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Endothelial Nitric Oxide Synthase Gene Polymorphism and the Risk of Diabetic Neuropathy in Asian Indian Patients with Type 2 Diabetes

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Abstract

Endothelial dysfunction plays a key role in the pathogenesis of diabetic vascular disease, including diabetic neuropathy (DNP). Endothelial-derived nitric oxide synthase (*eNOS*) gene polymorphisms affect *eNOS* activity and are associated with endothelial dysfunction. Studies examining association of *eNOS* gene polymorphism in type 2 diabetic patients (T2DM) with DNP and without DNP are lacking in Asian Indians. Thus, we investigated the association and interaction between two potentially functional single nucleotide polymorphisms (SNPs) of the *eNOS* gene (T-786C, G894T) and one repeat polymorphism (27VNTR) with DNP using validated PCR-RFLP assays in 1258 T2DM subjects belonging to two independent cohorts from north Indian Origin and also, we replicated our study in a 3rd independent cohort from south Indian population having T2DM. We also measured serum Nitric Oxide (NO) levels in these subjects and examined its correlation with DNP and *eNOS* genotypes. We observed that *eNOS* genotype carrying 'aa' genotype of 27VNTR (a/b) and *eNOS* haplotype C-a-G and C-a-T (allele of T-786C, 27VNTR a/b and G894T) carrying 'a' allele of 27VNTR (a/b) were associated with increased risk of DNP, in all the three cohorts. We also observed lower serum NO levels in T2DM subjects (both cases and controls) carrying ba+aa genotypes. Our study suggest that 27VNTR (a/b) *eNOS* polymorphism carrying 'a' allele alone and in association with T-786C and G894T *eNOS* polymorphism is associated with increased risk of DNP in Asian Indians T2DM patients.

Keywords: Diabetic neuropathy; *eNOS* (endothelial nitric-oxide synthase); Gene polymorphism; Type 2 diabetes

Introduction

Diabetic peripheral neuropathy (DPN) is one of the most commonly reported microvascular complication of diabetes, affecting up to 20%-40% of type 2 diabetic (T2DM) patients [1]. DPN is a heterogeneous disease which results from the direct damage of peripheral nerve components and of the associated vasa nervorum [1]. Also, it is a complex disorder emerging from the imbalance between multiple predisposing and protective genetic variants as well as following the interaction with environmental factors [2]. Although, chronic hyperglycemia, duration of diabetes and level of metabolic control are the main contributors to the development of DPN, however, endothelial dysfunction has been shown to be an important pathophysiologic denominator for DNP [3]. Nitric Oxide (NO) system through dramatically changed NO production and local release significantly contribute to endothelial dysfunction [4]. The process takes place by the modulation of the nitric oxide synthase (NOS) enzymes responsible for NO synthesis [5]. There are three distinct isoforms of NOS; endothelial constitutive NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) [6]. Endothelium derived NO plays a key role in the regulation of vascular tone [7], and has vaso-protective effects by scavenging superoxide radicals and suppressing platelet aggregation, leukocyte adhesion, and smooth muscle cell proliferation [7-10]. Moreover, previous studies suggest that dysfunctional endothelial nitric-oxide synthase (eNOS) might play a critical role in the pathogenic pathway leading to diabetic vascular complications including DNP [11,12]. Thus, eNOS genes became natural candidates for the study of DPN genetics. Furthermore, polymorphisms in the eNOS gene that lead to decreased eNOS expression have been found to be associated with advanced DNP suggesting that genetic polymorphisms of eNOS gene also may play a role in the NO abnormalities that contribute to the development and progression of DNP [13]. In comparison with the other chronic diabetes microvascular complications, data regarding the genetic background of DPN are rather scarce.

Several polymorphisms have been reported in *eNOS* promoter, exon and intron regions [14]. The most studied variants are the single nucleotide polymorphism (SNP) at position -786 represented by a base substitution from T to C (rs2070744), a polymorphism of *eNOS* in intron 4 (4a/4b) based on a variable 27-base pair tandem repeat; consisting of four (allele 4a) or five (allele 4b) repeats, SNP at position 894 represented by a base substitution from G to T (rs1799983). In previous studies it was shown that individuals with -786C, 4a allele had a reduced *eNOS* transcription rates, thus resulting in modulation of NOS3 enzymatic activity and, apparently, affecting plasma NO concentrations [15]. Study by Costacou et al. showed that G894T variant changes the *eNOS* protein sequence, probably leading to an alteration of enzyme activity [16]. Brouet et al. also suggested that G894T variant controls the NOS3 intracellular distribution by interacting with proteins that mediate its degradation [17].

Zotova et al. (2005) analyzed the influence of *eNOS* polymorphisms on DPN from Caucasian patients with Type 1 Diabetic Mellitus

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(T1DM) genotyped for rs2070744, rs1799983 and 4b/4a polymorphism and they showed that none of these polymorphisms were associated with DPN [18]. Heltianu et al. showed that *eNOS* 4a/4a genotype does not represent a risk or protective factor for T1DM-DPN [14]. Other T1DM reports revealed that 894GG (rs1799983) homozygous patients were associated with decreased incidence of DPN [19-21].

Manea et al. showed in T2DM patients belonging to Caucasian ethnicity that frequency of 4a allele was significantly lower in DPN patients as compared to T2DM patients without DPN [22]. Also, Mehrab-Mohseni et al. showed in Iranian population that frequency of 4a allele was significantly lower in DPN patients as compared to T2DM patients without DPN [23].

Since, Asian Indians, an ethnically distinct population, has the leading number of diabetic patients in the world [24,25] and studies examining association of eNOS gene polymorphism in type 2 diabetic patients with neuropathy and without diabetic neuropathy are lacking in Asian Indians. Moreover, there are significant genotypic differences between Indians belonging to different geographical regions [26]; thus, in the present study, we investigated the association and interaction between two potentially functional single nucleotide polymorphisms (SNPs) of the eNOS gene (T-786C,G894T) and one repeat polymorphism (27VNTR) with diabetic neuropathy in T2DM subjects belonging to two independent cohorts from north Indian Origin and also, we replicated our study in a 3rd independent cohort from south Indian population having T2DM. We have also examined association of these SNPs with serum NO levels as the graded differences in NO generation could play a significant role in the pathophysiological condition at the microvascular level.

Methods

Study population

Two independently ascertained T2DM cohorts of North Indian origin; visiting Endocrinology and Nephrology Clinics of Postgraduate Institute of Medical Education and Research, Chandigarh, India between June 2006 and September 2007 (cohort1) and July 2009 and March 2012 (cohort 2) were studied in this study.

DNP was defined as having sensory positive symptoms such as decreased sensation, dysaesthesia, paraesthesia, numbness and muscle cramp pain in both lower legs. DNP was also investigated by assessing patients' vibration perception thresholds, using Neurothesiometer; values \geq 25 V were diagnostic of DPN. None of the participants had myopathy, peripheral vascular disease or significant neurological diseases. Patients having vibration perception thresholds under 18 V were considered normal. Patients having bilateral values between >18 V and <25 V, who are considered DPN in presence of other important sign, were excluded from the study to avoid any misclassification. T2DM patients without DNP were considered as controls and healthy subjects without T2DM were not considered as control to avoid diabetes associated misclassification. Based upon this definition cohort 1 consisted of 139 patients with DNP and 356 T2DM patients without neuropathy (DM); cohort 2 had 133 patients with DNP and 342 patients had DM. Replication studies were performed in a population of 207 patients with DM and 81 DNP patients recruited from Madras Diabetes Research foundation, Chennai, South India that formed cohort 3. All the patients had same mean duration of diabetes (12.8 \pm 2.1). Also, 200 healthy subjects without diabetes or other co morbidities were screened to test deviation from Hardy Weinberg Equilibrium (HWE) for these variant.

All the subjects in both groups were age, sex and ethnicity matched. The study was approved by Post Graduate Institute of Medical Education and Research, Chandigarh and Madras Diabetes Research foundation ethics committees and written consent was obtained from participating subjects for genetic testing and the genetic tests to be performed were pre-specified at the time of the study design. Their ethnicity was confirmed on the basis of language spoken and ancestral history. No regional differences in disease prevalence or allele frequency were observed between recruitment sites.

Genotyping

SNPs in the *eNOS* gene selected for the present study are recorded in the public dbSNP database. The official gene symbol is NOS3, approved name is nitric oxide synthase 3 (endothelial cell) and location is 7q36. The SNP ID numbers and detailed sequence information are available publicly (http://www.ncbi.nlm.nih.gov/SNP/). SNPs selected for the present study were T–786C (rs 2070744), 27VNTR intron 4 a/b, and G894T (rs 1799983), they were located at the promoter, Intron 4 and Exon 7 of the *eNOS* gene (NOS3) based on their functional nature.

Genomic DNA was extracted from peripheral blood using phenolchloroform method. Two *eNOS* SNPs, namely, T-786C and G894T were genotyped using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Primers for T-786C SNP were designed using Primer_3 software, whereas primers for G894T SNP were same as previously used by Shimasaki et al. [27]. The 27VNTR is characterized by presence of either four 27-bp repeats (a allele) or five 27-bp repeats (b allele) and was genotyped using PCR assay with primers as previously used by Wang et al. [28]. Details including location of SNPs in the respective genes, primer sequences, PCR conditions and restriction enzyme with product sizes are presented in table 1. Positive and negative controls were used in each genotyping run, and 5% of randomly selected samples were re-genotyped by other lab personnel with 100% concordance. The genotypes were also confirmed by sequencing of some randomly samples.

Biochemical investigation

Serum NO was measured as nitrite/nitrate levels in subjects using Griess reagent (a 1:1 mixture of 1% sulfanilamide in 5% H_3PO_4 and 0.1% N-(1-naphthyl)-ethylenediamine), as described by Lepoivre et al. [29].

Statistical analysis

The statistical tests were performed, using the SPSS Inc., Chicago, IL version 11.0. We tested the genotype frequencies for deviation from Hardy-Weinberg equilibrium (HWE) proportions by using HWE calculator. Using a chi-square test the deviation of genotype distribution from HWE was considered significant at P<0.05. Discrete and continuous variables were compared between cases and controls using Pearson's χ^2 test and unpaired t test or Mann-Whitney U test as appropriate. Comparison of variables between different genotypes was performed using ANOVA for normally distributed data and Kruskal-Wallis test for skewed data. Power analysis was performed using Quanto (version 1.2; http://hydra.usc.edu/gxe). Based on this analysis our sample size had the power of 86% at a small effect size (0.2) and alpha level (0.04). Haplotype frequencies were estimated in various subject groups with the help of Phase Ver 2.1 software (http:// stephenslab.uchicago.edu/software.html). We have applied fisher exact probability test for estimating level of significance of haplotype

frequencies between the groups as it takes into account even the sample groups with a lower frequency, thus minimizing the error due to low frequencies. Linkage disequilibria (LD) were also estimated for the three polymorphisms in the study population, using Haploview software (http://www.broad.mit.edu/mpg/haploview/contact.php). Multivariate logistic regression was used to compute odds ratio for developing DNP by adjusting for potential confounders which include age, gender, HbA1c, duration of diabetes, duration of hypertension, smoking, systolic blood pressure, triglyceride levels, ACEI/ARB, serum creatinine and eGFR. We excluded the potential effects of these factors on the progression of DNP by selecting subjects which had same mean duration of diabetes, were age matched, assuming that these factors

are either protected or prone to developing severe microvascular complications.

Results

Baseline characteristics of study participants

Baseline characteristics of the subjects with T2DM enrolled in the three cohorts are given in table 2. In all the three cohorts, patients with DNP had significantly higher systolic blood pressure, HbA1c, serum creatinine and lower eGFR and duration of HT as compared to DM. The mean duration of T2DM was similar in DNP and DM group.

SNP (rs#)	Primers	Amplicon (bp)	Annealing temp. (°C)/[MgCl ₂] (mmol)	Restriction enzyme/allele size
T-786C (rs 2070744) promoter	P1: 5'-GCA TGC ACT CTG GCC TGA AGT G-3' P2: 5'-CAG GAA GCT GCC TTC CAG TGC-3'	223	61.5/2	Mspl (Bioron)/T=162, 61 bp C=116, 61, 46 bp
27 VNTR Intron 4	P1: 5'-AGG CCC TAT GGT AGT GCC TTT-3' P2: 5'-TCT CTT AGT GCT GTG GTC AC-3'	393/420	59.2/3.5	a allele=393 bp b allele=420 bp
G894T (rs 1799983) Exon 7	P1: 5'-AAG GCA GGA GAC AGT GGA TGG A-3' P2: 5'-CCC AGT CAA TCC CTT TGG TGC TCA-3'	457	62.9/3	BanII (New England Biolabs Inc.) G=137, 320 bp T=457 bp

Table 1: Standard PCR conditions used in genotyping T-786C, G894T and 27VNTR SNPs of the eNOS gene.

		Cohort 1			Cohort 2			Cohort 3	
Baseline characteristics (Mean ± SD)	DM(356)	DNP (139)	P value	DM (342)	DNP (133)	P value	DM (207)	DNP (81)	P value
Age (years)	59.3 ± 6.2	60.0 ± 5.8	0.42	58.0 ± 5.2	57.4 ± 6.6	0.51	54.3 ± 7.3	53.6 ± 4.9	0.36
Sex M/F (n)	142/214 (40/60)	57/82 (41/59)	0.98	161/ 181 (47/53)	64/69 (48/52)	0.81	106/101 (51/49)	40/41 (49/51)	0.85
Duration of diabetes (Years)	14.9 ± 4.2	15.2 ± 4.8	0.61	16.2 ± 5.1	16.7 ± 3.9	0.39	15.0 ± 3.8	14.8 ± 3.6	0.55
Duration of HTN (years)	8.3 ± 4.9	5.6 ± 3.6	<0.001	8.0 ± 4.9	5.4 ± 4.0	0.001	8.5 ± 4.4	5.5 ± 4.7	<0.001
BMI (kg/m2)	24.1 ± 3.0	28.2 ± 3.3	0.001	25.7 ± 3.6	25.8 ± 4.0	0.59	25.1 ± 3.9	24.7 ± 4.0	0.21
SBP (mm Hg)	136.2 ± 14.6	146.3 ± 18.0	<0.001	130.2 ± 12.3	142.6 ± 15.2	0.001	128.9 ± 15.6	145.5 ± 18.0	0.001
DBP (mm Hg)	83.3 ± 7.6	88.9 ± 8.9	0.001	81.0 ± 7.4	81.5 ± 7.4	0.25	76.9 ± 10.2	84.6 ± 10.0	<0.001
HbA1c (%)	8.1 ± 2.3	8.9 ± 1.4	<0.001	8.2 ± 1.6	8.9 ± 2.0	0.002	8.2 ± 1.9	8.9 ± 1.6	<0.001
S.creat (mg/dl)	1.18 ± 0.15	4.12 ± 2.01	<0.001	1.5 ± 0.3	4.0 ± 1.3	<0.001	1.0 ± 0.3	4.6 ± 1.2	<0.001
eGFR (ml/min)	98.4 ± 18.6	58.7 ± 33.7	0.002	98.3 ± 16.3	57.3 ± 35.5	0.003	98.6 ± 17.7	54.8 ± 18.0	0.002
тс	260.1 ± 42.3	255.3 ± 46.3	0.85	264.3 ± 40.6	253.6 ± 48.8	0.77	251.1 ± 44.0	255.9 ± 36.6	0.89
TG (mg%)	208.3 ± 41.2	204.6 ± 32.8	0.79	207.4 ± 40.2	204.3 ± 30.1	0.65	203.6 ± 42.3	207.7 ± 31.0	0.75
HDL (mg%)	61.3 ± 14.4	64.4 ± 14.0	0.46	62.1 ± 13.8	63.7 ± 11.8	0.35	61.7 ± 12.5	62.7 ± 13.5	0.71
LDL-C (mg/dl)	98.6 ± 30.1	102.3 ± 41.6	0.66	97.6 ± 29.0	101.3 ± 27.7	0.36	97.1 ± 26.5	101.0 ± 26.3	0.65

M: Male; F: Female; BMI: Body Mass Index; HTN: Hypertension; SBP: Systolic Blood Pressure; DBP: Diastolic Blood Pressure; S.creat: Serum Creatinine; eGFR: Estimated Glomerular Filtration Rate by MDRD Formula; TC: Total Cholesterol; TG: Triglyceride; LDL-C: Low Density Lipoprotein Cholesterol; HDL-C: High Density Lipoprotein Cholesterol; provide is significant).

*P value is after adjustment for multivariate regression analysis. Values are mean ± SD; unless indicated otherwise

 Table 2: Baseline characteristics of study subjects.

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Genetic analysis

All the polymorphisms examined in the present study were in Hardy–Weinberg equilibrium in all the three Groups from all the three cohorts (p>0.05) except T-786C (rs 2070744) where significant deviation from HWE of genotype distribution was observed in the present population. Significant deviation from Hardy Weinberg equilibrium was also observed in 200 healthy subjects without diabetes or other co morbidities in T-786C (rs 2070744). Genotype frequencies of the T-786C (rs 2070744), G894T (rs 1799983) and 27VNTR (a/b) SNPs are given in table 3. In all the three cohorts, the frequency of ba, aa and ba+aa genotype of 27VNTR(a/b) was found to be significantly higher as compared to bb genotype in Groups compared with and without DNP, with a high-risk ratio in the DNP Group as compared to DM Group. No association with increased or decreased risk of DNP was observed with respect to other *eNOS* genotypes (T-786C, G894T) in any of the three cohorts.

Biochemical analysis

In all the three cohorts, serum NO levels were significantly decreased in DNP Group as compared to DM Group. We observed lower mean NO levels in T2DM subjects (both with and without DNP) carrying ba+aa Genotype of 27VNTR (a/b) as compared to bb genotype. No significant difference in serum NO levels was observed with respect to other *eNOS* genotypes i.e. T-786C, G894T (Table 4) in any of the three cohorts.

Haplotype analysis

In all the three cohorts, Frequency of haplotypes C-a-G and C-a-T (allele of T-786C, 27VNTR a/b and G894T) carrying 'a' allele of 27VNTR (a/b) and C allele of T-786C was significantly higher in patients having DNP as compared to patients having DM and these haplotypes were associated with a increased risk of DNP. No association with increased or decreased risk of DNP was observed with respect to other six *eNOS* haplotypes (Table 5) in any of the three cohorts.

			Cohort 1			Cohort 2			Cohort 3	
Polymorphisms	Healthy Controls (n=200)	DM(356)	DNP (139)	OR (95%CI) P	DM (342)	DNP (133)	OR (95%CI) P	DM (207)	DNP (81)	OR (95%CI) P
T-786C (rs 2070744)	TT=131 (0.66) TC=67 (0.33) CC=2 (0.01) TC+CC=69 (0.34) T=329 (0.82) C=71 (0.18)	TT=199 (0.56) TC=121 (0.34) CC=36 (0.10) TC+CC=157 (0.44) T=519 (0.73) C=193 (0.27)	TT=72 (0.52) TC=50 (0.36) CC=17 (0.12) TC+CC=67 (0.48)\ T=194 (0.70) C=84 (0.30)	1.22 (0.76- 1.89) 0.79 1.30 (0.65- 2.90) 0.72 1.11 (0.70- 1.90) 0.61 1.16 (0.86- 1.58) 0.37	TT=171 (0.50) TC=130 (0.38) CC=41 (0.12) TC+CC=166 (0.50) T=472 (0.69) C=212 (0.31)	TT=66 (0.50) TC=52 (0.39) CC=15 (0.11) TC+CC=67 (0.50) T=184 (0.69) C=82 (0.31)	1.12 (0.80- 1.55) 0.98 1.15 (0.66- 1.90) 1.02 1.12 (0.81- 1.55) 0.80 0.99 (0.73- 1.35) 1	TT=107 (0.52) TC=75 (0.36) CC=25 (0.12) TC+CC=100 (0.48) T=289 (0.70) C=125 (0.30)	TT=40 (0.49) TC=31 (0.39) CC=10 (0.12) TC+CC=41 (0.51) T=111 (0.69) C=51 (0.31)	1.22 (0.85- 1.45) 0.90 1.11 (0.62- 1.98) 0.94 1.20 (0.83- 1.61) 1.02 1.06 (0.72- 1.57) 0.84
G894T (rs 1799983)	GG=102 (0.51) GT=80 (0.40) TT=18 (0.09) GT+TT=98 (0.49) G=284 (0.71) T=116 (0.29)	GG=156 (0.44) GT=164 (0.46) TT=36 (0.10) GT+TT=200 (0.56) G=476 (0.67) T=236 (0.33)	GG=65 (0.47) GT=60 (0.43) TT=14 (0.10) GT+TT=74 (0.53) G=190 (0.68) T=88 (0.32)	0.94 (0.59- 1.60) 0.79 0.94 (0.42- 222) 0.89 0.95 (0.59- 1.52) 0.78 0.93 (0.69- 1.26) 0.71	GG=157 (0.46) GT=144 (0.42) TT=41 (0.12) GT+TT=185 (0.54) G=458 (0.67) T=226 (0.33)	GG=63 (0.47) GT=57 (0.43) TT=13 (0.10) GT+TT=70 (0.53) G=183 (0.69) T=83 (0.31)	1.08 (0.72- 1.52) 1.05 0.80 (0.41- 1.52) 0.62 1.20 (0.77- 1.48) 0.95 0.92 (0.68- 1.25) 0.64	GG=97 (0.47) GT=87 (0.42) TT=23 (0.11) GT+TT=110 (0.53) G=281 (0.68) T=133 (0.32)	GG=39 (0.48) GT=34 (0.42) TT=8 (0.10) GT+TT=42 (0.52) G=112 (0.69) T=50 (0.31)	1.03 (0.75- 1.51) 0.89 0.97 (0.42- 1.51) 0.87 0.90 (0.71- .1.42) 0.95 0.94 (0.64- 1.40) 0.84
27 VNTR	bb=7 (0.03) ba=49 (0.25) aa=144 (0.72) ba+aa=193 (0.97) b=63 (0.16) a=337 (0.84)	bb=217 (0.61) ba*=121 (0.34) aa*=18 (0.05) ba+aa*=139 (0.39) b=555 (0.78) a*=157(0.22)	bb=61 (0.44) ba*=63 (0.45) aa*=15 (0.11) ba+aa*=78 (0.56) b=185 (0.67) a*=93 (0.33)	1.85 (1.22- 2.81) 0.005 2.96 (1.41- 3.22) 0.006 2.00 (1.34- 2.97) 0.008 1.77 (1.31- 2.41) 0.003	bb=202 (0.59) ba*=121 (0.35) aa*=21 (0.06) ba+aa*=142 (0.41) b=523 (0.76) a*=161 (0.24)	bb=59 (0.44) ba*=57 (0.43) aa*=17 (0.13) ba+aa*=74 (0.56) b=175 (0.66) a*=91 (0.34)	1.61 (1.05- 2.47) 0.04 2.77 (1.37- 3.59) 0.006 1.78 (1.19- 2.67) 0.006 1.69 (1.24- 2.30) 0.001	bb=117 (0.57) ba*=75 (0.36) aa*=14 (0.07) ba+aa*=89 (0.41) b=310 (0.75) a*=104 (0.25)	bb=37 (0.46) ba*=33 (0.41) aa*=11 (0.13) ba+aa*=44 (0.54) b=107 (0.66) a*=55 (0.34)	1.39 (1.07- 2.42) 0.03 2.48 (1.84- 3.92) 0.006 1.56 (1.10- 2.61) 0.62 1.53 (1.13- 2.27) 0.004

*P<0.05 P values for the model are adjusted for potential confounders which include age, gender, HbA1c, duration of diabetes, duration of hypertension, smoking, systolic blood pressure, triglyceride levels, ACEI/ARB, serum creatinine and eGFR

Table 3: Genotype and Allele frequencies of eNOS variants in T2DM patients (DM vs. DNP).

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	Cohort 1										(Cohort	2					Cohort 1									
NO levels (nmol/l)	T-786	6C		27VN	ITR		G894	т		T-78	6C		27VN	ITR		G894	ŧТ		T-78	6C		27VN	ITR		G894	Г	
	TT	TC+CC	Р	bb	ba+aa	P*	TT	TC+CC	Р	TT	TC+CC	Р	bb	ba+aa	P*	TT	TC+CC	Р	TT	TC+CC	Р	bb	ba+aa	P*	TT	TC+CC	Ρ
DM	0.62	0.64 ±	0.15	0.75	0.50 ±	0.03	0.63	0.65 ±	0.22	0.60	0.62 ±	0.14	0.76	0.52 ±	0.02	0.66	0.67 ±	0.18	0.64	0.67 ±	0.14	0.70	0.55 ±	0.01	0.65	0.63 ±	0.20
	±	0.14		±	0.17		±	0.20		±	0.10		±	0.15		±	0.14		±	0.15		±	0.14		±	0.10	
	0.12			0.18			0.18			0.11			0.12			0.19			0.14			0.11			0.15		
DNP	0.33	0.35 ±	0.12	0.42	0.28 ±	0.04	0.38	0.36 ±	0.20	0.32	0.33 ±	0.10	0.43	0.29 ±	0.03	0.40	0.37 ±	0.10	0.35	0.36 ±	0.11	0.40	0.30 ±	0.02	0.37	0.38 ±	0.18
	± 0.13	0.15		± 0.17	0.17		± 0.14	0.12		± 0.11	0.12		± 0.15	0.17		± 0.12	0.14		± 0.14	0.14		± 0.12	0.16		± 0.16	0.14	

Values are mean ± standard deviation, *P<0.05 is significant

Table 4: eNOS genotypes and NO levels.

		Cohort 1			Cohort 2		Cohort 3				
Haplotype ^a	DNP (139)	DM(356)	OR (95%CI) P	DNP (133)	DM (342)	OR (95%CI) P	DNP (81)	DM (207)	OR (95%CI) P		
T-b-G	17 (0.12)	46 (0.13)	Reference	15 (0.11)	44 (0.13)	Reference	10 (0.12)	29 (0.14)	Reference		
T-b-T	18 (0.14)	53 (0.15)	1.09 (0.82-1.55) 0.88	16 (0.12)	48 (0.14)	0.92 (0.70-1.32) 0.88	11 (0.14)	33 (0.16)	0.99 (0.68-1.36) 0.22		
C-b-G	13 (0.09)	39 (0.11)	1.23 (0.99-1.59) 0.21	15 (0.11)	44 (0.13)	1.13 (1.02-1.80) 0.12	8 (0.10)	25 (0.12)	1.25 (0.92-1.77) 0.31		
T-a-G	13 (0.09)	39 (0.11)	0.69(0.30-1.37) 0.55	13 (0.10)	41 (0.12)	1.22 (0.79-1.54) 0.91	8 (0.10)	27 (0.13)	0.93 (0.55-1.34) 0.77		
C-b-T	22 (0.16)	61 (0.17)	0.69 (0.35-1.44) 0.67	21 (0.17)	65 (0.19)	0.79 (0.44-1.14) 0.37	12 (0.15)	35 (0.17)	0.77 (0.42-1.11) 0.45		
T-a-T	24 (0.17)	68 (0.19)	1.12 (0.88-1.81) 0.13	23 (0.17)	58 (0.17)	1.55 (1.21-1.92) 0.12	13 (0.16)	38 (0.18)	0.99 (0.59-1.55) 1.02		
C-a-G*	17 (0.12)	25 (0.07)	1.55 (1.22-2.80) 0.01	15 (0.11)	21 (0.06)	1.37 (1.20-2.94) 0.01	10 (0.12)	10 (0.05)	1.65 (1.42-2.91) 0.01		
C-a-T*	15 (0.11)	25 (0.07)	1.71 (1.31-2.96) 0.008	15 (0.11)	21 (0.06)	1.71 (1.47-2.98) 0.009	9 (0.11)	10 (0.05)	1.77 (1.57-2.94) 0.007		

^a Order of SNPs in *eNOS* haplotypes: T-786C, a 27VNTR b and G894T. *P<0.05

Table 5: eNOS gene Haplotype Frequency Distribution (DM vs. DNP).

Cohort 1						Col	nort 2			Cohort 3				
Variant 1	Variant 2	D'	LOD	r ²	Variant 1	Variant 2	D'	LOD	r ²	Variant 1	Variant 2	D'	LOD	r ²
T-786C	27VNTR	0.02	0.04	0.00	T-786C	27VNTR	0.03	0.06	0.01	T-786C	27VNTR	0.02	0.07	0.00
T-786C	G894T	0.10	0.14	0.01	T-786C	G894T	0.09	0.22	0.01	T-786C	G894T	0.08	0.20	0.01
27VNTR	G894T	0.03	0.04	0.00	27VNTR	G894T	0.02	0.03	0.00	27VNTR	G894T	0.02	0.02	0.01

Table 6: Measures of LD (D', LOD, and r²), observed as a pair-wise comparison for the three polymorphisms of the eNOS gene.

Linkage Disequilibria (LD) analysis

LD values were generated to look for association among the three studied polymorphisms (Figure 1). A pair-wise comparison of the polymorphisms, depicting the LD measures is represented in table 6. In all the three cohorts, no significant LD was observed among the polymorphisms (T-786C, 27VNTR, G894T) (Table 6).

Discussion

We observed that *eNOS* genotype carrying 'aa', 'ba', 'ba+aa' genotype of 27VNTR (a/b), 'a' allele of 27VNTR (a/b) and *eNOS* haplotype C-a-G and C-a-T (allele of T-786C, 27VNTR a/b and G894T) carrying 'a' allele of 27VNTR (a/b) and C allele of T-786C were associated with increased risk of DNP. Moreover, the present study fulfills most of the criteria of a good genetic association study, as suggested by Hattersley and McCarthy [30]. All the study population was in HWE, however, significant deviation from HWE of genotype distribution in the present population in T-786C gene polymorphism may be due to moderate population size. High frequency of mutation occurring at the specific loci can also cause deviation from equilibrium

of genotype distribution in the present population. Moreover, we excluded the possibility of a typing error (LOD Score>zero). Also, we screened one control group composed by 200 healthy subjects without diabetes or other co morbidities to help us elucidate the plausible fact that Asian Indians are not in HWE for this variant, moreover, as we used the same conditions for the PCR-RFLP method as described by Ahluwalia et al. [31] and genotype distribution in their study was also not in HWE. Moreover, positive association observed between *eNOS* SNPs and DNP was not due to chance, as this association was replicated in three independent cohorts and persisted even after the influence of confounding factors was corrected, also the sample size was predetermined for the three polymorphisms to avoid type 1 error [32,33]. Also, our study has the statistical power to detect influence of these polymorphisms on the course of DNP.

The mechanisms responsible for the association between the *eNOS* gene polymorphisms and the risk of DNP are not completely known. We observed lower serum NO levels in T2DM subjects (both cases and controls) carrying ba+aa genotypes. The graded differences in NO generation could play a significant role in the pathophysiological

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condition at the microvascular level. Lower NO level could accelerate the progression of microvascular complications of diabetes, presumably through mechanisms, such as increased vascular tone and enhancement of angiotensin II effects [34,35]. aa 27VNTR bb polymorphism is known to affect the activity of eNOS and has been found to be associated with a significant reduction in the eNOS gene promoter activity [36]; lower eNOS mRNA and serum nitrite/nitrate levels have been found in individuals with the 27VNTR aa variant in some studies [37-39] and 27VNTR aa variant has also been found to be associated with a significant reduction in the eNOS gene promoter activity [36]. However, we were aware that serum NO is not necessarily correlated with eNOS activity due to the fact of presence of three NOS activities that may contribute in the serum NO level; eNOS, inducible NOS (iNOS) and neuronal NOS (nNOS). In fact, NO level is affected more by the iNOS activity which might be enhanced in systemic inflammation associated with the disease. This might explain why this study contradicts our previous work performed with the participation of the same cohort of patients [40], where in the mean NO levels in T2DM subjects were significantly higher in carriers of the "ba+aa" genotype of 27VNTR gene compared to "bb" genotype which contradicts the principal that SNP may affect the enzyme in one direction only or it may be nonfunctional.

Our study was in disagreement with two previous studies by Manea et al. [22] and Mehrab-Mohseni et al. [23] from Caucasian and Iranian population respectively who observed significantly higher proportion of 4a allele carriers in free complications patients than in DNP patients. However, Asian Indians, an ethnically distinct population, has the leading number of diabetic patients in the world and there are significant genotypic differences between Asian Indians and other ethnic groups belonging to different geographical regions [26]. Nevertheless, additional data to confirm these findings are needed. Moreover, we maintained stringent criteria to define DNP, no regional differences in disease prevalence or allele frequency were observed between recruitment sites. The relatively medium size of our case–control study is a limitation that could introduce type 1 errors; however, very few investigators have access to a large sample size. Haplotype analyses of *eNOS* gene may not reliable due to the small number of subjects in several haplotypes and positive associations observed in the interaction models may be due to combination of SNPs showing positive associations. Also, we were aware that combining the three cohorts would increase the statistical power of the study; however, we were unable to merge the three cohorts as they were independent cohorts enrolled at different time interval.

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