did reveal a significant association between Glu298Asp gene polymorphism and occurrence of CAD.[12,13] In our study, there was no definite association of eNOS gene polymorphism Glu298Asp with STEMI however, in the subset of STEMI patients >40 years of age, the mutant TT genotype had a higher frequency. The reason for this conflicting evidence in terms of association between eNOS gene polymorphism and occurrence of CAD includes (a) genetic and environmental heterogeneity among different population group, (b) Glu298Asp locus may be a marker of functional polymorphism at other genetic loci and the T894 allele could be in linkage disequilibrium with genetic polymorphism which has an effect on CAD and ACS, (c) CAD and ACS may be associated with other genetic mutations and a stronger genetic effect at another loci may be masking the effect of eNOS gene polymorphism.

Genetic parameters usually play a more significant role during early years of life than during later ages. In our study, we also compared the genotypic frequency among subjects with premature CAD (STEMI patients age<40 years). Findings of our study reported that eNOS gene polymorphism Glu298Asp was not significantly different between patients with ACS <40 years and their age matched controls. Similar findings were reported by Kara and colleagues[14] who found that no association existed between eNOS gene polymorphism Glu298Asp and occurrence of early onset CAD. This finding was in contrast to the data

from Turkey[15] and China[16] where Asp allele was independent risk factor for premature CAD and STEMI. However, in these studies, the population studied were half a decade older than our CAD patients. Additionally, other non-conventional risk factors may be masking the effect of eNOS gene polymorphism in our subset of patients. There has been an association between eNOS gene polymorphism and CAD severity. Previous studies by Colombo et al.[17,18] and Andrikopoulos GK et al.[19] have reported a significant difference in the frequency of TT allele between patients with SVD as compared to those with TVD. Similar findings were reported in our study too wherein the genotype frequency correlated with severity of disease based on angiographic characteristics.

In our study, we also attempted to analyse the functional significance of eNOS gene polymorphism by analysing NO levels which were significantly lower in STEMI patients >40 years of age as compared to the control population. This difference in NO levels were not apparent in patients with premature CAD further supporting the possibility of some unknown factors contributing to the development of ACSin patients younger than 40 years. In a study from Egypt, mean levels of NO was significantly lower in patients with CAD than in controls.[20] Our study had few limitations which included (a) single centre study with relatively small sample size thereby findings cannot be generalised to the entire population, and (b) not assessing non-conventional risk factors in premature CAD. The present study depicted the association between eNOS genetic polymorphisms in patients with STEMI >40 years of age. However, there was a lack of association between eNOS genetic polymorphisms in patients <40 years of age (premature CAD). To the best of our knowledge, this is one of the first studies comparing the role of eNOS genetic polymorphisms in young (<40 years of age) and older patients (>40 years of age) with STEMI in the South-East Asian population group. However, there is a need for large scale, multi-centre randomised studies to highlight the role of these novel genetic polymorphism on occurrence of cardiovascular diseases.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ihj.2024.01.017.

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