Association Between Fatty Acid Binding Protein 3 Gene Variants and Essential Hypertension in Humans

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BACKGROUND

We have earlier identified a quantitative trait locus (QTL) on rat chromosome 5 that appears to be primarily under the control of the sympathetic nervous system. Because sympathetic overactivity is related to both hypertension and insulin resistance, *FABP3* is a candidate gene for the link between this QTL and blood pressure regulation. In this study, therefore, we explored the role of *FABP3* genetic variations in essential hypertension (EH) in humans.

METHODS

We evaluated two single-nucleotide polymorphisms (SNPs) (rs2279885 and rs2271072) in 758 patients with EH and 726 controls. Polymorphism-related genotypes were determined using TaqMan assays, while haplotypes were estimated from the genotype data.

RESULTS

The frequencies of occurrence of the G allele of rs2279885 and the C allele of rs2271072 were significantly higher in subjects with EH than in normotensive (NT) subjects ($P = 0.0339$, $P = 0.0209$, respectively). However, the genotype distributions did not exhibit any significant differences.

CONCLUSION

We found an association between *FABP3* gene polymorphisms and EH in a Japanese population, thereby suggesting that *FABP3* is a susceptibility locus for EH.

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Hypertension is a disease of multifactorial origin, involving environmental factors as well as genetic factors[1] arising from the inheritance of a number of susceptibility genes. These genes may contribute to between 30 and 50% of the variations in blood pressure seen among individuals.[2]

In order to understand the genetic components of hypertension, many studies have used rats that are crosses between inbred strains, so as to dissect out quantitative trait loci (QTLs) that are linked to blood pressure.[3–5] We have earlier established pharmacogenetic methods of evaluating the contribution of blood pressure regulatory systems to blood pressure maintenance. This novel approach systematically analyzes the cosegregation of genetic loci for acute cardiovascular responses to drugs that influence the renin–angiotensin system, sympathetic nervous system, or nitric oxide system in F2 populations derived from cross-breeding Prague hypertensive-hyperlipidemic rats with normotensive (NT) Lewis rats. In this analysis, the disappearance of QTLs for mean arterial pressure after blockade of blood pressure regulation reveals the contribution of the blocked system to baseline mean arterial pressure. We have identified a QTL on rat chromosome 5 that is correlated with basal blood pressure. The correlation disappeared after pentolinium administration, thereby suggesting modulation by the sympathetic nervous system.[6,7]

In recent decades, several intracellular fatty acid binding proteins (FABPs) have been identified. FABPs appear to modulate intracellular lipid metabolism and regulation of gene expression, as well as intracellular shuttling of fatty acids.[8–12] Several FABPs have been implicated in the development of insulin resistance and metabolic syndrome.

Fatty acid binding protein 3 (FABP3) has been suggested to be the major FABP in skeletal muscle.[13,14] Blaak *et al.* demonstrated reduced FABP3 expression in patients with type 2 diabetes, correlating with diminished fatty acid uptake in skeletal muscle. They also showed a positive correlation between the degree of weight loss and increase in FABP3 content.[15] Because

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sympathetic overactivity is related to both hypertension and insulin resistance,[16,17] *FABP3* is an attractive candidate gene for blood pressure regulation in the QTL that we have earlier identified on rat chromosome 5.[6,7]

Despite recent significant advances in genomic and statistical tools, a genetics-based approach to human hypertension remains a major challenge. The aim of this study was to investigate the association between human FABP3 and hypertension by analyzing single-nucleotide polymorphisms (SNPs) in the human *FABP3* gene.

METHODS

Case–control study: study in collaboration with the hypertension section of the Japanese Millennium Project. The study population consisted of 758 patients with essential hypertension (EH) and 726 NT healthy control subjects, recruited through a subgroup collaboration with the hypertension section of the Japanese Millennium Project. Six medical institutes took part in the collaborative study and collected data on hypertensive patients and controls. Hypertensive patients were defined as those having a systolic blood pressure ≥140 mm Hg or a diastolic blood pressure of ≥90 mm Hg, or those receiving chronic antihypertensive medication. In order to increase the statistical power of the present study, additional inclusion criteria were set for the hypertensive subjects: 60 years of age, or onset of hypertension at 50 years of age; a family history of hypertension; and absence of obesity (body mass index ≤26 kg/m^2). The inclusion criteria for the NT subjects were: systolic blood pressure/diastolic blood pressure ≤130/85 mm Hg; no family history of hypertension; and absence of obesity. Both groups were recruited from throughout Japan, and informed consent was obtained from each individual as per the protocol approved by each institution's human studies committee.

Genotyping. On the basis of information from the web site of the National Center for Biotechnology Information (NCBI) regarding frequency of occurrence of alleles, two SNPs in the human *FABP3* gene, with minor alleles >15% were selected. SNPs containing relatively more minor alleles have demonstrated utility as genetic markers in genetic case–control studies. Both the SNPs were confirmed using the NCBI web site (accession numbers, rs2279885 and rs2271072) (**Figure 1a**). We examined the association between EH and these 2 SNPs.

Genotypes were determined using Assay-on-Demand kits (Applied Biosystems, Foster City, CA) together with TaqMan PCR. TaqMan Universal Master Mix (PE Biosystems, Foster City, CA) was used for PCR in a 25 μl reaction volume containing 50 ng of DNA, 700 nmol/l primer, and 100 nmol/l probe final concentrations. Thermal cycling conditions were: 95 °C for 10 min, followed by 40 cycles at 92 °C for 15 s, and 60 °C for 1 min in a GeneAmp 9700 system. Fluorescence levels of the PCR products were measured using an ABI PRISM 7700 Sequence Detector (Applied Biosystems), resulting in clear identification of three genotypes for the two alleles.

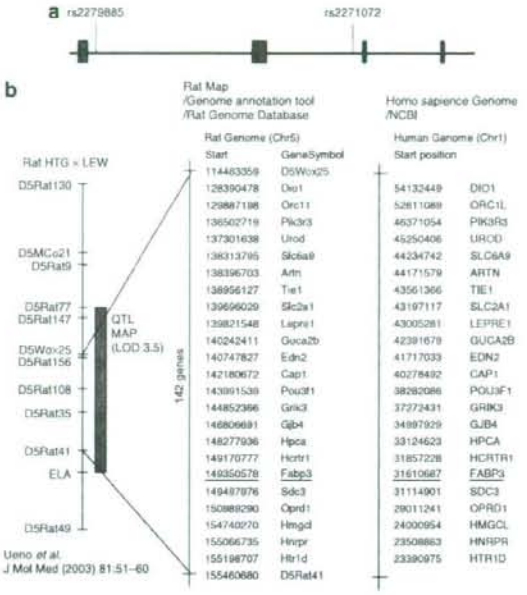

Figure 1 | Fatty acid binding protein 3 gene structure and comparative map of rat chromosome 5 and human chromosome 1. (**a**) Organization of the human fatty acid binding protein 3 gene and location of the SNPs analyzed in the present case–control study. Solid boxes, exons; and solid line, introns. (**b**) Comparative map of rat chromosome 5 and human chromosome 1. Comparative mapping was performed using HTG×LEW map,[18] the genome annotation tool of the Rat Genome Database (http://rgd.mcw/edu/), and the Homo Sapiens Genome Map of National Center for Biotechnology Information (NCBI) (http://www.ncbi.nih.gov). Dio1, deiodinase, iodothyronine, type I; Orc1l, origin recognition complex, subunit 1-like; Pik3r3, phosphatidylinositol 3 kinase, regulatory subunit, polypeptide 3; Urod, uroporphyrinogen decarboxylase; solute carrier family 6, member 9; Artn, artemin; Tie1, tyrosine kinase with immunoglobulin-like and EGF-like domains 1; Slc2a1, solute carrier family 2, member 1; Lepre1, leprecan 1; Guca2b, guanylate cyclase activator 2b; Edn2, endothelin 2; Cap1, adenylate cyclase-associated protein 1; Pou3f1, POU domain, class 3, transcription factor 1; Girk3, potassium inwardly rectifying channel, subfamily J, member 9; Hpca, hippocalcin; Hcrtr1, hypocretin receptor 1; Fabp3, fatty acid binding protein 3; Sdc3, syndecan 3; Oprd1, opioid receptor, delta 1; Hmgcl, 3-hydroxy-3-methylglutaryl-Coenzyme A lyase; Hnrpr, heterogeneous nuclear ribonucleoprotein R; Htr1d, 5-hydroxytryptamine receptor 1D.

Comparative mapping was performed using HTG×LEW map,[18] the genome annotation tool of the Rat Genome Database (http://rgd.mcw.edu/), and the Homo Sapiens Genome Map of the NCBI (http://www.ncbi.nih.gov).

Linkage disequilibrium analysis. SNPAlyze version 3.2 (DYNACOM, Yokohama, Japan) was used for determining haplotype and linkage disequilibrium (http://www.dynacom.co.jp/products/package/snpalyze/index.html).

Statistical analysis. The data are shown as mean values ± s.d. All statistical analyses were carried out using StatView 5.0 (SAS, Cary, NC) and Dr SPSS II (SPSS, Chicago, IL). Hardy–Weinberg equilibrium was assessed by two analysis. The overall

Table 1 | Clinical characteristics of NT and EH

	EH	NT
Number of subjects	758	726
Male (%)	564 (74.4)	550 (75.8)
Age	59.0 ± 11.0*	62.8 ± 9.4
Body mass index (kg/m^2)	23.6 ± 3.0*	22.7 ± 2.9
Systolic blood pressure (mm Hg)	163.5 ± 24.6*	115.9 ± 12.0
Diastolic blood pressure (mm Hg)	100.3 ± 15.7*	72.0 ± 7.6
Antihypertensive medication, *n* (%)	499 (65.8)	

EH, essential hypertension; NT, normotensive control.
*$P < 0.05$ by Mann–Whitney's *U*-test.

distributions of the genotypes or alleles were analyzed by two analysis using 2 3 or 2 2 contingency tables between EH patients and NT controls. In order to assess the quantitative effects of covariates, multiple logistic regression analysis was carried out using SPSS II. Statistical significance was established at $P = 0.05$.

RESULTS

We had earlier identified a QTL on rat chromosome 5, between D5Wox25 and D5Rat41,[18] that appeared to affect blood pressure regulation. One hundred and forty two known genes were mapped to this locus and annotated using the genome-annotation tool of the Rat Genome Database (http://rgd.mcw.edu/). FABP3 was located in the middle area of the region. This rat locus was syntenic with a region on human chromosome 1. The mapping data for the human locus were obtained from the NCBI (http://www.ncbi.nih.gov) (**Figure 1b**).

The baseline characteristics of the patient and control groups are given in **Table 1**. The EH patients had significantly higher values of body mass index as well as of systolic and diastolic blood pressure. Of the 758 patients with EH, 499 were on treatment with oral antihypertensive agents.

We performed the case–control study for the rs2279885 and rs2271072 SNPs using 758 EH patients and 726 NT controls. The observed and expected genotypic frequencies of occurrence of each SNP in the NT subjects were in good agreement with the predicted Hardy–Weinberg equilibrium values (**Table 2**).

The genotypic frequency of occurrence of rs2279885 ($P = 0.0450$), and the distribution in its recessive model (CC vs. GG + GC, $P = 0.0160$), showed significant differences between the EH and NT groups.

The genotypic frequency of occurrence of rs2271072 and the distribution in its dominant model (CC + GC vs. GG) also showed significant differences between the EH and NT groups, with the G allele of rs2271072 occurring more frequently in EH subjects than in NT subjects ($P = 0.0130$).

The frequencies of occurrence of haplotypes were calculated, and a statistically significant difference was found between the EH and NT groups with respect to the H2 haplotype (C-G) (**Table 3**).

Table 2 | Genotype distribution in NTs and patients with EH

	rs2279885			rs2271072	
	NT	EH		NT	EH
Genotype					
CC	11 (0.015)	27 (0.036)	CC	27 (0.038)	21 (0.028)
GC	190 (0.265)	191 (0.253)	GC	215 (0.301)	190 (0.252)
GG	516 (0.720)	536 (0.711)	GG	472 (0.661)	543 (0.720)
P		0.0450			0.046
Dominant					
CC + GC	201 (0.280)	218 (0.289)	CC + GC	242 (0.339)	211 (0.280)
GG	516 (0.720)	536 (0.711)	GG	472 (0.661)	543 (0.720)
OR		1.044			0.758
95% CI		0.832–1.310			0.607–0.946
P		0.7090			0.0140
Recessive					
CC	11 (0.015)	27 (0.036)	CC	27 (0.038)	21 (0.028)
GG + GC	706 (0.985)	727 (0.964)	GG + GC	687 (0.962)	733 (0.972)
OR		2.384			0.729
95% CI		1.173–4.842			0.408–1.302
P		0.0160			0.2850
Allele					
C	212 (0.148)	245 (0.162)	C	269 (0.188)	232 (0.154)
G	1222 (0.852)	1263 (0.838)	G	1159 (0.812)	1276 (0.846)
OR		1.118			0.783
95% CI		0.915–1.366			0.646–0.950
P		0.2740			0.0130

EH, essential hypertension; NT, normotensive control.

Table 3 | Haplotype frequencies in NT and EH

	Frequency			
Haplotype	EH	NT	χ^2	*P* value
H1	0.687	0.681	0.248	0.6184
H2	0.149	0.175	5.352	0.0207
H3	0.163	0.145	2.963	0.0852

H1: rs2279885 G / rs2271072 G
H2: rs2279885 C / rs2271072 G
H3: rs2279885 G / rs2271072 C

EH, essential hypertension; NT, normotensive control.

DISCUSSION

We performed a genetic case–control study in a Japanese population, and found that SNPs in the *FABP3* gene were associated with EH. To our knowledge, this is the first study that relates *FABP3* gene polymorphisms to hypertension in humans.

Recently attention has been focused on attempts to identify loci for drug responses by candidate gene and genome-scanning approaches in experimental animals and humans. Prague

hypertriglyceridemic (HTG) rats represent a novel strain that develops several major features of metabolic syndrome including hypertension, hypertriglyceridemia, hyperinsulinemia, and impaired glucose tolerance.[19,20] We have established a new pharmacogenetic approach to estimate the contribution of blood pressure regulatory systems by analyzing QTLs for mean arterial pressure. Using this approach, we determined earlier that baseline blood pressure in HTG rats is controlled by two loci through the sympathetic nervous system.[6,7]

Dense gene maps have been established for the rat by Rat Genome Database and for the mouse by Mouse Genome Database. The human genome project has enabled construction of a human genome map that is currently available from the NCBI. These maps can be used for projecting the results of quantitative genetic analyses of rat chromosomes onto the human genome. We have previously reported a strategy to extrapolate data from rat quantitative trait genetics onto the human genome, using a comparative mapping approach.[18] In this study, we applied our comparative mapping approach to our previously identified QTL for baseline mean arterial pressure on rat chromosome 5, and we selected *FABP3* as a possible candidate gene for blood pressure regulation. Cytoplasmic FABPs have been found in tissues that are actively involved in the uptake or utilization of fatty acids. FABPs increase the solubility of long-chain fatty acids, facilitate diffusion, and protect against toxicity associated with these fatty acids.[8–12] FABP3 is present in a wide variety of tissues, the highest concentration being present in cardiac and skeletal muscle.[21] It has been shown that intracellular accumulation of triglycerides in skeletal muscle is correlated with insulin resistance.[22–24] Blaak *et al.* found reduced FABP3 expression in skeletal muscles of patients with type 2 diabetes, correlating with diminished fatty acid uptake by the skeletal muscle. They also showed a positive correlation between the degree of weight loss and FABP3 content.[15] Additionally, thiazolidinedione PPAR agonists increased the FABP3 expression in cultured human skeletal muscle cells.[25] In recent years a considerable number of studies with animals and humans have shown that the use of thiazolidinediones is usually associated with small but significant reductions in blood pressure levels.[26]

Skeletal muscle constitutes the largest insulin-sensitive tissue in the body and is the primary site for insulin-stimulated glucose utilization.[27] Skeletal muscle resistance to insulin is fundamental to the metabolic dysregulation associated with obesity and physical inactivity, and it contributes to the development of metabolic syndrome.[28–30] Taken together, these observations support our hypothesis that hypertension arising from skeletal muscle insulin resistance is mediated by FABP3. Recent results additionally suggest that stronger sympathetic nervous reactivity is associated with severe insulin resistance before the onset of hypertension, and support the hypothesis that insulin-mediated stimulation of the sympathetic nervous system is involved in the development of cardiovascular diseases related to altered glucose metabolism.[16,17]

In summary, we have examined polymorphisms of the *FABP3* gene in a case–control study within the Japanese population. This finding needs further confirmation in a variety of ethnic groups, and functional studies are required in order to elucidate the mechanisms underlying the association of FABP3 polymorphisms with EH.

Disclosure: The authors declared no conflict of interest.

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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 (χ^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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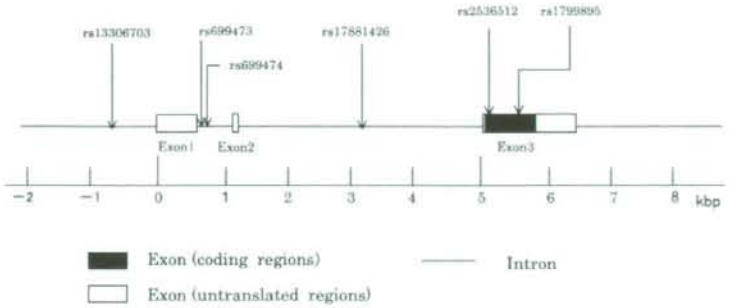

Fig. 1. *Organization of the gene encoding human extracellular superoxide dismutase and the location of the single-nucleotide polymorphisms (SNPs) that were used in the present association study. Closed boxes indicate exons; lines represent introns.*

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 tetratic 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	*p*-value	NT	EH	*p*-value	NT	EH	*p*-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	*p*-value	NT	EH	*p*-value	NT	EH	*p*-value
Number of participants		251	243		162	155		89	88	
Variants										
rs13306703										
Genotype	C/C	195 (0.777)	190 (0.782)		124 (0.765)	120 (0.774)		71 (0.798)	70 (0.795)	
	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)	0.800	4 (0.025)	3 (0.019)	0.944	1 (0.011)	0 (0.000)	0.611
	C/C and C/T	246 (0.980)	240 (0.988)		158 (0.975)	152 (0.981)		88 (0.989)	88 (1.000)	
	T/T	5 (0.020)	3 (0.012)	0.505	4 (0.025)	3 (0.019)	0.747	1 (0.011)	0 (0.000)	0.319
	C/C	195 (0.777)	190 (0.782)		124 (0.765)	120 (0.774)		71 (1.000)	70 (0.795)	
	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
Allele	C	441 (0.878)	430 (0.885)		282 (0.870)	272 (0.877)		159 (0.893)	158 (0.898)	
	T	61 (0.122)	56 (0.115)	0.760	42 (0.130)	38 (0.123)	0.789	19 (0.107)	18 (0.102)	0.876
rs699473										
Genotype	C/C	121 (0.482)	122 (0.502)		86 (0.531)	78 (0.503)		35 (0.393)	44 (0.500)	
	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)	0.466	18 (0.111)	13 (0.084)	0.512	11 (0.124)	7 (0.080)	0.308
	C/C and C/T	222 (0.884)	223 (0.918)		144 (0.889)	142 (0.916)		78 (0.876)	81 (0.920)	
	T/T	29 (0.116)	20 (0.082)	0.217	18 (0.111)	13 (0.084)	0.414	11 (0.124)	7 (0.080)	0.332
	C/C	121 (0.482)	122 (0.502)		86 (0.531)	78 (0.503)		35 (0.393)	44 (0.500)	
	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
Allele	C	343 (0.683)	345 (0.710)		230 (0.710)	220 (0.710)		113 (0.635)	125 (0.710)	
	T	159 (0.317)	141 (0.290)	0.363	94 (0.290)	90 (0.290)	0.996	65 (0.365)	51 (0.290)	0.131
rs17881426										
Genotype	A/A	4 (0.016)	3 (0.012)		4 (0.025)	3 (0.019)		0 (0.000)	0 (0.000)	
	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)	0.454	124 (0.765)	126 (0.813)	0.585	69 (0.775)	72 (0.818)	0.478
	A/A and A/T	58 (0.231)	45 (0.185)		38 (0.235)	29 (0.187)		20 (0.225)	16 (0.182)	
	T/T	193 (0.769)	198 (0.815)	0.209	124 (0.765)	126 (0.813)	0.301	69 (0.775)	72 (0.818)	0.478
	A/A	4 (0.016)	3 (0.012)		4 (0.025)	3 (0.019)		0 (0.000)	0 (0.000)	
	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)	—
Allele	A	62 (0.124)	48 (0.099)		42 (0.130)	32 (0.103)		20 (0.112)	16 (0.091)	
	T	440 (0.876)	438 (0.901)	0.217	282 (0.870)	278 (0.897)	0.301	158 (0.888)	160 (0.909)	0.504
rs2536512										
Genotype	A/A	22 (0.088)	17 (0.070)		14 (0.086)	12 (0.077)		8 (0.090)	5 (0.057)	
	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)	0.640	85 (0.525)	75 (0.484)	0.685	42 (0.472)	44 (0.500)	0.643
	A/A and A/G	124 (0.494)	124 (0.510)		77 (0.475)	80 (0.516)		47 (0.528)	44 (0.500)	
	G/G	127 (0.506)	119 (0.490)	0.718	85 (0.525)	75 (0.484)	0.467	42 (0.472)	44 (0.500)	0.709
	A/A	22 (0.088)	17 (0.070)		14 (0.086)	12 (0.077)		8 (0.090)	5 (0.057)	
	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
Allele	A	146 (0.291)	141 (0.290)		91 (0.281)	92 (0.297)		55 (0.309)	49 (0.278)	
	G	356 (0.709)	345 (0.710)	0.980	233 (0.719)	218 (0.703)	0.659	123 (0.691)	127 (0.722)	0.528
rs1799895										
Genotype	C/C	226 (0.900)	219 (0.901)		144 (0.889)	140 (0.903)		82 (0.921)	79 (0.898)	
	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)	0.975	0 (0.000)	0 (0.000)	0.676	0 (0.000)	0 (0.000)	0.584
	C/C and C/G	251 (1.000)	243 (1.000)		162 (1.000)	155 (1.000)		89 (1.000)	88 (1.000)	
	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)	
	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
Allele	C	477 (0.950)	462 (0.951)		306 (0.944)	295 (0.952)		171 (0.961)	167 (0.949)	
	G	25 (0.050)	24 (0.049)	0.976	18 (0.056)	15 (0.048)	0.685	7 (0.039)	9 (0.051)	0.593

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±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	*p*-value	Haplotype	Overall	NT (251)	EH (243)	χ^2	*p*-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%, χ^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%, χ^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-base 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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Original Article

Haplotype-Based Case-Control Study of the Human CYP4F2 Gene and Essential Hypertension in Japanese Subjects

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CYP4F2 acts primarily as an enzyme that converts arachidonic acid to 20-hydroxyeicosatetraenoic acid (20-HETE), a metabolite involved in the regulation of blood pressure in humans. The aim of the present study was to assess the association between the human CYP4F2 gene and essential hypertension (EH) using a haplotype-based case-control study that included separate analysis of the two gender groups. The 249 EH patients and 238 age-matched controls were genotyped for 5 single-nucleotide polymorphisms (SNPs) of the human CYP4F2 gene (rs3093105, rs3093135, rs1558139, rs2108622, rs3093200). Data were analyzed for 3 separate groups: all subjects, and men and women separately. For the total population and for male subjects, the distribution of the dominant model of rs1558139 (CC *vs.* CT+TT) differed significantly between the EH patients and control subjects ($p=0.037$ and $p=0.005$, respectively), with a higher percentage of EH patients showing the CC genotype. Logistic regression showed that, for men, the CC genotype of rs1558139 was more prevalent in the EH patients than in the control subjects ($p=0.026$), while, for the total population, the difference disappeared ($p=0.247$). For men, the overall distribution of the haplotypes was significantly different between the EH patients and the control subjects ($p=0.042$), and the frequency of the T-T-G haplotype was also significantly lower for EH patients than for control subjects ($p=0.009$). In conclusion, the present results indicate that rs1558139 might be a genetic marker for EH and the T-T-G haplotype might be a protective genetic marker for EH in Japanese men. (*Hypertens Res* 2008; 31: 1719–1726)

***Key Words*: CYP4F2, single-nucleotide polymorphism, haplotype, case-control study, androgen**

Introduction

Essential hypertension (EH) is a major risk factor for many common causes of morbidity and mortality including stroke, myocardial infarction, congestive heart failure, and end-stage renal disease (*1*). The etiology and pathogenesis of EH are likely comprised of a multifactorial disorder that results from the inheritance of several susceptibility genes as well as from multiple environmental determinants. A variety of gene variants have been shown to be associated with EH (*2*).

Cytochrome P450 (CYP) is a superfamily of cysteinato-

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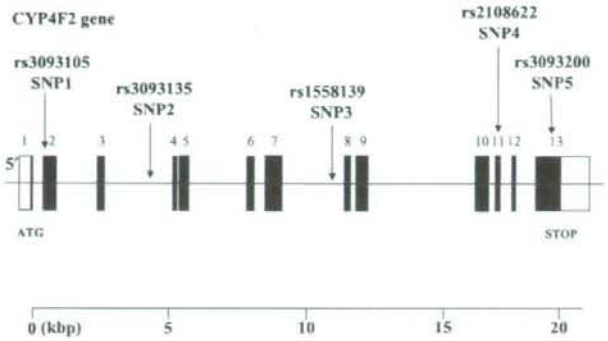

Fig. 1. *Structure of the human CYP4F2 gene. This gene consists of 13 exons separated by 12 introns. Boxes indicate exons, and lines indicate introns and intergenic regions. Filled boxes indicate coding regions. Arrows mark the polymorphism locations.*

heme enzymes that are not only involved in the metabolism of xenobiotics, but also metabolize a host of endobiotics. CYP enzymes are classified into families, subfamilies, and individual isoenzymes, based on similarities in their amino acid sequence (*3*). To date, the 5 human CYP4F members that have been identified are CYP4F2, CYP4F3, CYP4F8, CYP4F11, and CYP4F12, all of which are located on chromosome 19 (*4*). The CYP4F2 gene is located at 19pter–p13.11, spans approximately 20 kbp, and contains 13 exons.

The CYP4F2 family is expressed at high levels in the kidney and liver and is involved not only in the metabolism of leukotriene B4 but also arachidonic acid (AA) (*5*). CYP4F2 converts AA to 20-hydroxyeicosatetraenoic acid (20-HETE), which exhibits potent biological effects on renal tubular and vascular functions and on the long-term control of arterial pressure (*6*). It has been well established that alterations in renal 20-HETE production contribute to the development of hypertension in both rodent models and humans (*7, 8*). Recently, Stec *et al.* identified a functional variant in the human CYP4F2 gene (rs2108622, V433M) that results in a decrease in 20-HETE production from AA (*9*). However, large, population-based studies are needed to determine whether the functional variants of the CYP4F2 gene are related to hypertension. Given the potential importance of 20-HETE in the regulation of hypertension, the aim of the present study was to assess the association between EH and the human CYP4F2 gene in Japanese subjects using a haplotype-based case-control method.

Methods

Subjects

Subjects diagnosed with EH were recruited at Nihon University Itabashi Hospital and other neighboring hospitals in Tokyo from 1993 to 2003. A total of 249 EH patients were enrolled in the present study, with a male/female (m/f) ratio of 1.94. EH was diagnosed based on the following criteria: seated systolic blood pressure (SBP) > 160 mmHg or diastolic blood pressure (DBP) > 100 mmHg on 3 occasions within 2 months after the first blood pressure (BP) reading. None of the EH patients were receiving antihypertensive medication and patients diagnosed with secondary hypertension were excluded. A total of 238 normotensive (NT) age-matched individuals (m/f ratio = 1.88) were enrolled as control subjects. Controls were members of the New Elder Citizen Movement in Japan, and all subjects lived in Tokyo or the suburbs of Tokyo. All controls were confirmed to have grade 0 on the modified Rankin Scale. In this study group, participants with cancer or autoimmune diseases were excluded. None of the control subjects had a family history of hypertension, and all had a SBP of < 130 mmHg and a DBP of < 85 mmHg. The sample size was thought to be large enough because our previous studies successfully isolated EH susceptibility markers using a similar sample size (*10, 11*). Informed consent was obtained from each subject in accordance with the protocol approved by the Human Studies Committee of Nihon University.

Genotyping

There are 225 single-nucleotide polymorphisms (SNPs) for the human CYP4F2 gene listed in the National Center for Biotechnology Information SNP database Build 126 (http://www.ncbi.nlm.nih.gov/SNP). We screened the data for the Tag SNPs on the International HapMap Project website (http://www.hapmap.org/index.html.ja) and used a cut off level of $r^2 \geq 0.5$, along with a cut off level of ≤ 0.1 for the minor allele frequency (MAF). According to the above criteria we selected rs3093135, rs1558139, and rs2108622 as SNPs for this gene. rs3093135 and rs1558139 were located in introns, and rs2108622 was located in an exon with a nonsyn-

Table 1. Characteristics of Study Participants

	Total			Men			Women		
	EH patients (n=249)	Control subjects (n=238)	p-value	EH patients (n=165)	Control subjects (n=155)	p-value	EH patients (n=84)	Control subjects (n=83)	p-value
Age (years)	51.1±5.6	51.3±8.9	0.766	50.9±5.7	52.0±6.8	0.123	51.4±5.3	50.0±11.7	0.294
BMI (kg/m^2)	24.6±3.4	22.8±3.3	<0.001	24.6±3.3	23.1±3.3	<0.001	24.5±3.6	22.4±3.4	<0.001
SBP (mmHg)	173.6±20.1	112.4±10.6	<0.001	171.2±19.0	113.0±10.2	<0.001	178.1±21.4	111.4±11.3	<0.001
DBP (mmHg)	106.3±12.9	69.4±8.6	<0.001	106.5±12.7	70.1±8.1	<0.001	105.8±13.2	68.2±9.3	<0.001
Pulse rate (beats/min)	77.3±15.0	72.9±12.6	0.002	76.6±15.6	72.7±14.0	0.045	78.9±13.8	73.2±9.5	0.009
Creatinine (mg/dL)	0.8±0.2	0.8±0.2	0.192	0.9±0.2	0.9±0.2	0.148	0.7±0.2	0.7±0.1	0.915
Total cholesterol (mg/dL)	210.1±35.8	202.8±40.9	0.041	204.3±32.7	198.2±37.4	0.132	220.6±38.8	211.2±45.6	0.154
HDL cholesterol (mg/dL)	56.1±17.4	57.4±18.1	0.468	52.9±16.6	55.1±16.9	0.261	61.9±17.3	61.6±19.6	0.920
Uric acid (mg/dL)	5.7±1.6	5.4±1.4	0.037	6.2±1.5	5.8±1.4	0.016	4.6±1.3	4.5±1.2	0.571
Hyperlipidemia (%)	26	19	0.055	22	15	0.102	34	26	0.263
Diabetes (%)	10	3	<0.001	11	3	0.005	8	2	0.090
Drinking (%)	59	45	0.014	73	63	0.093	29	11	0.014
Smoking (%)	54	42	0.013	66	52	0.025	32	23	0.257

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

onymous substitution amino acid change. Variations of rs2108622 that can alter the production of 20-HETE have been identified (*9*). In the current study, we also looked at rs3093105 and rs3093200, which are located in the exons and are known to be nonsynonymous SNPs. We designated these SNPs as SNP1 (rs3093105), SNP2 (rs3093135, C_27482167_10), SNP3 (rs1558139, C_2583813_10), SNP4 (rs2108622, C_16179493_40), and SNP5 (rs3093200), in order of increasing distance from the 5′ end of the gene (Fig. 1). SNP2, SNP3 and SNP4 were genotyped using a kit from Applied Biosystems Inc. (ABI; Foster City, USA). Since SNP1 and SNP5 were not registered in the genotyping kit from ABI, we purchased and used a Custom TaqMan® SNP Genotyping Assay for these SNPs.

Blood samples were collected from all participants, and genomic DNA was extracted from the peripheral blood leukocytes by phenol and chloroform extraction.

Genotyping was performed using the TaqMan® SNP Genotyping Assay (ABI). TaqMan® SNP Genotyping Assays were performed using Taq amplification. In the 5′ nuclease assay, polymerase chain reaction (PCR) discriminates between SNPs because allele-specific fluorogenic probes that hybridize to the template are cleaved by the 5′ nuclease activity of Taq polymerase. The probes contain a 3′ minor groove–binding group (MGB) that hybridizes to single-stranded targets with greater sequence specificity than is seen with ordinary DNA probes. This reduces nonspecific probe hybridization, and results in low background fluorescence in the 5′ nuclease PCR assay (TaqMan®, ABI). Cleavage results in increased emission of the reporter dye. Each 5′ nuclease assay requires 2 unlabeled PCR primers and 2 allele-specific probes. Each probe is labeled with 2 reporter dyes at the 5′ end. In the present study, VIC and carboxyfluorescein (FAM) were used as the reporter dyes. The primers and probes used in the TaqMan® SNP Genotyping Assays (ABI) were chosen based on 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 a 5-μL final reaction volume containing 2 ng of DNA, 0.046 μL of TaqMan® SNP Genotyping Assay Mix (40×), primers at a concentration of 331 nmol/L each, and probes at a final concentration of 73.6 nmol/L each. Thermal cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 62°C for 1 min. Thermal cycling was performed using the GeneAmp 9700™ system.

Each 96-well plate contained 80 DNA samples of an unknown genotype and 4 reaction mixtures containing reagents but no DNA (control). The control samples without DNA are a necessary part of the Sequence Detection System (SDS) 7700™ signal processing, as outlined in the TaqMan Allelic Discrimination Guide (ABI). The 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 scatter-plot of the SDS software. The genotypes were also determined automatically by the software's signal processing algorithms. The results of each scoring method were saved in 2 separate

Table 2. Genotype and Allele Distributions for the Patients with EH and the Control Subjects

		Total			Men			Women		
		EH patients (*n* (%))	Control subjects (*n* (%))	*p*-value	EH patients (*n* (%))	Control subjects (*n* (%))	*p*-value	EH patients (*n* (%))	Control subjects (*n* (%))	*p*-value
rs3093105 (SNP1)										
Genotype	T/T	197 (79.1)	190 (79.8)		130 (78.8)	124 (80.0)		67 (79.8)	66 (79.5)	
	T/G	47 (18.9)	43 (18.1)	0.972	30 (18.2)	27 (17.4)	0.952	17 (20.2)	16 (19.3)	0.597
	G/G	5 (2.0)	5 (2.1)		5 (3.0)	4 (2.6)		0 (0.0)	1 (1.2)	
dominant model	TT	197 (79.1)	190 (79.8)	0.845	130 (78.8)	124 (80.0)	0.789	67 (79.8)	66 (79.5)	0.969
	TG+GG	52 (20.9)	48 (20.2)		35 (21.2)	31 (20.0)		17 (20.2)	17 (20.5)	
recessive model	GG	5 (2.0)	5 (2.1)	0.943	5 (3.0)	4 (2.6)	0.808	0 (0.0)	1 (1.2)	0.313
	TG+TT	244 (98.0)	233 (97.9)		160 (97.9)	151 (97.4)		84 (100.0)	82 (98.8)	
Allele	T	441 (88.6)	423 (88.9)	0.878	290 (87.9)	275 (88.7)	0.744	151 (89.9)	148 (89.2)	0.829
	G	57 (11.4)	53 (11.1)		40 (12.1)	35 (11.3)		17 (10.1)	18 (10.8)	
rs3093135 (SNP2)										
Genotype	T/T	196 (78.7)	190 (79.8)		129 (78.2)	124 (80.0)		67 (79.8)	66 (79.5)	
	T/A	48 (19.3)	43 (18.1)	0.942	31 (18.8)	27 (17.4)	0.917	17 (20.2)	16 (19.3)	0.597
	A/A	5 (2.0)	5 (2.1)		5 (3.0)	4 (2.6)		0 (0.0)	1 (1.2)	
dominant model	TT	196 (78.7)	190 (79.8)	0.761	129 (78.2)	124 (80.0)	0.690	67 (79.8)	66 (79.5)	0.969
	TA+AA	53 (21.3)	48 (20.2)		36 (21.8)	31 (20.0)		17 (20.2)	17 (20.5)	
recessive model	AA	5 (2.0)	5 (2.1)	0.943	5 (3.0)	4 (2.6)	0.808	0 (0.0)	1 (1.2)	0.313
	TA+TT	244 (98.0)	233 (97.9)		160 (97.0)	151 (97.4)		84 (100.0)	82 (98.8)	
Allele	T	440 (88.4)	423 (88.9)	0.802	289 (87.6)	275 (88.7)	0.658	151 (89.9)	148 (89.2)	0.829
	A	58 (11.6)	53 (11.1)		41 (12.4)	35 (11.3)		17 (10.1)	18 (10.8)	
rs1558139 (SNP3)										
Genotype	C/C	125 (50.2)	97 (40.7)		92 (55.8)	62 (40.0)		33 (39.3)	35 (42.2)	
	C/T	93 (37.3)	113 (47.5)	0.068	54 (32.7)	74 (47.7)	0.013	39 (46.4)	39 (47.0)	0.786
	T/T	31 (12.5)	28 (11.8)		19 (11.5)	19 (12.3)		12 (14.3)	9 (10.8)	
dominant model	CC	125 (50.2)	97 (40.7)	0.037	92 (55.8)	62 (40.0)	0.005	33 (39.3)	35 (42.2)	0.705
	CT+TT	124 (49.8)	141 (59.3)		73 (44.2)	93 (60.0)		51 (60.7)	48 (57.8)	
recessive model	TT	31 (12.5)	28 (11.8)	0.817	19 (11.5)	19 (12.3)	0.837	12 (14.3)	9 (10.8)	0.502
	CT+CC	218 (87.5)	210 (88.2)		146 (88.5)	136 (87.7)		72 (85.7)	74 (89.2)	
Allele	C	343 (68.9)	307 (64.5)	0.147	238 (72.1)	198 (63.9)	0.025	105 (62.5)	109 (65.7)	0.547
	T	155 (31.1)	169 (35.5)		92 (27.9)	112 (36.1)		63 (37.5)	57 (34.3)	
rs2108622 (SNP4)										
Genotype	G/G	143 (57.4)	128 (53.8)		95 (57.6)	86 (55.5)		48 (57.1)	42 (50.6)	
	G/A	74 (29.7)	91 (38.2)	0.059	50 (30.3)	58 (37.4)	0.188	24 (28.6)	33 (39.8)	0.271
	A/A	32 (12.9)	19 (8.0)		20 (12.1)	11 (7.1)		12 (14.3)	8 (9.6)	
dominant model	GG	143 (57.4)	128 (53.8)	0.418	95 (57.6)	86 (55.5)	0.706	48 (57.1)	42 (50.6)	0.397
	GA+AA	106 (42.6)	110 (46.2)		70 (42.4)	69 (44.5)		36 (42.9)	41 (49.4)	
recessive model	AA	32 (12.9)	19 (8.0)	0.080	20 (12.1)	11 (7.1)	0.129	12 (14.3)	8 (9.6)	0.355
	GA+GG	217 (87.1)	219 (92.0)		145 (87.9)	144 (92.9)		72 (85.7)	75 (90.4)	
Allele	G	360 (72.3)	347 (72.9)	0.831	240 (72.7)	230 (74.2)	0.675	120 (71.4)	117 (70.5)	0.849
	A	138 (27.7)	129 (27.1)		90 (27.3)	80 (25.8)		48 (28.6)	49 (29.5)	
rs3093200 (SNP5)										
Genotype	C/C	248 (99.6)	238 (100.0)		164 (99.4)	155 (100.0)		84 (100.0)	83 (100.0)	
	C/A	1 (0.4)	0 (0.0)	—	1 (0.6)	0 (0.0)	—	0 (0.0)	0 (0.0)	—
	A/A	0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	
dominant model	CC	248 (99.6)	238 (100.0)	0.328	164 (99.4)	155 (100.0)	0.332	84 (100.0)	83 (100.0)	—
	CA+AA	1 (0.4)	0 (0.0)		1 (0.6)	0 (0.0)		0 (0.0)	0 (0.0)	
recessive model	AA	0 (0.0)	0 (0.0)	—	0 (0.0)	0 (0.0)	—	0 (0.0)	0 (0.0)	—
	CA+CC	249 (100.0)	238 (100.0)		165 (100.0)	155 (100.0)		84 (100.0)	83 (100.0)	
Allele	C	497 (99.8)	476 (100.0)	0.328	329 (99.7)	310 (100.0)	0.332	168 (100.0)	166 (100.0)	—
	A	1 (0.2)	0 (0.0)		1 (0.3)	0 (0.0)		0 (0.0)	0 (0.0)	

SNP, single-nucleotide polymorphism; EH, essential hypertension. The *p*-value of genotypes and alleles were calculated using Fisher's exact test.

Table 3. Odds Ratios and 95% Confidence Intervals (CI) for Each Risk Factor and SNP Genotype Associated with Essential Hypertension

Risk factor	Total			Men			Women		
	Odds ratios	95% CI	*p*-value	Odds ratios	95% CI	*p*-value	Odds ratios	95% CI	*p*-value
rs1558139 CC genotype	0.775	0.504–1.193	0.247	1.829	1.076–3.111	0.026	1.659	0.757–3.638	0.206
BMI	0.853	0.797–0.913	0.000	0.884	0.814–0.959	0.003	0.770	0.670–0.884	0.000
DM	1.985	0.810–4.866	0.134	2.426	0.839–7.017	0.102	1.029	0.173–6.126	0.975
Smoking	0.582	0.378–0.896	0.014	1.885	1.101–3.230	0.021	0.713	0.295–1.725	0.453

SNP, single-nucleotide polymorphism; BMI, body mass index; DM, diabetes mellitus.

output files for later comparison (*12*).

Biochemical Analysis

We measured the plasma concentration of total cholesterol and high-density lipoprotein (HDL) cholesterol and the serum concentration of creatinine and uric acid using standard methods employed by the Clinical Laboratory Department of Nihon University Hospital (*13*).

Statistical Analysis

All continuous variables are expressed as mean±SD. Differences in continuous variables between EH patients and control subjects were analyzed using the Mann-Whitney *U*-test. Differences in categorical variables were analyzed using Fisher's exact test. Hardy-Weinberg equilibrium was assessed by χ^2 analysis. Differences in distributions of genotypes and alleles between EH patients and control subjects were analyzed using Fisher's exact test. Based on the genotype data of the genetic variations, we performed linkage disequilibrium (LD) analysis and haplotype-based case-control analysis, using the expectation maximization (EM) algorithm (*14*) and the software SNPAlyze version 3.2 (Dynacom Co., Ltd., Yokohama, Japan) (*15*). The SNP5 showed little heterogeneity as only one heterozygosity was found in the EH patients, therefore, SNP5 was not available for the haplotype-based case-control study. Pairwise LD analysis was performed using SNP1, SNP2, SNP3, and SNP4. We used $|D'|$ values of ≥0.5 to assign SNP locations to 1 haplotype block. SNPs with an r^2 value of <0.5 were selected as tagged, which means they were available for the haplotype. In the haplotype-based case-control analysis, haplotypes with a frequency of <0.01 were excluded. With regard to this method, the general consensus is that the effect of less frequent haplotypes should be excluded (*10*, *12*). The analysis was also performed for each of the 3 groups: total population, men, and women. The frequency distribution of the haplotypes was calculated by χ^2 analysis. In addition, logistic regression analysis was performed to assess the contribution of the major risk factors. Statistical significance was established at $p<0.05$. Statistical analyses were performed using SPSS software for Windows, version 12 (SPSS Inc., Chicago, USA).

Results

Table 1 shows the clinical characteristics of the study participants. For men, women, and total subjects, the following values were significantly higher in the EH patients than in the control subjects: body mass index (BMI), SBP, DBP, and pulse rate. For men and total subjects, serum uric acid and the prevalence of diabetes mellitus and smoking were significantly higher for the EH patients than the control subjects. For women and total subjects, the prevalence of drinking was higher in the EH group than in the control group. For the total subject group, total cholesterol was higher in the EH group as compared to the control group. There was no significant difference for any of the following variables between the EH patients and the control subjects: serum creatinine and the prevalence of hyperlipidemia.

Table 2 shows the distribution of the genotypes and alleles of the 5 SNPs. The genotype distributions for each of the SNPs in the control subjects were in agreement with the predicted Hardy-Weinberg equilibrium values (data not shown). For the male subjects, the genotype distribution of SNP3 differed significantly between the EH patients and the control subjects ($p=0.013$). For the total population and the men, the distribution of the dominant model of rs1558139 (CC *vs.* CT+TT) differed significantly between the EH patients and the control subjects ($p=0.037$, $p=0.005$, respectively), with the CC genotype higher in the EH patients than in the control subjects. For the male subjects, the C allele was significantly higher in the EH patients as compared to control subjects ($p=0.025$).

Table 3 shows the results of the logistic regression analysis. For the men, the frequency of the CC genotype of rs1558139 differed significantly between the EH patients and control subjects ($p=0.026$), even after an adjustment for confounding factors such as BMI, diabetes mellitus, and smoking. However, for the total group, there were no differences noted for the frequency of the CC genotype of rs1558139 after adjustment for the aforementioned confounding factors ($p=0.247$).

Table 4 shows patterns of linkage disequilibrium for the CYP4F2 gene with $|D'|$ and r^2 values. All 4 SNPs were located in 1 haplotype block because all $|D'|\geq 0.5$. Because the r^2 value of SNP1–SNP2≥0.5, it is not very effective for a

Table 4. Pairwise Linkage Disequilibrium ($|D'|$ above Diagonal and r^2 below Diagonal) for the Four SNPs

		$\|D'\|$							
		EH patients				Control subjects			
	SNP	SNP1	SNP2	SNP3	SNP4	SNP1	SNP2	SNP3	SNP4
r^2	SNP1		1.000	1.000	0.972		0.979	1.000	0.938
	SNP2	0.981		1.000	0.973	0.958		1.000	0.907
	SNP3	0.058	0.060		0.341	0.069	0.069		0.438
	SNP4	0.319	0.326	0.099		0.297	0.278	0.130	

SNP, single-nucleotide polymorphism; EH, essential hypertension. Underline: $|D'| \geq 0.5$ or $r^2 \geq 0.5$.

Table 5. Haplotype Analysis for the Patients with EH and the Control Subjects

Haplotypes	CYP4F2 polymorphism			Overall p value			Frequency in total			Frequency in men			Frequency in women		
	SNP1	SNP3	SNP4	Total	Men	Women	EH patients	Control subjects	p value	EH patients	Control subjects	p value	EH patients	Control subjects	p value
				0.470	0.042	0.457									
H1	T	C	G				0.578	0.536	0.192	0.593	0.529	0.099	0.524	0.559	0.509
	Mj	Mj	Mj												
H2	T	T	G				0.156	0.191	0.152	0.132	0.209	0.009	0.199	0.146	0.190
	Mj	Mn	Mj												
H3	G	C	A				0.109	0.108	0.978	0.010	0.000	0.094	0.096	0.098	1.000
	Mn	Mj	Mn												
H4	T	T	A				0.158	0.165	0.753	0.119	0.108	0.661	0.181	0.187	0.887
	Mj	Mn	Mn												
H5	T	C	A				—	—	—	0.145	0.154	0.798	—	—	—
	Mj	Mj	Mn												
H6	G	T	A				—	—	—	—	—	—	0.000	0.011	0.156
	Mn	Mn	Mn												

EH, essential hypertension. Haplotypes with frequency >0.01 were estimated using SNPAlyze software. The p values were calculated by χ^2 analysis.

haplotype-based case-control study when SNP1 and SNP2 are used simultaneously. In our haplotype-based case-control study, although the minor allele frequency of SNP1 in the controls was equal to that of SNP2, the minor allele frequency of SNP1 in the NCBI data was larger than that for SNP2. Therefore, we constructed haplotypes using SNP1, SNP3, and SNP4.

In the haplotype-based case-control study, using SNP1, SNP3, and SNP4, 4 haplotypes were established in the total group, and 5 haplotypes were established in both the groups for men and women (Table 5). For men, the overall distribution of the haplotypes was significantly different between the EH patients and the control subjects ($p=0.042$). In addition, for men, the frequency of the T-T-G haplotype was significantly lower in the EH patients as compared to that seen in the control subjects ($p=0.009$).

Discussion

It has been demonstrated that many CYP subfamilies are associated with EH, for example, CYP2J2, CYP2C9 (EET synthesis), CYP3A5 (metabolizes cortisol into 6β-hydroxycortisol), CYP4A11 (20-HETE synthesis), CYP8A (prostacyclin synthesis), and CYP11B2 (aldosterone synthesis) (*8*, *16–20*). In humans, the CYP4 subfamily mainly catalyzes AA to 20-HETE, which acts either in a prohypertensive or antihypertensive manner, depending on whether it is expressed at renovascular or tubular sites. In the renal tubule, 20-HETE blocks sodium transport and acts primarily as a natriuretic, antihypertensive substance. In the renal vasculature, 20-HETE has vasoconstricting and prohypertensive effects (*6*, *21*). CYP4A11 was first described as being able to catalyze AA into 20-HETE. Additionally, it was reported that the C-to-T mutation in the CYP4A11 gene influenced the production of 20-HETE, and that this was associated with EH in large human populations (*8*). In our previous haplotype-based case-control study, we succeeded in identifying a haplotype of the CYP4A11 gene that was associated with EH in Japanese men (*10*). Recently, immunoprecipitation studies using human kidney microsomes have shown that the CYP4F2

accounts for up to 70% of the 20-HETE production in humans. Therefore, CYP4F2-catalyzed 20-HETE formation is thought to be quantitatively more important than CYP4A11-catalyzed 20-HETE formation (*5*). The major difference between CYP4A11 and CYP4F2 with regard to function is that the CYP4F2 isoform is expressed in the liver, lung, and white blood cells and is also the main enzyme responsible for the ω-hydroxylation of leukotriene B4 (LTB4) (*22*). A functional variant of the human CYP4F2 gene (rs2108622, V433M) that is capable of altering the production of 20-HETE has recently been identified (*9*). Alterations in renal 20-HETE production that might contribute to the development of hypertension have been demonstrated in both animals and humans (*7, 8*). Large population-based studies are needed to determine whether the functional variants of the CYP4F2 gene are related to the development of hypertension in humans. In our haplotype-based case-control study in Japanese subjects, we genotyped 5 SNPs in the CYP4F2 gene (including rs2108622) and assessed the association between the CYP4F2 gene and EH.

In the present study, we did not find any significant difference in the genotypic and allele distribution of SNP4 (rs2108622) in the total population, men, and women groups, while the distribution of the dominant model of SNP3 (rs1558139, CC *vs.* CT+TT) differed significantly between the EH patients and control subjects in the total population and in men ($p=0.037$ and $p=0.005$, respectively). When the logistic regression was adjusted for the BMI, diabetes mellitus, and smoking, the same results were observed in men ($p=0.026$). However, for the total group, the difference between the EH patients and control subjects disappeared ($p=0.247$). For men, the C allele was also significantly higher in EH patients compared to the control subjects ($p=0.025$). These results indicate that the risk of EH is increased in men with the CC genotype and the C allele of SNP3. Additionally, the findings suggest that SNP3 might be a gender-specific genetic marker for EH. Only 1 CA heterozygous genotype of SNP5 (rs3093200) was found in 1 male EH patient. The clinical features for this patient were not different from the other EH patients.

Morris and Kaplan found that for genes with multiple susceptibilities, analysis based on haplotypes have advantages over analysis based on individual SNPs, particularly when linkage disequilibria between the SNPs are weak (*23*). For men, the overall distribution of the haplotypes were significantly different between the EH patients and the control subjects ($p=0.042$). In addition, the frequency of the T-T-G haplotype constructed with SNP1, SNP3, and SNP4 for men was also significantly lower for EH patients as compared to the control subjects, and thus, the T-T-G haplotype is a protective haplotype in men. In the present study, although SNP4 (rs2108622) was not associated with EH, we successfully isolated the significant haplotypes. This result suggests that there is a possible mutation that has an effect on the production of 20-HETE associated with EH rather than SNP4 because the haplotype-based case-control study is a more powerful tool than the simple case-control study that uses each SNP (*24*). This mutation may be linked to the significant haplotype.

Some case-control studies have identified gene variants associated with gender-specific susceptibility to EH (*24, 25*). Animal experiments have revealed that the expression of the number of P450 enzymes is sex-dependent (*26, 27*). The present haplotype-based case-control study also showed gender-specific (for men only) genotype and haplotype significant differences between the EH patients and the control subjects. This is very interesting in light of previous findings that showed that male cyp4a14$^{-/-}$ or cyp4a10$^{-/-}$ mice have higher BP than female cyp4a14$^{-/-}$ or cyp4a10$^{-/-}$ mice, respectively. The male cyp4a14$^{-/-}$ mice exhibited increases in plasma androgen, and their hypertensive phenotype was found to be androgen sensitive (*28, 29*). Cyp4a14 and cyp4a10 do not exist in humans, but as human CYP4A11 is 72.69% identical with murine cyp4a14 and 73.02% identical with murine cyp4a10 by amino acid sequence, murine cyp4a14 and cyp4a10 can therefore be regarded as being homologous to human CYP4A11. Unfortunately, there has yet to be a knockout animal model established for the CYP4F gene that includes cyp4f2. In the current experiment, we also did not obtain plasma androgen data because we were not able to obtain informed consent to collect blood samples for the purpose of measuring the plasma androgen levels. When all of the current results are taken together, our findings suggest that SNP3 (rs1558139) and the T-T-G haplotype might be associated with the androgen-mediated activity of the CYP4F2 enzyme.

In conclusion, the CC genotype and the C allele of rs1558139 in the human CYP4F2 gene might be susceptibility markers. In addition, the T-T-G haplotype appears to be a protective marker for EH in Japanese men. However, due to the moderate size of the population examined in this study, in order to be able to conclusively discuss the statistical significance of the interaction between the genotype (or haplotype) and the phenotype, further studies based on larger populations are needed. Such studies would allow us to isolate the functional mutations of the CYP4F2 gene that regulate BP, and to evaluate the function of the CYP4F2 variants that are involved in the metabolism of sex hormones.

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