Lack of Association between T-786→C Mutation in the 5’-Flanking Region of the Endothelial Nitric Oxide Synthase Gene and Essential Hypertension

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Accumulating evidence strongly suggests that an alteration in nitric oxide metabolism is involved in the pathogenesis of hypertension. We recently found 2 polymorphisms in the endothelial nitric oxide synthase (eNOS) gene, a Glu298Asp missense variant in exon 7 and a T-786→C variant in the 5’-flanking region, which are not linked to each other. In our previous reports, we showed a positive association between the Glu298Asp variant and essential hypertension, myocardial infarction, and coronary spastic angina. We also revealed that the T-786→C variant is strongly associated with coronary spastic angina and leads to the reduction of the eNOS gene promoter activity. To further investigate the genetic involvement of the eNOS gene in essential hypertension, we examined the frequency of T-786→C variant in two independent populations of persons with essential hypertension in Kyoto (n=215) and Kumamoto (n=186) and compared the frequency with that in each age- and gender-matched control (233 controls in Kyoto and 223 controls in Kumamoto). In both groups, the frequency of T-786→C variant was similar in patients with hypertension and normal controls. In conclusion, the T-786→C variant was not positively associated with essential hypertension. Given the evidence of positive association of another polymorphism in the eNOS gene, the Glu298Asp polymorphism, with essential hypertension, special attention will be required to interpret the results of a case-control study for genetic risk factors. (Hypertens Res 2000; 23: 561-565)

Key Words: genes, endothelial nitric oxide synthase, essential hypertension, polymorphism

Introduction

Nitric oxide (NO) synthesis in the vasculature is important for the regulation of vasodilator tone and the control of blood pressure in humans and experimental animals (1-3). A recent study using mice with disrupted endothelial nitric oxide synthase (eNOS) gene revealed that the disruption of the eNOS gene leads to the establishment of hypertension (4). Moreover, recent reports demonstrate that whole-body NO production in patients with essential hypertension is diminished under basal conditions based on the measurement of urinary and plasma nitrate (5). In addition, offspring of hypertensive patients exhibit a reduced response to acetylcholine linked to a defect in the nitric oxide pathway (6). These results strongly implicate
genetic alterations in the eNOS gene in the pathogenesis of human essential hypertension.

We recently identified two distinct variants in the eNOS gene that are not linked to each other. One is a G to T conversion at nucleotide position 894 in exon 7 of eNOS cDNA that results in a replacement of glutamic acid by asparatic acid at codon 298 (Glu298Asp), which is associated with essential hypertension as well as coronary spastic angina and myocardial infarction (7-9). The other variant is a T to C conversion at nucleotide position-786 (T-786→C) in the 5’-flanking region of the eNOS gene that is strongly associated with coronary spastic angina (CSA), and the mutation altered the eNOS gene expression in our in vitro transcription assay (10). The genetic involvement of the T-786→C variant in hypertension is not yet known.

To further elucidate the genetic involvement of the eNOS gene in essential hypertension, we examined the possible association between the T-786→C variant and essential hypertension in two Japanese populations. We report here a polymodal genetic influence of the eNOS gene variants on essential hypertension.

**Methods**

**Patient Population: Hypertensive and Control Subjects**

A total of 215 patients (109 men, 106 women) with essential hypertension were selected from the outpatient clinics at Kyoto University Hospital and its affiliated hospitals in Kyoto according to the following criteria: 1) patients were more than 20 years of age, 2) the onset of hypertension occurred at less than 50 years of age, 3) established hypertension was defined either by long-term treatment of the disease or, in those previously untreated as systolic/diastolic blood pressure greater than 140/90 mmHg on two consecutive visits, and 4) secondary forms of hypertension were absent, as determined through extensive workup. Blood pressure was measured in the supine position using a sphygmomanometer. A group of 233 normotensive control subjects (134 men, 99 women) were selected from the same clinics according to the following criteria: 1) patients were more than 30 years of age, 2) patients had a systolic/diastolic blood pressure less than 140/90 mmHg, and 3) patients were not receiving any antihypertensive treatment.

A second study population of 186 individuals (121 men, 65 women) with essential hypertension and 223 normotensive control subjects (133 men, 90 women) were also selected at the Kumamoto University Hospital in Kumamoto according to the same criteria just listed. Kyoto and Kumamoto are capital cities of two different prefectures located on different islands of Japan and are approximately 500 miles apart from each other.

At the time of recruitment, informed consent was obtained from each person according to a protocol approved by the Human Study Committee of Kyoto University or Kumamoto University.

**Identification of the Genotype of the eNOS Gene**

Hypertensive patients and normotensive controls were genotyped for a common T-786→C variant in the promoter of the eNOS gene by polymerase chain reaction (PCR) (with primers 5’-TCAGTCTATGAGGTCTCGATAAG-3’ and 5’-CTTCCTTGAGTCTGACATTAGGG-3’) followed by mutant allele-specific endonuclease digestion with Msp I and agarose gel electrophoresis. We also genotyped the same population for the missense Glu298Asp variant in exon7 by PCR-restriction fragment length polymorphism (RFLP) as previously described (5-7).

**Analysis of Differences in the Clinical Parameters between Hypertensive and Normotensive Subjects**

Clinical parameters were analyzed in hypertensive and normotensive control samples; differences in the frequen-

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**Table 1. Clinical Parameters of Hypertensive and Normotensive Subjects in the Kyoto Study Group**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normotensive (n=233)</th>
<th>Hypertensive (n=215)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex Men/Women</td>
<td>134/99</td>
<td>109/106</td>
</tr>
<tr>
<td>Age (y)</td>
<td>54.0±13.2</td>
<td>56.5±11.5</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.0±2.6</td>
<td>23.9±5.1†</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>125.5±11.3</td>
<td>161.8±18.5*</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>73.7±9.6</td>
<td>93.6±13.7*</td>
</tr>
<tr>
<td>T-Cho (mmol/l)</td>
<td>5.14±0.97</td>
<td>5.22±0.98</td>
</tr>
<tr>
<td>HDL-Cho (mmol/l)</td>
<td>1.26±0.46</td>
<td>1.30±0.45</td>
</tr>
<tr>
<td>HbAlc (%)</td>
<td>5.9±1.3</td>
<td>5.6±1.3</td>
</tr>
<tr>
<td>Cre (mmol/l)</td>
<td>83±23</td>
<td>83±88</td>
</tr>
<tr>
<td>UA (mmol/l)</td>
<td>315±83</td>
<td>339±83†</td>
</tr>
<tr>
<td>PRA (ng/l/s)</td>
<td>0.30±0.30</td>
<td>0.33±0.70</td>
</tr>
<tr>
<td>PAC (pmol/l)</td>
<td>1,842±1,485</td>
<td>2,127±2,108</td>
</tr>
<tr>
<td>ANP (pmol/l)</td>
<td>8.5±2.8</td>
<td>12.5±9.9†</td>
</tr>
<tr>
<td>BNP (pmol/l)</td>
<td>3.7±2.8</td>
<td>13.0±9.9†</td>
</tr>
<tr>
<td>SVI+RV5 (mV)</td>
<td>2.2±1.0</td>
<td>3.1±1.1*</td>
</tr>
<tr>
<td>LVMI (g/m²)</td>
<td>111±27</td>
<td>136±41*</td>
</tr>
</tbody>
</table>

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; T-Cho, serum total cholesterol; HDL-Cho, serum HDL-cholesterol; Cre, serum creatinine; UA, uric acid; PRA, plasma renin activity; PAC, plasma aldosterone concentration; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; SVI+RV5, voltage in electrocardiogram; LVMI, left ventricular mass index. Data are means±SD. *p<0.0001, †p<0.01, ‡p<0.03.
cies of the following quantitative variables were tested using unpaired, two-tailed Student's t tests: age, systemic blood pressure, diastolic blood pressure, body mass index, plasma total cholesterol, high-density lipoprotein (HDL) cholesterol, creatinine, blood urinary nitrates, plasma renin activity, plasma aldosterone concentrations, plasma atrial natriuretic peptide (ANP) concentrations, plasma brain natriuretic peptide (BNP) concentrations, an amplitude of SV1 plus RV5 in electrocardiogram, and left ventricular mass index.

Analysis of Clinical Parameters between Normotensive and Hypertensive Subjects

All clinical parameters are expressed as mean ± SD. Except sex ratios, all clinical parameters were compared with Student's t test. Sex ratios were tested by χ² test with 1 df. P values less than 0.05 were considered statistically significant.

Analysis of Genotype Frequencies for eNOS Variants

For each biallelic marker, allele frequencies were calculated from the genotypes in the hypertensive and normotensive groups. Deviation from Hardy-Weinberg equilibrium was assessed using a χ² test with 1 df. Differences in autosomal dominant genotype distribution between hypertensive and control subjects were tested using Fisher's exact test. P values of 0.05 or less were considered significant for single testing.

Results

Clinical Characteristics of Study Subjects

The clinical characteristics of the 215 hypertensive and 233 normotensive subjects in Kyoto are summarized in Table 1. No differences between the two groups were noted with respect to age, body mass index, total serum cholesterol levels, serum HDL-cholesterol levels, serum creatinine levels, plasma renin activity, or plasma aldosterone concentrations. Serum uric acid levels were significantly higher in the hypertensive group than in the control group (p<0.01). Plasma ANP and BNP levels in the hypertensive group were significantly higher than those in the control group (p<0.01 and p<0.01, respectively). Moreover, SV1 plus RV5 in the electrocardiogram and left ventricular mass index estimated by echocardiography were also significantly higher in the hypertensive group than in the control group (p<0.0001 and p<0.0001, respectively). These data reflected cardiac hypertrophy associated with hypertension.

Association between Essential Hypertension and the Polymorphisms of the eNOS Gene

The T-786→C variant in the eNOS gene was analyzed in hypertensive and normotensive subjects in Kyoto (Table 2). The genotype frequencies in each group satisfied the Hardy-Weinberg equilibrium law. In the Kyoto population, there was no difference between hypertensive and normotensive subjects in the frequencies of heterozygotes and homozygotes for the C-786 variant (p=0.606). According to Schlesselman's equation for statistical power (II), power was 7.3% in the present study. The genotype frequencies of the homozygotes and heterozygotes for the C-786 variant in normotensive and hypertensive subjects were 21.9% and 20.5%, respectively.

We also investigated a Japanese population from Kumamoto prefecture and observed similar results in the frequency of the T-786→C variant between hypertensive and normotensive subjects (Table 2). Genotypic frequency of the T-786→C variant was similar in the Kyoto and Kumamoto populations. In contrast, as we previously reported, the Asp298 variant showed a significant effect of genotype on hypertension (p<0.01) with the odds ratio.
polymorphism in a candidate gene is not sufficient to de-
clearly indicate that a case-control study using only one
associated with essential hypertension. These findings
and as previously reported the G1u298Asp variant is not
-C variant and the G1u298Asp variant (data not shown),
tions, there is no linkage disequilibrium between the T-786
associated with essential hypertension in Japanese sub-
jects.

In the present study, we failed to find a positive associ-
ation between the T-786→C variant and essential hyperten-
sion in study populations in Kyoto, Japan, and Kumamoto,
Japan. In case-control studies of the candidate gene
approach, special care must be taken to minimize sam-
ping bias, which is why we used two independent popula-
tions in Kyoto and Kumamoto, 500 miles from each
other, with the same entry criteria. Using these popula-
tions, we recently reported that a missense variant in
exon 7 of the eNOS gene, the Glu298Asp variant, is sig-
ificantly associated with essential hypertension, though
the single nucleotide polymorphism (SNP) in either intron
18 or intron 23 is not (7). These results are consistent
with recent reports by Bonnardeaux et al. (12), showing a
negative association between SNPs in introns 18 and 23
and essential hypertension in a European population, and
by Yasujima et al. (13), showing a positive association be-
tween the Glu298Asp variant and essential hypertension
in Japan. Moreover, allele frequency of each of these
polymorphisms, including the T-786→C variant, was simi-
lar in the Kyoto and Kumamoto populations. All these
findings suggest that our populations are little biased and
are therefore suitable for genetic analyses.

Based on Schlesselman's equation for statistical power,
our sample size is too small to get a statistically significant
association between the T-786→C variant and essential
hypertension, because the odds ratio and frequency of the
T-786→C variant were 0.9 and 20.6%, respectively, in the
present study. To get a positive association between the
T-786→C variant and hypertension with power stronger
than 70%, 10,035 patients and 10,035 healthy control sub-
jects would be required if the odds ratio and frequency
would be the same as the present population. Using this
sample size, if the odds ratio was more than 1.5, power
for positive association would be more than 70%, which
is a high enough power for case-control studies. It is
therefore unlikely that the T-786→C variant is strongly
associated with essential hypertension in Japanese sub-
jects.

The present results together with our previous results
show that only the Glu298Asp variant is significantly
associated with essential hypertension. In our popula-
tions, there is no linkage disequilibrium between the T-786
→C variant and the Glu298Asp variant (data not shown),
and as previously reported the Glu298Asp variant is not
linked with either SNP in intron 18 or 23. It is therefore
reasonable to conclude that the T-786→C variant is not
associated with essential hypertension. These findings
clearly indicate that a case-control study using only one
polymorphism in a candidate gene is not sufficient to de-
termine the polymorphism's involvement in the patho-
genesis of a disease.

Recently we also reported that the T-786→C variant is
strongly associated with coronary artery spastic angina
(odds ratio of 6.0) (10) and acute myocardial infarction
without organic coronary artery stenosis (14). We also re-
vealed that replication protein A1, known as a single-
stranded DNA binding protein responsible for DNA rep-
lication, recombination and repair, binds to the T-786→C
mutant eNOS allele and functions as a repressor to re-
duce eNOS gene transcription (15). However, the T-786→C
variant was not linked with essential hypertension in the
present study. Considering the evidence that the reduced
NO production seems to be related to the pathogenesis of
both CSA and hypertension, discussion of why the T-786
→C variant is associated with coronary spasm but not
with essential hypertension is warranted. One possible ex-
planation is that affected arteries are different in diameter
in patients with CSA and those with essential hyperten-
sion. CSA results from abnormal over constriction of
coronary conductance vessels, while the pathogenesis of
hypertension is related to vasoconstriction of resistance
arterioles. Further studies of the eNOS expression level in
vascular endothelial cells of various vessels with different
diameters are needed. Understanding the expression
levels may be a clue to understanding the underling
mechanism for the positive and negative association of
the T-786→C variant with CSA and essential hyperten-
sion, respectively. The finding of no strong association
between CSA and hypertension (16) is consistent with the
present data.

In conclusion, we demonstrated that the T-786→C
variant in the eNOS gene is not positively associated with
essential hypertension in a Japanese population. These
data indicate that the T-786→C variant may not be a
genetic susceptibility factor to essential hypertension, in
contrast to the Glu298Asp variant of the eNOS gene.
These results should be closely examined with the candi-
date gene approach using multiple genetic variants or
mutations.

**Abbreviations**

Abbreviations used in this paper: eNOS, endothelial nitric
oxide synthase; HTN, hypertensive subjects; NTN, normo-
tensive control subjects; NO, nitric oxide; VNTRs, variable
number of tandem repeats; PCR, polymerase chain reaction;
RFLP, restriction fragment length polymorphism; bp, base
pair; ANP, atrial natriuretic peptide; BNP, brain natriuretic
peptide.

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References