Original Article

A Haplotype-Based Case-Control Study Examining Human Extracellular Superoxide Dismutase Gene and Essential Hypertension

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It has been reported that oxidative stress is involved in the pathophysiology of essential hypertension (EH), which is a multifactorial disorder. Extracellular superoxide dismutase (EC-SOD) protects the human body from oxidative stress by converting the toxic superoxide anion (O_2^-) into less toxic hydrogen peroxide (H_2O_2). In EC-SOD knockout mice, blood pressure was reported to be significantly higher than that seen in wild-type mice. The aim of this study was thus to investigate the relationship between EH and the human EC-SOD gene by using single-nucleotide polymorphisms (SNPs) in a haplotype-based case-control study. We selected 6 SNPs within the human EC-SOD gene (rs13306703, rs699473, rs699474, rs17881426, rs2536512 and rs1799895), and then performed case-control studies in 243 EH patients and 251 age-matched normotensive (NT) subjects. In Japanese subjects, no heterogeneity was found for rs699474, and no significant differences were observed between the EH and NT groups for the overall distribution of the genotypes or the alleles for each of the SNPs. However, in the haplotype-based case-control study that used rs13306703 and rs2536512, significant differences were observed in the overall distribution ($\chi^2=14.26$, $p=0.003$). The frequency of the T-A haplotype was significantly higher in the EH group than in the NT group (2.4% vs. 0.0%, $p<0.001$). Based on the results of our haplotype-based case-control study, the T-A haplotype may be a genetic marker for EH, and thus the EC-SOD gene might be a susceptibility gene for EH. (*Hypertens Res* 2008; 31: 1533–1540)

Key Words: essential hypertension, extracellular superoxide dismutase, single-nucleotide polymorphism, haplotype, case-control study

Introduction

Superoxide, one of the most abundant reactive oxygen species (ROS) produced by the mitochondria, plays an essential role in the human defense system by protecting the body from invading bacteria and viruses (1). However, the superoxide

anion (O_2^-) can react with nitric oxide (NO) to form peroxynitrite ($ONOO^-$). Exposure to ROS such as O_2^- and $ONOO^-$ leads to oxidative stress and a breakdown of cellular macromolecules (2), which can then cause various diseases such as cancer, leukemia, cerebral infarction (CI), myocardial infarction (MI) and hypertension (3, 4). In the human body, superoxide dismutase (SOD) converts superoxide to hydrogen

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Fig. 1. Organization of the gene encoding human extracellular superoxide dismutase and the location of the single-nucleotide polymorphisms (SNPs) that were used in the present association study. Closed boxes indicate exons; lines represent introns.

peroxide (H_2O_2), which is then removed by glutathione peroxidase or catalase (5). Therefore, ROS do not normally present a problem, as SOD prevents ROS levels from increasing to the point where they can cause cellular damage.

McCord and Fridovich characterized SOD more than 30 years ago (6). Since then, three distinct mammalian isoforms of SOD have been reported (6). Initially, a copper- and zinc-containing superoxide dismutase (CuZn-SOD: SOD1) was discovered in 1969. CuZn-SOD is localized in the cytoplasmic and nuclear compartments (7). It was subsequently discovered that manganese superoxide dismutase (Mn-SOD: SOD2), a tetratric manganese-containing enzyme, was localized within the mitochondria (8). More recently, an extracellular superoxide dismutase (EC-SOD: SOD3) was discovered (9). The human EC-SOD gene, which is located on chromosome 4p16.3–q21, contains three exons and two introns. A gene distinct from CuZn-SOD is responsible for encoding human EC-SOD, which is composed of 240 amino acids and harbors an 18-peptide segment that targets the protein to the extracellular compartment (8, 10). Although the function of the amino terminal half of the mature EC-SOD is unclear, amino acid residues 96–193 show a high level of sequence homology to CuZn-SOD, and also contain the critical amino acids for catalytic activity. There was no sequence homology between Mn-SOD and EC-SOD (10).

In the human body, EC-SOD is present only in small amounts in most tissues. However, within the vascular tissues the presence increases to 30 to 50% of the total SOD (11, 12). It has been reported that the blood pressure in EC-SOD knockout (EC-SOD^{-/-}) mice is higher than that observed for wild-type mice after a dose-dependent administration of angiotensin II (12–14), suggesting that EC-SOD is important in blood pressure regulation.

In most populations, hypertension affects 25% of adults and is a major risk factor for death from CI, MI, and congestive heart failure (15). The most prevalent form of hypertension is essential hypertension (EH), which is considered to be a multifactorial disease that results from an interaction

between genetic and environmental factors (16).

The aim of this study was to investigate the relationship between EH and the human EC-SOD gene by using single-nucleotide polymorphisms (SNPs) and a haplotype-based case-control study.

Methods

Subjects

The 243 EH subjects enrolled in this study were diagnosed using the following criteria: a seated systolic blood pressure (SBP) ≥ 160 mmHg or a diastolic BP (DBP) ≥ 100 mmHg on 3 occasions, within 2 months of the subject's first medical examination. None of the EH subjects was using antihypertensive medications or Cox inhibitors (NSAIDs), and all patients with secondary forms of hypertension were excluded. Diagnosis of secondary forms of hypertension was made on the basis of clinical and laboratory examinations that included: 1) measurement of fasting blood sugar, glycosylated hemoglobin A1c, plasma aldosterone, plasma renin activity and plasma catecholamines and 2) computed tomography and magnetic resonance imaging, which were used to assess the condition of the adrenal glands and to check for pituitary tumors. For comparison, we included 251 healthy normotensive (NT) controls. None of the NT subjects had a family history of hypertension, and all NT subjects had a SBP of <130 mmHg and a DBP of <85 mmHg. A family history of hypertension was defined as a prior diagnosis of hypertension in a grandparent, uncle, aunt, parent, or sibling. Both groups were recruited from the Tokyo area in Japan. Informed consent was obtained from each subject according to a protocol approved by the Human Studies Committee of Nihon University (17).

Biochemical Analysis

Blood samples were drawn in the morning from fasting sub-

Table 1. Characteristics of Study Participants

	Total			Men			Women		
	NT	EH	<i>p</i> -value	NT	EH	<i>p</i> -value	NT	EH	<i>p</i> -value
Number of subjects	251	243		162	155		89	88	
Age (years)	52.4±10.1	51.1±5.5	0.070	52.1±6.7	51.0±5.6	0.059	52.6±14.3	51.2±5.3	0.410
BMI (kg/m ²)	22.8±3.3	24.5±3.5	<0.001	23.1±3.3	24.7±3.3	<0.001	22.3±3.3	24.2±3.8	<0.001
SBP (mmHg)	112.4±10.6	174.0±20.1	<0.001	113.1±10.3	171.7±19.2	<0.001	111.1±11.1	178.1±21.1	<0.001
DBP (mmHg)	69.4±8.5	106.4±12.9	<0.001	70.3±8.0	106.7±12.9	<0.001	67.9±9.3	105.7±13.0	<0.001
Pulse (beats/min)	73.3±13.0	77.3±15.4	0.007	72.8±13.9	76.9±16.0	0.031	74.2±11.1	77.9±14.3	0.100
Creatinine (mg/dL)	0.82±0.21	0.85±0.25	0.248	0.89±0.21	0.94±0.23	0.091	0.69±0.18	0.68±0.18	0.784
Total cholesterol (mg/dL)	198.9±46.2	208.8±40.3	0.014	193.7±49.7	201.1±39.9	0.144	208.4±43.9	221.7±37.8	0.036
HDL cholesterol (mg/dL)	56.3±17.7	56.6±17.8	0.872	54.3±16.5	53.2±17.2	0.561	60.1±19.5	62.3±17.5	0.465
Uric acid (mg/dL)	5.41±1.78	5.61±1.58	0.206	5.83±1.37	6.15±1.50	0.518	4.62±2.14	4.65±1.22	0.904
Hyperlipidemia (%)	18.3	25.5	0.053	7.9	10.4	0.178	23.6	33.0	0.167
Diabetes (%)	3.2	9.1	0.006	3.1	10.3	0.010	3.4	6.8	0.297
Drinking (%)	60.6	68.3	0.112	71.9	83.2	0.029	37.5	42.0	0.599
Smoking (%)	42.9	53.6	0.035	51.4	66.9	0.011	26.3	35.4	0.259

EH, essential hypertension; NT, normotension; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high density lipoprotein. Continuous variables are expressed as mean±SD. Categorical variables were expressed as percentage. *p*-values of continuous variables were calculated by Mann-Whitney *U* test. *p*-values of categorical variables were calculated by Fisher's exact test.

jects who had rested in a sitting position for at least 30 minutes. The clinical laboratory of our university hospital measured the total cholesterol and the high-density lipoprotein (HDL) cholesterol concentrations in the plasma, as well as the creatinine and uric acid concentrations in the serum (15, 17, 18).

Genotyping

Because the SNP detail data for the EC-SOD gene on the HapMap website were not clear, we used information on SNP allelic frequencies from the website of the National Center for Biotechnology Information (NCBI) instead. We selected 6 SNPs for the human EC-SOD gene. All the SNPs used were considered minor alleles with frequencies >5%, and there was at least one SNP from the promoter, intron, and exon regions. Using these 6 SNPs, we examined their association with EH, confirming all information for the 6 SNPs on the NCBI website. The SNP accession numbers were rs13306703, rs699473, rs699474, rs17881426, rs2536512 and rs1799895 (Fig. 1). Genotypes were determined using Assays-on-Demand kits (Applied Biosystems [ABI], Branchburg, USA) in addition to TaqMan PCR assays (ABI) (17). The TaqMan® SNP Genotyping Assays were performed using the Taq amplification method. In the 5' nuclease assay, the allele-specific fluorogenic probes that are hybridized to the template are cleaved by the 5' nuclease activity of the Taq polymerase, allowing for discrimination during the PCR assay. The probes contain a 3' minor groove-binding group (MGB) that hybridizes to single-stranded targets with increased sequence specificity compared with ordinary DNA

probes. This reduces nonspecific probe hybridization, which results in low background fluorescence during the 5' nuclease PCR assay (TaqMan®, ABI). When cleavage occurs, there is a net increase in the emission of the reporter dye. The 5' nuclease assay requires two unlabeled PCR primers and two allele-specific probes, with each probe labeled with two reporter dyes at the 5' end. In the current study, VIC and FAM were used as the reporter dyes. The primers and probes used in the TaqMan® SNP Genotyping Assays (ABI) were chosen from the information available on the ABI website (<http://myscience.appliedbiosystems.com>).

PCR amplification was performed using 2.5 µL of TaqMan® Universal Master Mix, No AmpErase® UNG (2×) (ABI) in 5 µL final reaction volumes, along with 2 ng DNA, 2.375 µL ultrapure water, 0.079 µL Tris-EDTA (TE) buffer (1×), 0.046 µL TaqMan® SNP Genotyping Assay Mix (40×) containing a 331.2 nmol/L final concentration of primers, and a 73.6 nmol/L final concentration of probes. The thermal cycling conditions were 95°C for 10 min, followed by 50 cycles of 92°C for 15 s, and finally 60°C for 1 min. Thermal cycling was performed using the GeneAmp 9700™ system.

Each 96-well plate contained 80 samples of an unknown genotype and 4 control samples that contained reagents without any DNA template. As outlined in the TaqMan Allelic Discrimination Guide (ABI), negative control samples lacking DNA were necessary for signal processing by the Sequence Detection System (SDS) 7700™. These plates were read on the SDS 7700 instrument using the end-point analysis mode of the SDS version 1.6.3 software package (ABI). The genotypes were determined visually based on the dye-component fluorescent emission data depicted in the *X-Y* scatterplot

Table 2. Genotype and Allele Distributions in Normotensives and Patients with EH

		Total			Men			Women		
		NT	EH	<i>p</i> -value	NT	EH	<i>p</i> -value	NT	EH	<i>p</i> -value
Number of participants		251	243		162	155		89	88	
Variants										
rs13306703										
Genotype	C/C	195 (0.777)	190 (0.782)	0.800	124 (0.765)	120 (0.774)	0.944	71 (0.798)	70 (0.795)	0.611
	C/T	51 (0.203)	50 (0.206)		34 (0.210)	32 (0.206)		17 (0.191)	18 (0.205)	
	T/T	5 (0.020)	3 (0.012)		4 (0.025)	3 (0.019)		1 (0.011)	0 (0.000)	
	C/C and C/T	246 (0.980)	240 (0.988)	158 (0.975)	152 (0.981)	88 (0.989)	88 (1.000)	0.319		
	T/T	5 (0.020)	3 (0.012)	4 (0.025)	3 (0.019)	1 (0.011)	0 (0.000)			
	C/C	195 (0.777)	190 (0.782)	124 (0.765)	120 (0.774)	71 (1.000)	70 (0.795)			
Allele	C/T and T/T	56 (0.223)	53 (0.218)	0.893	38 (0.235)	35 (0.226)	0.853	0 (0.000)	18 (0.205)	0.970
	C	441 (0.878)	430 (0.885)	0.760	282 (0.870)	272 (0.877)	0.789	159 (0.893)	158 (0.898)	0.876
	T	61 (0.122)	56 (0.115)		42 (0.130)	38 (0.123)		19 (0.107)	18 (0.102)	
rs699473										
Genotype	C/C	121 (0.482)	122 (0.502)	0.466	86 (0.531)	78 (0.503)	0.512	35 (0.393)	44 (0.500)	0.308
	C/T	101 (0.402)	101 (0.416)		58 (0.358)	64 (0.413)		43 (0.483)	37 (0.420)	
	T/T	29 (0.116)	20 (0.082)		18 (0.111)	13 (0.084)		11 (0.124)	7 (0.080)	
	C/C and C/T	222 (0.884)	223 (0.918)	144 (0.889)	142 (0.916)	78 (0.876)	81 (0.920)	0.332		
	T/T	29 (0.116)	20 (0.082)	18 (0.111)	13 (0.084)	11 (0.124)	7 (0.080)			
	C/C	121 (0.482)	122 (0.502)	86 (0.531)	78 (0.503)	35 (0.393)	44 (0.500)			
Allele	C/T and T/T	130 (0.518)	121 (0.498)	0.657	76 (0.469)	77 (0.497)	0.626	54 (0.607)	44 (0.500)	0.153
	C	343 (0.683)	345 (0.710)	0.363	230 (0.710)	220 (0.710)	0.996	113 (0.635)	125 (0.710)	0.131
	T	159 (0.317)	141 (0.290)		94 (0.290)	90 (0.290)		65 (0.365)	51 (0.290)	
rs17881426										
Genotype	A/A	4 (0.016)	3 (0.012)	0.454	4 (0.025)	3 (0.019)	0.585	0 (0.000)	0 (0.000)	0.478
	A/T	54 (0.215)	42 (0.173)		34 (0.210)	26 (0.168)		20 (0.225)	16 (0.182)	
	T/T	193 (0.769)	198 (0.815)		124 (0.765)	126 (0.813)		69 (0.775)	72 (0.818)	
	A/A and A/T	58 (0.231)	45 (0.185)	38 (0.235)	29 (0.187)	20 (0.225)	16 (0.182)	0.478		
	T/T	193 (0.769)	198 (0.815)	124 (0.765)	126 (0.813)	69 (0.775)	72 (0.818)			
	A/A	4 (0.016)	3 (0.012)	4 (0.025)	3 (0.019)	0 (0.000)	0 (0.000)			
Allele	A/T and T/T	247 (0.984)	240 (0.988)	0.736	158 (0.975)	152 (0.981)	0.749	89 (1.000)	88 (1.000)	—
	A	62 (0.124)	48 (0.099)	0.217	42 (0.130)	32 (0.103)	0.301	20 (0.112)	16 (0.091)	0.504
	T	440 (0.876)	438 (0.901)		282 (0.870)	278 (0.897)		158 (0.888)	160 (0.909)	
rs2536512										
Genotype	A/A	22 (0.088)	17 (0.070)	0.640	14 (0.086)	12 (0.077)	0.685	8 (0.090)	5 (0.057)	0.643
	A/G	102 (0.406)	107 (0.440)		63 (0.389)	68 (0.439)		39 (0.438)	39 (0.443)	
	G/G	127 (0.506)	119 (0.490)		85 (0.525)	75 (0.484)		42 (0.472)	44 (0.500)	
	A/A and A/G	124 (0.494)	124 (0.510)	77 (0.475)	80 (0.516)	47 (0.528)	44 (0.500)	0.709		
	G/G	127 (0.506)	119 (0.490)	85 (0.525)	75 (0.484)	42 (0.472)	44 (0.500)			
	A/A	22 (0.088)	17 (0.070)	14 (0.086)	12 (0.077)	8 (0.090)	5 (0.057)			
Allele	G/G and A/G	229 (0.912)	226 (0.930)	0.466	148 (0.914)	143 (0.923)	0.770	81 (0.910)	83 (0.943)	0.399
	A	146 (0.291)	141 (0.290)	0.980	91 (0.281)	92 (0.297)	0.659	55 (0.309)	49 (0.278)	0.528
	G	356 (0.709)	345 (0.710)		233 (0.719)	218 (0.703)		123 (0.691)	127 (0.722)	
rs1799895										
Genotype	C/C	226 (0.900)	219 (0.901)	0.975	144 (0.889)	140 (0.903)	0.676	82 (0.921)	79 (0.898)	0.584
	C/G	25 (0.100)	24 (0.099)		18 (0.111)	15 (0.097)		7 (0.079)	9 (0.102)	
	G/G	0 (0.000)	0 (0.000)		0 (0.000)	0 (0.000)		0 (0.000)	0 (0.000)	
	C/C and C/G	251 (1.000)	243 (1.000)	162 (1.000)	155 (1.000)	89 (1.000)	88 (1.000)	0.584		
	G/G	0 (0.000)	0 (0.000)	0 (0.000)	0 (0.000)	0 (0.000)	0 (0.000)			
	C/C	226 (0.900)	219 (0.901)	144 (0.889)	140 (0.903)	82 (0.921)	79 (0.898)			
Allele	G/G and C/G	25 (0.100)	24 (0.099)	0.975	18 (0.111)	15 (0.097)	0.676	7 (0.079)	9 (0.102)	0.584
	C	477 (0.950)	462 (0.951)	0.976	306 (0.944)	295 (0.952)	0.685	171 (0.961)	167 (0.949)	0.593
	G	25 (0.050)	24 (0.049)		18 (0.056)	15 (0.048)		7 (0.039)	9 (0.051)	

EH, essential hypertension; NT, normotension.

SNP		rs699473	rs17881426	rs2536512	rs1799895
rs13306703	$ D' $	1.000	0.886	0.517	0.860
	r^2	0.064	0.771	0.015	0.005
rs699473	$ D' $		0.896	0.819	1.000
	r^2		0.052	0.593	0.043
rs17881426	$ D' $			0.859	0.890
	r^2			0.043	0.006
rs2536512	$ D' $				1.000
	r^2				0.002

$|D'| \geq 0.50$
 $r^2 \geq 0.25$

Fig. 2. Pairwise LD in the human EC-SOD gene, as evaluated by $|D'|$ and r^2 . Pairwise LD was computed for the 5 marker pairs that were studied in the human EC-SOD gene. Pairs in LD ($|D'| \geq 0.5$ or $r^2 \geq 0.25$) are shown as grayshaded values.

of the SDS software. The software also automatically determined the genotypes through the use of signal processing algorithms. The results of each scoring method were saved in two separate output files for later comparison (18–20).

Statistical Analysis

Data are shown as means $\pm$ SD. The Hardy-Weinberg equilibrium (HWE) was assessed using χ^2 analysis. The overall distribution of alleles was analyzed using 2×2 contingency tables, with the distribution of the genotypes between the EH patients and the NT control subjects analyzed using a two-sided Fisher's exact test (21, 22). For this haplotype-based case-control study, a linkage disequilibrium (LD) analysis based on the genotype data of the genetic variations was performed using the expectation maximization (EM) algorithm of the SNPalyze software program, version 3.2 (Dynacom Co., Ltd., Yokohama, Japan). A pairwise LD analysis was completed using the SNP pairs. $|D'|$ values ≥ 0.5 were used to assign the SNP locations to one haplotype block. Tagged SNPs were selected by omitting one SNP from an SNP pair that showed an $r^2 \geq 0.25$ for each haplotype block. In this haplotype-based case-control study, haplotypes with a frequency < 0.02 were excluded. The distribution of the haplotypes' frequencies was calculated using the χ^2 test. A probability level of $p < 0.05$ was considered to indicate statistical significance. Differences in clinical data between the EH and NT groups were assessed using an analysis of variance followed by Fisher's protected least significant difference test (20).

Results

Table 1 shows the clinical features of the EH patients and NT control subjects. There were no significant differences in age, serum concentration of creatinine, plasma concentration of

HDL cholesterol, uric acid, hyperlipidemia or drinking between the 2 groups.

Table 2 shows the distribution of genotypic and allelic frequencies of the SNPs in each group. rs699474 was excluded because there was no heterogeneity; *i.e.*, the genotype for all of the participants was G/G. The observed and expected genotypic frequencies for each of the SNPs in the total study group and between both men and women in the NT group were in good agreement with the predicted Hardy-Weinberg equilibrium values (data not shown). In addition, there were no significant differences between the total EH and total NT groups for the overall distribution of the genotype and the allele for all 5 SNPs.

In Fig. 2, patterns of the LD are shown with their $|D'|$ and r^2 values. Because all the $|D'|$ values were large, all the polymorphisms were located in one haplotype block. In addition, since the r^2 of rs13306703–rs17881426 and rs699473–rs2536512 were ≥ 0.25 , we constructed a haplotype-based association study using rs13306703, rs2536512 and rs1799895.

Table 3 shows the distribution of the individual haplotypes. Significant differences between the total EH and total NT groups for the overall distribution were observed for two combinations, rs13306703–rs2536512 and rs13306703–rs2536512–rs1799895 ($\chi^2 = 14.26$, $p = 0.003$ and $\chi^2 = 13.98$, $p = 0.007$). The T-A haplotype constructed with the rs13306703–rs2536512 in EH was significantly higher than that seen for the NT group ($\chi^2 = 12.45$, $p < 0.001$). The T-A-C haplotype constructed with the rs13306703–rs2536512–rs1799895 in EH was significantly higher than that seen for the NT group ($\chi^2 = 12.40$, $p < 0.001$).

Discussion

While some papers have reported a relationship between the

Table 3. Distribution of Individual Haplotypes

Combination of SNPs	Overall distribution		Distribution of individual haplotypes					
	χ^2	<i>p</i> -value	Haplotype	Overall	NT (251)	EH (243)	χ^2	<i>p</i> -value
rs13306703–rs2536512	14.26	0.003	C-G	0.611	0.598	0.619	0.45	0.501
			T-G	0.098	0.114	0.091	1.53	0.216
			C-A	0.270	0.287	0.266	0.58	0.446
			T-A	0.020	0.000	0.024	12.45	<0.001
rs13306703–rs1799895	0.10	0.953	C-C	0.832	0.829	0.835	0.08	0.778
			T-C	0.118	0.122	0.115	0.09	0.760
			C-G	0.050	0.050	0.049	<0.01	0.976
rs2536512–rs1799895	<0.01	0.999	G-C	0.660	0.659	0.660	<0.01	0.970
			A-C	0.290	0.291	0.290	<0.01	0.980
			G-G	0.050	0.050	0.049	<0.01	0.976
rs13306703–rs2536512–rs1799895	13.98	0.007	C-G-C	0.563	0.552	0.571	0.31	0.580
			T-G-C	0.097	0.113	0.090	1.34	0.247
			C-A-C	0.269	0.286	0.265	0.53	0.465
			T-A-C	0.022	0.000	0.025	12.40	<0.001
			T-A-G	0.050	0.048	0.049	0.01	0.942

EH, essential hypertension; NT, normotension.

human EC-SOD gene and EH (7, 12–14), there have been no reports detailing association studies using haplotypes. Thus, this is the first time an association study between the human EC-SOD gene and EH using a haplotype has been reported in the literature.

rs2536512 and rs1799895, which are located in the amino acid coding region in the human EC-SOD gene, are nonsynonymous, *i.e.*, Ala40Thr (GCG→ACG) and Arg213Gly (CGG→GGG). In a functional SNP study, normally one or two SNPs are used. Tamai *et al.* (23) previously reported an association between the EC-SOD gene SNP rs2536512 (Ala40Thr) and type 2 diabetes. In that study, both the frequency of the Thr allele and the number of subjects with the Thr allele (Ala/Thr+Thr/Thr) were higher in diabetic patients than in non-diabetic subjects. Also, patients with the Thr allele had a higher prevalence of hypertension than those without the allele. Those researchers also hypothesized that rs2536512 was not a functional SNP but might be a genetic marker for susceptibility to type 2 diabetes and hypertension. Moreover, Chu *et al.* (24) reported finding a vascular effect for rs1799895 using an expression vector that included the variant. They made EC-SOD_{Arg213Gly} using a recombinant adenovirus that expressed mutagenesis of rs1799895. In order to investigate how EC-SOD_{Arg213Gly} affected the vascular functions, they injected EC-SOD_{Arg213Gly} or normal EC-SOD into spontaneously hypertensive rats (SHRs). In contrast to the SHRs that were injected with normal EC-SOD, no significant protective effect against changes in arterial pressure, vascular function, or vascular levels of oxidative stress were noted for SHRs injected with EC-SOD_{Arg213Gly}. However, that report presented rat data, and because there have been no case-control studies that examined the differences between EH and

rs1799895 (Arg213Gly) in human EC-SOD, it is unclear whether those data are transferable to the human variant. If the data are transferable, it would suggest that the rs1799895 SNP is a functional SNP for EH. In the present report, we performed an association study using the rs13306703, rs2536512 and rs1799895 in a case-control study. Our results show that the T-A haplotype at rs13306703–rs2536512 was significantly more frequent in the EH group than in the NT group (2.4% vs. 0.0%, $\chi^2=12.45$, $p<0.001$). Moreover, the T-A-C haplotype at rs13306703–rs2536512–rs1799895 was significantly more frequent in the EH group than in the NT group (2.5% vs. 0.0%, $\chi^2=12.40$, $p<0.001$). rs1799895 is not considered an important genetic marker for EH because the T-A haplotype at rs13306703–rs2536512 and the T-A-C haplotype at rs13306703–rs2536512–rs1799895 also both show significant differences. Our data suggest that the T-A haplotype is indeed a genetic marker for EH regardless of whether the haplotype itself is functional in mediating the factors associated with EH, *i.e.*, arterial pressure, vascular function and oxidative stress (O_2^- and ONOO $^-$). Thus, there may be a mutation that is responsible for linking the T-A haplotype. As this possible mutation might be related to the activity of EC-SOD, it may subsequently affect vascular function.

Since the first human genome draft sequence was completed in 2001, the methodologies and strategies for performing genetic research have changed dramatically. SNPs are now used for the positional cloning of susceptibility genes by performing whole-genome-wide scanning (25). With regard to these changes, haplotype analysis has changed more than any other technique. Recent studies have shown that the human genome has a haplotype block structure that can be divided into discrete blocks of limited haplotype diversity. In

each block, a small fraction of SNPs, referred to as “tag SNPs,” can be used to distinguish a large fraction of the haplotypes. These tag SNPs have the potential to be extremely useful in association studies, as their use relieves the burden of genotyping all the SNPs. Thus, a haplotype-based case-control study is now considered to be much more effective than a marker-by-marker analysis (26). In genes with multiple susceptibility alleles, in particular when the LD between the polymorphisms is weak, a haplotype-based association study has advantages over an analysis that is based on individual polymorphisms (27). In the present study, the haplotype-based case-control study was constructed using rs13306703–rs2536512, which exhibited a significant difference. Haplotype analysis has been used to successfully localize the susceptibility genes for some multifactorial diseases (28, 29). Based on such findings, we hypothesized that haplotype analysis would be useful for assessing the association between haplotypes and EH, resulting in the present study in which we attempted to use SNPs to establish the haplotypes of the EC-SOD gene. We found that the low-frequency haplotype in the EH group exhibited a significant difference in the haplotype-based case-control study. Since several studies have indicated that this phenomenon might occur frequently, we believe our results confirm the usefulness of case-control studies in the examination of multifactorial diseases (19, 30).

In conclusion, the T-A haplotype may be a genetic marker for EH, and therefore the human EC-SOD gene could very well be a susceptibility gene for EH. Further studies are needed, however, to clarify the causal/susceptibility mutation of the EC-SOD gene and/or the neighboring genes in EH.

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