Lack of Evidence for Association Between the Endothelial Nitric Oxide Synthase Gene and Hypertension

Norihiro Kato, Takao Sugiyama, Hiroyuki Morita, Toru Nabika, Hiroki Kurihara, Yukio Yamori, Yoshio Yazaki

Abstract—Significant association between a Glu298Asp polymorphism of the endothelial nitric oxide synthase (eNOS) gene and essential hypertension was recently reported in Japanese populations, with the 298Asp variant showing a higher prevalence in hypertensive patients (10.3% to 12.0%) than in normotensive subjects (5.0% to 5.8%). In contrast, another study demonstrated that the 298Glu variant was significantly associated with hypertension in a Caucasian population. We therefore undertook an extensive association study in Japanese to resolve these contradictory claims. A total of 1165 individuals were selected from clinic outpatients and hospital staff in a single institution. The relevance of the Glu298Asp polymorphism to hypertension in this population was tested in 2 ways. First, a case-control study was conducted in 549 hypertensive and 513 normotensive subjects within the study population, with the χ² statistic used to test the significance of an association between eNOS genotype and the presence of hypertension. Second, an ANOVA was used to test the significance of an association between eNOS genotype and the level of blood pressure within the entire population except for 167 hypertensive subjects who had been under treatment for hypertension. No significant association was observed in either of the statistics tested. Allele frequencies of 298Asp were concordant across the panels: 8.4% in hypertensive subjects, 8.2% in normotensive subjects, and 7.9% and 9.5% in 2 additional sample populations used as reference panels. Taken together, our results do not support the previous observation that the molecular variant of the eNOS gene may confer principal susceptibility for essential hypertension but rather suggest the existence of sampling variation. (Hypertension. 1999;33:933-936.)

Key Words: hypertension, essential ■ Japanese ■ genetics ■ nitric oxide synthase

A number of components in known physiological pathways regulating blood pressure (BP) have been explored through the candidate gene approach to see whether they are involved in the pathogenesis of essential hypertension (EH).1 Suggestions of linkage and association with EH have been observed for the components in the renin-angiotensin-aldosterone pathway, such as the angiotensinogen2 and angiotensin I–converting enzyme gene3 loci. Causative genes for several secondary forms of hypertension have been identified through the candidate gene approach.4 Among the genes thus investigated, the endothelial nitric oxide synthase (eNOS) gene has drawn considerable attention because of its substantial contributions to BP regulation. For example, (1) eNOS mediates the release of nitric oxide, a potent vasodilator, from endothelial cells5–7; (2) inhibition of NOS elevates BP in healthy humans8; and (3) the disruption of the eNOS gene leads to hypertension in mice.9 Several lines of evidence have also supported that impaired nitric oxide production is responsible for the BP regulation in humans.10,11 Hence, several investigators have examined the eNOS gene as a potential candidate for EH.12–17 The gene encoding eNOS is located on chromosome 7 and contains 26 exons spanning 21-kb of genomic DNA.7 So far, 5 polymorphic sites—3 single nucleotide polymorphisms (SNPs), 1 variable number of tandem repeat and 1 CA-repeat polymorphisms—have been identified in the eNOS gene.7,12 Among them, only the SNP located in exon 7 results in amino acid substitution, which substitutes Asp for Glu at amino acid residue 298 (Glu298Asp). Five previous studies tested the association between either of the above eNOS polymorphisms and EH.12,14–17 Two studies implicated significant association of the Glu298Asp polymorphism in Japanese16 and in Caucasians,17 respectively, whereas the absence of association was reported for 27-bp tandem repeats (intron 4),16 CA-repeat polymorphisms (intron 13),14,15 A27→C (intron 18),12,16 and G10→T (intron 23)12,16,17 polymorphisms. It must be noted, however, that the alternate allele of Glu298Asp appeared to be associated with hypertension in each of the 2 study groups. On the other hand, 2 independent studies12,13 showed the lack of evidence for linkage between the eNOS CA-repeat polymorphism and EH.
in white populations by affected sib-pair analysis. Furthermore, although the functional significance of Glu298Asp has not been reported to date, this polymorphism has been recently shown to be significantly associated with the presence of acute myocardial infarction in a Japanese population.\(^\text{18}\)

Thus we conducted a case-control study of a relatively large size to clarify the uncertain picture about association of the Glu298Asp polymorphism of the eNOS gene with EH. All participants were Japanese and ascertained in a single institution to minimize geographical and socioeconomic differences in the study population. Genotype distribution and allele frequencies of Glu298Asp were compared between 549 hypertensive and 513 normotensive subjects with \(\chi^2\) statistics, and the association was also tested with BP as a continuous variable.

**Methods**

**Patients and Control Subjects**

This study was approved by an institutional review committee. Participants consisted of a total number of 1165 individuals selected from outpatients and hospital staff at the Institute for Adult Diseases Asahi Life Foundation, Tokyo. Subjects were consecutively enrolled in a clinic, and hospital staff taking annual medical examination were also enrolled. Two other panels, which were composed of 552 employees of a company in Tokyo (reference panel I) and 179 healthy students volunteered at Shimane Medical University (reference panel II), were used as random population controls. Informed consent for participation was obtained from all subjects. Two BP measurements were taken with subjects in the seated position with the use of a sphygmomanometer on separate visits and averaged as the individual’s readings except for those in the reference panels, in which only age and gender of the participants were recorded as the individual’s information.

Criteria of hypertension for participants are defined as follows: (1) age \(>20\) years, (2) onset of hypertension \(<60\) years, (3) systolic blood pressure \(\geq160\) mm Hg and/or diastolic blood pressure \(\geq95\) mm Hg on 2 consecutive visits for those untreated, (4) patients under chronic antihypertensive treatment, (5) absence of secondary form of hypertension through extensive workup, including serum creatinine and electrolytes, chest radiography, ECG, urinalysis, and other hematological screening tests, and (6) subjects with a history of diabetes mellitus and renal failure were excluded from the present study. Here, the age onset of hypertension was defined as the time when BP readings exceeded the above criteria on consecutive visits before starting medication or when antihypertensive medication was initiated. People with systolic blood pressure \(\geq160\) mm Hg and/or diastolic blood pressure \(<90\) mm Hg, and age \(>30\) years were categorized into a control group; otherwise, subjects were regarded as unknown phenotype in the case-control study. Table 1 displays the clinical characteristics of participants according to hypertension status.

<table>
<thead>
<tr>
<th>Hypertensive</th>
<th>Normotensive</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (Men/Women)</td>
<td>549 (305/244)</td>
</tr>
<tr>
<td>Age, y</td>
<td>63.1 ± 9.8*</td>
</tr>
<tr>
<td>BMI, kg/m(^2)</td>
<td>23.7 ± 2.8*</td>
</tr>
<tr>
<td>Systolic Blood pressure, mm Hg</td>
<td>164.0 ± 18.2*</td>
</tr>
<tr>
<td>Diastolic</td>
<td>100.8 ± 9.2*</td>
</tr>
<tr>
<td>Smoking, %</td>
<td>37.9</td>
</tr>
<tr>
<td>Serum creatinine, (\mu)mol/L</td>
<td>74.0 ± 17.3</td>
</tr>
<tr>
<td>Serum cholesterol, mmol/L</td>
<td>5.10 ± 0.57</td>
</tr>
<tr>
<td>Serum triglycerides, mmol/L</td>
<td>1.61 ± 0.98*</td>
</tr>
<tr>
<td>Serum HDL-C, mmol/L</td>
<td>1.56 ± 0.40</td>
</tr>
</tbody>
</table>

*P < 0.0001, †P < 0.01, hypertensive vs normotensive subjects.

PCR was performed in PTC-100 (MJ Research Inc.) in a 15-\(\mu\)L reaction volume containing 160 nmol/L of each of FP191G and FP191T, 200 nmol/L of RP191, 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 25 \(\mu\)mol/L each of dNTPs, 0.4 U Ampli-Taq DNA Polymerase (Perkin Elmer), and 1.5 mmol/L MgCl\(_2\). Initial denaturation for 3 minutes at 95°C was followed by 35 cycles of denaturation for 20 seconds at 94°C, annealing for 30 seconds at 60°C, and extension for 30 seconds at 72°C. The size of PCR products was 126 bp and 122 bp for the 298Glu and 298Asp alleles, respectively, which were electrophoresed in 6% polyacrylamide/7 mol/L urea gels on the model S2 sequencing apparatus (Life Technologies Inc.) and blotted onto nylon membranes (Pall Inc). The membranes were hybridized in 7% polyethylene glycol/10% SDS at 42°C for 3 hours with the RP191 primer labeled with \(^{32}\)P-dCTP by terminal transferase (Boehringer Mannheim). After hybridization, the membranes were rinsed in 2× SSC, 0.1% SDS, washed in 2× SSC, 0.1% SDS at room temperature for 15 minutes, wrapped in plastic, and exposed directly to film for 2 hours at –80°C.

**Statistical Analysis**

Statistical analysis was performed in 2 ways as follows: First, the likelihood ratio \(\chi^2\) statistics were calculated between genotype distribution (or allele frequencies) and hypertension status. Confounding influences of age and body mass index (BMI) were assessed in a multiple logistic regression model with the JMP statistical package (SAS Institute Inc). Second, BP was considered as a continuous variable, and association of the 298Asp variant with BP was tested with 1-way ANOVA with all typed individuals except for those who had started their antihypertensive drugs without definite hospital records of BP readings. For hypertensive patients, an initial value for BP at the onset of hypertension (ie, the time when BP readings exceeded the above criteria on consecutive visits before starting medication) was used in the analysis.

Approximate 95% confidence intervals (CIs) of the odds ratio were given by Woolf’s method.\(^\text{22}\) All values were expressed as mean ± SD unless otherwise indicated.

**Results**

Table 2 displays results for genotyping of the Glu298Asp polymorphism in each study group. No significant association was observed in the comparison of either genotype distribution...
Table 2. Glu298Asp Genotypes in Each Study Group

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Hypertensive (n=549)</th>
<th>Normotensive (n=513)</th>
<th>Reference Panel*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I (n=552)</td>
<td>II (n=179)</td>
<td></td>
</tr>
<tr>
<td>Glu/Glu</td>
<td>461</td>
<td>433</td>
<td>471</td>
</tr>
<tr>
<td>Glu/Asp</td>
<td>84</td>
<td>76</td>
<td>75</td>
</tr>
<tr>
<td>Asp/Asp</td>
<td>4</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Frequency of Asp allele</td>
<td>0.084</td>
<td>0.082</td>
<td>0.079</td>
</tr>
</tbody>
</table>

*Reference panel I comprises 552 company employees (491 men and 61 women) without serious health problems, age 44.1±7.0 years; Reference panel II comprises 179 healthy students (130 men and 49 women), age 24.4±1.8 years.

$\chi^2=0.057, df=2; P=0.97$ or allele frequencies ($\chi^2=0.026, df=1; P=0.87$) between hypertensive and normotensive groups. To evaluate influences of the age difference between case and control groups, normotensive subjects were divided into 2 subgroups: people ≥50 years of age (subgroup 1, n=331) and those <50 years of age (subgroup 2, n=182). Age and BMI were 61.1±7.6 years and 22.5±2.7 kg/m² in subgroup 1 and 42.4±6.0 years and 22.1±3.0 kg/m² in subgroup 2. Allele frequencies of 298Asp (and genotype distribution—Glu homozygote/heterozygote/Asp homozygote) were 8.5% (278/50/3) in subgroup 1 and 7.7% (155/26/1) in subgroup 2, neither of which were significantly different from the 298Asp frequency in hypertensive subjects (8.4%). The lack of observed association was independent of age and BMI in the logistic regression analysis. The prevalence of the eNOS alleles in each of the 4 study groups was consistent with Hardy-Weinberg equilibrium. When tested with ANOVA, association was still not significant between the Glu298Asp polymorphism and BP measurements (Table 3). Here, 167 hypertensive subjects were not included in the analysis because their BP readings had not been clearly documented before a start of antihypertensive medication (see Methods).

The odds ratio for 298Asp versus 298Glu allele frequencies was 1.03 (95% CI 0.76 to 1.40) in the present study.

**Discussion**

Two previous studies, one in 2 geographically distinct Japanese populations16 and the other in a white population,17 independently implicated significant association between the Glu298Asp polymorphism of eNOS and EH. However, the results appeared to be contradictory because the allele frequency of 298Asp in hypertensive subjects was increased in the Japanese populations, whereas that of 298Glu was increased in the white population. Two interpretations can be proposed for such ambiguous results. First, the discrepancy may stem from the nature of sampling variability in case-control study design. Second, it reflects different genetic backgrounds between 2 ethnic groups. The present study has assessed these interpretations and has provided evidence against the eNOS association as discussed below.

It is unlikely that the identical molecular variant (the 298Asp variant in this case) exerts BP-elevating effects in one population and BP-lowering effects in another population. Accordingly, the reported associations can be justified only if we speculate a functional mutation (or mutations) other than the Glu298Asp substitution polymorphism. In theory, it could not be impossible that such putative mutation is in linkage disequilibrium with the alternate allele of Glu298Asp in each of 2 ethnic groups distantly separated in human evolutionary history. However, it is also possible that either (or both) of the positive results for association would be a case of random error, a spurious association.

All participants in this study were selected from the same institution in Tokyo, Japan, with classification criteria no less stringent than those used for the original studies.16,17 Moreover, the trial size is larger than the sum of 2 Japanese populations previously investigated—Kyoto and Kumamoto cohorts.16 Allele frequencies of 298Asp proved to be very similar between case (8.4%) and control (8.2%) groups in our population, whereas they were in between the previously reported prevalences for hypertensive (10.3% to 12.0%) and normotensive (5.0% to 5.8%) subjects in Japanese. One may argue that our negative results for association could be rather biased as the result of sampling variation in the control group. To evaluate this possibility, we separately recruited a random control panel (reference panel I) in the same area under investigation. Another possible explanation for the diverse allele frequencies is that the prevalence of the 298Asp variant could be innately low in Kyoto and Kumamoto areas compared with that in Tokyo area because of the geographic (or population structure) difference. Since the 3 study bases were 300 to 500 miles apart from each other in the country (the order is Tokyo, Kyoto, and Kumamoto from east to west), we examined the 298Asp allele frequency in another panel of healthy students (reference panel II) ascertained in the area between Kyoto and Kumamoto. Concordant results for the allele frequencies across a series of panels are reassuring and refute the above 2 possibilities (Table 2). The robustness of allele frequency estimation in our study thus rests on the relatively clear criteria of hypertension, the larger number of control

Table 3. Clinical Characteristics of Participants According to Genotypes at Residue 298 of eNOS

<table>
<thead>
<tr>
<th>Variable</th>
<th>Total</th>
<th>Glu/Glu</th>
<th>Glu/Asp</th>
<th>Asp/Asp</th>
<th>ANOVA (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (Men/Women)</td>
<td>998 (570/428)</td>
<td>844 (486/358)</td>
<td>146 (80/66)</td>
<td>8 (4/4)</td>
<td></td>
</tr>
<tr>
<td>Blood pressure, mm Hg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>138.6±27.2</td>
<td>138.4±26.9</td>
<td>140.1±28.7</td>
<td>142.4±36.5</td>
<td>0.73</td>
</tr>
<tr>
<td>Diastolic</td>
<td>86.1±15.4</td>
<td>85.9±15.4</td>
<td>87.4±15.6</td>
<td>84.4±14.9</td>
<td>0.52</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>22.9±3.0</td>
<td>22.9±3.0</td>
<td>23.0±2.8</td>
<td>22.5±5.6</td>
<td>0.84</td>
</tr>
<tr>
<td>Age, y</td>
<td>58.6±12.2</td>
<td>58.5±12.4</td>
<td>58.8±11.0</td>
<td>65.7±7.9</td>
<td>0.24</td>
</tr>
</tbody>
</table>
subject, and the use of reference panels. Dichotomous classification of participants into case and control groups would somewhat decrease the statistical power to detect modest genetic susceptibility for hypertension. Therefore we additionally tested potential influences of the 298Asp variant on BP readings with ANOVA, resulting in the lack of association (Table 3).

Taken together, our data indicate that 298Asp of the eNOS gene may not confer principal predisposition to EH in Japanese. The present study itself, however, does not directly answer the question of whether the association between 298Glu and EH seen in a white population is attributable to so-called ethnic differences. To facilitate comparisons among 3 study results, we calculated the odds ratio for 298Asp versus 298Glu allele frequencies, which appeared symmetrically distributed about the dashed line representing an odds ratio = 1.0 (Figure). A mirrored pattern was observed when the odds ratio for 298Glu versus 298Asp was calculated. Because of the small number of studies included and because of the different criteria and clinical characteristics among studies, we cannot deduce any conclusions from this figure alone. To examine the assumption that studies in different ethnic groups are estimating the same value for genetic effects of the eNOS variant, further investigation should be done in each ethnic group separately and then results of individual populations compared.

In summary, the present study does not support the relevance of the eNOS locus to EH. Nevertheless, there remains the possibility that as-yet unidentified functional mutations exist in the eNOS gene, which need to be screened by systematic searches of SNPs. The selection of participants based on the limited number of BP measurements cannot exclude the possibility of misclassification in the case-control study of hypertension. Comprehensive genetic approaches including linkage analysis and family-based tests for association, together with a number of replication studies with large sample size, should be performed before making conclusive claims about the pathophysiological involvement of a given candidate gene in EH.

References
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