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The 27-bp repeat polymorphism in intron 4 (27 bp-VNTR) of endothelial nitric oxide synthase (*eNOS*) gene is associated with albumin to creatinine ratio in Mexican Americans

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Abstract

The T-786C, Glu298Asp, and 27 bp variable number of tandem repeats (27 bp-VNTR-a/b) polymorphsims of the endothelial nitric oxide synthase (*eNOS*) gene are thought to alter nitric

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oxide production and contribute to the development of vascular and renal disease risk. The objective of this study is to investigate whether these three polymorphisms examined previously by others are associated with cardiovascular and renal disease risk in Mexican Americans. Study participants (N = 848; 21 families) were genotyped for T-786C, Glu298Asp, and 27 bp-VNTR-a/b polymorphisms by PCR followed by restriction digestion. Association analyses were performed by a measured genotype approach implemented in the program SOLAR. Of the phenotypes (type 2 diabetes, hypertension, body mass index, waist circumference, total cholesterol, high density lipoprotein cholesterol, triglycerides, systolic and diastolic blood pressure, albumin to creatinine ratio (ACR), and estimated glomerular filtration rate) examined for association, the 27 bp-VNTR-a/b variant exhibited statistically significant association with ACR (P = 0.047) after accounting for the trait specific covariate effects. In addition, the promoter variant (T-786C) showed a significant association with triglycerides (P = 0.034) after accounting for covariate influences. In conclusion, the present study adds evidence to the role of *eNOS* candidate gene polymorphisms in modulating the risk factors related to cardiovascular-renal disease in Mexican Americans although the magnitude of the genetic effect is small.

Keywords

eNOS; Genetic polymorphisms; Association analyses; ACR; Triglycerides; Mexican Americans

Introduction

Nitric oxide (NO) is a gaseous free radical and an important molecular mediator of many physiologic processes in virtually every organ. 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. The gene encoding *eNOS* is located on human chromosome 7q36, a genetic region previously linked to the metabolic syndrome, cardiovascular and renal disease risk factors [1–3]. Due to the importance of NO production in the endothelium and its regulation of vascular and renal function [4, 5], the *eNOS* gene has been considered as a logical target for DNA sequence variations that may contribute to the pathophysiology of cardiovascular and renal diseases. Of the polymorphisms thus far identified in the *eNOS* locus, a T to C single nucleotide polymorphism (SNP) in the promoter region (T-786C, rs2070744), a G to T SNP in exon 7 (Glu298Asp, rs1799983), and a 27 bp variable number of tandem repeats (27 bp-VNTR-a/b) in intron 4, have gained recent attention. The functional effect of these polymorphisms is thought to be a reduction in NO production (27 bp-VNTR-a/b) [6] through reduced mRNA expression (T-786C) [7] or altered eNOS function (Glu298ASP) [8].

Because, the polymorphisms identified in *eNOS* are thought to affect NO production, several epidemiological studies investigated whether the T-786C, G/T (Glu298Asp) and 27 bp-VNTR-a/b polymorphisms affect cardiovascular and renal disease possibly by altered NO availability [9]. Furthermore, the association results, which are controversial, have been mostly conducted in Caucasians and Asians. Therefore, in the present study, we investigated the effects of these variants on individual's susceptibility to type 2 diabetes as well as to risk factors related to cardiovascular and renal diseases in a cohort of Mexican American subjects.

Materials and methods

Subjects and phenotypic data

The San Antonio Family Heart Study (SAFHS) family member recruitment and data collection procedures from more than 40 extended families were described previously [10].

Briefly, probands for the SAFHS were selected randomly from a census tract in San Antonio of low-income Mexican Americans regardless of preexisting medical conditions. A variety of metabolic, hemodynamic, anthropometric, and demographic variables were collected from more than 40 extended Mexican American families [10]. Although a total of 1,400 patients were recruited for SAFHS from 40 families, kidney-related phenotypic data were available for only 848 participants from 21 families, who came to the clinic during their 3rd visit. Therefore, this study involves the 848 subjects from 21 families for whom genotypic and phenotypic data are available. Estimation of glomerular filtration rate (eGFR) by the modification of diet in renal disease (MDRD) equation, and albumin to creatinine ratio (ACR) has already been described [11]. The quantitative trait values were inverse-normalized and used in 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, and all subjects gave informed consent.

Genetic variants genotyping

T-786C promoter polymorphism of eNOS—The genotypes of T-786C (rs2070744) polymorphism located in the 5′ flanking region of *eNOS* was 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—Genotypes of G/T (Glu298Asp; rs1799983) polymorphisms in exon 7 of *eNOS* were 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 G (Glu298) allele, but fails to cleave the 248 bp fragment containing the T (Asp298) allele. Restricted products were separated on 2% agarose gel electrophoresis.

27 bp-VNTR polymorphism in intron 4—The 27-VNTR polymorphism in intron 4 was 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.

In order to assure accuracy of the genotyping, coded blind replicate samples (10%) were included in each assay. Genotypic data with 0% of genotyping error and 0% of inheritance error were subjected to statistical association analyses.

Statistical association analyses

The genotypic data were checked for Mendelian pedigree inconsistencies using the program INFER and GENTEST as implemented in PEDSYS [12]. Association analysis in our family data was carried out using the measured genotype approach (MGA) within the variance components analytical framework [13]. A P value ≤ 0.05 is considered significant. Based on the number of participants, there is 83% power to detect an association that accounts for as little as 1% of the phenotypic variation. Prior to performing MGA, the quantitative transmission disequilibrium test (QTDT) was used to examine hidden population

stratification [14]. All statistical techniques described above were implemented in the program SOLAR [13].

Results and discussion

The clinical characteristics of the genotyped individuals ($N = 848$) are shown in Table 1. The mean age of study participants ($N = 848$; 21 large families) was 45 years, and 61% were females. Of the examined individuals from 21 families, 52%, 22%, and 14% had hypertension, T2DM, and albuminuria, respectively. Genotypic data of T-786C, Glu298Asp, and 27 bp-VNTR-a/b variants were consistent with the Hardy–Weinberg Equilibrium expectations. In order to protect against potential effects of hidden population stratification, we performed the QTDT [14]. This approach permits a formal test of likely population heterogeneity on a marker-specific basis [15]. Using this approach, we found no evidence for such population stratification or admixture factor. Additionally, the results of the QTDT analyses were consistent with the more powerful measured genotype analyses. Allele and genotypic frequencies for T-786C, G/T (Glu298Asp), and 27 bp-VNTR-a/b are presented in Table 2.

Before performing statistical association analysis, we estimated the pairwise linkage disequilibrium (LD; r^2) between all the three variants. The pairwise LD between variants ranged from 0 to 0.25 and the highest pairwise LD found among the *eNOS* SNPs were T-786C—G/T (Glu298Asp; $r^2 = 0.25$), 27 bp-VNTR—G/T (Glu298Asp $r^2 = 0.03$), T-786C—27 bp-VNTR ($r^2 = 0.004$). Of the cardiovascular and renal-related risk factors [T2DM, body mass index, blood pressure measures, total cholesterol, high density lipoprotein-cholesterol, eGFR, TGL, and ACR] examined for association, the 27 bp-VNTR-a/b variant exhibited a statistically significant association only with ACR ($P = 0.047$) after adjusting for the covariate effects of age, sex, diabetes, duration of diabetes, systolic blood pressure, and antihypertensive medications (Table 3). Our results are consistent with several reports that the 27 bp repeat polymorphism significantly predicted different forms of renal disease including end stage renal disease [16–21]. Average values for the phenotypes of ACR and TGL were estimated for the individuals with no risk allele and all three risk alleles. The average ACR values with no risk allele and all three risk alleles were 0.001 and 0.07, respectively. The average TGL values with no risk allele and all three risk alleles were 73.5 and 131.3 mg/dl, respectively.

The mechanism responsible for the association between the 27 bp-VNTR-a/b variant of *eNOS* and variation in albuminuria needs to be elucidated. However, NO is involved in the regulation of renal function including glomerular hemodynamics [22], and mediation of pressure natriuresis [23]. Significantly, deficient renal NO synthesis has been implicated in the pathogenesis of hypertension, albuminuria, and diabetic nephropathy. Prevailing experimental and clinical data suggest that generalized endothelial dysfunction, frequently characterized by decreased NO bioavailability, actually precedes the development of microalbuminuria [24]. In addition, decreased renal NO production accelerates the progression of diabetic nephropathy, presumably through mechanisms such as increased renal vascular tone and potentiation of angiotensin II effects [25]. Taken together, it is reasonable to speculate that the association between the 27 bp-VNTR-a/b and ACR is due to low bioavailability of NO in the kidney [6].

Association analyses also indicated that the T-786C variant located in the promoter region revealed a significant association with TGL ($P = 0.034$) after accounting for the covariates effects of age, sex, diabetes, duration of diabetes, BMI, and lipid medications (Table 3). Although the functional significance of this association needs to be elucidated, endothelial nitric oxide is considered to be an important atheroprotective mediator, and acquired defects

in the generation of NO due to T-786C variation in *eNOS* gene are believed to be associated with the increase in cardiovascular risk [9]. Association analyses failed to find a statistically significant association between the G/T (Glu298Asp) polymorphism and any of the cardiovascular and renal-related risk factors examined (Table 3). The data are in agreement with several reports including another cohort of Mexican Americans [26] and Hispanics [27].

While, the current study is advantageous as it measures multiple cardiovascular-renal related traits in the same individuals in a relatively large sample of Mexican American families, it has limitations in that it attempted to replicate the three variants examined previously by several studies of the *eNOS* gene and has not attempted comprehensive tagging of all common variation within the *eNOS*. In addition, the eGFR estimated by the MDRD formula has not been validated in Mexican Americans. However, the MDRD equation is commonly used to estimate GFR and has been employed in several genetic studies [3, 28, 29]. It is to be noted that while allowing for multiple testing of SNPs preserves the significance of our two best associations, we, however, do not consider corrections for tests across multiple phenotypes since each phenotype can be considered to reflect an unique hypothesis.

In conclusion, the present study adds evidence to the role of *eNOS* candidate gene polymorphisms in modulating the risk factors related to cardiovascular-renal disease in Mexican Americans, although the magnitude of the genetic effects appears to be small.

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Table 1

Clinical characteristics of the genotyped SAFHS participants

Variables	Mean ± SD or %
Females	63
Type 2 diabetes	22
Age (years)	47.9 ± 14.8
Systolic blood pressure (mm Hg)	124.4 ± 19.0
Diastolic blood pressure (mm Hg)	69.8 ± 10.3
Body mass index (kg/m^2)	31.8 ± 7.2
Total cholesterol (mg/dl)	180.6 ± 38.5
High density lipoprotein-cholesterol (mg/dl)	48.2 ± 13.6
Triglycerides (mg/dl)	129.2 ± 86.6
Albumin to creatinine ratio (ACR)	0.06 ± 0.3

Sample size varies from 780 (ACR) to 848 (Age)

Table 2

Allele and Genotype frequencies of the eNOS variants genotyped in SAFHS participants

Polymorphisms	Major/minor allele (%)	Genotype (%)		
T (−786) C	T (78)/C (22)	TT (59)	TC (34)	CC (7)
27 bp-VNTR—a(4 repeats)/b(5 repeats)	b (91)/a (9)	bb (84)	ab (15)	aa (1)
G/T (Glu298Asp)	G (82)/T (18)	GG (63)	GT (31)	TT (6)

VNTR Variable number of tandem repeats

Table 3

Association analysis between eNOS variants and Cardiovascular and Renal-related risk factors in SAFHS

Phenotypes	T(−786)C *P* value	27 bp-VNTR *P* value	G/T (Glu298Asp) *P* value
Type 2 diabetes[a]	0.591	0.207	0.985
Body mass index[b]	0.137	0.337	0.489
Total cholesterol[c]	0.594	0.841	0.711
High density lipoprotein cholesterol[c]	0.697	0.403	0.875
Triglycerides[c]	*0.034*	0.606	0.739
Systolic blood pressure[d]	0.228	0.744	0.870
Diastolic blood pressure[d]	0.646	0.174	0.753
Albumin to creatinine ratio[e]	0.423	*0.047*	0.445
Estimated glomerular filtration rate[e]	0.958	0.620	0.496

[a]Data adjusted for age and sex terms

[b]Data adjusted for age, sex and diabetes

[c]Data adjusted for age, sex, diabetes, duration of diabetes, body mass index, and lipid medication

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

[e]Data adjusted for age, sex, diabetes, duration of diabetes, systolic blood pressure and antihypertensive treatment; Glomerular filtration rate was estimated by MDRD equation