Endothelial nitric oxide synthase (eNOS) gene polymorphisms and their association with type 2 diabetes-related traits in Mexican Americans

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Abstract

Genetic variants of the endothelial nitric oxide synthase (eNOS) gene such as T-786C, Glu298Asp and 27bp-VNTR have been examined for their association with type 2 diabetes (T2DM)-related traits in various 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) were found to have 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.


Key words: association analyses, eNOS gene, genetic polymorphisms, Mexican Americans, type 2 diabetes.

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, leucocyte adhesion to vascular endothelium and oxidation of low-density lipoprotein (LDL).1 Endothelium-derived NO is synthesised from L-arginine by NO synthase encoded by the endothelial NO synthase (eNOS or NOS3) gene, mapped to chromosome 7q36. Upon release, NO diffuses rapidly through the cell membrane and relaxes neighbouring vascular smooth cells through the production of cyclic guanine 3’5’-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, which contributes to the development of insulin resistance, type 2 diabetes mellitus (T2DM), chronic renal failure, and cardiovascular complications including hypertension and hypercholesterolaemia.2 Due to the importance of eNOS in the generation of NO that regulates endothelium-dependent vasodilation in many organs, several epidemiological studies are in the process of 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

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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 repeats (27b-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. The Glu298Asp polymorphism causes a structural change of the eNOS protein and reduces eNOS activity. In the 27-bp repeat of intron 4, two alleles have been identified, the larger of which, eNOS-4b, has five tandem 27-bp repeats (GAAGTCTAGACCTGCTGC(A/G) GGGGTGAG) and the smaller, eNOS-4a, has four repeats. The 27bp-VNTR reduces the plasma concentration of nitric oxide. However, genetic studies examining the association of T-786C, Glu298Asp and 27bp-VNTR polymorphisms with cardiovascular and renal diseases have yielded conflicting conclusions. 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 for one study that examined the relevance of Glu298Asp polymorphism only with insulin-resistant measures in Mexican Americans.

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

Methods
Study subjects and phenotypic data
The SAFDGS family member recruitment and data collection procedures have been described previously. Briefly, probands were low-income Mexican Americans with T2DM, and all first-, second- and third-degree relatives of probands were invited to participate in the study. A variety of metabolic, haemodynamic, anthropometric and demographic variables were collected from about 700 individuals drawn from 59 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 (TG) and high-density lipoprotein cholesterol (HDL-C), and they were collected again two hours after a standardised oral glucose load to measure plasma glucose. Measurement of all these phenotypes has been described elsewhere. Diabetes status was defined by the 1999 criteria of the World Health Organization (i.e. fasting glucose levels ≥ 126 mg/dL and/or two-hour glucose levels ≥ 200 mg/dL). Participants who did not meet these criteria but who reported that they were being treated 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 the albumin to creatinine ratio (ACR) and analysed as a continuous variable. The quantitative trait values of triglycerides (ln TGL) and ACR (ln ACR) were log-transformed for the association analyses since their raw data were not normally distributed. The Institutional Review Board of the University of Texas Health Science Center at San Antonio approved all procedures. All subjects gave their 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 polymerase chain reaction (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, US) at 37 °C, producing fragments of 140 and 40 bp for the T allele, or fragments of 90, 50 and 40 bp for the C allele. Fragments were separated by 2% agarose gel electrophoresis containing ethidium bromide and visualised 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’-AGG GCA GGA GAC TAT GGT AGT GCC TTT-3 (forward) and 5-TCT CTT AGT GTG TAC CCC A-3 (reverse). The PCR products were restricted by the enzyme Ban II (New England Biolabs, MA, US). 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 CCA GGA GAC AGT CCA TGG A-3 (forward) and 5’-CCC AGT CAA TCC CTT TGC TGG TCA-3 (reverse). The PCR products were digested with the enzyme Ban II (New England Biolabs, MA, US). 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.

Statistical genetic analysis
The genotypic data were checked for Mendelian pedigree inconsistencies using the programs INFER and GENTEST (PEDSYS programs). 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. The VC-based approach accounts for the non-independence among family members. In this approach, VC are modelled as random effects (e.g. additive genetic effects and random environmental effects), whereas the effects of measured covariates such as age and sex are modelled 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. The effect of this measured genotype (association parameter) together with other covariate effects (such as 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 is fixed at zero. Twice the difference in the log-likelihoods of these models yields a test statistic that is asymptotically distributed, approximating a $\chi^2$ distribution with one degree of freedom. A p value < 0.05 is considered significant. Prior to performing MGA, the quantitative transmission disequilibrium test (QTDT) was used to examine hidden population stratification. All statistical techniques described above were implemented in the computer program SOLAR [http://www.sph.sc.edu/cmpb/solar/].

Results
Table 1 shows the clinical characteristics of the SAFDGS sub-
jects who provided 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. Of the total sample, 29% of the subjects had T2DM (table 1).

Genotyping of T-786C (rs2070744), Glu(298)Asp (rs1799983) and 27bp-VNTR polymorphisms was carried out, as described earlier. The allele and genotype frequencies are presented in table 2. Genotypic data of T-786C, G/T and 27bp-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. A number of phenotypes were examined for association—T2DM, body mass index (BMI), systolic blood pressure (SBP), diastolic blood pressure (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 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 (four 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 to vascular and renal diseases. The variants in the promoter region (T-786), intron-4 (27bp-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 conducted mostly in Caucasian and Asian populations. To our knowledge, the genetic epidemiological studies examining these polymorphisms in Mexican Americans for their association with cardiovascular and renal disease-related traits are very limited.

There is a marked ethnic difference in the distribution of eNOS variants. Tanus-Santos et al. examined the distribution of the T-786C, Glu298Asp and 27bp-VNTR variants of

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### Table 1. Clinical characteristics of the genotyped SAFDGS participants used in the present study.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Mean ± SD or %</th>
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</thead>
<tbody>
<tr>
<td>Females</td>
<td>61</td>
</tr>
<tr>
<td>Type 2 diabetes</td>
<td>29</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>44.8±16.2</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>127.1±16.8</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>70.3±9.6</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>30.9±7.0</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>194.0±37.9</td>
</tr>
<tr>
<td>High-density lipoprotein cholesterol (mg/dL)</td>
<td>46.0±12.0</td>
</tr>
<tr>
<td>Triglycerides (log-transformed)</td>
<td>4.9±0.6</td>
</tr>
<tr>
<td>Albumin to creatinine ratio (log-transformed)</td>
<td>2.4±0.8</td>
</tr>
</tbody>
</table>

**Key:** SAFDGS = San Antonio Family Diabetes/Gallbladder Study; SD = standard deviation

To convert cholesterol from mg/dL to mmol/L, multiply by 0.0259; To convert triglycerides from mg/dL to mmol/L, multiply by 0.01129; Sample size varies from n=610 (cholesterol) to n=670 (age)

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### Table 2. Allele and genotype frequency of the selected variants from the NOS3 gene

<table>
<thead>
<tr>
<th>Polymorphisms</th>
<th>Major/minor allele (%)</th>
<th>Genotype (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T (-786) C</td>
<td>T (76) / C (24)</td>
<td>TT (58)</td>
</tr>
<tr>
<td>G / T (Glu298Asp)</td>
<td>G (80) / T (20)</td>
<td>GC (65)</td>
</tr>
<tr>
<td>27bp-VNTR – a (4 repeats)/b (5 repeats)</td>
<td>b (90) / a (10)</td>
<td>aa (1)</td>
</tr>
</tbody>
</table>

**Key:** VNTR = Variable Number of Tandem Repeats
the eNOS gene in a sample of 305 individuals of different ethnicity (100 Caucasians, 100 African Americans and 105 Asians). They found that the -786C variant was more common in Caucasians (42%) 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 27bp-VNTR polymorphism, the minor allele frequency of a allele (four repeats) was more common in African Americans (27%) than in Caucasians (16%) or Japanese (13%). The frequency of a allele (four 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 the 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-786C, Glu298Asp and 27bp-VNTR polymorphisms of the 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 DBP 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 27bp-VNTR four repeats (a allele) carriers were found to have lower HDL-C values and higher DBP values. Few studies19,20 have reported that the T-786C and 27bp-VNTR polymorphisms are in perfect linkage disequilibrium (LD). Such a LD pattern between these two polymorphisms was not seen in our study.

This study suggests that 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 the 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 contribute to any future meta-analyses validating these polymorphisms.

In conclusion, of the three eNOS polymorphisms

### Table 3. Association analysis between NOS3 gene polymorphisms and diabetes-related traits in SAFDGS data

<table>
<thead>
<tr>
<th>Phenotypes</th>
<th>T(-786)C p value</th>
<th>Glu(298)Asp p value</th>
<th>27bp-VNTR p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 2 diabetesa</td>
<td>0.49</td>
<td>0.64</td>
<td>0.57</td>
</tr>
<tr>
<td>Body mass indexb</td>
<td>0.58</td>
<td>0.47</td>
<td>0.79</td>
</tr>
<tr>
<td>Total cholesterolb</td>
<td>0.86</td>
<td>0.09</td>
<td>0.30</td>
</tr>
<tr>
<td>High-density lipoprotein cholesterolb</td>
<td>0.98</td>
<td>0.20</td>
<td>0.04</td>
</tr>
<tr>
<td>Triglycerides (log-transformed)b</td>
<td>0.82</td>
<td>0.62</td>
<td>0.52</td>
</tr>
<tr>
<td>Systolic blood pressurec</td>
<td>0.81</td>
<td>0.17</td>
<td>0.42</td>
</tr>
<tr>
<td>Diastolic blood pressurec</td>
<td>0.19</td>
<td>0.27</td>
<td>0.02</td>
</tr>
<tr>
<td>Albumin to creatinine ratio (log-transformed)d</td>
<td>0.64</td>
<td>0.40</td>
<td>0.85</td>
</tr>
</tbody>
</table>

**Key:** SAFDGS = San Antonio Family Diabetes/Gallbladder Study; aData adjusted for age and sex; bData adjusted for age, sex, diabetes and lipid-lowering medications; cData adjusted for age, sex, diabetes and antihypertensive treatment; dData 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

<table>
<thead>
<tr>
<th>Phenotypesa</th>
<th>Mean ± SE by genotypes</th>
<th>a/a (4 repeats)</th>
<th>a/b</th>
<th>b/b (5 repeats)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDL-C (mg/dL)</td>
<td>37.9±2.0</td>
<td>40.5±1.5</td>
<td>43.1±1.0</td>
<td></td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>79.1±1.9</td>
<td>77.0±1.1</td>
<td>74.9±0.8</td>
<td></td>
</tr>
</tbody>
</table>

**Key:** aHDL-C = high-density lipoprotein cholesterol; DBP = diastolic blood pressure
To convert HDL-C from mg/dL to mmol/L, multiply by 0.0259
examined, there is some evidence for a minor contribution of the 27bp-VNTR polymorphism to variation in T2DM-related subclinical cardiovascular traits in Mexican Americans.

Acknowledgements

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Conflicts of interest statement

None declared.

References


