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Original Research Article

Endothelial nitric oxide synthase (*eNOS*) VNTR as a probable marker in type 2 diabetes mellitus

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Abstract

Background: The gene encoding *eNOS* is located on chromosome 7q36, a genetic region previously linked to metabolic syndrome, cardiovascular and renal diseases. Generally, in diabetes there are numerous genes involved, each being a small contributor in type 2 diabetes mellitus (T2DM) manifestation. A 27 bp variable number of tandem repeat (27 bp VNTR-a/b) in intron 4 of *eNOS* gene has gained attention and this polymorphism may affect the expression of *eNOS*. We studied the association of *eNOS*-27 bp VNTR with T2DM in north Indian population.

Material and methods: Blood samples were collected in 0.5 M EDTA from 200 T2DM patients and 210 age/sex matched healthy controls. Genomic DNA was extracted from peripheral blood mononuclear cells (PBMCs) using the salting out method. The 27-VNTR polymorphism was determined by standard PCR amplification using forward and reverse primers 5'-AGGCCCTATGGTAGTGCCTTT-3' and 5'-TCTCTTAGTGCTGTGGTCAC-3' respectively. The genotypes were determined by analyzing the amplified products on 2% agarose gels. Genotypic and allelic frequencies were calculated by SPSS (version 15.0).

Results: Clinical and biochemical profiles of healthy controls and T2DM cases as well as gender wise comparisons showed significant association in certain parameters ($P < 0.001$). Five different alleles (I, II, IV, V and VI) were found in the study population. The genotypic frequency was significantly associated with T2DM ($P < 0.001$).

Conclusion: A significant role of allele 'I' in T2DM susceptibility was an interesting observation. Therefore, The 27 bp VNTR in *eNOS* gene polymorphism can be used as a probable marker in determining susceptibility to T2DM in north Indian population.

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Key words

Endothelial nitric oxide synthase, eNOS, Type 2 diabetes mellitus, 27 bp VNTR polymorphism, North Indian population.

Introduction

Type 2 diabetes mellitus (T2DM) is a metabolic disorder which is characterized by hyperglycemia, insulin resistance and insulin deficiency. It is a chronic disease caused by genetic and environmental factors which decreases the life span up to ten years [1, 2]. According to International Diabetes Federation (IDF) Diabetes Atlas 5th edition, 2012 update, 371 million people have been reported with T2DM and the number is expected to rise to >552 million by 2030. The 2012 Indian statistics showed 63.0 million diabetic cases and a prevalence of 8.37% in adult population [3] while a 4.0% prevalence of T2DM was reported in North Indian population [2].

The process of endothelial dysfunction takes place by modulation of nitric oxide synthase (NOS) enzymes responsible for NO synthesis, an important molecular mediator of many physiological processes in virtually every organ. The three distinct isoforms of NOS are endothelial constitutive NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) [4, 5, 6]. Endothelium-derived NO is produced from L-arginine by endothelial nitric oxide synthase (eNOS). Impaired NO production has been implicated in the pathogenesis of several diseases. Endothelial dysfunction caused by nitric oxide (NO) impairment is also regarded as an early step in the development of insulin resistance, atherosclerosis and T2DM [7, 8, 9, 10, 11]. NO inhibits platelet aggregation, leukocyte adhesion to vascular endothelium and oxidation of low density lipoprotein (LDL) which in case of T2DM gets trapped in the arteries. This internalization of Ox-LDL in the subendothelial spaces of arteries leads to formation of foam cells and cholesterol engorged cells, the hallmark of early atherosclerotic lesions [12].

The *eNOS* gene is located on chromosome 7q36, it is linked to several complications like metabolic syndrome, cancer, cardiovascular and renal diseases [12, 13, 14]. Generally in diabetes there are numerous genes involved, each being a small contributor to T2DM manifestation [15, 16, 17, 18, 19]. A 27 bp variable number of tandem repeat (27 bp VNTR-a/b) in intron 4 of *eNOS* gene has gained attention and this polymorphism is possibly because of altered NO availability [20, 21, 22, 23, 24, 25]. Lots of controversies have been associated with the study of 27 bp VNTR polymorphism in Caucasians and Asians [26, 27]. Therefore, the present study was undertaken to see the effect of this VNTR on T2DM susceptibility in North Indian population.

Material and methods

Patient selection and clinical evaluation

T2DM patients (n=200) were enrolled from the outpatient Diabetes Clinic of King George's Medical University (KGMU), Lucknow, India, under the supervision of expert clinicians. Normal controls (n=210) matched for age and sex were screened from healthy staff members of both universities. The study was approved by the Institutional Ethics Committee of KGMU (No-1234/R-Cell-10; Dated 18/08/10; Ref. Code XLIVECM/A-P6) and written informed consent was taken from all subjects enrolled in the study. Controls showing a normal oral glucose tolerance test were included in the study, whereas those having a history of coronary artery disease or other metabolic disorders were

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excluded. Subjects with fasting glucose concentrations >126 mg/dl or 2-h glucose concentrations >200 mg/dl after a 75 g oral glucose tolerance test were categorized in the diabetes group [28]. Medical records of these patients were reviewed to ascertain diabetes-associated complications. A self-administered questionnaire was used to record the clinical history of diabetes, associated complications such as hypertension *etc*. All patients were on oral hypoglycemic agents to maintain a normal glucose level in their blood. Plasma glucose (mg/dl), serum insulin (mg/dl), and lipid profile were estimated using commercially available *Ecoline* kits (Merck) by a double-beam spectrophotometer. Height, weight, and waist circumference were measured to calculate body mass index and waist–hip ratio. Clinical details of patients and controls were recorded.

DNA extraction and genotyping

Blood samples were collected using 0.5 M EDTA as anticoagulant and stored at -20^{0}C until further use. Genomic DNA was extracted from peripheral blood mononuclear cells (PBMCs) using the salting out method with slight modifications [29]. The 27 bp-VNTR polymorphism in intron 4 of *eNOS* gene was determined by standard PCR amplification using the primers 5'-AGGCCCTATGGTAGTGCCTTT-3 (forward) and 5'-TCTCTTAGTGCTGTGGTCAC-3 (reverse). The genotypes were determined by PCR products visualized on 2% agarose gels. In order to ensure accuracy of genotyping, coded blind replicate samples (20%) were included in each assay. Genotypic data was subjected to statistical analyses.

Statistical analysis

Allele frequency was calculated as the number of occurrences of test allele in the population divided by the total number of alleles. Carriage rate was calculated as the number of individuals carrying at least one copy of test allele divided by the total number of individuals. Allele frequencies, genotype frequencies and carriage rates of the alleles in all the groups were compared by using Fisher's exact test. The Hardy-Weinberg equilibrium at individual locus was assessed by $\chi 2$ statistics using SPSS (version 15.0) and clinical association was calculated by paired *t*-test. All P-values were two sided and differences were considered statistically significant for $p < 0.05$. Odds ratio (OR) at 95% confidence interval (CI) was determined to describe the strength of association by logistic regression model. Multiple logistic regression analysis was performed to compare the biochemical parameters with individual genotypes.

Results

Clinical and biochemical profiles of healthy controls and T2DM cases were as per **Table - 1**. Age, fasting glucose (FG), post-prandial (PP) glucose and low density lipoproteins (LDL) showed highly significant association in T2DM cases when compared to controls. Gender wise comparisons also showed significant association ($P < 0.001$). **(Table - 1)** Body Mass Index (BMI) and Total Cholesterol (TC) showed highly significant association in the study population. In males, TC, TGL and VLDL showed significant association while in females, BMI and LDL showed highly significant association ($p < 0.001$). **(Table - 1)**

eNOS VNTRs were successfully genotyped and the representative gels were as per **Figure - 1**. The wild type allele (five copies of 27 bp repeats-'b' allele) and mutant allele (four copies of 27 bp repeats-'a'allele) generated 420 and 393 bp fragments respectively. All allele and genotype frequencies were found to be in Hardy–Weinberg equilibrium.The number of each type of allele and combinations in both cases and controls were as per **Table - 2**.

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Genotypic frequency showed significant association in our population and increased the T2DM susceptibility up to 1.226 times (p=0.006). **(Table - 2)** No association was observed in allele frequencies. However, carriage rate of allele 'I' showed significant association in our population. **(Table - 2)** In the clinical and biochemical profiles of healthy controls and T2DM cases with particular genotypes of *eNOS* VNTR, allele 'I' showed significant association with PP, LDL; allele 'IV' with age, BMI, F, PP, LDL; allele 'V' with age, F, PP, TC, TGL, LDL, VLDL and allele 'II/V' with age, F, PP, TC, LDL. **(Figure - 2)**

Discussion

Endothelial nitric oxide synthase, a key regulator of vascular nitric oxide production, has been investigated extensively to determine the relevance of DNA variants in *eNOS* gene to vascular and renal diseases [27, 30]. Endothelium derived NO plays a key role in the regulation of vascular tone and has vasoprotective effects by scavenging superoxide radicals and suppressing platelet aggregation, leukocyte adhesion and smooth muscle cell proliferation [31, 32]. Several polymorphisms have been reported in *eNOS* promoter, exonic and intronic regions. The variants in the promoter region (T-786), intron-4 (27bp-VNTR) and exon-7 (Glu298Asp) have been explored in several epidemiological studies [20, 22, 33, 34, 35, 36]. Furthermore, polymorphisms in the *eNOS* gene that lead to decreased *eNOS* expression and NO abnormalities contribute to the development and progression of complications such as advanced diabetic peripheral neuropathy (DPN) [37]. Studies showed that the *eNOS* minor "4a" allele was significantly higher in Slovenian patients with proliferative diabetic retinopathy (PDR) [38]. However, *eNOS* 27-bp repeat polymorphism was not found to be associated with diabetic retinopathy (DR) in either of the studies [39]. Further, the 27 bp-VNTR exhibited statistically significant association with albumin to creatinine ratio (ACR) in modulating the risk factors related to cardiovascular-renal disease in Mexican Americans [23]. Another study revealed significantly high risk of essential hypertension for individuals who were obese. Although the intron 4b/a polymorphism of *eNOS* gene did not reveal any association with essential hypertension in general, males with a/a genotype showed significantly high risk for developing hypertension [22, 24].

The genetic association studies examining these polymorphisms have been conducted mostly in Caucasians and Asian populations (20, 26, 27, 35, 38, 40]. In the present finding we found that *eNOS* VNTR polymorphism plays a significant role in north Indian population. An interesting observation was that the carriage rate of allele 'I' of *eNOS* 27-bp VNTR has a significant association with T2DM and may increase disease susceptibility. Genetic studies on *eNOS* gene polymorphisms that contribute to T2DM and related complications are in progress.

Conclusion

A significant role of allele 'I' in T2DM susceptibility was an interesting observation. Therefore, The 27 bp VNTR in *eNOS* gene polymorphism can be used as a probable marker in determining susceptibility to T2DM in north Indian population.

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Table - 1: Clinical and biochemical profiles of healthy controls and T2DM cases.

Clinical parameters	Age	WHR	BMI	F	PP	TC	TGL	HDL	LDL	VLDL	SCRT
Controls (n=201)	40.122 ± 9.647	1.093 ± 1.914	22.969 ± 2.707	83.881 ± 7.035	139.444 ± 9.816	190.560 ± 23.941	119.291 ± 27.586	45.490 ± 8.809	60.040 ± 18.889	23.858 ± 5.517	1.023 ± 0.130
Cases (n=200)	49.689 ± 10.147	0.977 ± 0.683	24.634 ± 4.970	172.619 ± 73.776	265.498 ± 107.415	209.877 ± 43.600	116.216 ± 21.229	45.335 ± 8.141	135.984 ± 53.591	23.286 ± 4.326	1.050 ± 0.097
***p*-value**	**<0.001**	0.453	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.251	0.867	**<0.001**	0.290	0.059
Controls (Males) (n=123)	40.737 ± 10.285	0.938 ± 0.046	23.051 ± 2.186	83.000 ± 6.621	140.000 ± 9.624	190.293 ± 25.026	121.653 ± 32.864	46.622 ± 10.148	61.751 ± 20.407	24.331 ± 6.572	1.028 ± 0.148
Cases (Males) (n=101)	51.380 ± 10.738	0.947 ± 0.065	22.805 ± 3.703	166.497 ± 73.550	259.480 ± 111.588	218.203 ± 39.539	113.039 ± 19.872	44.668 ± 8.625	148.471 ± 50.075	22.608 ± 3.974	1.061 ± 0.101
***p*-value**	**<0.001**	0.222	0.719	**<0.001**	**<0.001**	**<0.001**	**0.010**	0.183	**<0.001**	**0.010**	0.287
Controls (Females) (n=87)	39.019 ± 8.360	1.370 ± 3.198	22.820 ± 3.469	85.265 ± 7.502	138.571 ± 10.148	191.043 ± 22.061	115.012 ± 12.797	43.440 ± 5.105	56.940 ± 15.472	23.002 ± 2.559	1.015 ± 0.090
Cases (Females) (n=99)	47.927 ± 9.222	1.006 ± 0.961	26.401 ± 5.401	179.160 ± 73.961	272.884 ± 102.853	200.793 ± 46.168	119.682 ± 22.213	46.062 ± 7.560	121.623 ± 54.201	24.027 ± 4.589	1.035 ± 0.092
***p*-value**	**<0.001**	0.316	**<0.001**	**<0.001**	**<0.001**	0.152	0.164	**0.026**	**<0.001**	0.138	0.241

Age (years); BMI - body mass index (kg/m2); F - fasting blood sugar (mg/dl); PP - post prandial blood sugar (mg/dl); TC - total cholesterol (mg/dl); TGL - triglycerides (mg/dl); HDL - high density lipoproteins (mg/dl); LDL - low density lipoproteins (mg/dl); VLDL - very low density lipoproteins (mg/dl); SCRT - serum creatinine (mg/dl); WHR - waist hip ratio

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Table - 2: Genotypic, allelic and carriage rate frequencies of *eNOS* 27 bp-VNTR polymorphism in healthy controls (n=210) and T2DM cases (n=200).

Genotype Frequency				
	Controls	**Cases**	***p*-Value**	**OR CI (95%)**
I	2	4	**0.006**	**1.226 (1.059-1.420)**
IV	112	86		
V	85	82		
VI	0	2		
I/IV	0	6		
II/IV	0	2		
II/V	11	6		
IV/V	0	11		
V/VI	0	1		
Allele Frequency				
I	4	14	0.925	1.010 (0.819-1.245)
II	11	8		
IV	224	191		
V	181	182		
VI	0	5		
Carriage Rate				
I (+)	2	10	**0.030**	**0.183 (0.040-0.844)**
I (-)	208	190		
II (+)	11	8	0.552	1.327 (0.522-3.369)
II (-)	199	192		
IV (+)	112	105	0.866	1.034 (0.702-1.524)
IV (-)	98	95		
V (+)	96	99	0.443	0.859 (0.583-1.266)
V (-)	114	101		
VI (+)	0	3	0.999	0.000 (0.000-~)
VI (-)	210	197		

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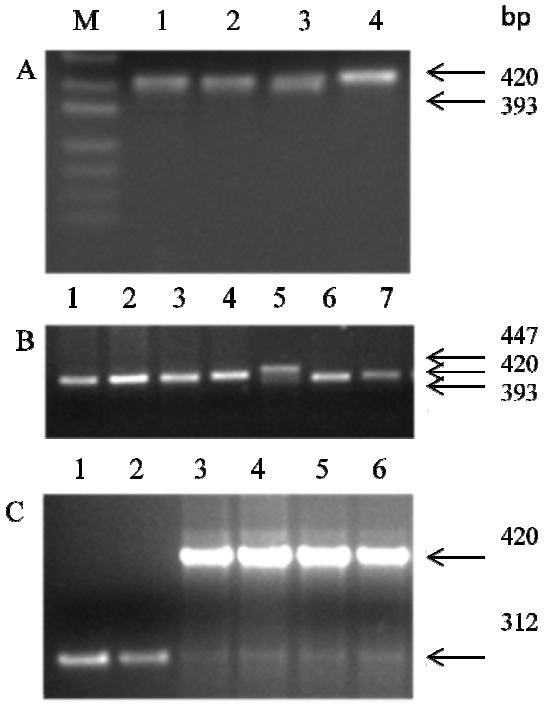

Figure 1: 27 bp VNTRs of *eNOS* gene in 2% agarose gel.
(A) Lanes 1-3 (allele 'IV' 393bp) and Lane 4 (allele 'V' 420bp)
(B) Lanes 1, 2, 6 (allele 'IV' 393bp), Lanes 3, 4, 7 (allele 'V'420bp) and Lane 5 (allele 'VI' 447bp)
(C) Lanes 1, 2 (allele 'I' 312bp) and Lanes 3-6 (allele 'V' 420bp)
M- 50bp DNA Ladder

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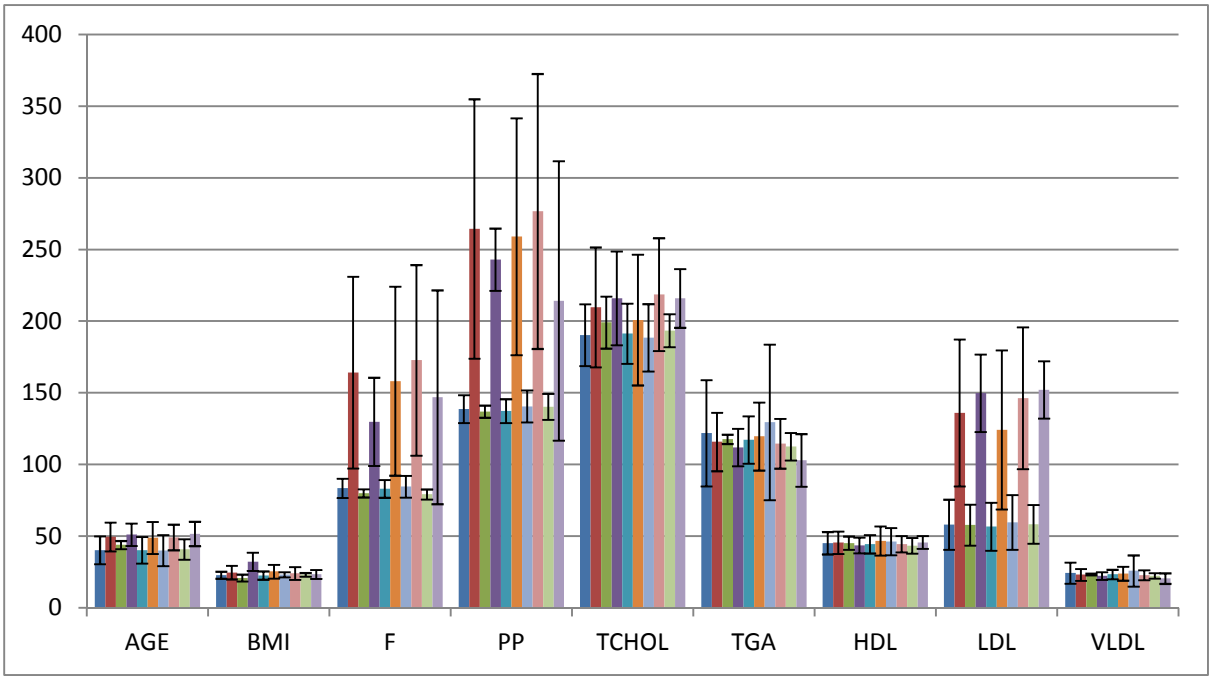
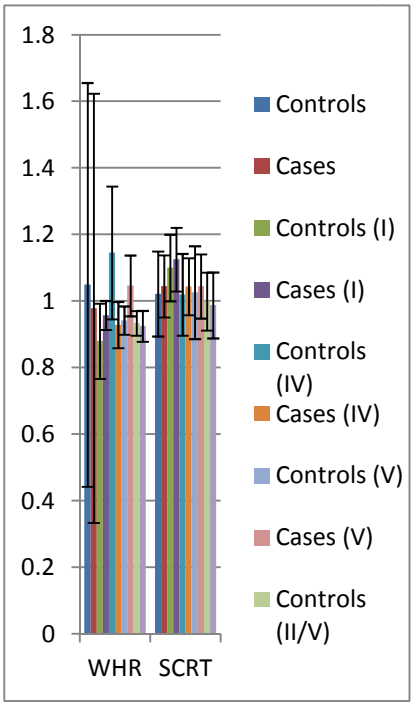
Figure - 2: Clinical and biochemical profile of healthy controls and T2DM cases of individual genotypes of eNOS VNTRs.

Age (years); BMI - body mass index (kg/m2); F - fasting blood sugar (mg/dl); PP - post prandial blood sugar (mg/dl); TC - total cholesterol (mg/dl); TGL - triglycerides (mg/dl); HDL - high density lipoproteins (mg/dl); LDL - low density lipoproteins (mg/dl); VLDL - very low density lipoproteins (mg/dl); SCRT - serum creatinine (mg/dl); WHR - waist hip ratio.





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