Association Analyses of Endothelial Nitric Oxide Synthase Gene Polymorphisms in Essential Hypertension

Adam V. Benjafeld and Brian J. Morris

Endothelial nitric oxide synthase (eNOS), encoded by NOS3, is a potent regulator of vasomotor tone and peripheral resistance. Congenic experiments indicate that a chromosomal segment containing the rat eNOS gene contributes to rat spontaneous hypertension (HT). A role for NOS3 in onset of essential hypertension (HT) is, however, controversial. We therefore decided to test NOS3 polymorphisms in a set of patients who have an accentuated ability to show an existing genetic association. The 112 HT subjects had two HT parents and the normotensive (NT) subjects had two NT parents. All were Anglo-Celtic whites. The two most promising polymorphisms, viz, a biallelic variable number of tandem repeats (VNTR) in intron 4 and an exon 7 variant that leads to an amino acid change (Glu298Asp), were genotyped by PCR (and BanII digestion in the case of the latter). Frequency of the minor allele of the VNTR was 0.11 in the NT and 0.10 in the HT subjects (P = .9). For the exon 7 variant, Asp298 frequency was 0.30 and 0.32 in each respective group (P = .6). Tracking was seen for the Asp298 allele with elevation in body mass index (P = .034), and the minor allele of the VNTR with elevation in LDL (P = .007) and reduction in HDL (P = .048). In conclusion, we saw no association of NOS3 markers with HT in the population studied. However, possible genotypic effects on plasma lipids and body mass index might warrant further studies, especially in view of possible associations with heart disease. Am J Hypertens 2000; 13:994–998 © 2000 American Journal of Hypertension, Ltd.

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intron 4,\textsuperscript{15} and a (CA)-repeat in intron 13.\textsuperscript{15} In whites, linkage\textsuperscript{20,21} and association\textsuperscript{22} involving the latter have proved negative for HT, as have intronic SNP.\textsuperscript{20,23} In Japanese an association was seen in HT without left ventricular hypertrophy.\textsuperscript{24} Although the exon 7 (Glu298Asp) variant, detected as a Ban\textsuperscript{II} restriction fragment-length polymorphism (RFLP), has shown an association with HT in two\textsuperscript{25,26} of three\textsuperscript{27} Japanese studies and in one study of whites,\textsuperscript{23} this concerned contrasting alleles. The intron 4 VNTR was associated with plasma nitrite and nitrate,\textsuperscript{28} and with HT in one Japanese study,\textsuperscript{29} but not in two others,\textsuperscript{25,26} nor with HT in whites with coronary artery disease (CAD).\textsuperscript{30}

The present study tested the two most promising polymorphisms in a well-studied cohort having two HT parents and early-onset, moderate to severe disease.\textsuperscript{31,32}

**MATERIALS AND METHODS**

**Subjects** These, their characteristics, and methods for determination of various parameters were as described previously.\textsuperscript{31,32}

**Genotyping** DNA was isolated from whole blood using a QIAamp DNA Mini Kit (Qiagen). Genotypes for the VNTR ‘\textit{\(4a/b\)}’ polymorphism were determined by polymerase chain reaction (PCR) using the primers (sense) 5’-AGG CCC TAT GTT AGT GCC TTT-3’, (antisense) 5’-TCT CTT AGT GCC GTG CTC AC-3’, synthesized by GeneWorks (Adelaide). Steps were: 94°C for 5 min, then 35 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min in 20 \(\mu\)L (100 ng genomic DNA, 13.5 pmol each primer, 3 mmol/L each dNTP, 0.1 U AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, CT), 63 mmol/L KCl, 13 mmol/L Tris-HCl, pH 8.3, 2 mmol/L MgCl\(_2\)). PCR products of 393 bp (\(4a\) allele) and 420 bp (\(4b\) allele) were seen on 3% agarose gel electrophoresis. Genotypes for the G/T (Glu298Asp) variant were determined by PCR using primers (sense) 5’-TCC CTG AGG AGG GCA TGA GGC T-3’, (antisense) 5’-TGA GGG TCA CAC AGG TTC CT-3’, synthesized by GeneWorks. After 94°C for 5 min, 30 cycles of 94°C, 61°C, and 72°C for 1 min each were performed in 20 \(\mu\)L (100 ng genomic DNA, 13.5 pmol each primer, 3 mmol/L each dNTP, 0.1 U AmpliTaq DNA polymerase, 63 mmol/L KCl, 13 mmol/L Tris-HCl, pH 8.3, 2 mmol/L MgCl\(_2\)). PCR products (20 \(\mu\)L) were digested with 2 U Ban\textsuperscript{II} (Promega, Madison, WI) at 37°C for 8 h. With T (Asp298) present the 457-bp PCR product was not cut, but was for the G (Glu298) allele, to fragments of 137 and 320 bp.

**Statistical Analyses** \(\chi^2\) and ANOVA was performed with StatView (Abacus Concepts, Berkeley, CA). Linkage disequilibrium was tested by the method of Hill.\textsuperscript{33}

**RESULTS**

Genotype data for each variant are shown in Table 1. Genotype frequencies did not deviate from Hardy-Weinberg expectations. No significant association with HT was seen. For a relative risk of 1.6, our study had 97% power to detect significant association of the Glu298Asp variant with HT, given the \(P\) value we obtained, and for the VNTR marker was 96%. For the VNTR, no HT subjects were homozygous for the \(4a\) allele, which is rare: frequency = 3% in whites.\textsuperscript{30} Examination of haplotypes of the VNTR and G/T

<table>
<thead>
<tr>
<th>VNTR</th>
<th>Genotype Frequencies</th>
<th>Allele Frequencies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group n</td>
<td>(4b/4b)</td>
<td>(4b/4a)</td>
</tr>
<tr>
<td>NT</td>
<td>147</td>
<td>35</td>
</tr>
<tr>
<td>(0.80)</td>
<td>(0.19)</td>
<td>(0.01)</td>
</tr>
<tr>
<td>HT</td>
<td>89</td>
<td>23</td>
</tr>
<tr>
<td>(0.79)</td>
<td>(0.21)</td>
<td>(0)</td>
</tr>
<tr>
<td>(\chi^2)</td>
<td>1.3</td>
<td>0.52</td>
</tr>
<tr>
<td>(P)</td>
<td>0.02</td>
<td>0.90</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>G/T (Glu298Asp) variant</th>
<th>Genotype Frequencies</th>
<th>Allele Frequencies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group n</td>
<td>G G</td>
<td>G T</td>
</tr>
<tr>
<td>NT</td>
<td>70</td>
<td>68</td>
</tr>
<tr>
<td>(0.47)</td>
<td>(0.46)</td>
<td>(0.07)</td>
</tr>
<tr>
<td>HT</td>
<td>40</td>
<td>43</td>
</tr>
<tr>
<td>(0.44)</td>
<td>(0.47)</td>
<td>(0.09)</td>
</tr>
<tr>
<td>(\chi^2)</td>
<td>0.29</td>
<td>0.87</td>
</tr>
<tr>
<td>(P)</td>
<td>0.26</td>
<td>0.61</td>
</tr>
</tbody>
</table>

\(\text{VNTR} = \text{variable number of tandem repeats; NT} = \text{normotensive subjects; HT} = \text{hypertensive subjects.}

Values in parentheses are fractions.
Lipid values are mmol/L. 

We found no associations with essential HT, consistent with findings in other white populations.20,30 In studies by others, genotype frequencies for the VNTR30 and \( G/T \) (Glu298Asp) variants in HT23 and CAD34 groups resembled those we saw, but a lower Glu289 frequency in controls (0.56), cf, 0.65 in HT, in the European study may explain their positive \((P = .004)\) finding.23 Moreover, a lack of association with BP might suggest an artifact of population stratification. 

Comparison of body mass index (BMI) and plasma lipid concentrations across genotypes in HT revealed, for the VNTR, tracking of the \( 4a \) allele with lower HDL and higher LDL; for the RFLP, tracking of the \( T \) (Asp298) allele with elevation in BMI and a trend for rises in triglyceride were noted (Table 2). A similar spread of medications was seen within each genotype.

## DISCUSSION

We found no associations with essential HT, consistent with findings in other white populations.20,30 In studies by others, genotype frequencies for the VNTR30 and \( G/T \) (Glu298Asp) variants in HT23 and CAD34 groups resembled those we saw, but a lower Glu289 frequency in controls (0.56), cf, 0.65 in HT, in the European study may explain their positive \((P = .004)\) finding.23 Moreover, a lack of association with BP might suggest an artifact of population stratification.

To demonstrate a positive association in complex polygenic diseases requires \( \geq 10^2 \) per group,35 and our power estimates confirmed the capacity of our study to do this. Our cohort has, moreover, shown strong associations for markers in other genes.31,32

Thus the defect in NO production in HT could involve a gene other than NOS3. A role in other conditions cannot be ruled out. The VNTR \( 4a \) allele is associated with smoking-dependent risk of CAD in whites,30 but not with acute myocardial infarction (AMI) in Japanese.36 Moreover, Asp298 homozygotes were elevated in Japanese with AMI,36,37 but more Glu298 homozygotes were seen in French,18 but not Irish,18 British,38 or Australian44 white AMI patients, and no association was seen with CAD.34,38

Because high BMI and dyslipidemia are associated with CAD and AMI,39 it was of interest that we saw tracking of the VNTR \( 4a \) allele with higher LDL and lower HDL, and of the Asp298 variant with elevation in triglyceride and BMI, and lower HDL. However, these could be chance findings due to multiple comparisons, so that a firm conclusion would be premature pending more extensive investigations. Any causative variant will also need to be identified. In this regard an association of the \( T/4a \) variant with reduced promoter activity and coronary spasm in Japanese17 is of interest.

## ACKNOWLEDGMENTS

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