Original Article

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

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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'-TCAGTCTATGAGGTCTCGAA-3' and 5'-GTCCTTGAGTCTGACATTAGGG-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-

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

Parameter	Normotensive (n=233)	Hypertensive (n=215)
Sex Men/ Women	134/99	109/106
Age (y)	54.0±13.2	56.5±11.5
BMI (kg/m ²)	23.0±2.6	23.9±5.1 [‡]
SBP (mmHg)	125.5±11.3	161.8±18.5*
DBP (mmHg)	73.7±9.6	93.6±13.7*
T-Cho (mmol/l)	5.14±0.97	5.22±0.98
HDL-Cho (mmol/l)	1.26±0.46	1.30±0.45
HbA1c (%)	5.9±1.3	5.6±1.3
Cre (mmol/l)	83±23	83±88
UA (mmol/l)	315±83	339±83 [§]
PRA (ng/l/s)	0.30±0.30	0.33±0.70
PAC (pmol/l)	1,842±1,485	2,127±2,108
ANP (pmol/l)	8.5±2.8	12.5±9.9 [§]
BNP (pmol/l)	3.7±2.8	13.0±9.9 [§]
SV1+RV5 (mV)	2.2±1.0	3.1±1.1*
LVMI (g/m ²)	111±27	136±41*

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; SV1+RV5, voltage in electrocardiogram; LVMI, left ventricular mass index. Data are means±SD. **p*<0.0001, [§]*p*<0.01, [‡]*p*<0.03.

Table 2. Comparison of Genotype and Allele Frequencies for the T⁻⁷⁸⁶→C Variant in Hypertensive and Normotensive Subjects in the Kyoto and Kumamoto Study Groups

	Kyoto		Kumamoto	
	NTN 233 (100)	HTN 215 (100)	NTN 223 (100)	HTN 186 (100)
Genotypes				
AA	182 (78.1)	171 (79.5)	180 (80.7)	153 (82.3)
Aa+aa	51 (21.9)	44 (20.5)	43 (19.3)	33 (17.7)
Significance*	$p=0.606$		$p=0.39$	
Odds Ratio (95% CI)	0.9 (0.8-1.1)		0.9 (0.8-1.1)	

NTN, normotensive subjects; HTN, hypertensive subjects. Genotype and allele frequencies are indicated in absolute values with percentages in parentheses. "A" and "a" represent each allele of the polymorphisms. *Test of comparison of autosomal dominant genotype distributions (Fisher's exact test).

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 χ^2 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 χ^2 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 (11), 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

2.0 (1.2–3.4 in 95% Confidence Index) in both population groups.

Discussion

In the present study, we failed to find a positive association between the T⁻⁷⁸⁶→C variant and essential hypertension 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 sampling bias, which is why we used two independent populations in Kyoto and Kumamoto, 500 miles from each other, with the same entry criteria. Using these populations, we recently reported that a missense variant in exon 7 of the eNOS gene, the Glu298Asp variant, is significantly 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 between the Glu298Asp variant and essential hypertension in Japan. Moreover, allele frequency of each of these polymorphisms, including the T⁻⁷⁸⁶→C variant, was similar 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 subjects 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 subjects.

The present results together with our previous results show that only the Glu298Asp variant is significantly associated with essential hypertension. In our populations, 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 pathogenesis 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 revealed that replication protein A1, known as a single-stranded DNA binding protein responsible for DNA replication, recombination and repair, binds to the T⁻⁷⁸⁶→C mutant eNOS allele and functions as a repressor to reduce 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 explanation is that affected arteries are different in diameter in patients with CSA and those with essential hypertension. 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 underlying mechanism for the positive and negative association of the T⁻⁷⁸⁶→C variant with CSA and essential hypertension, 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 candidate gene approach using multiple genetic variants or mutations.

Abbreviations

Abbreviations used in this paper: eNOS, endothelial nitric oxide synthase; HTN, hypertensive subjects; NTN, normotensive 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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