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RESEARCH ARTICLE

Association of eNOS gene variant with insulin resistance in Metabolic Syndrome

Parineeta Samant[1], Padma Chavan[1]*, Sandeep Rai[2]

1Department of Biochemistry, MGM Medical College, Navi-Mumbai, India.
2Department of Medicine, MGM Medical College, Navi-Mumbai, India.

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Corresponding Author

Dr.Padma Chavan

Abstract

INTRODUCTION

Metabolic syndrome (MS) is a cluster of manifestations including central obesity, increased BMI, high waist to hip ratio (WHR) high blood pressure, increased fasting blood glucose level, increased serum concentration of triglycerides (TGs) and decreased serum concentration of high-density lipoprotein cholesterol (HDL-C). Individuals with MS are at high risk of developing type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD) (Eckel et al.,2005). In addition to environmental factors such as a poor diet and physical inactivity (Parket al.,2003), several studies suggested a genetic etiology for MS (Groop, 2000 and Kissebah etal.,2000). Animal models indicate that lack of endothelial nitric oxide synthase (eNOS) is related to hypertension, hypertriglyceridemia and insulin resistance (Duplain et al.,2001).

Endothelial nitric oxide synthase (eNOS) is involved in the production of nitric oxide (NO), a small molecule having molecular weight of 30 Daltons, which is a potent vasodilator and responsible for maintaining normal endothelial function. (Nishevitha et al., 2009) It acts as a key factor in the anti-atherosclerotic properties of the endothelium. It is also involved in neuronal transmission, smooth muscle relaxation and immunity (Bredt, 1999).

In humans, eNOS is coded by the NOS3 gene located on chromosome 7q35-36 (Marsden et al.,1993). The eNOS enzyme constitutively synthesizes nitric oxide (NO) via the conversion of L-arginine into L-citrulline, involving the transfer of five electrons provided by NADPH (Mayer et al.,1997). Because of the role of NO in vascular homeostasis, both the enzyme and its gene has attracted the attention of many researchers (Bhanoori,2011). Several polymorphisms were identified in the eNOS3 gene. Much attention was focused on putatively functional variants: T-786C (rs2070744), G894T (Glu298Asp) (rs1799983) and the intron 4b/a VNTR (27-bp variable number of tandem repeats) (Stuehr, 1997). The exon 7 polymorphism (894G ----→ T) that specifies substitution of a glutamate residue for an aspartate at position 298 in the human eNOS protein is known to be associated with hypertension and insulin resistance. This polymorphism is of particular interest because substitution of the conserved amino acid (Glutamate) within the oxygenase domain of eNOS may influence eNOS function (Fairchild et al., 2001).

Insulin increases NO production, leading to vasodilatation and increased blood perfusion and it also has anti-apoptotic and pro-survival effects on the ischemic/reperfused heart. Impairment of the phosphatidylinositide 3-kinases (PI3K) – protein kinase B (AKT) – eNOS – NO pathway as a manifestation of insulin resistance contributes

to endothelial dysfunction, predisposing the endothelium to hyper-inflammatory and thrombotic states. (Zanatta et al., 2008)

It would be beneficial to identify Insulin resistance, the hallmark of MS at an early stage. Therefore, the aim of the present study was to verify the contribution of G894T eNOS gene single nucleotide polymorphisms to NO production, insulin resistance, microalbuminuria and risk for MS.

Material & Methods

Study design: The study consisted of 110 subjects which were divided into two groups: Group I- Control (n=50) and Group II- Metabolic syndrome (MS, n=60). Individuals who visited medicine OPD for routine health checkup and those who were within the NCEP ATP III criteria were enrolled as cases for MS. The study protocol was approved by the institutional ethics review committee. The purpose of the study was explained to all the volunteers and informed written consent was obtained from them. The study was carried out in department of Biochemistry and central research laboratory.

Cases of MS were selected according to the modified Third Report of the National Cholesterol Education Program's Adult Treatment Panel (NCEP ATP III) (NCEP ATP III.,2011). which defines MS as the presence of at least 3 of the following: (1) a fasting plasma glucose (FPG) of ≥110 mg/dl; (2) serum triglycerides of ≥150 mg/dl; (3) serum HDL-C of < 40 mg/dl in men and<50 mg/dl in women; (4) a blood pressure of ≥ 130/85 mmHg; and (5) a waist circumference (WC) of > 90 cm in men and > 80 cm in women. The three features considered for inclusion of subjects in MS group were BP, FPG, and TGs. Anthropometric measurements were obtained during a complete physical examination. Weight was measured in kilograms on weighing scale without shoes and wearing light clothing. Height was measured in centimeters for all the participants. BMI was derived from the formula of weight/height^2 (kg/m^2). Blood pressure was measured at the right brachial artery using a random-zero sphygmomanometer with the participant in a sitting position.

Biochemical analyses: In all the subjects overnight fasting venous blood samples were collected. Plasma glucose levels were estimated by GOD-POD enzymatic method. Standard enzymatic methods were used to measure serum concentrations of total cholesterol, high density lipoprotein (HDL) cholesterol and triglycerides. LDL and VLDL cholesterol concentration was calculated by Friedwald's formula (Friedewald.,1972). Fasting plasma insulin levels were estimated by ELISA method (Kit by Immunoshop), HbA1c levels were estimated by antigen antibody method. Serum nitric oxide levels were measured by modified Griess method. Insulin resistance was measured by employing homeostasis model assessment of insulin resistance (HOMA-IR).

DNA extraction: Genomic DNA was isolated from EDTA blood using DNA extraction kit of Sigma. The isolated DNA was checked for integrity by gel electrophoresis using 1.5% agarose (Hi-Media,Mumbai) and quantified by UV spectrophotometer (Thermofischer).

PCR analysis of G894T SNP: PCR analysis was carried out using Thermo cycler peq start**.** Genomic DNA (~ 50 ng) was incubated in a total reaction volume of 30µl containing equal concentration of the forward primer -5' AAGGCAGGAGACAGTGGATGGA 3'and reverse primers - 5'CCCCTCCATCCCACCCAGTCAATC 3'(Fermentas). A 261 bp fragment of exon 7 of eNOS gene was amplified by PCR by taking 50 ng of genomic DNA and 10 pmol/µl of primers. Super mix containing Taq polymerase, dNTPs, $MgCl_2$ was added to make a final volume of 30 µl. PCR was cycled 35 times at 94°C for 45 sec, 66°C for 45 sec and 72°C for 45 sec, with initial 5 min denaturation at 94°C and final extension at 72°C for 6 min. Negative control was included in every experiment to monitor for contamination. The amplification of 261 bp PCR product was verified on 1.5% agarose gel using 5µl of PCR product.

Restriction analysis of G894T SNP: Restriction digestion was performed using 10 µl of PCR mixture, 18 µl of nuclease free water, 2 µl of Tango buffer and 1 µl (10 units/ µl) of Mbo I restriction enzyme (Thermoscietific). Samples were then incubated for 5 h at 37°C and the digested PCR products were separated by 1.5 per cent polyacrylamide gel electrophoresis stained with ethidium bromide (Figure 1). The products of the restriction digest were viewed under transilluminator and then documented using gel documentation system.

Statistical analysis: Data reported as mean ±SD were analyzed using SPSS 17 software. Pearson chi-square ($\chi 2$) test was performed to find the statistical significance between the genotypes and the gene frequency was calculated by allele counting.

Result:

Table 1 summarizes the descriptive characteristics of participants with and without MS. BMI, waist circumference, systolic and diastolic blood pressure, triglycerides, fasting glucose, insulin and HOMA-IR were higher in MS group than in control group. Serum nitric oxide and HDL-C were significantly lower in MS group compared to control group. The distribution of G894T eNOS genotypes in the control group was as per Hardy-Weinberg equilibrium ($\chi 2$ test = 2.26, degree of freedom=1, P>0.05). The tests for association (Table 2) showed a significant presence of TT homozygotes in MS group (OR=2.58; 95% CI:1-12-7- 11, table 2). Analysis of variance with post-hoc Bonferroni and t tests showed that the TT homozygotes had higher values of waist circumference, systolic BP, triglycerides (TG)

Figure 1. Gel electrophoresis of RFLP

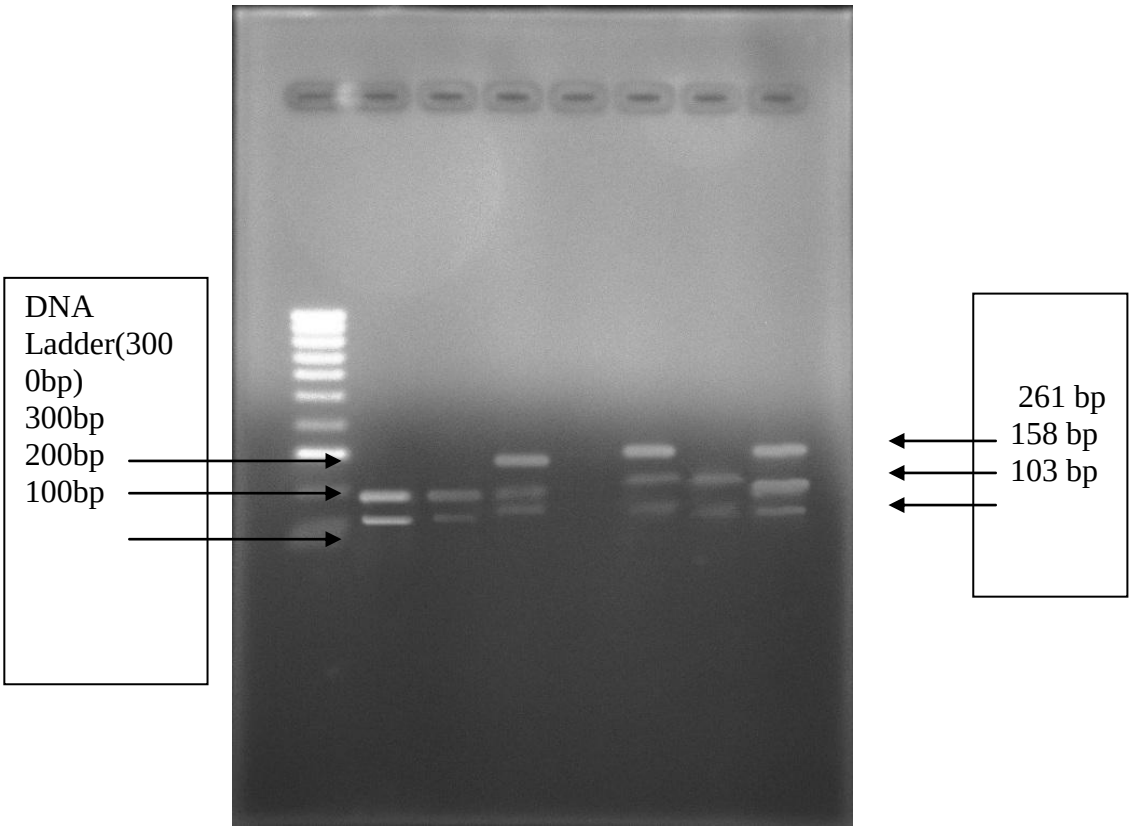

Table 1 Descriptive analysis of Biochemical parameters

Parameter	Group I	Group II	P value
BP (Systolic) mm/Hg	115 ± 6.98	134 ± 2.86	0.0001**
BP (Diastolic) mm/Hg	76.7 ± 4.98	96 ± 0.60	0.0001**
BMI in kg/m^2	23.3 ± 1.39	29.6 ± 0.53	0.0001**
WHR	0.802 ±0.03	0.99±0.01	0.0001**
FBG mg/dl	88.1 ±5.06	113 ± 1.19	0.0001**
PP mg/dl	123.2 ± 4.91	149 ± 31	0.0001**

HbA1c %	4.34 ± 0.33	5.8 ± 0.5	0.0001**
Fasting plasma insulin µIU/ml	10.2 ± 3.2	22 ± 0.11	0.0001**
HOMA IR	2.23 ± 0.69	6.14 ± 0.09	0.0001**
Cholesterol mg/dl	146.3±10.59	174 ±15	0.0001**
TG mg/dl	110± 17.2	189 ±7.5	0.0001**
HDL mg/dl	44.5 ±3.6	40±3.7	0.0001**
VLDL mg/dl	21.59± 2.72	36±2.1	0.0001**
LDL mg/dl	83.92± 14.2	103±22.2	0.0001**
Nitric Oxide µmol/l	0.29 ± 0.052	0.27±.05	0.03*
Urinary microalbumin mg/dl	5.02 ± 1.86	5.52±3.26	0.07

All data is expressed as Mean ± SD
Student t test was applied to data to find statistical significance
*P<0.05 is statistically significant
**p<0.0001 is highly statistically significant;

Table 2. Allelic frequency of SNP at G894T

Allele	Group I	Group II
G Allele	90 %	78%
T Allele	10 %	22%

Table 3.Genotype frequency of eNOS SNP and its association with disease

Groups	Genotypes			χ^2 Test	Fischer Exact Test	
	GG	**GT**	**TT**		**Odds Ratio 95% CI**	**P value**
Control group I(N=50)	84%	16%	-	-		
Metabolic syndrome Group II (N=60)	65%	27%	8%	5.07	2.82 (1.12 - 7.11)	<0.05

Table 4. Genotype : Phenotype association

Parameters	Genotype	Control (Group I)	Metabolic Syndrome (Group II)
SerumNitric Oxide µmol/l	GG	0.33±0.04	0.29±0.09
	GT+TT	0.32±0.04	0.22±0.06
Urinary Micro-albuminuria mg/dl	GG	4.93±1.48	5.81±3.77
	GT+TT	5.47±2.58	5.0±2.1
HOMA IR	GG	2.2±0.66	5.97±0.95
	GT+TT	2.2±2.32	6.47±0.73

Genotype specific Mean ±SD for each group

Table 5. Within Group pair wise Comparison

Parameters	Control (Group I)	MS (Group II)
	GG vs GT+TT	GG vs GT+TT
SerumNitric Oxide, µmol/l	P<0.89	P<0.02
Urinary Microalbuminuria, mg/dl	P<0.39	P<0.35
HOMA-IR	P<0.63	P<0.037
Based on estimated marginal means		
a. Adjustment for multiple comparisons: Bonferroni.		
*. The mean difference is significant at the .05 level.		

Discussion

Endothelial nitric oxide synthase, a key regulator of vascular nitric oxide production, has been investigated extensively to determine the relevance of variants in the eNOS gene on vascular diseases. The variants Glu298Asp in exon 7 of eNOS gene has been explored in several epidemiological studies but very few association studies are documented in Indian population (Thameem et al.,2008).

In the present study, the genotype frequency of eNOS gene G894T was 84% GG, 16% GT, 0% TT in control and 65% GG, 27% GT and 8% TT in the MS group as shown in table 3. In our study, the prevalence of heterozygous (GT) and homozygous TT genotype for eNOS - G894T SNP was found to be higher in study group (MS) as compared to controls. The presence of high frequency of eNOS gene genotype GT and TT in study group may be indicative of occurrence of the disease**.**

Fischer's exact test and 2x2 contigency table (for odds ratio) were employed as the suitable statistical tool for genetic analysis. Due to low frequency of TT in our population the GT+TT genotypes were combined for genotype:phenotype analysis. The frequencies of GG and (GT+TT) genotype of control (Group I) and MS (Group II) when compared by Chi -square analysis showed an odds ratio of 2.82 for MS indicating high chance for occurrence of MS among the healthy population ($p<0.05$).

Angeline et al., (2011) in their study reported 71.62% and 29.37% frequencies for GG and GT genotypes respectively in healthy controls in south Indian population and found that the homozygous TT genotype was absent. Shankarishan et al., (2011) studied the prevalence of eNOS gene variant among both the tea garden sect and Indigenous community of Assam. The frequencies of the GG, GT and TT genotypes observed among the Tea garden community were 77.0% , 21.0% and 2.0% per cent respectively and those among the Indigenous Assamese community were 65.0%, 32.0% and 3.0% respectively (Shankarishan et al.,2011). In a study about prevalence of eNOS Glu298Asp polymorphism in healthy volunteers from Northern India region, Srivastava et al., (2005) reported the distribution of GG, GT and TT to be 71.22 %, 28.06% and 0.72%, respectively. Tripathi et al,, (2010) reported 74.5%, 24.2% and 1.3 % frequency for GG, GT and TT genotypes respectively in the north Indian population. The frequencies of these genotypes observed in our healthy control population is in agreement with the genotype frequencies reported by Angeline et al., (2011) and Shankarishan et al., (2011). In a study done by Liu et al.,(2009) on association of eNOS gene polymorphism with metabolic risk factors, the genotype frequencies of GG, GT and TT genotype was found to be 77.6%, 22.1% and 0.3% respectively. The frequency of GT genotype for eNOS gene in metabolic syndrome patients reported by us is in agreement with that of Liu et al., (2009). Since the frequency of GG genotype is higher in healthy control it can be stated that this may be a protective genotype against endothelial damage.

In addition, our work suggests the role of genetic variant of the eNOS gene in triggering some of the metabolic abnormalities of insulin-resistant state, which may predispose an individual to type 2 diabetes and cardiovascular disease. In an attempt to study the effect of genetic variants of eNOS gene to this insulin resistant state, we studied

the association between the variant and nitric oxide levels, urinary micralbumin and insulin resistance using HOMA IR model.

Metabolic alterations typical of the insulin resistance syndrome, i.e., increased insulin secretion and a marked degree of insulin resistance, are known to be associated with polymorphic variants of the eNOS gene. These characteristics highly resemble a state of "preglycemic diabetes" in which hyperinsulinemia and/or insulin resistance precedes the development of type 2 diabetes (Reaven, 1999; Zimmet and Zimmet,1993). In healthy control subjects we demonstrated increased NO levels in all the genotypes whereas it was markedly diminished in MS subjects with these variants. Moreover. the MS subjects with homozygous GG genotype had higher NO levels than those of heterozygous (GT+TT) genotype, shown in table 4.

NO plays a important physiological role at the level of kidney. It controls renal and glomerular hemodynamics, at multiple and physiologically critical steps of nephron function. (Mount and Power, 2006) NO is important for maintenance of endothelial health which in turn is indicated by the presence of urinary microalbumin and therefore we estimated urinary microalbumin levels in both the groups and compared the GG genotypes of both the control and metabolic syndrome groups with those of (GT +TT) genotype of these groups. We found no statistically significant correlation of any of the genotypes with urinary microalbumin levels. Thus it can be stated that variance of G894T has no association with urinary microalbumin both in controls and MS.

HOMA IR an accepted indicator of insulin resistance was measured and correlated with eNOS gene polymorphism. High values of HOMA IR were observed in (GT+TT) genotype of MS subjects when compared with (GG) genotype in the same group. (6.47 vs 5.97, $p<0.05$) Also, both the GG and GT+TT genotypes of MS exhibited higher HOMA IR values as compared to control group with these genotypes. (refer to table 4 & 5). This may be because insulin resistance impairs the production of NO and favors the production of vasoconstrictors like edothelin-1 which have effect on isolated arterioles (Verrotti et al., 2003). Thus, metabolic syndrome subjects with homozygous GG genotype are probably protected from endothelial damage. This finding suggests an association between genotype variant and the phenotypic characteristics such as decreased NO levels and insulin resistance in metabolic syndrome subjects. A previously reported study by Monti et al.,(2003) also suggested similar association between genotype variant and phenotypes such as increased NO levels, reduced NO activity, hyperinsulinemia, and insulin resistance in type 2 diabetic patients and their non diabetic first-degree relatives, as well as in insulin-resistant subjects (Piatti et al., 2000; Zavaroni et al., 2000).

It is known that the loss of insulin sensitivity or development of insulin resistance which is a hallmark of metabolic syndrome and underlying cause for type 2 diabetes, results in serious vascular complications, including endothelial dysfunction (Zavaroni et al., 2000). Insulin has direct vascular effects and it is mediated through stimulation of nitric oxide production in endothelial cells (Kuboki et al., 2000). In the insulin-resistant state, the ability of insulin to stimulate nitric oxide production in the endothelium is diminished. In our study we have found association of GT and TT genotype with insulin resistance and decreased levels of NO. Hence it can be said that individuals who exhibit IR state may have diminished NO production by endothelial cells. Previous clinical study done by Dosenko et al., (2006) have suggested that G894T eNOS gene polymorphism is associated with diminished activity of eNOS enzyme (Gabbai et al., 2001). Because of this, the vasodilatory effects of insulin have been extensively studied and shown to be mediated essentially through NO signaling.

We suggest that lower levels of NO seen in MS subjects with GT+TT genotype could be presumably due to diminished activity the eNOS enzyme. A single nucleotide change may be a cause for such an important effect on the endothelial function. When a T is present instead of a G at nucleotide position 894, the NOS3 gene encodes for a protein product containing aspartate, instead of glutamate, it increases the predisposition risk for development of metabolic syndrome. Our study is limited by the fact that this SNP was not correlated with the nitric oxide synthase enzyme activity.

Conclusion

Our results show that the G894T polymorphism is associated with insulin resistant state. The lower values of serum nitric oxide and increased levels of HOMA IR in T allele carriers is suggestive of early endothelial damage in metabolic syndrome. Thus individuals with presence of GT and TT genotype G894Tsingle nucleotide polymorphism are genetically susceptible to develop MS with increased risk of development of type 2 diabetes.

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Competing Interests
The authors have no competing interests.

Ethical Approval
All authors hereby declare that approval from Institutional ethics review committee was obtained and study was carried out as per standards.

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