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Endothelial Nitric Oxide Synthase *(eNOS)* gene polymorphisms and their association with Type 2 diabetes related traits in Mexican Americans

Farook Thameem[1,3,*], **Sobha Puppala**[5], **Nedal H. Arar**[1,3,4], **Michael P. Stern**[2], **John Blangero**[5], **Ravindranath Duggirala**[5], and **Hanna E. Abboud**[1,3,4]

[1]Division of Nephrology, Department of Medicine, University of Texas Health Science Center, San Antonio, TX 78229, USA

[2]Division of Clinical Epidemiology, Department of Medicine, University of Texas Health Science Center, San Antonio, TX 78229, USA

[3]George O'Brien Kidney Research Center, University of Texas Health Science Center, San Antonio, TX 78229, USA

[4]South Texas Veterans Healthcare System, San Antonio, TX 78229, USA

[5]Department of Genetics, Southwest Foundation for Biomedical Research, San Antonio, TX 78227, USA

Abstract

Genetic variants of the *eNOS* gene such as T-786C, Glu298Asp, and 27bp-VNTR have been examined for their association with type 2 diabetes (T2DM)-related traits in different populations but not in Mexican Americans. However, the results from such studies have been controversial. This study investigated whether these three polymorphisms are associated with T2DM and its related traits in Mexican Americans, a population at high risk for T2DM and its complications. The study participants (N = 670; 39 families) were genotyped for the three polymorphisms using polymerase chain reaction followed by restriction fragment length polymorphism assay. Association analyses between these polymorphisms and T2DM and its related phenotypes were carried out using a measured genotype approach as implemented in the computer program SOLAR. Of the variants examined, only the 27bp-VNTR variant exhibited significant association with high density lipoprotein cholesterol (HDL-C) ($P = 0.04$) and diastolic blood pressure (DBP) levels ($P = 0.02$) after accounting for trait-specific covariates. The carriers of the rare allele (27bp-VNTR-4a) are associated with decreased HDL-C and increased DBP levels. In conclusion, of the genetic polymorphisms examined at the *eNOS* locus, only 27bp-VNTR appears to be a minor contributor to the variation in T2DM-related traits in Mexican Americans.

Keywords

eNOS gene; type 2 diabetes; genetic polymorphisms; association analyses; Mexican Americans

[*]Corresponding author: Farook Thameem Ph.D., Assistant Professor of Medicine, Division of Nephrology; Department of Medicine, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, Texas 78229; USA, Phone: +1-210-567-4787, FAX: +1- 210-567-4712, thameem@uthscsa.edu.

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Introduction

Nitric oxide (NO) plays a fundamental role in the regulation of endothelial function and vascular tone in many organs including the kidney. NO inhibits platelet aggregation, leukocyte adhesion to vascular endothelium, and oxidation of LDL[1]. Endothelium-derived NO is synthesized from L-arginine by NO synthase encoded by endothelial NO synthase (eNOS or NOS3) gene, mapped to chromosome 7q36. Upon release, NO diffuses rapidly through the cell membrane and relaxes neighboring vascular smooth cells through the production of guanine 3'5'-cyclic monophosphate (cGMP). cGMP then activates the protein kinase G family, leading to a cascade of responses at the levels of transcription and translation. The increase in cGMP levels forms the basis for regulation of several physiological functions including relaxation of vascular smooth muscle cells. Impairment of NO production causes endothelial dysfunction contributing to the development of insulin resistance, T2DM, chronic renal failure, and cardiovascular complications including hypertension and hypercholesterolemia[2]. Due to the importance of eNOS in the generation of NO that regulates endothelial-dependent vasodilation in many organs, several epidemiological studies are evaluating whether genetic polymorphisms in the *eNOS* gene, and alteration in eNOS activity and NO availability are associated with T2DM, cardiovascular, and renal diseases.

Over the last few years, several polymorphisms of the *eNOS* gene have been identified, and their association with various diseases has been explored. In particular, a single-nucleotide polymorphism (SNP) in the promoter region (T-786C), a G to T substitution at nucleotide 894 in exon 7 of *eNOS*, which leads to an amino acid change from Glu to Asp at codon 298 (Glu298Asp), and a 27 bp variable number of tandem repeat (27bp-VNTR) polymorphism in intron 4 have received most of the attention, because of their functional relevance to eNOS activity. The T-786C promoter polymorphism reduces the promoter activity and thus affects eNOS protein expression and eNOS activity[3]. The Glu298Asp polymorphism causes a structural change of the eNOS protein and reduces eNOS activity[4]. In the 27-bp repeat of intron 4, two alleles have been identified, the larger of which, *eNOS-4b*, has 5 tandem 27-bp repeats (GAAGTCTAGACCTGCTGC(A/G)GGGGTGAG) and the smaller, *eNOS-4a*, has four repeats. The 27 bp-VNTR reduces plasma concentration of nitric oxide[5]. However, genetic association studies examining T-786C, Glu298Asp, and 27bp-VNTR polymorphisms with cardiovascular and renal diseases have yielded conflicting conclusions[1]. Further, the genetic association studies have been mostly conducted in Caucasian and Asian populations. To date, the genetic epidemiological studies examining these polymorphisms in Mexican Americans for their association with cardiovascular and renal disease related traits are very limited except one study that examined the relevance of only Glu298Asp polymorphism with insulin resistant measures in Mexican Americans[6].

The aim of the present study is therefore to investigate whether the three polymorphisms discussed above are associated with T2DM and relevant subclinical cardiovascular and renal-related phenotypes, using data from the San Antonio Family Diabetes/Gallbladder Study (SAFDGS), a family study of Mexican Americans that explores genetic susceptibility to diabetes and gall bladder disease.

Methods

Study Subjects and Phenotypic data

The SAFDGS family member recruitment and data collection procedures were described previously[7]. Briefly, probands were low income Mexican-Americans with T2DM, and all 1st, 2nd, and 3rd degree relatives of probands were invited to participate in the study. A variety of metabolic, hemodynamic, anthropometric, and demographic variables were

collected from about 700 individuals drawn from 39 large Mexican American families. Studies were conducted at the General Clinical Research Center (GCRC) Laboratory at the South Texas Veterans Health Care System, Audie L. Murphy Memorial Hospital Division, San Antonio, Texas, using standard procedures. Blood samples were obtained after a 12-hour fast for assessment of various phenotypes including glucose, total cholesterol, triglycerides (TGL), and high density lipoprotein cholesterol (HDL-C), and they were collected again 2 h after a standardized oral glucose load to measure plasma glucose. Measurement of all these phenotypes has been described elsewhere[7]. Diabetes status was defined by the 1999 criteria of the World Health Organization (i.e., fasting glucose levels ≥ 126 mg/dl and/or 2-hr glucose levels ≥ 200 mg/dl). Participants who did not meet these criteria but reported to be under treatment with either oral antidiabetic agents or insulin and who gave a history of diabetes were also considered to have T2DM. Urine albumin excretion was expressed as albumin to creatinine ratio (ACR) [8] and analyzed as a continuous variable. The quantitative trait values of triglycerides (*ln* TGL) and ACR (*ln* ACR) was log transformed for the association analyses since their raw data were non-normally distributed. The Institutional Review Board of the University of Texas Health Science Center at San Antonio approved all procedures. All subjects gave informed consent.

Molecular variants genotyping

T-786C promoter polymorphism of eNOS—The genotypes of T-786C polymorphism (rs2070744) located in the 5' flanking region of *eNOS* were determined by standard PCR followed by restriction digestion using the following primers: 5'-TGG AGA GTG CTG GTG TAC CCC A-3 (forward) and 5'-GCC TCC ACC CCC ACC CTG TC-3 (reverse). The amplified products of 180 bp were digested with Msp I (New England Biolabs, MA, USA) at 37 °C producing fragments of 140 and 40 bp for the T allele, or fragments of 90, 50, and 40 bp for C allele. Fragments were separated by 2% agarose gel electrophoresis containing ethidium bromide and visualized under UV light.

Glu298Asp polymorphism in exon 7—The Glu298Asp polymorphisms (rs1799983) in exon 7 of eNOS was determined by PCR followed by restriction digestion using the following primers 5'-AAG GCA GGA GAC AGT GGA TGG A-3 (forward) and 5'-CCC AGT CAA TCC CTT TGG TGC TCA-3 (reverse). The PCR products were restricted by the enzyme *Ban* II (New England Biolabs, MA, USA). The Ban II digestion of the 248 bp PCR amplicon produces 163 bp and 85 bp products for the Glu298 allele, but fails to cleave the 248 bp fragment containing the Asp298 allele. Restricted products were separated on 2% agarose gel electrophoresis.

27bp-VNTR polymorphism in intron 4—Genotypes of the 27-VNTR in intron 4 were determined by standard PCR amplification using the primers 5'-AGG CCC TAT GGT AGT GCC TTT-3 (forward) and 5-TCT CTT AGT GCT GTG GTC AC-3 (reverse). The genotypes were determined by fragments visualized in 3% agarose gel. The wild type allele (five copies of 27 bp repeats – b allele) generated a 420 bp band and the mutant allele (four copies of 27 bp repeats – a allele) generated 393 bp band.

Statistical genetic analysis

The genotypic data were checked for Mendelian pedigree inconsistencies using the programs INFER and GENTEST (PEDSYS programs) [9]. Allele frequencies were estimated, and all polymorphisms were tested for Hardy–Weinberg Equilibrium. We performed association analysis in our family data using the measured genotype approach (MGA) within the variance components (VC) analytical framework[10]. The VC-based approach accounts for the non-independence among family members. In this approach, VCs are modeled as random effects (e.g. additive genetic effects and random environmental effects), whereas the

effects of measured covariates such as age and sex are modeled as fixed effects on the trait mean. The marker genotypes were incorporated in the mean effects model as a measured covariate, assuming additivity of allelic effects[10–12]. The effect of this measured genotype (i.e., association parameter) together with other covariate effects (e.g., age and sex) and VCs were estimated by maximum likelihood techniques. The hypothesis of no association is tested by comparing the likelihood of a model in which the effect of the measured genotype is estimated with a model where the effect of the measured genotype was fixed at zero. Twice the difference in the log-likelihoods of these models yields a test statistic that is asymptotically distributed, approximating a χ^2 distribution with one degree of freedom. A p value ≤ 0.05 is considered significant. Prior to performing MGA, the quantitative transmission disequilibrium test (QTDT) [13] was used to examine hidden population stratification. All statistical techniques described above were implemented in the computer program SOLAR [http://www.sfbr.org/solar/] [11].

Results

Table 1 shows the clinical characteristics of the SAFDGS subjects that have genotypic and phenotypic data for this study. Of the genotyped individuals, the mean age of the study subjects was 45 years, and 61% of them were females. The available phenotypic data for each phenotype varied from 610 subjects for cholesterol to 670 subjects for age. In the total sample, 29% of the subjects had T2DM (Table 1).

Genotyping of T-786C (rs2070744), Glu(298)Asp (rs1799983), and 27bp-VNTR polymorphisms were carried out as described earlier. The allele and genotype frequencies are presented in Table 2. Genotypic data of T-786C, G/T, and 27-bp VNTR were consistent with the Hardy-Weinberg Equilibrium expectations, and there was no evidence for hidden population stratification in the data as tested by QTDT (Table 2).

Association analysis in our family data was performed using the MGA. Of the phenotypes examined for association [T2DM, BMI, systolic blood pressure (SBP), DBP, total cholesterol, HDL-C, *ln* TGL, and *ln* ACR], after adjusting for the trait-specific covariate effects (Table 3), only HDL-C ($P = 0.04$), and DBP ($P = 0.02$) exhibited significant association only with 27bp-VNTR polymorphism (Table 3). Neither T-786C nor the Glu298Asp polymorphism showed significant association with any of the phenotypes examined. Table 4 shows the 27bp-VNTR genotype class-specific mean values of HDL-C and DBP levels. As can be seen, the carriers of a allele (4 repeats of 27bp-VNTR) were found to have decreased HDL-C and increased DBP levels.

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 the *eNOS* gene and vascular-renal diseases. The variants in the promoter region (T-786), intron-4 (27-bp VNTR), and exon-7 (Glu298Asp) have been explored in several epidemiological studies but the association findings have been inconsistent. Also, the genetic association studies examining these polymorphisms have been mostly conducted in Caucasian and Asian populations. To our knowledge, the genetic epidemiologic studies examining these polymorphisms in Mexican Americans for their association with cardiovascular and renal disease-related traits are very limited[6].

There is a marked ethnic difference in the distribution of *eNOS* variants. Tanus-Santos et al.[14] examined the distribution of T-786C, Glu298Asp and 27 bp-VNTR variants of *eNOS* gene in a sample of 305 ethnically different individuals (100 Caucasians, 100 African

Americans, and 105 Asians). They found that the -786C variant was more common in Caucasians (42.0%) than in African Americans (18%) or Asians (14%). In this study, we observed a frequency of 24% for -786C in Mexican Americans. The Asp298 variant was found to be more frequent in Caucasians (35%) than in African Americans (16%) or Asians (9%). The frequency of Asp298 (20%) variant in our Mexican American cohort was comparable to that found in another Mexican American sample[6]. With respect to 27 bp-VNTR polymorphism, the minor allele frequency of a allele (4 repeats) was more common in African Americans (27%) than in Caucasians (16%) or Japanese (13%). The frequency of a allele (4 repeats) in Mexican Americans (10%) was similar to that reported in a Venezuelan Hispanic population[15]. Such ethnic differences in the occurrence of genetic variants at *eNOS* locus may relate to the ethnic-specific predisposition to T2DM and its correlated phenotypes.

In this study, we evaluated the phenotypic association between measures of T2DM and its correlated cardiovascular, renal-related traits and genotypes of T-786, Glu298Asp and 27bp-VNTR polymorphisms of *eNOS* gene in a Mexican American population at high risk for the development of T2DM and related risk factors. While our study failed to find significant association between the T-786C and Glu298Asp polymorphisms and the phenotypes examined, the 27bp-VNTR exhibited nominally significant association with HDL-C and **D**BP measures after adjusting for the trait-specific covariate effects. Our results are in agreement with other reports in Asians and Caucasians[1,16–18]. The 27 bp-VNTR 4 repeats (a allele) carriers were found to have lower HDL-C values and higher DBP values. Few studies[19,20] have reported that the T-786C and 27 bp-VNTR polymorphisms are in perfect linkage disequlibrium (LD). Such a LD pattern between these two polymorphisms was not seen in our study.

This study suggests further examination of the association found between 27bp-VNTR and HDL-C or DBP levels is warranted. There are limitations of the findings in this study. First, we only investigated three polymorphisms that have been extensively examined by other studies, instead of examining comprehensive tagging of all common variations within *eNOS* gene that could influence phenotypic variation in our population. Second, once the issue of multiple testing is considered, the two nominally significant associations found in this study become insignificant. However, we believe that our findings could potentially contribute to any future meta-analyses validating these polymorphisms. In conclusion, of the three *eNOS* polymorphisms examined, there is some evidence for the minor contribution of the 27bp-VNTR polymorphism to the variation in T2DM-related subclinical cardiovascular traits in Mexican Americans.

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References

1. Casas JP, Cavalleri GL, Bautista LE, Smeeth L, Humphries SE, Hingorani AD. Endothelial nitric oxide synthase gene polymorphisms and cardiovascular disease: a HuGE review. Am J Epidemiol. 2006; 164:921–35. [PubMed: 17018701]
2. Wang XL, Wang J. Endothelial nitric oxide synthase gene sequence variations and vascular disease. Mol Genet Metab. 2000; 70:241–51. [PubMed: 10993711]

3. Nakayama M, Yasue H, Yoshimura M, et al. T-786-->C mutation in the 5'-flanking region of the endothelial nitric oxide synthase gene is associated with coronary spasm. Circulation. 1999; 99:2864–70. [PubMed: 10359729]
4. Hingorani AD. Endothelial nitric oxide synthase polymorphisms and hypertension. Curr Hypertens Rep. 2003; 5:19–25. [PubMed: 12530931]
5. Tsukada T, Yokoyama K, Arai T, et al. Evidence of association of the ecNOS gene polymorphism with plasma NO metabolite levels in humans. Biochem Biophys Res Commun. 1998; 245:190–3. [PubMed: 9535806]
6. Guo X, Cheng S, Taylor KD, et al. Hypertension genes are genetic markers for insulin sensitivity and resistance. Hypertension. 2005; 45:799–803. [PubMed: 15699455]
7. Puppala S, Dodd GD, Fowler S, et al. A genomewide search finds major susceptibility loci for gallbladder disease on chromosome 1 in Mexican Americans. Am J Hum Genet. 2006; 78:377–92. [PubMed: 16400619]
8. Lehman DM, Leach RJ, Johnson-Pais T, et al. Evaluation of tight junction protein 1 encoding zona occludens 1 as a candidate gene for albuminuria in a Mexican American population. Exp Clin Endocrinol Diabetes. 2006; 114:432–7. [PubMed: 17039425]
9. Dyke, B. Tech rep. Vol. 2. Population Genetics Laboratory, Department of Genetics, Southwest Foundation for Biomedical Research; San Antonio: 1996. PEDSYS: a pedigree data management system.
10. Boerwinkle E, Chakraborty R, Sing CF. The use of measured genotype information in the analysis of quantitative phenotypes in man. I. Models and analytical methods. Ann Hum Genet. 1986; 50:181–94. [PubMed: 3435047]
11. Almasy L, Blangero J. Exploring positional candidate genes: linkage conditional on measured genotype. Behav Genet. 2004; 34:173–7. [PubMed: 14755182]
12. Richardson DK, Schneider J, Fourcaudot MJ, et al. Association between variants in the genes for adiponectin and its receptors with insulin resistance syndrome (IRS)-related phenotypes in Mexican Americans. Diabetologia. 2006; 49:2317–28. [PubMed: 16955209]
13. Abecasis GR, Cookson WOC, Cardon LR. Pedigree tests of transmission disequilibrium. Eur J Hum Genet. 2000; 8:545–51. [PubMed: 10909856]
14. Tanus-Santos JE, Desai M, Flockhart DA. Effects of ethnicity on the distribution of clinically relevant endothelial nitric oxide variants. Pharmacogenetics. 2001; 11:719–25. [PubMed: 11692081]
15. Hoffmann IS, Tavares-Mordwinkin R, Castejon AM, Alfieri AB, Cubeddu LX. Endothelial nitric oxide synthase polymorphism, nitric oxide production, salt sensitivity and cardiovascular risk factors in Hispanics. J Hum Hypertens. 2005; 19:233–40. [PubMed: 15565175]
16. Benjafield AV, Morris BJ. Association analyses of endothelial nitric oxide synthase gene polymorphisms in essential hypertension. Am J Hypertens. 2000; 13:994–98. [PubMed: 10981549]
17. Pulkkinen A, Viitanen L, Kareinen A, Lehto S, Vauhkonen I, Laakso M. Intron 4 polymorphism of the endothelial nitric oxide synthase gene is associated with elevated blood pressure in type 2 diabetic patients with coronary heart disease. J Mol Med. 2000; 78:372–79. [PubMed: 11043380]
18. Ohtoshi K, Yamasaki Y, Gorogawa S, et al. Association of (-)786T-C mutation of endothelial nitric oxide synthase gene with insulin resistance. Diabetologia. 2002; 45:1594–601. [PubMed: 12436344]
19. Yoshimura M, Yasue H, Nakayama M, et al. Genetic risk factors for coronary artery spasm: Significance of endothelial nitric oxide synthase gene T-786 C and missense Glu298Asp variants. J Investig Med. 2000; 48:367–74.
20. Asakimori Y, Yorioka N, Taniguchi Y, Ito T, Ogata S, Kyuden Y, Kohno N. T(-786) C polymorphism of the endothelial nitric oxide synthase gene influences the progression of renal disease. Nephron. 2002; 91:747–51. [PubMed: 12138283]

Table 1

Clinical characteristics of the genotyped SAFDGS participants used in the present study[a]

Variables	Mean ± SD or %
Females	61
Type 2 diabetes	29
Age (yrs)	44.8 ± 16.2
Systolic blood pressure (mm Hg)	127.1 ± 16.8
Diastolic blood pressure (mm Hg)	70.3 ± 9.6
Body mass index (kg/m^2)	30.9 ± 7.0
Total Cholesterol (mg/dl)	194.0 ± 37.9
High density lipoprotein-Cholesterol (mg/dl)	46.0 ± 12.0
Triglycerides (log transformed)	4.9 ± 0.6
Albumin to Creatinine Ratio (log transformed)	2.4 ± 0.8

[a]Sample size varies from 610 (cholesterol) to 670 (Age).

Table 2

Allele and Genotype Frequency of the selected variants from *NOS3* gene

Polymorphisms	Major/minor allele (%)	Genotype (%)		
T (-786) C	T (76) / C (24)	TT (58)	TC (36)	CC (6)
G / T (Glu298Asp)	G (80) / T (20)	GG (65)	GT (30)	TT (5)
27bp-VNTR – a(4 repeats)/b(5 repeats)	b (90) / a (10)	aa (1)	ab (18)	bb (81)

Table 3

Association analysis between *NOS3* gene polymorphisms and diabetes-related traits in SAFDGS data

Phenotypes	T(-786)C *P* value	Glu(298)Asp *P* value	27bp-VNTR *P* value
Type 2 diabetes[a]	0.49	0.64	0.57
Body mass index[b]	0.58	0.47	0.79
Total Cholesterol[b]	0.86	0.09	0.30
High density lipoprotein cholesterol[b]	0.98	0.20	**0.04**
Triglycerides (log transformed)[b]	0.82	0.62	0.52
Systolic blood pressure[c]	0.81	0.17	0.42
Diastolic blood pressure[c]	0.19	0.27	**0.02**
Albumin to creatinine ratio (log transformed)[d]	0.64	0.40	0.85

[a]Data adjusted for age and sex terms;

[b]Data adjusted for age, sex, diabetes and lipid lowering medications;

[c]Data adjusted for age, sex, diabetes and antihypertensive treatment;

[d]Data adjusted for age, sex, diabetes, duration of diabetes, systolic blood pressure and antihypertensive treatment

Table 4

Mean values of HDL-C and DBP levels by 27bp-VNTR genotype category

Phenotypes[a]	Mean ± SE by genotypes		
	a/a (4 repeats)	a/b	bb (5 repeats)
HDL-C (mg/dl)	37.9 ± 2.6	40.5 ± 1.5	43.1 ± 1.0
DBP (mm Hg)	79.1 ± 1.9	77.0 ± 1.1	74.9 ± 0.8

[a]HDL-C - High density lipoprotein-Cholesterol; DBP - Diastolic blood pressure