rs699664, leads to the substitution of Gln for Arg at amino acid 325.

We previously genotyped three SNPs, −1639G>A, 1173C>T, and 3730G>A in *VKORC1*, in 3652 population-based individuals [20]. This analysis obtained a minor allele frequency of 0.086 for all SNPs. Three SNPs were in tight LD with a pair-wise r^2 value of 0.98. Two SNPs in particular, −1639G>A and 1173C>T, were in complete LD in the study population. Therefore, −1639G>A and 3730G>A were used for additional analysis to estimate the influence of *VKORC1* genotypes of warfarin dosage.

To estimate the contribution of each SNP to variabilities in warfarin dosages, we performed univariate regression analyses for four SNPs, *VKORC1* −1639G>A and 3730G>A, *GGCX* 8016G>A, and *CYP2C9* 42613A>C (*CYP2C9*3*) (Table 3). The R^2 values determined for *VKORC1* −1639G>A and 3730G>A were 0.086 and 0.082, respectively. The equivalent R^2 value observed in the model of *GGCX* 8016G>A ($R^2 = 0.081$) was higher than that of *CYP2C9* 42613A>C ($R^2 = 0.064$).

Multiple regression analysis was performed to estimate the relative contributions of age, sex, weight, and three genetic polymorphisms to the inter-individual variations in warfarin dose. These results were shown in Table 4. The model included age, sex, weight, and three genetic polymorphisms, 6 variables in total, as the independent variables and accounted for 33.3% of total variations in warfarin dose. The contribution, P_i, to inter-individual variation in warfarin dose was 5.9% for *VKORC1* −1639G>A, 5.2% for *CYP2C9* 42613A>C, and 4.6% for *GGCX* 8016G>A.

Discussion

In this study, we have examined the contribution of four genes to the warfarin maintenance dose required in Japanese patients following ischemic stroke. The patients were controlled in the target INR of 1.6–2.6. A previous study on the optimal intensity of warfarin therapy for secondary prevention of stroke in patients with non-valvular atrial fibrillation showed that the low-intensity warfarin (INR 1.5 to 2.1) treatment seemed to be safer than the conventional-intensity (INR 2.2 to 3.5) treatment [18]. The annual rate of ischemic stroke was low in both groups (1.1% per year in the conventional-intensity group and 1.7% per year in the low-intensity group) and did not differ significantly. Based on this result and the guideline of the Japanese Circulation Society for the treatment of atrial fibrillation, we adopted the target INR of 1.6–2.6. Daily warfarin dose of each patient was properly controlled to meet target INR. As a result, the range of the warfarin dose was between 1 and 10 mg.

Table 3 Univariate regression analyses for warfarin daily dosage

Variables	R^2	P
VKORC1-1639G>A*	0.086	0.004
VKORC1 3730G>A*	0.082	0.006
GGCX 8016G>A	0.081	0.022
CYP2C9 42613A>C	0.064	0.015

R^2 and P values were calculated by univariate regression analyses. *These two SNPs were in linkage disequilibrium.

Table 4 Multiple regression analysis for estimating the relative contributions of age, sex, weight, and selective genetic variations with warfarin dose

Independent	Std $\beta^{\dagger}$	$P_i \times 100$
Age	−0.141	1.69
Sex	0.786	8.12*
Weight	0.374	7.78*
VKORC1 −1639G>A	0.735	5.88**
GGCX 8016G>A	−0.451	4.60**
CYP2C9 42613A>C	−0.847	5.19**

†: Standardized regression coefficient.
*: $P<0.01$, **: $0.01 \leq P<0.05$.

Warfarin is the most prescribed oral anticoagulant. Warfarin targets VKORC1 and antagonizes vitamin K, an essential cofactor for the modification of specific glutamic acid to γ-carboxyglutamic acid in coagulation factors II, VII, IX and X. Warfarin is metabolized by CYP2C9. Patients with *CYP2C9*2* and *CYP2C9*3* alleles have lower mean daily warfarin doses and a greater risk of bleeding [16,23]. Recent studies on *VKORC1* showed that SNPs in *VKORC1* have a more important function than the *CYP2C9* variations in terms of inter-individual variability of warfarin. It has been reported that the *VKORC1* haplotype accounted for 21% of inter-individual variability of warfarin and the *CYP2C9* genotype explained 6% [6]. Subsequent studies reached the similar conclusion that the *VKORC1* genotype affects inter-individual variability of warfarin more greatly than the *CYP2C9* genotype [5,8–11]. Inclusion of non-genetic factors such as age, sex, body surface area, body weight, and drug interaction with genotype information accounted for up to 60% of inter-individual variability of warfarin [5,8–11]. The remaining 40% of warfarin dosing variability remains unexplained.

In our study, *VKORC1* −1639G>A explained 5.9% of the inter-individual variabilities in warfarin dose, while CYP2C9*3 explained 5.2% (Table 4). We also detected a significant association between *GGCX* 8016G>A (R325Q) and warfarin dosage, which explained 4.6% of the variability seen in our subjects (Table 4). We have recently reported that *GGCX* 8016G>A influences the inter-individual variations in

protein C activity in the general population of Japan; women with the GG genotype exhibit approximately 5% higher plasma protein C activity ($p=0.002$) than those with either the GA or AA genotypes [20]. The R325Q mutation is predicted by the topological model to reside within the cytoplasmic domain of GGCX [24]. In this domain, amino acids 343–355 mediate GGCX enzyme/substrate interactions; residues 343–345 of CVY are necessary for both substrate binding and γ-carboxylase activity [25].

Recent studies reported the association of a microsatellite marker in intron 6 of *GGCX* with warfarin dose [26,27]. In 45 warfarin-treated Japanese patients, 10, 11, and 13 CAA repeats were detected. Three individuals heterozygous for the 13 repeat allele required higher maintenance doses than patients with fewer repeats [26]. In 183 warfarin-treated Swedes, a group of individuals bearing both alleles with 13 repeats or those with 14–16 repeats required significantly higher maintenance doses than patients with fewer repeats. Taken together, GGCX is a promising candidate influencing warfarin maintenance doses significantly. Further studies with larger populations and additional ethnic groups are required to elucidate the association between variations in warfarin dosages and the *GGCX* 8016G>A genotype.

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References

[1] Rost S, Fregin A, Ivaskevicius V, Conzelmann E, Hortnagel K, Pelz HJ, et al. *VKORC1* cause warfarin resistance and multiple coagulation factor deficiency type 2. *Nature* 2004;**427**:537–41.

[2] Li T, Chang CY, Jin DY, Lin PJ, Khvorova A, Stafford DW. Identification of the gene for vitamin K epoxide reductase. *Nature* 2004;**427**:541–4.

[3] Wajih N, Sane DC, Hutson SM, Wallin R. The inhibitory effect of calumenin on the vitamin K-dependent γ-carboxylation system. Characterization of the system in normal and warfarin-resistant rats. *J Biol Chem* 2004;**279**:25276–83.

[4] D'Andrea G, D'Ambrosio RL, Di Perna P, Chetta M, Santacroce R, Brancaccio V, et al. A polymorphism in the *VKORC1* gene is associated with an interindividual variability in the dose-anticoagulant effect of warfarin. *Blood* 2005;**105**:645–59.

[5] Bodin L, Verstuyft C, Tregouet DA, Robert A, Dubert L, Funck-Brentano C, et al. Cytochrome P450 2C9 *(CYP2C9)* and vitamin K epoxide reductase *(VKORC1)* genotypes as determinants of acenocoumarol sensitivity. *Blood* 2005;**106**: 135–40.

[6] Rieder MJ, Reiner AP, Gage BF, Nickerson DA, Eby CS, McLeod HL, et al. Effect of *VKORC1* haplotypes on transcriptional regulation and warfarin dose. *N Engl J Med* 2005;**352**:2285–93.

[7] Yuan HY, Chen JJ, Lee MT, Wung JC, Chen YF, Charng MJ, et al. A novel functional VKORC1 promoter polymorphism is associated with inter-individual and inter-ethnic differences in warfarin sensitivity. *Hum Mol Genet* 2005;**14**: 1745–51.

[8] Sconce EA, Khan TI, Wynne HA, Avery P, Monkhouse L, King BP, et al. The impact of *CYP2C9* and *VKORC1* genetic polymorphism and patient characteristics upon warfarin dose requirements: proposal for a new dosing regimen. *Blood* 2005;**106**:2329–33.

[9] Veenstra DL, You JH, Rieder MJ, Farin FM, Wilkerson HW, Blough DK, et al. Association of Vitamin K epoxide reductase complex 1 *(VKORC1)* variants with warfarin dose in a Hong Kong Chinese patient population. *Pharmacogenet Genomics* 2005;**15**:687–91.

[10] Wadelius M, Chen LY, Downes K, Ghori J, Hunt S, Eriksson N, et al. VKORC1 and GGCX polymorphisms associated with warfarin dose. *Pharmacogenomics J* 2005;**5**:262–70.

[11] Takahashi H, Wilkinson GR, Nutescu EA, Morita T, Ritchie MD, Scordo MG, et al. Different contributions of polymorphisms in VKORC1 and CYP2C9 to intra- and inter-population differences in maintenance dose of warfarin in Japanese, Caucasians and African-Americans. *Pharmacogenet Genomics* 2006;**16**:101–10.

[12] Vecsler M, Loebstein R, Almog S, Kurnik D, Goldman B, Halkin H, et al. Combined genetic profiles of components and regulators of the vitamin K-dependent γ-carboxylation system affect individual sensitivity to warfarin. *Thromb Haemost* 2006;**95**:205–11.

[13] Mushiroda T, Ohnishi Y, Saito S, Takahashi A, Kikuchi Y, Saito S, et al. Association of *VKORC1* and *CYP2C9* polymorphisms with warfarin dose requirements in Japanese patients. *J Hum Genet* 2006;**51**:249–53.

[14] Lee SC, Ng SS, Oldenburg J, Chong PY, Rost S, Guo JY, et al. Interethnic variability of warfarin maintenance requirement is explained by *VKORC1* genotype in an Asian population. *Clin Pharmacol Ther* 2006;**79**:197–205.

[15] Montes R, Ruiz de Gaona E, Martinez-Gonzalez MA, Alberca I, Hermida J. The c.−1639G>A polymorphism of the *VKORC1* gene is a major determinant of the response to acenocoumarol in anticoagulated patients. *Br J Haematol* 2006;**133**: 183–7.

[16] Higashi MK, Veenstra DL, Kondo LM, Wittkowsky AK, Srinouanprachanh SL, Farin FM, et al. Association between *CYP2C9* genetic variants and anticoagulation-related outcomes during warfarin therapy. *JAMA* 2002; **287**:1690–8.

[17] Nasu K, Kubota T, Ishizaki T. Genetic analysis of *CYP2C9* polymorphism in a Japanese population. *Pharmacogenetics* 1997;**7**:405–9.

[18] Yamaguchi T. Optimal intensity of warfarin therapy for secondary prevention of stroke in patients with nonvalvular atrial fibrillation: a multicenter, prospective, randomized trial. Japanese Nonvalvular Atrial Fibrillation-Embolism Secondary Prevention Cooperative Study Group. *Stroke* 2000;**31**:817–21.

[19] Chimowitz MI, Lynn MJ, Howlett-Smith H, Stern BJ, Hertzberg VS, Frankel MR, et al. Comparison of warfarin and aspirin for symptomatic intracranial arterial stenosis. *N Engl J Med* 2005;**352**:1305–16.

[20] Kimura R, Kokubo Y, Miyashita K, Otsubo R, Nagatsuka K, Otsuki T, et al. Polymorphisms in vitamin K-dependent

γ-carboxylation-related genes influence interindividual variability in plasma protein C and protein S activity in general population. Int J Hematol in press.

[21] Antonarakis SE. Recommendations for a nomenclature system for human gene mutations. Nomenclature Working Group. *Hum Mutat* 1998;**11**:1–3.

[22] Tanaka C, Kamide K, Takiuchi S, Miwa Y, Yoshii M, Kawano Y, et al. An alternative fast and convenient genotyping method for the screening of angiotensin converting enzyme gene polymorphisms. *Hypertens Res* 2003;**26**:301–6.

[23] Sanderson S, Emery J, Higgins J. *CYP2C9* gene variants, drug dose, and bleeding risk in warfarin-treated patients: a HuGEnet systematic review and meta-analysis. *Genet Med* 2005;**7**:97–104.

[24] Tie J, Wu SM, Jin D, Nicchitta CV, Stafford DW. A topological study of the human γ-glutamyl carboxylase. *Blood* 2000;**96**:973–8.

[25] Pudota BN, Hommema EL, Hallgren KW, McNally BA, Lee S, Berkner KL. Identification of sequences within the γ-carboxylase that represent a novel contact site with vitamin K-dependent proteins and that are required for activity. *J Biol Chem* 2001;**276**:46878–86.

[26] Shikata E, Ieiri I, Ishiguro S, Aono H, Inoue K, Koide T, et al. Association of pharmacokinetic *(CYP2C9)* and pharmacodynamic (*factors II, VII, IX,* and *X*; *proteins S* and *C*; and *γ-glutamyl carboxylase*) gene variants with warfarin sensitivity. *Blood* 2004;**103**:2630–5.

[27] Chen LY, Eriksson N, Gwilliam R, Bentley D, Deloukas P, Wadelius M. γ-Glutamyl carboxylase (GGCX) microsatellite and warfarin dosing. *Blood* 2005;**106**:3673–4.

[28] Sullivan-Klose TH, Ghanayem BI, Bell DA, Zhang ZY, Kaminsky LS, Shenfield GM, et al. The role of the CYP2C9-Leu359 allelic variant in the tolbutamide polymorphism. *Pharmacogenetics* 1996;**6**:341–9.

Association of genetic polymorphisms of *ACADSB* and *COMT* with human hypertension

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Objectives **Genetically hypertensive rats provide an excellent model to investigate the genetic mechanisms of hypertension. We previously identified three differentially expressed genes, Acadsb (short/branched chain acyl-CoA dehydrogenase), Comt (catecholamine-*O*-methyltransferase), and Pnpo (pyridoxine 5′-phosphate oxidase), in hypertensive and normotensive rat kidneys as potential susceptibility genes for rat hypertension. We examined the association of human homologues of these genes with human hypertension.**

Methods **We sequenced three genes using samples from 48 or 96 hypertensive patients, identified single nucleotide polymorphisms, and genotyped them in a population-based sample of 1818 Japanese individuals (771 hypertensive individuals and 1047 controls).**

Results **After adjustments for age, body mass index, present illness (hyperlipidaemia, diabetes mellitus), and lifestyle (smoking, alcohol consumption), multivariate logistic regression analysis revealed that −512A>G in *ACADSB* was associated with hypertension in women (AA vs AG + GG: odds ratio = 0.70, 95% confidence interval = 0.53–0.94). This single nucleotide polymorphism was in tight linkage disequilibrium with −254G>A. Furthermore, −1187G>C in *COMT* was associated with hypertension in men (GG vs CG + CC: odds ratio = 0.69, 95% confidence interval = 0.52–0.93) and was in tight linkage disequilibrium with 186C>T. After adjustments described above, −512 A>G and −254G>A in *ACADSB* were associated with variations in systolic blood pressure. *ACADSB* was in tight linkage disequilibrium with *MGC35392* across a distance of 18.3 kb. *COMT* was not in linkage disequilibrium with any adjacent genes. Analysis indicated that two haplotypes of *COMT* were significantly associated with hypertension in men.**

Conclusion **Our study suggests the possible involvement of genetic polymorphisms in *ACADSB* and *COMT* in essential hypertension in the Japanese population.**
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Keywords: catecholamine-*O*-methyltransferase, gene polymorphism, hypertension, salt sensitivity, short/branched-chain acyl-CoA dehydrogenase

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Introduction

The identification of genes contributing to essential hypertension in humans is difficult because hypertension is a multifactorial disease resulting from both environmental and genetic factors. To overcome this difficulty and facilitate genetic analyses, genetically hypertensive rats such as spontaneously hypertensive rats and Dahl salt-sensitive (Dahl-S) rats have been utilized. Some genes that cause phenotypes such as hypertension and insulin resistance will be differentially expressed, and therefore candidates are sought from among genes found to be differentially expressed [1–3].

To identify candidate genes responsible for hypertension in Dahl-S rats, we previously utilized an oligonucleotide microarray analysis and identified differentially expressed genes in the kidneys of salt-loaded Dahl-S and Lewis rats [4]. To examine the association of these genes with variations in blood pressure, we obtained 101 F_2 males from Dahl-S and Lewis rats and performed precise blood pressure measurements by telemetric monitoring at 14 weeks of age following 9 weeks of salt loading. Correlation analyses of genotypes of 12 differentially expressed genes, and blood pressure variation in the F_2 rats, indicated that short/branched chain acyl-CoA dehydrogenase (*Acadsb*), catecholamine-*O*-methyltransferase (*Comt*), pyridoxine 5′-phosphate oxidase (*Pnpo*), and *Sah* (medium-chain acyl-CoA synthetase) showed a significant association with

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blood pressure variation. To extend these studies to hypertension in humans, it is important to know whether human homologues of these genes cause susceptibility to hypertension in humans.

The human chromosome is divided into discrete blocks, called haplotype blocks, separated by hot spots of recombination [5]. In the haplotype blocks, a small number of common haplotypes are present. The International HapMap Project was completed in 2005 and catalogued the patterns of more than 1 million single nucleotide polymorphisms (SNPs) [6]. It determined that most inter-SNP distances are less than 10 kb, although some are over 20 kb. Once a candidate polymorphism associated with a phenotype is identified, genotyping of SNPs in adjacent genes is highly important. If the haplotype block consists of multiple genes, the phenotype-causing SNP might be present in an adjacent gene.

In the present study, we attempted to evaluate three potential hypertension-causing genes, obtained from an earlier study in rats, using a population-based sample of 1818 Japanese (771 individuals with hypertension and 1047 controls). Since the *Sah* gene has already been studied extensively [7], we did not analyse it in here. We first identified genetic variations, primarily SNPs, in all the exons of three human homologues of the potential hypertension susceptibility genes, *ACADSB*, *COMT*, and *PNPO*. We next examined the association of the SNPs and their haplotypes of these candidate genes with the presence of hypertension and blood pressure variation in the general Japanese population. We also studied linkage disequilibrium at the candidate gene loci.

Methods

Participants

For the sequencing of DNA, patients with essential hypertension were recruited at the outpatient clinic of the Division of Hypertension and Nephrology, National Cardiovascular Center, Suita, Japan. For genotyping, 1818 individuals, including 771 patients with hypertension (396 men, 375 women) and 1047 controls (439 men, 608 women), were used as a population-based sample for the Suita study. The selection criteria and design of the Suita study have been described previously [8,9]. Only individuals who provided written informed consent for genetic analyses were included in this study, and the study protocol was approved by the Ethical Review Committee of the National Cardiovascular Center.

Measurements

Blood pressure measurements were taken after at least 10 min of rest in a sitting position. The recorded systolic and diastolic blood pressures were the means of two measurements recorded at least 3 min apart. Hypertension was defined as a systolic blood pressure (SBP) of at least 140 mmHg and/or a diastolic blood pressure (DBP) of at least 90 mmHg, or the current use of antihypertensive medication. Diabetes mellitus was defined as a fasting plasma glucose concentration greater than 7.0 mmol/l (126 mg/dl), a nonfasting plasma glucose concentration above 11.1 mmol/l (200 mg/dl), taking antidiabetic medication, or a HbA1c value of at least 6.5%. Hyperlipidaemia was defined as a total cholesterol concentration greater than 5.68 mmol/l (220 mg/dl) or the taking of antihyperlipidaemia medication.

Blood samples drawn from the participants after 12 h of fasting were collected in tubes containing ethylenediamine tetraacetic acid. We measured the total cholesterol and high-density lipoprotein-cholesterol levels with an autoanalyser (Toshiba TBA-80; Toshiba, Tokyo, Japan) in accordance with the Lipid Standardization Program of the US Centers for Disease Control and Prevention through the Osaka Medical Center for Health Science and Promotion, Japan.

Direct sequencing for single nucleotide polymorphism discovery, database searches for single nucleotide polymorphisms, and polymorphism genotyping

We sequenced the entire coding regions of three candidates for genes causing susceptibility to hypertension, *ACADSB*, *COMT*, and *PNPO*, in 48 or 96 hypertensive individuals in which we predicted the hypertension-susceptive SNPs would be found. Our methods for direct sequencing were described previously [10,11]. SNPs with a minor allele frequency of greater than 5% were considered candidates for genotyping using the TaqMan polymerase chain reaction system [12,13]. Since a missense mutation may cause direct susceptibility to hypertension, several missense mutations with a minor allele frequency of less than 5% were also genotyped. As a consequence, we genotyped five, seven, and two SNPs in *ACADSB*, *COMT*, and *PNPO*, respectively, from the general population.

The HapMap Project revealed that the inter-SNP distances in certain regions were greater than 20 kb [6]. Genotyping other polymorphisms in such a haplotype block is highly important. Within a region of 200 kb surrounding the *ACADSB* locus, 10 genes (*MGC45962*, *LOC118670*, *FLJ13490*, *MGC35392*, *PEGASUS*, *LOC340784*, *LOC387716*, *LOC387717*, *BUB3*, and *LOC390009*) are present. Seven genes (*TBX1*, *GNB1L*, *FL21125*, *TXNRD2*, *ARVCF*, *DKFZp761P1121*, and *DGCR8*) are located within approximately 200 kb of *COMT*. We determined SNPs in these genes using the database of Japanese Single Nucleotide Polymorphisms (http://snp.ims.u-tokyo.ac.jp/) [14,15] and genotyped the following 14 SNPs using the TaqMan polymerase chain reaction system: rs1891110-GA (*MGC45962*), rs3736583-AG (*MGC35392*), rs3736582-CG (*MGC35392*), rs11190-AC (*MGC35392*), rs752920-TA (*LOC390009*), rs2301558-CT (*TBX1*), rs2073767-CT

(*GNB1L*), rs1139793-GA (*TXNRD2*), rs1005873-AG (*TXNRD2*), rs2073747-GA (*ARVCF*), rs1990277-GA (*ARVCF*), rs1054215-CT (*DKFZp761P1121*), rs1640297-TC (*DGCR8*), and rs720012-AG (*DGCR8*).

Statistical analysis

Analysis of variance was used to compare mean values between groups and, if overall significance was demonstrated, the intergroup difference was assessed using a general linear model. Frequencies were compared using a chi-squared analysis.

The relationships between genotypes and the presence of hypertension were expressed in terms of odds ratios adjusted for several possible confounding effects, including age, body mass index, present illness (hyperlipidaemia and diabetes mellitus), and lifestyle choices (smoking and drinking). For multivariate risk predictors, the adjusted odds ratios were determined using 95% confidence intervals. For each gender, analysis of any association between genotype and blood pressure were also investigated using a logistic regression analysis that considered potential confounding risk variables, including age, body mass index, present illness (hyperlipidaemia and diabetes mellitus), lifestyle choices (smoking and alcohol consumption), and antihypertensive medication. All analyses were performed using SAS statistical software (release 6.12; SAS Institute Inc., Cary, North Carolina, USA) [16]. Linkage disequilibrium and haplotype analyses were conducted using SNPAlyze version 2.1 (DYNACOM Co., Ltd., Mohara, Japan). The pairwise linkage disequilibrium value, D', was obtained between the SNP and −512A>G at the *ACADSB* locus, and between the SNP and −1187G>C at the *COMT* locus. Haplotype frequencies were estimated from genotype data using an expectation maximization algorithm. Controlling for deviation from Hardy–Weinberg equilibrium gave nonsignificant results for all the SNPs examined in the current study.

Results

General characteristics of study participants

The characteristics of the 1818 individuals (835 men and 983 women) are summarized in Table 1. Age, SBP, DBP, body mass index, percentages of current smokers and drinkers, prevalence of hypertension, and prevalence of diabetes mellitus were significantly higher in the men than in the women. Total cholesterol, high-density lipoprotein-cholesterol, and the percentage of hyperlipidaemic patients were significantly higher in the women than in the men.

Table 1 Basic characteristics of the participants

Characteristic	Women ($n=983$)	Men ($n=835$)
Age (years)	63.3 ± 11.0	66.3 ± 11.1*
Systolic blood pressure (mmHg)	128.0 ± 19.6	131.9 ± 19.5*
Diastolic blood pressure (mmHg)	76.6 ± 9.8	79.7 ± 10.7*
Body mass index (kg/m^2)	22.3 ± 3.2	23.3 ± 3.0*
Total cholesterol (mmol/l)	5.57 ± 0.79*	5.10 ± 0.78
High-density lipoprotein-cholesterol (mmol/l)	1.67 ± 0.40*	1.42 ± 0.36
Current smokers (%)	6.3	30.1†
Current drinkers (%)	29.3	67.0†
Present illness (%)		
Hypertension	38.2	47.4†
Hyperlipidaemia	55.2†	27.4
Diabetes mellitus	5.2	12.6†

Values presented as the mean ± SD or the percentage. The indications for each condition were as follows: hypertension, systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg, or antihypertensive medication; hyperlipidaemia, total cholesterol ≥ 5.68 mmol/l (220 mg/dl) or antihyperlipidaemia medication; and diabetes, fasting plasma glucose ≥ 7.0 mmol/l (126 mg/dl), nonfasting plasma glucose ≥ 11.1 mmol/l (200 mg/dl), or antidiabetic medication. *$P<0.05$ between females and males with Student's *t*-test. †$P<0.05$ between females and males with a chi-squared test.

Polymorphisms in *ACADSB*, *COMT*, and *PNPO*, and single nucleotide polymorphism genotyping

We sequenced either 96 or 182 alleles from 48 or 96 Japanese hypertensive patients for the *ACADSB*, *COMT*, and *PNPO* genes, and identified 14, 14, and five polymorphisms, respectively (Table 2). There were two and three missense mutations in *ACADSB* and *COMT*, respectively. The R13K mutation in *ACADSB* and the A72S and V158M mutations in *COMT* were common, with minor allele frequencies of 0.125, 0.093, and 0.279, respectively. The V158M mutation in *COMT* is known to be functional; the enzyme containing Met has one-quarter the activity of the Val-containing enzyme [17]. The H31R mutation in *ACADSB* showed a minor allele frequency of 0.021, and the K212T mutation in *COMT* showed a minor allele frequency of 0.005. Considering the allele frequencies and linkage disequilibrium, we selected five, seven, and two SNPs in *ACADSB*, *COMT*, and *PNPO*, respectively, and genotyped them using large-scale population-based samples.

Association of single nucleotide polymorphisms with hypertension

Multivariate logistic regression analysis, after adjustments for age, body mass index, current illness (hyperlipidaemia and diabetes mellitus), and lifestyle (smoking and alcohol consumption), revealed that −512A>G and −254G>A in *ACADSB* in tight linkage disequilibrium showed an association with the presence of hypertension in women (−512A>G: AA vs AG + GG: odds ratio = 0.70, 95% confidence interval = 0.53–0.94, $P=0.0163$; −254G>A: GG vs GA + AA, odds ratio = 0.70, 95% confidence interval = 0.53–0.94, $P=0.0171$) (Table 3). In addition, −1187G>C and 186C>T in *COMT* in tight linkage disequilibrium were associated with hypertension in men (−1187G>C: GG vs GC + CC, odds ratio = 0.69, 95% confidence interval = 0.52–0.93, $P=0.0122$; 186C>T: CC vs CT + TT, odds ratio = 0.69, 0.69, 95% confidence interval = 0.52–0.92, $P=0.0116$) (Table 3). A functional SNP in *COMT*, 1222G>A, accompanied by the V158M substitution, was marginally associated with hypertension ($P=0.0742$).

Table 2 List of polymorphisms and their allele frequencies in *ACADSB*, *COMT*, and *PNPO*, as identified by direct sequencing

Single nucleotide polymorphism	LD	Amino acid change	Region	Allele frequency		Flanking sequence	Taqman	dbSNP ID
				Allele 1	Allele 2			
ACADSB								
−512A>G	a		Promoter	0.714	0.286	ccctccggctaa[a/g]gaggtcccgggc	Taqman	rs2277249
−254G>A	a		Promoter	0.714	0.286	accgtcacagtc[g/a]ccgccgccatct	Taqman	rs2277250
−211C>A			Promoter	0.995	0.005	ccttcccgcccc[c/a]ctgccttgctca		
−107G>A	b		Promoter	0.979	0.021	gcagggattaag[g/a]gggggtgtgtgc		
−80G>C			Promoter	0.995	0.005	ggcgggtactga[g/c]tgggcggggcct		
−22A>G			Promoter	0.995	0.005	ccagaggcgcag[a/g]gcggagaggcct		
38G>A		R13K	Exon 1	0.875	0.125	TGCGCGGCAGCA[G/A]GCTGGTGAGTGC	Taqman	
89delG			Intron 1	0.995	0.005	agggcgaccttg[g/-]cccctggaatcg		
25376A>G	b	H31R	Exon 2	0.979	0.021	AGATTCCTCCTC[A/G]TGTCTCAAAATC	Taqman	
31341delTAA	c		Intron 3	0.196	0.804	aaataataataa[taa/-]atatggttacag		
31379G>A			Intron 3	0.989	0.011	ttgttcatgcaa[g/a]aaatttccccat		
32308C>T		H213H	Exon 5	0.896	0.104	CAGTGCTGAGCA[C/T]GCAGGGCTCTTT		
43942A>G	c		Intron 9	0.198	0.802	gccactaacagt[a/g]aatccatgttgc	Taqman	rs2421166
44814C>T			3′-UTR	0.979	0.021	TGGGAGTAAGTG[C/T]CTTGCGTGGGAA		
COMT								
−20878A>G			Promoter	0.990	0.010	accctcacgagg[a/g]caccccggccgc		
−20531G>A			Intron 1	0.984	0.016	gtggggaattcg[g/a]accgctgtgaag		
−1187G>C	d		Intron 2	0.724	0.276	ggtacagattcc[g/c]gcccggtgcatg	Taqman	rs165656
−98A>G	e		Intron 2	0.728	0.272	ttgccccctctgc[a/g]aacacaaggggg		rs6269
186C>T	d	H62H	Exon 3	0.717	0.283	CATCCTGAACCA[C/T]GTGCTGCAGCAT	Taqman	rs4633
214G>T		A72S	Exon 3	0.907	0.093	GAGCCCGGGAAC[G/T]CACAGAGCGTGC	Taqman	rs6267
379A>G	e		Intron 3	0.725	0.275	tgttatcacccc[a/g]tttccaggggc		rs2239393
971G>A			Intron 3	0.995	0.005	aggtgggggcc[g/a]tgcctggggatc		
1158C>G	e	L136L	Exon 4	0.716	0.284	AGGGGCGAGGCT[C/G]ATCACCATCGAG	Taqman	rs4818
1222G>A	d	V158M	Exon 4	0.721	0.279	GATTTCGCTGGC[G/A]TGAAGGACAAGg	Taqman	rs4680
1755G>A		P199P	Exon 5	0.941	0.059	CCGGTACCTGCC[G/A]GACACGCTTCTC		rs769224
1848G>C			Intron 5	0.856	0.144	agcctctccaaa[g/c]agccaggcattc	Taqman	rs4646315
6029A>C		K212T	Exon 6	0.995	0.005	GCCTGCTGCGGA[A/C]GGGGACAGTGCT		
6220–6221insC			3′-UTR	0.468	0.532	GACTGCCCCCCCC[-/C]GGCCCCCCTCTC	Taqman	rs362204
PNPO								
−139A>C			Promoter	0.989	0.011	ttggctccgagg[a/c]cttaggacctgt		
1657C>T		S55S	Exon 2	0.840	0.160	TCATCTGACCTC[C/T]CTTGACCCAGTG	Taqman	
3848C>T			Intron 3	0.379	0.621	tcctctccctgt[c/t]ctgatggctggc	Taqman	rs4491575
4119G>A			Intron 4	0.995	0.005	acagagaggaac[g/a]gggcctgtgctg		
4308T>C		D180D	Exon 5	0.995	0.005	TGTGATCCCTGA[T/C]CGGGAGgtgagt		

ACADSB, acyl-Coenzyme A dehydrogenase, short/branched chain (10q25-q26); *COMT*, catechol-O-methyltransferase (22q11.2); *PNPO*, pyridoxine-5-prime-phosphate oxidase (17); UTR, untranslated region. The apparent linkage disequilibrium (LD), defined by $r^2 > 0.5$, is indicated by 'a–e' in the LD column. Single nucleotide polymorphisms for large-scale genotyping are indicated by 'Taqman'. The A of the ATG of the initiating Met codon is denoted nucelotide + 1, following recommendations by the Nomenclature Working Group [29]. Localization of the human chromosome is shown in parentheses. The nucleotide sequences (GenBank accession number NT_030059.12 for *ACADSB*, NT_011519.10 for *COMT*, and NT_010783.14 for *PNPO*) were used as reference sequences. Uppercase and lowercase letters in the flanking sequences are sequences in extron and intron regions, respectively.

Table 3 Odds ratio of polymorphisms in *COMT* and *ACADSB*

Gene	SNPs (allele frequency)	Genotype	Women		Men	
			Odds ratio (95% confidence interval)*	*P* value	Odds ratio (95% confidence interval)*	*P* value
ACADSB	−512A>G[b]	AA	1	0.0163	1	0.3832
	(0.738/0.262)	AG + GG	0.70 (0.53–0.94)		1.13 (0.85–1.51)	
		AA + AG	1	0.5695	1	0.4850
		GG	0.84 (0.46–1.54)		1.21 (0.71–2.07)	
ACADSB	−254G>A[b]	GG	1	0.0171	1	0.3785
	(0.738/0.262)	GA + AA	0.70 (0.53–0.94)		1.14 (0.86–1.51)	
		GG + GA	1	0.5676	1	0.3899
		AA	0.84 (0.46–1.54)		1.27 (0.74–2.18)	
COMT	−1187G>C[a]	GG	1	0.2791	1	0.0122
	(0.703/0.297)	GC + CC	1.18 (0.88–1.56)		0.69 (0.52–0.93)	
		GG + GC	1	0.6844	1	0.1573
		CC	0.89 (0.52–1.54)		0.70 (0.43–1.15)	
COMT	186C>T[a]	CC	1	0.3097	1	0.0116
	(0.704/0.296)	CT + TT	1.16 (0.87–1.54)		0.69 (0.52–0.92)	
		CC + CT	1	0.4891	1	0.1555
		TT	0.83 (0.48–1.43)		0.70 (0.43–1.15)	
COMT	1222G>A[a]	GG	1	0.1522	1	0.0742
	(0.695/0.305)	GA + AA	1.23 (0.92–1.64)		0.77 (0.58–1.03)	
		GG + GA	1	0.4946	1	0.4935
		AA	0.83 (0.50–1.41)		0.85 (0.52–1.37)	

* Conditional logistic analysis, adjusted for age, body mass index, present illness (hyperlipidaemia and diabetes mellitus), and lifestyle (smoking and drinking). The apparent linkage disequilibrium, defined by $r^2 > 0.5$, is indicated by 'a' and 'b' in the single nucleotide polymorphisms (SNPs) column.

Table 4 Association of genotypes with blood pressure variation

Gene	Single nucleotide polymorphism	Allele 1/2 (allele frequency)	Sex	BP	Genotype group	BP, mean ± SD (mmHg)	*P* value*	Variation of mean BP (mmHg)
ACADSB	−512A>G[a]	A/G (0.738/0.262)	Women	SBP	AA	128.77 ± 0.69	0.0302	2.29
					AG + GG	126.48 ± 0.80		
ACADSB	−254G>A[a]	G/A (0.738/0.262)	Women	SBP	GG	128.82 ± 0.69	0.0264	2.35
					GA + AA	126.47 ± 0.79		
ACADSB	38G>A (Arg13Lys)	G/A (0.878/0.122)	Women	DBP	GG + GA	76.46 ± 0.30	0.0235	5.91
					AA	82.37 ± 2.59		

BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure. [a] The apparent linkage disequilibrium, defined by $r^2 > 0.5$. * Conditional logistic analysis, adjusted for age, body mass index, present illness (hyperlipidaemia and diabetes mellitus), and lifestyle (smoking and drinking).

SBP was 2.29 mmHg higher in women with the *ACADSB* AA genotype −512A>G than women with the AG + GG genotype ($P = 0.030$), and 2.35 mmHg higher in women with the *ACADSB* GG genotype −254G>A than women with the GA + AA genotype ($P = 0.026$), after adjusting for the factors described above (Table 4). In addition, DBP was 5.90 mmHg higher in women with the *ACADSB* GG + GA genotype 38G>A than women with the AA genotype ($P = 0.024$) (Table 4). This SNP results in the amino acid substitution R13K and appears to be of functional significance.

Table 5 presents the results of the analysis of haplotype frequency for the SNPs of these three genes between hypertensive individuals and normotensive individuals. We identified haplotypes three and seven of *COMT* as having a significantly lower ($P = 0.006$) and higher frequency ($P = 0.029$) in hypertensive men than in normotensive men, respectively.

Taken together, *ACADSB* was associated with both hypertension and blood pressure variation, and *COMT* was associated with hypertension.

Linkage disequilibrium of *ACADSB* and *COMT* with adjacent genes

It is possible that the polymorphisms in *ACADSB* and *COMT* that are significantly associated with hypertension are in linkage disequilibrium with other genes in their vicinities and compose a haplotype block. To evaluate the haplotype block structure in these regions, we genotyped 14 additional SNPs present within approximately 200 kb. The pairwise linkage disequilibrium parameters, D', calculated from the genotyping data are shown in Fig. 1. These methods revealed that at the *ACADSB* locus, IMS-JST080977 in *MGC35392*, which is 18.3 kb from −512A>G in *ACADSB*, exhibited a D' value of 0.997, while IMS-JST080979 in *MGC35392*, which is 25.2 kb from −512A>G in *ACADSB*, showed a D' value of 0.928, indicating a large haplotype block at this locus. The haplotype structure of the *ACADSB* locus suggests the association of this block with the presence of hypertension. *COMT*, on the other hand, was not in linkage disequilibrium with any adjacent genes.

Discussion

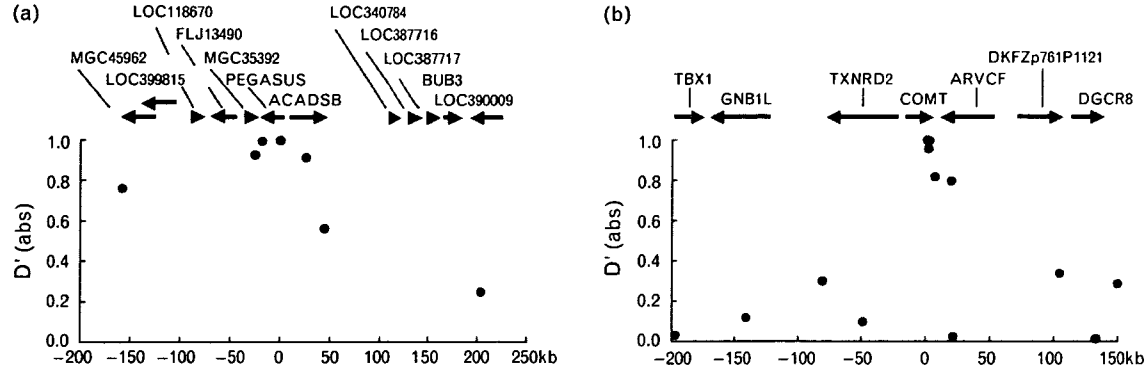
We previously identified differentially expressed genes in the kidneys of salt-loaded Dahl-S and Lewis rats [4].

Table 5 Haplotype frequency of *COMT*, *ACADSB*, and *PNPO* genes in hypertensive individuals (HT) and normotensive individuals (NT)

Gene	Haplotype		Men (%)				Women (%)			
			HT (812 alleles)	NT (902 alleles)	χ^2	*P*	HT (772 alleles)	NT (1242 alleles)	χ^2	*P*
COMT		−1187/186/214/1158/1222/1848/6221insC								
	1	G/C/G/C/G/G/−	22.8	23.6	0.166	0.684	20.9	21.7	0.184	0.668
	2	G/C/G/G/G/G/C	20.1	18.4	0.768	0.381	21.6	21.3	0.040	0.842
	3	C/T/G/C/A/G/C	12.4	17.2	7.638	0.006	14.9	15.1	0.022	0.883
	4	C/T/G/C/A/C/C	12.2	12.4	0.020	0.888	14.0	11.8	1.977	0.160
	5	G/C/G/G/G/G/−	9.5	9.5	0.001	0.971	11.3	9.5	1.611	0.204
	6	G/C/T/C/G/G/−	10.2	8.3	1.854	0.173	7.5	8.3	0.397	0.529
	7	G/C/G/C/G/G/C	9.0	6.2	4.748	0.029	6.1	8.0	2.565	0.109
	8	G/C/G/C/A/G/−	1.7	1.3	0.443	0.506	1.2	1.5	0.059	0.809
ACADSB		−512/38/25376/43942								
	1	A/G/A/G	63.5	65.5	0.762	0.383	69.6	66.3	2.488	0.125
	2	G/G/A/A	15.1	13.1	1.426	0.232	10.3	12.7	2.646	0.104
	3	G/A/A/G	13.0	12.0	0.406	0.524	11.0	12.5	1.030	0.310
	4	A/G/A/A	5.5	7.1	1.684	0.194	6.3	6.7	0.097	0.756
	5	A/G/G/G	1.4	0.7	2.110	0.146	1.9	1.0	2.678	0.102
	6	G/G/A/G	1.2	1.4	0.135	0.713	0.8	0.8	0.016	0.898
PNPO		1657/4308								
	1	C/T	60.3	61.1	0.139	0.709	59.5	59.3	0.015	0.904
	2	C/C	22.9	22.0	0.199	0.656	24.7	23.8	0.231	0.631
	3	T/C	16.6	16.5	0.010	0.920	15.8	16.9	0.449	0.503

Haplotypes with frequency ≥ 1.0% are shown.

Fig. 1



Pairwise linkage disequilibrium at the *ACADSB* (a) and *COMT* (b) loci. The pairwise linkage disequilibrium value, *D'*, was obtained between the single nucleotide polymorphism and −512A>G at the *ACADSB* locus, and between the single nucleotide polymorphism and −1187G>C at the *COMT* locus.

In these experiments, we obtained 101 F_2 male rats from Dahl-S and Lewis rats and performed precise measurements of blood pressure by telemetric monitoring at 14 weeks of age, following 9 weeks of salt loading. Correlation analyses of the genotypes of 12 differentially expressed genes and the variations in blood pressure in F_2 rats indicated that *Acadsb*, *Comt*, *Pnpo*, and *Sah* are significantly associated with blood pressure. In the current study, we have examined 1818 individuals for a relationship between the genes, *ACADSB*, *COMT*, and *PNPO*, and hypertension or blood pressure variation. These three genes were originally selected based on studies in the Dahl-S rat. We determined that two SNPs in *ACADSB*, −512A>G and −254G>A, which are in tight linkage disequilibrium, were associated with both hypertension and blood pressure variation. Two SNPs in *COMT*, −1187G>C and 186C>T, which are also in tight linkage disequilibrium, were associated with hypertension. These candidate genes were selected from the salt-loaded rats, and therefore the genetic association of these genes with hypertension might be greater if we had selected patients with salt-sensitive hypertension.

In this study, we genotyped 14 SNPs in total; therefore, after applying the Bonferroni correction for multiple testing, the level of significance was $P < 0.004$ (0.05/14 for 14 loci). Unfortunately, none of the SNPs appeared to be significant with the use of a strict Bonferroni correction. As described, however, two SNPs in *ACADSB* were associated with both hypertension and blood pressure variation. In addition, one SNP and two haplotypes in *COMT* were significantly associated with hypertension. These two genes were therefore considered valid as hypertensive candidates.

This study was undertaken to prove that candidate susceptibility genes for hypertension in the Dahl-S rat studies might also be applicable to humans. The genes *Acadsb* and *Comt* were associated with hypertension in humans, but *Pnpo* was not. *Sah* was the first example of a possible link between a differentially expressed gene in rats and human hypertension [7]. Our study is another example linking candidate susceptibility genes for hypertension identified in rats, to humans, and it also revealed genetic differences between humans and rats, particularly in salt-loaded Dahl-S rats, in terms of sensitivity to hypertension. The population of F_2 rats and the general population in this study may not be large enough to provide good statistical power. As stated above, when a human study is performed using a subgroup of salt-sensitive patients, stronger associations may become apparent.

ACADSB, short/branched chain acyl-CoA dehydrogenase, is a member of the acyl-CoA dehydrogenase family. Acyl-CoA dehydrogenases with specificity for different chain-lengths of fatty acids carry out the first step of β-oxidation in the mitochondria, each round of which removes two-carbon units as acetyl-CoA for entry into the tricarboxylic acid cycle. Acyl-CoA dehydrogenases are mitochondrial enzymes involved in the metabolism of fatty acids and branched-chain amino acids, which are required to meet physiologic energy requirements during illness and periods of fasting or under physiologic stress. In addition, two other important kidney-specific genes involved in fatty acid metabolism, *SAH* and *KS* (kidney specific) have acyl-CoA synthetase activity for medium-chain fatty acids. Both genes were isolated by differential screening from a genetically hypertensive rat strain, the spontaneously hypertensive rat [1,7,18]. Moreover, polymorphism of *SAH* was associated with cardiovascular diseases, including hypertension, hypertriglyceridaemia, hypercholesterolemia, and obesity [7]. Both *ACADSB* and *SAH* are therefore related to fatty acid metabolism and their products may exhibit some link or cross-talk that could be involved in hypertension.

Human *ACADSB* is located at 10q25-26, which corresponds to 1q35 in rats. This rat locus is reportedly related to hypertension [19], and the genomic structure of *ACADSB* indicates that *ACADSB* is located close to *PEGASUS* in a head-to-head fashion (Fig. 1). Two SNPs in *ACADSB*, −512A>G and −254G>A, which are both associated with hypertension and blood pressure variation, correspond to −9893T>C in intron 1 and −10151C>T in the 5′-untranslated region of *PEGASUS*, respectively. In searching for a transcription factor-binding motif, we determined that the nucleotide change −254G>A would give rise to the AP-1 transcription factor-binding motif. PEGASUS is a member of the Ikaros family of transcription factors, and is expressed not only in haematopoietic cell lines, as are other Ikaros family members, but also in other tissues, including the brain, heart, skeletal muscle, kidney, and liver [20]. The PEGASUS study is highly limited, and no direct links between *PEGASUS* and blood pressure have been reported. Taken together, we consider *ACADSB/PEGASUS* to be a susceptibility gene for hypertension.

COMT is a ubiquitous enzyme that catalyses the transfer of a methyl group from *S*-adenosylmethionine to catecholamines. The substrates of COMT are catechol neurotransmitters (e.g. dopamine, epinephrine, and norepinephrine), catechol estrogens (e.g. carcinogenic 4-hydroxyestradiol), indolic intermediates in melanin metabolism, xenobiotic catechols (e.g. carcinogenic flavonoids), and drugs (e.g. levodopa). COMT therefore plays an important role in the pathophysiology of Parkinson's disease, depression, oestrogen-induced cancers, and hypertension [21]. A recent study indicated that *Comt* gene-disrupted mice showed resistance to salt-induced hypertension, and the sodium-induced increase in blood pressure in wild-type mice was completely normalized by treatment with the COMT inhibitor nitecapone [22]. At baseline, 24-h urinary excretion of dopamine was increased in *Comt*-deficient mice compared with wild-type mice. In *Comt*-deficient and wild-type mice, a high-sodium diet increased urinary dopamine excretion by 405 and 660% (reflected as 102 and 212% increases in dopamine excretion), respectively. COMT can therefore regulate blood pressure, sodium excretion, and renal dopaminergic tone [22].

A functional polymorphism, 1222G>A, encoding V158M, has been reported in *COMT*. The enzyme containing Met is unstable at 37°C and has one-quarter the activity of the Val-containing enzyme [17]. In the present study, the allele frequencies of 1222G>A were 0.695 and 0.305, respectively ($n = 1818$) (Table 3). This functional SNP showed marginal significance in the case–control setting (Table 3), and it also showed linkage disequilibrium with −1187G>C and 186C>T in *COMT* (Table 2). A recent study showed that this SNP was associated with myocardial infarction in a hypertensive population, in which the low activity *COMT* genotype is protective against myocardial infarction [23].

In summary, we have studied the association between the presence of hypertension or variation in blood pressure and candidate genes selected based on experiments with the Dahl-S hypertensive rat previously reported by our group [4]. *ACADSB/PEGASUS* was associated with both hypertension and blood pressure variation, and *COMT* was associated with hypertension. Due to false positives, false negatives, and true variability between different populations, association studies are not consistently reproducible [24]. Confirmation of these results using additional cohorts is therefore required.

Perspective

Since essential hypertension is a multifactorial disease, genetic influence is thought to play an important role in its initial stages and progression. Multiple approaches have been used to detect causative genetic polymorphisms [25–28]. The candidate gene approach is the most popular method, but crucial genetic polymorphisms are still only poorly understood. We therefore attempted to identify genetic polymorphisms that cause susceptibility to hypertension on the basis of the results of expression studies previously performed in a hypertensive rat model. We revealed that two SNPs in *ACADSB/PEGASUS* and SNPs of *COMT* might cause susceptibility to essential hypertension. These results were obtained from one population. Further replication of these results in an independent population is therefore necessary. Although functional analyses are needed to clarify the association of these SNPs with the pathogenesis of hypertension, we plan to apply this information in a gene evaluation system that will develop individualized treatment for hypertension.

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References

1 Iwai N, Inagami T. Isolation of preferentially expressed genes in the kidneys of hypertensive rats. *Hypertension* 1991; **17**:161–169.
2 Aitman TJ, Glazier AM, Wallace CA, Cooper LD, Norsworthy PJ, Wahid FN, *et al.* Identification of Cd36 (Fat) as an insulin-resistance gene causing defective fatty acid and glucose metabolism in hypertensive rats. *Nat Genet* 1999; **21**:76–83.
3 Cicila GT, Lee SJ. Identifying candidate genes for blood pressure quantitative trait loci using differential gene expression and a panel of congenic strains. *Hypertens Res* 1998; **21**:289–296.
4 Okuda T, Sumiya T, Iwai N, Miyata T. Pyridoxine 5′-phosphate oxidase is a candidate gene responsible for hypertension in Dahl-S rats. *Biochem Biophys Res Commun* 2004; **313**:647–653.

5 Goldstein DB. Islands of linkage disequilibrium. *Nat Genet* 2001; **29**:109–111.
6 Consortium TIH. A haplotype map of the human genome. *Nature* 2005; **437**:1299–1320.
7 Iwai N, Katsuya T, Mannami T, Higaki J, Ogihara T, Kokame K, *et al.* Association between SAH, an acyl-CoA synthetase gene, and hypertriglyceridemia, obesity, and hypertension. *Circulation* 2002; **105**:41–47.
8 Mannami T, Baba S, Ogata J. Strong and significant relationships between aggregation of major coronary risk factors and the acceleration of carotid atherosclerosis in the general population of a Japanese city: the Suita Study. *Arch Intern Med* 2000; **160**:2297–2303.
9 Mannami T, Katsuya T, Baba S, Inamoto N, Ishikawa K, Higaki J, *et al.* Low potentiality of angiotensin-converting enzyme gene insertion/deletion polymorphism as a useful predictive marker for carotid atherogenesis in a large general population of a Japanese city: the Suita study. *Stroke* 2001; **32**:1250–1256.
10 Okuda T, Fujioka Y, Kamide K, Kawano Y, Goto Y, Yoshimasa Y, *et al.* Verification of 525 coding SNPs in 179 hypertension candidate genes in the Japanese population: identification of 159 SNPs in 93 genes. *J Hum Genet* 2002; **47**:387–394.
11 Yang J, Kamide K, Kokubo Y, Takiuchi S, Tanaka C, Banno M, *et al.* Genetic variations of regulator of G-protein signaling 2 in hypertensive patients and in the general population. *J Hypertens* 2005; **23**:1497–1505.
12 Tanaka C, Kamide K, Takiuchi S, Miwa Y, Yoshii M, Kawano Y, *et al.* An alternative fast and convenient genotyping method for the screening of angiotensin converting enzyme gene polymorphisms. *Hypertens Res* 2003; **26**:301–306.
13 Kamide K, Kokubo Y, Yang J, Tanaka C, Hanada H, Takiuchi S, *et al.* Hypertension susceptibility genes on chromosome 2p24–p25 in a general Japanese population. *J Hypertens* 2005; **23**:955–960.
14 Haga H, Yamada R, Ohnishi Y, Nakamura Y, Tanaka T. Gene-based SNP discovery as part of the Japanese Millennium Genome Project: identification of 190,562 genetic variations in the human genome. Single-nucleotide polymorphism. *J Hum Genet* 2002; **47**:605–610.
15 Hirakawa M, Tanaka T, Hashimoto Y, Kuroda M, Takagi T, Nakamura Y. JSNP: a database of common gene variations in the Japanese population. *Nucl Acids Res* 2002; **30**:158–162.
16 Kokubo Y, Kamide K, Inamoto N, Tanaka C, Banno M, Takiuchi S, *et al.* Identification of 108 SNPs in *TSC*, *WNK1*, and *WNK4* and their association with hypertension in a Japanese general population. *J Hum Genet* 2004; **49**:507–515.
17 Lotta T, Vidgren J, Tilgmann C, Ulmanen I, Melen K, Julkunen I, *et al.* Kinetics of human soluble and membrane-bound catechol-O-methyltransferase: a revised mechanism and description of the thermolabile variant of the enzyme. *Biochemistry* 1995; **34**:4202–4210.
18 Hilgers KF, Nagaraj SK, Karginova EA, Kazakova IG, Chevalier RL, Carey RM, *et al.* Molecular cloning of KS, a novel rat gene expressed exclusively in the kidney. *Kidney Int* 1998; **54**:1444–1454.
19 Frantz S, Clemitson JR, Bihoreau MT, Gauguier D, Samani NJ. Genetic dissection of region around the Sa gene on rat chromosome 1: evidence for multiple loci affecting blood pressure. *Hypertension* 2001; **38**:216–221.
20 Perdomo J, Holmes M, Chong B, Crossley M. Eos and pegasus, two members of the Ikaros family of proteins with distinct DNA binding activities. *J Biol Chem* 2000; **275**:38347–38354.
21 Xie T, Ho SL, Ramsden D. Characterization and implications of estrogenic down-regulation of human catechol-O-methyltransferase gene transcription. *Mol Pharmacol* 1999; **56**:31–38.
22 Helkamaa T, Mannisto PT, Rauhala P, Cheng ZJ, Finckenberg P, Huotari M, *et al.* Resistance to salt-induced hypertension in catechol-O-methyltransferase-gene-disrupted mice. *J Hypertens* 2003; **21**:2365–2374.
23 Eriksson AL, Skrtic S, Niklason A, Hulten LM, Wiklund O, Hedner T, *et al.* Association between the low activity genotype of catechol-O-methyltransferase and myocardial infarction in a hypertensive population. *Eur Heart J* 2004; **25**:386–391.
24 Lohmueller KE, Pearce CL, Pike M, Lander ES, Hirschhorn JN. Meta-analysis of genetic association studies supports a contribution of common variants to susceptibility to common disease. *Nat Genet* 2003; **33**:177–182.
25 Doris PA. Hypertension genetics, single nucleotide polymorphisms, and the common disease: common variant hypothesis. *Hypertension* 2002; **39**:323–331.
26 Hopkins PN, Hunt SC. Genetics of hypertension. *Genet Med* 2003; **5**:413–429.
27 Garcia EA, Newhouse S, Caulfield MJ, Munroe PB. Genes and hypertension. *Curr Pharm Des* 2003; **9**:1679–1689.
28 Ruppert V, Maisch B. Genetics of human hypertension. *Herz* 2003; **28**:655–662.
29 Nomenclature Working Group. Recommendations for a nomenclature system for human gene mutations. *Hum Mut* 1998; **11**:1–3.

Original Article

Association of Genetic Polymorphisms of Endothelin-Converting Enzyme-1 Gene with Hypertension in a Japanese Population and Rare Missense Mutation in Preproendothelin-1 in Japanese Hypertensives

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Endothelin-1 (EDN1), a 21-amino acid peptide, is a potent vasoconstrictor with various pharmacological responses. EDN1 is synthesized from a 212-amino acid precursor protein, preproEDN1, through multiple proteolytic steps. Endothelin-converting enzyme (ECE) cleaves a Trp73–Val74 peptide bond in big-EDN1 to give rise to mature EDN1. In this study, we examined the possible association of genetic variations in *ECE1* with hypertension in a general Japanese population and searched for missense mutations in and around the EDN1 polypeptide. We genotyped 5 single nucleotide polymorphisms (SNPs) in the *ECE1* gene in 1,873 individuals from a general Japanese population and identified one SNP associated with hypertension in women (rs212528: TT *vs.* TC+CC: odds ratio=1.40; 95% confidence intervals: 1.04–1.89; p=0.026), after adjusting for confounding factors. The systolic blood pressure in women with the CC genotype was 6.44 mmHg higher than that in those with the TT genotype (p=0.007), after adjusting for the same factors. Next, to identify the missense mutations that may influence the biological activity of EDN1, we sequenced the genomic region that encodes EDN1 in 942 Japanese hypertensive patients. We identified a novel missense mutation, G36R, in one hypertensive patient, but no mutations were observed in EDN1. A gene polymorphism in *EDN1*, Lys198Asn, has been reported to be associated with hypertension in obese subjects. Taken together, these findings reveal that the EDN-ECE pathway is an important system involved in essential hypertension in Japanese. (*Hypertens Res* 2007; 30: 513–520)

***Key Words*: endothelin, endothelin-converting enzyme, gene variants, hypertension, general population**

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Introduction

The endothelin (EDN) system is comprised of 4 active EDNs, with EDN1 being the predominant isoform in the cardiovascular system (*1*). Because of the potent vasoconstricting and mitogenic effects of EDN1 and its involvement in various cardiovascular diseases, biosynthesis of EDN1 has received considerable attention. EDN1 is synthesized from a 212-amino acid precursor protein, preproEDN1, through multiple proteolytic steps. In the first step, preproEDN1 is cleaved by a signal peptidase, resulting in the formation of proEDN1, which is then cleaved by a furin-like enzyme to yield the 38-amino acid protein known as big-EDN1 (amino acids 53–92) or other intermediates. Big-EDN1 is subsequently cleaved by a unique type II metalloprotease, EDN-converting enzyme-1 (ECE1), to yield EDN1 (amino acids 53–73) (*2*).

The EDN system is a promising target for the genetic analysis for hypertension. The missense mutation Lys198Asn has been identified in preproEDN1, and several reports have described that this polymorphism showed a positive association with blood pressure elevation in overweight people (*3–5*), although no significant difference in the EDN1 levels between the Asn-type and Lys-type transfectant was observed in an expression analysis (*6*). As for *ECE1*, an association between the −338C>A polymorphism in *ECE1* and blood pressure levels in women but not in men has recently been reported (*7*). This C>A polymorphism is associated with increased promoter activity, as demonstrated in a promoter assay analysis (*8*).

Complex traits such as hypertension, diabetes mellitus, and hyperlipidemia are suggested to be caused by common sequence variants that may have a small to moderate phenotypic effect (*9–11*). On the other hand, accumulating data has shown that most Mendelian disorders are caused by a set of different mutations that often reside in coding regions. These rare variants tend to have strong phenotypic effects. Several recent studies have shown that rare genetic variations in *ABCA1*, *APOA1*, and *LCAT* collectively contribute to the variation in plasma levels of high-density lipoprotein (HDL) cholesterol in the general population (*12*, *13*). We hypothesized that rare genetic variations in hypertension candidate genes could collectively contribute to hypertension. To investigate this hypothesis, we have been identifying such mutations in Japanese hypertensive subjects; to date, we have identified missense mutations in the β- or γ-subunit of the amiloride-sensitive epithelial sodium channel encoded by *SCNN1B* and *SCNN1G* (*14*), a causative gene for pseudohypoaldosteronism type II encoded by serine-threonine kinase *WNK4* (*15*), the regulator of G-protein signaling 2 (*RGS2*) (*16*), and the mineralocorticoid receptor encoded by *NR3C2* (*17*). As the next hypertension candidate gene, we have begun to sequence the *EDN1* gene and to search for missense mutations (*18*).

In present study, we genotyped the genetic polymorphisms of one of the EDN-converting enzymes, the *ECE1* gene, in a general Japanese population to examine whether the *ECE1* gene is a susceptibility gene for hypertension. Secondly, to evaluate the EDN system in essential hypertension in Japanese, we re-sequenced the EDN1 polypeptide in the *EDN1* gene in Japanese hypertensive patients to identify missense mutations that may deleteriously affect EDN1 function.

Methods

General Population

The selection criteria and design of the Suita study have been described previously (*19*, *20*). Only those who gave written informed consent for genetic analyses were included in this study. The study protocol was approved by the Ethical Review Committee of the National Cardiovascular Center. In this study, the genotypes of 1,873 samples were determined. The characteristics of the 1,873 participants (863 men and 1,010 women) are shown in Table 1. Routine blood examinations that included total serum cholesterol, HDL cholesterol, triglyceride, and glucose levels were performed. A physician or nurse interviewed each patient regarding smoking and alcohol drinking habits and personal history of cardiovascular disease, including angina pectoris, myocardial infarction, and/or stroke. Blood pressure was measured after at least 10 min of rest in a sitting position. Systolic and diastolic blood pressures (SBP and DBP) were the means of two measurements by well-trained doctors (recorded >3 min apart). Hypertension was defined as SBP of ≥140 mmHg, DBP of ≥90 mmHg, or the current use of antihypertensive medication (*20*). Diabetes mellitus was defined as fasting plasma glucose ≥7.0 mmol/L (126 mg/dL), non-fasting plasma glucose ≥11.1 mmol/L (200 mg/dL), current use of antidiabetic medication, or HbA1c ≥6.5%. Hyperlipidemia was defined as total cholesterol ≥5.68 mmol/L (220 mg/dL) or antihyperlipidemia medication. Body mass index (BMI) was calculated as weight (in kg) divided by height (in m) squared.

Hypertensive Subjects

A total of 942 hypertensive subjects (518 men and 424 women; average age: 65.1±10.5 years) were recruited from the Division of Hypertension and Nephrology at the National Cardiovascular Center. Ninety-two percent of study subjects (870 subjects) were diagnosed with essential hypertension, and the rest had secondary hypertension, including renal hypertension (36 subjects), renovascular hypertension (23 subjects), primary aldosteronism (11 subjects) and hypothyroid-induced hypertension (2 subjects) (*14–17*). The hypertension criteria were blood pressure above 140 and/or 90 mmHg or the use of antihypertensive agents. Blood pressure was the average of three measurements taken in a sitting position after at least 5 min of rest on each occasion. About one-third of the hypertensive subjects had hypertensive cardiovas-

Table 1. Basic Characteristics of Subjects in Japanese General Population (Suita Study)

	Women (n=1,010)	Men (n=863)
Age (yeasr old)	63.3±11.0	66.3±11.1*
Systolic blood pressure (mmHg)	128.0±19.6	131.9±19.5*
Diastolic blood pressure (mmHg)	76.6±9.8	79.7±10.7*
Body mass index (kg/m^2)	22.3±3.2	23.3±3.0*
Total cholesterol (mmol/L)	5.57±0.79*	5.10±0.78
HDL-cholesterol (mmol/L)	1.67±0.40*	1.42±0.36
Current smokers (%)	6.3	30.1†
Current drinkers (%)	29.3	67.0†
Present illness (%)		
Hypertension	38.2	47.4†
Hyperlipidemia	55.2†	27.4
Diabetes mellitus	5.2	12.6†

Values are mean±SD or percentage. Hypertension: systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg or antihypertensive medication; hyperlipidemia: total cholesterol ≥220 mg/dL or antihyperlipidemia medication; diabetes: fasting plasma glucose ≥126 mg/dL or non-fasting plasma glucose ≥200 mg/dL or HbA1c ≥6.5% or antidiabetic medication. *$p<0.05$ between women and men by Student's t-test. †$p<0.05$ between women and men by χ^2 test. HDL, high-density lipoprotein.

Table 2. General Characteristics of Patients with Hypertension and/or Renal Failure

Number	942
Age (years)	65.1±10.5
Gender (M/F)	518/424
Body mass index (kg/m^2)	24.2±3.3
Systolic blood pressure (mmHg)	145.5±19.2
Diastolic blood pressure (mmHg)	84.8±13.4
Essential hypertension	870
Secondary hypertension	72
Renal hypertension	36
Renovascular hypertension	23
Primary aldosteronism	11
Hypothyroid-induced hypertension	2
Renal impairment*	110
Ischemic heart disease	102
Stroke**	145

Values are expressed as mean±SD. *Patients who had serum creatinine ≥1.4 mg/dL. **Silent cerebral infarction was included. M, male; F, female.

cular complications. The clinical features of the patients in this study are summarized in Table 2.

All of the participants for the genetic analysis in the present study gave their written informed consent. The study protocol was approved by the Ethical Review Committee of the National Cardiovascular Center.

Genotyping of Mutations of Single Nucleotide Polymorphisms of the *ECE1* Gene in the General Population

We obtained genetic polymorphisms in the *ECE1* gene using the database of Japanese Single Nucleotide Polymorphisms (JSNP) (http://snp.ims.u-tokyo.ac.jp/) (*21*, *22*) and genotyped the following 5 single nucleotide polymorphisms (SNPs) by the TaqMan-PCR system: rs212548-TC (IMS-JST017298 in intron 4), rs212528-TC (IMS-JST004319 in intron 5), rs212526-CT (IMS-JST009090 in intron 6), rs2038090-AC (IMS-JST004325 in intron 17), and rs2038089-AG (IMS-JST004324 in intron 17). The primers and probes of the TaqMan-PCR system are available on request. Hereafter, SNPs are described according to the RS nomenclature system.

Screening of Mutations in Exon 2 of the *EDN1* Gene

Blood samples were obtained from each subject and genomic DNA was isolated from peripheral blood leukocytes using an NA-3000 nucleic acid isolation system (KURABO, Osaka, Japan). The region of exon 2 was amplified by polymerase chain reaction (PCR) using a pair of specific primers, 5′-CTGATGGCAGGCTGTGTGCTT-3′ and 5′-CCCCATCAG ATGCCACTGTGA-3′, which flank the 612-bp region containing exon 2. The PCR products were directly sequenced on an ABI PRISM 3700 DNA analyzer (Applied Biosystems, Foster City, USA) as described previously (*23*, *24*). The obtained sequences were examined for the presence of mutations using Sequencher software (Gene Codes Corporation, Ann Arbor, USA), followed by visual inspection (*25*).

Statistical Analysis

Analysis of variance was used to compare mean values between groups, and if overall significance was demonstrated, the intergroup difference was assessed by means of a general linear model. Frequencies were compared by χ^2 analysis. Association analyses between genotypes and blood pressure in each sex were performed through logistic regression analysis with consideration for potential confounding risk variables, including age, BMI, present illness (hyperlipidemia and diabetes mellitus), lifestyle (smoking and drinking), and antihypertensive medication. For multivariate risk predictors, the adjusted odds ratios were given with the 95% confidence intervals. The relationship between genotypes and risk of hypertension was expressed in terms of the odds ratios adjusted for possible confounding effects, including age, BMI, present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking). Odds ratios were calculated as a measure of the association between each genotype

Table 3. Odds Ratio of Polymorphisms in *ECE1*

SNP	Sex	Genotype	*n*	Odds ratio	(95% CI)	*p*	Genotype	*n*	Odds ratio	(95% CI)	*p*
rs212548	Women	TT	328	1	(reference)		TT+TC	821	1	(reference)	
		TC+CC	686	1.28	(0.94–1.74)	0.116	CC	193	1.21	(0.85–1.72)	0.293
	Men	TT	275	1	(reference)		TT+TC	692	1	(reference)	
		TC+CC	590	1.10	(0.82–1.50)	0.520	CC	173	0.98	(0.69–1.40)	0.924
rs212528	Women	TT	663	1	(reference)		TT+TC	980	1	(reference)	
		TC+CC	347	1.40	(1.04–1.89)	0.026	CC	30	1.63	(0.74–3.58)	0.227
	Men	TT	528	1	(reference)		TT+TC	827	1	(reference)	
		TC+CC	335	0.83	(0.62–1.11)	0.198	CC	36	0.75	(0.37–1.53)	0.428
rs212526	Women	CC	734	1	(reference)		CC+CT	996	1	(reference)	
		CT+TT	280	0.76	(0.55–1.05)	0.099	TT	18	0.77	(0.25–2.35)	0.650
	Men	CC	615	1	(reference)		CC+CT	842	1	(reference)	
		CT+TT	251	0.95	(0.70–1.30)	0.751	TT	24	1.40	(0.58–3.38)	0.455
rs2038090	Women	AA	774	1	(reference)		AA+AC	999	1	(reference)	
		AC+CC	239	1.17	(0.84–1.64)	0.348	CC	14	1.05	(0.30–3.61)	0.939
	Men	AA	676	1	(reference)		AA+AC	856	1	(reference)	
		AC+CC	189	1.00	(0.71–1.40)	0.989	CC	9	3.32	(0.67–16.45)	0.142
rs2038089	Women	AA	414	1	(reference)		AA+AG	880	1	(reference)	
		AG+GG	598	1.19	(0.89–1.59)	0.240	GG	132	1.21	(0.80–1.84)	0.358
	Men	AA	380	1	(reference)		AA+AG	788	1	(reference)	
		AG+GG	486	1.12	(0.84–1.49)	0.450	GG	78	1.33	(0.81–2.18)	0.264

*Conditional logistic analysis, adjusted for age, body mass index, present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking). SNP, single nucleotide polymorphism; CI, confidence interval.

Table 4. Association of Genotypes with Blood Pressure Variation

SNP	Genotype	Women					Men				
		n	DBP (mmHg)	*p**	SBP (mmHg)	*p**	*n*	DBP (mmHg)	*p**	SBP (mmHg)	*p**
rs212528	TT	663	76.49±0.37		126.89±0.64		528	79.98±0.43		131.94±0.75	
	TC	317	76.55±0.53		129.21±0.93		299	79.48±0.57		131.18±1.00	
	CC	30	77.57±1.72	0.698	133.33±3.02	0.007	36	80.93±1.66	0.931	133.83±2.89	0.941
	TT	663	76.49±0.37		126.89±0.64		528	79.98±0.43		131.94±0.75	
	TC+CC	347	76.63±0.51	0.823	129.56±0.89	0.016	335	79.64±0.54	0.840	131.47±0.94	0.698
	TT+TC	980	76.51±0.30		127.64±0.53		827	79.67±0.34		131.66±0.60	
	CC	30	77.57±1.72	0.545	133.33±3.02	0.066	36	80.93±1.66	0.458	133.83±2.89	0.463
rs212526	CC	734	76.56±0.35		128.07±0.61		615	79.41±0.40		131.67±0.69	
	CT	262	76.90±0.58		127.51±1.03		227	80.15±0.66		131.39±1.15	
	TT	18	70.08±2.19	0.344	120.04±3.87	0.175	24	84.13±2.06	0.048	138.16±3.59	0.422
	CC	734	76.56±0.35		128.07±0.61		615	79.41±0.40		131.67±0.69	
	CT+TT	280	76.45±0.56	0.874	127.02±0.99	0.371	251	80.52±0.63	0.135	132.03±1.09	0.780
	CC+CT	996	76.65±0.30		127.92±0.52		842	79.61±0.34		131.59±0.59	
	TT	18	70.08±2.19	0.003	120.04±3.87	0.044	24	84.13±2.06	0.030	138.16±3.59	0.071

Values are mean±SEM. *Conditional logistic analysis, adjusted for age, body mass index (BMI), present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking). SNP, single nucleotide polymorphism; DBP, diastolic blood pressure; SBP, systolic blood pressure.

and hypertension under the assumption of a dominant (with scores of 0 for patients homozygous for the major allele and 1 for carriers of the minor allele) or recessive (with scores of 0 for carriers of the major allele and 1 for patients homozygous

Table 5. Haplotype Frequency (Freq) of *ECE1* Gene in Hypertensives (HT) and Normotensives (NT)

Haplotype		All				Men				Women			
		Freq (%)	χ^2	*p* Asymptotic	*p* Permutation	Freq (%)	χ^2	*p* Asymptotic	*p* Permutation	Freq (%)	χ^2	*p* Asymptotic	*p* Permutation
H1 T/T/C/A/A	Overall	19.2	1.278	0.258	0.327	19.0	0.040	0.841	0.893	19.3	2.954	0.086	0.127
	NT	19.8				18.8				20.4			
	HT	18.4				19.2				17.3			
H2 C/C/C/A/A	Overall	16.2	1.305	0.253	0.284	17.5	0.193	0.661	0.669	15.1	2.991	0.084	0.091
	NT	15.5				17.9				14.0			
	HT	16.9				17.1				16.9			
H3 T/T/C/A/G	Overall	14.3	0.122	0.727	0.769	14.7	0.231	0.631	0.695	14.2	0.060	0.807	0.825
	NT	14.1				14.4				14.0			
	HT	14.5				15.2				14.4			
H4 C/T/C/A/A	Overall	11.8	0.181	0.670	0.716	11.9	0.033	0.857	0.867	11.8	0.250	0.617	0.699
	NT	12.0				12.1				12.1			
	HT	11.5				11.8				11.4			
H5 T/T/T/A/A	Overall	10.7	8.254	0.004	0.015	10.9	0.421	0.516	0.575	10.6	11.865	0.001	0.003
	NT	12.0				11.4				12.4			
	HT	9.0				10.4				7.5			
H6 T/T/C/C/G	Overall	8.3	0.317	0.574	0.618	7.8	0.327	0.568	0.624	9.0	0.001	0.974	0.978
	NT	8.1				8.1				9.0			
	HT	8.7				7.3				9.0			
H7 C/T/C/A/G	Overall	7.8	0.133	0.715	0.775	6.2	1.115	0.291	0.402	8.8	2.071	0.150	0.192
	NT	7.6				5.5				8.2			
	HT	7.9				6.7				10.0			

Haplotypes (rs212548/rs212528/rs212526/rs2038090/rs2038089) with frequencies of more than 5% are shown. One hundred thousand replicates were used for permutation test for all, men and women. Numbers of haplotypes in Overall, NT, and HT are 3,736, 2,150, 1,586 for All; 1,730, 914, 816 for men; and 2,030, 1,254, 776 for women, respectively.

for the minor allele) mode of inheritance. The *p* values were adjusted by Bonferroni correction. SAS statistical software (release 6.12; SAS Institute Inc., Cary, USA) was used for the statistical analyses. The data of linkage disequilibrium, haplotype blocks and coverage of HapMap SNPs were downloaded from the HapMap Consortium (http://www.hapmap.org). Haplotypes and permutation analyses were calculated using SNPAlyze version 4.0 software (DYNACOM Co., Mobara, Japan).

Results

Association between SNPs in the *ECE1* Gene and Hypertension

Five genetic polymorphisms in the *ECE1* gene were genotyped in 1,873 individuals. The genotype frequencies for each polymorphism were as follows: rs212548-T>C, 0.563/0.437; rs212528-T>C, 0.800/0.200; rs212526-C>T, 0848/0.152; rs2038090-A>C, 0.880/0.120; rs2038089-A>G, 0.655/0.345. None of the genotype frequencies were significantly different from those expected from the Hardy-Weinberg equilibrium ($p>0.05$). Multiple logistic regression analysis after adjusting for confounding factors of age, BMI, hyperlipidemia, diabetes mellitus, smoking, and drinking revealed that one polymorphism, rs212528, in intron 5 was significantly associated with hypertension in women (rs212528-T>C: TT *vs.* TC+CC; odds ratio=1.40; 95% confidence interval: 1.04–1.89; $p=0.026$) (Table 3). The SBPs in women with the TT, TC, and CC genotypes were 126.89±0.64 mmHg ($n=663$), 129.21±0.93 mmHg ($n=317$), and 133.33±3.02 mmHg ($n=30$) ($p=0.007$), after adjusting for the same confounding factors (Table 4). Thus, the difference in SBP was 6.44 mmHg between women with the CC genotype and those with the TT genotype. This association was still significant even after the Bonferroni correction.

Another polymorphism, rs212526, was associated with a significant difference in DBP: women having the CC+CT genotype had a DBP of 76.65±0.30 mmHg ($n=996$) and those with the TT genotype had a DBP of 70.08±2.19 mmHg ($n=18$) ($p=0.003$) after adjusting for the same confounding factors (Table 4). This polymorphism was also significantly associated with the SBP in women (CC+CT: 127.92±0.52 mmHg, $n=996$; TT: 120.04±3.87 mmHg, $n=18$; $p=0.044$). However, this polymorphism did not show a significant association with hypertension. In men, this polymorphism was

Table 6. List of 5 Polymorphisms and Their Allele Frequency in Exon 2 of *EDN1* Identified by Direct Sequencing of 942 Hypertensive Japanese

Allele 1 > allele 2	Amino acid change	region	Allele frequency		Flanking sequence	rs ID
			Allele 1	Allele 2		
1753G>A	G36R	exon 2	1.000	0.000	TGAGAACGGC[G/A]GGGAGAAACC	
1910G>T		intron 2	0.473	0.527	TGTAACCCTA[G/T]TCATTCATTA	rs2070699
1918T>A		intron 2	0.999	0.001	TAGTCATTCA[T/A]TAGCGCTGGC	
2008G>A		intron 2	0.999	0.001	GTGCCTCAGT[G/A]GGGACAGTTT	
2107G>A		intron 2	0.999	0.001	TACTCATGAT[G/A]GGACAAGCAG	

The A of the ATG of the initiator Met codon is denoted nucleotide +1, as recommended by the Nomenclature Working Group (*28*). The nucleotide number was according to the reference sequences GenBank Accession ID: NT_007592.

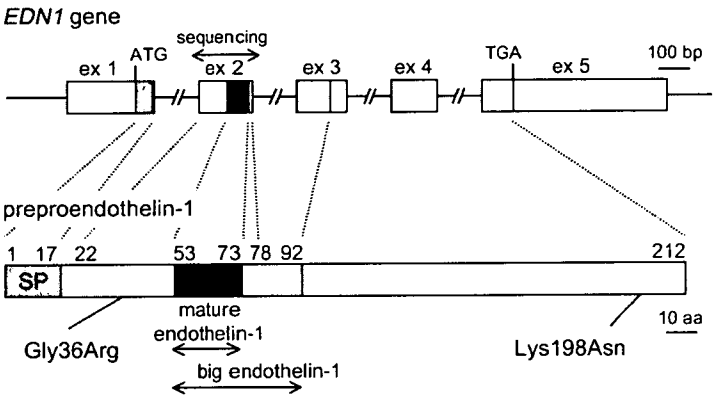

Fig. 1. *Genome and domain structure of human endothelin 1. Two missense mutations in endothelin-1, Gly36Arg (G36R) and Lys198Asn (K198N), are shown. The G36R mutation in preproendothelin-1 was identified in this study.*

significantly associated with DBP (CC+CT: 79.61±0.34 mmHg, n=842; TT: 84.13±2.06 mmHg, n=24; p=0.030).

The haplotypes composed of the 5 SNPs genotyped in this study are shown in Table 5. Seven inferred haplotypes with frequencies of more than 5% were examined to determine their association with hypertension in all patients and in two sub-populations (men and women). In women, the frequency of haplotype H5 in the hypertensive group was significantly lower than that in the normotensive group.

A Novel Missense Mutation in the preproEDN1 Polypeptide in Japanese Hypertensives

We sequenced the region of exon 2 of *EDN1* in 942 hypertensive patients with strong genetic background and secondary hypertension. The results are shown in Table 6. In this study, we were not able to detect any missense mutations within the mature EDN1 region. However, we identified one novel missense mutation, G36R, in *EDN1* in a heterozygous form in a male patient. The prevalence of this mutation was 0.05% in our Japanese hypertensive population. We tried to screen this missense mutation, G36R in *EDN1*, in our general population by the TaqMan-PCR method, but this genotyping failed due to technical problems.

Discussion

In this study, we used two different approaches to reveal the contribution of the EDN system to hypertension in two different populations, a general population and a hypertensive population, both from the Osaka region in Japan.

We genotyped 5 SNPs in *ECE1* and identified rs212528 as the hypertension/blood pressure susceptibility genetic variant. We used the currently available HapMap data from CHB-JPN to assess the coverage of haplotype blocks across the *ECE1* gene by 5 SNPs. The *ECE1* gene consisted of 6 haplotype blocks, in which rs212548 was present in block 2, two SNPs, rs212528 and rs212526, were present in block 3, and two SNPs, rs2038090 and rs2038089, were present in block 6, and the genotyped SNPs were estimated to cover approximately 90% of the haplotypes in block 2, 30% of those in block 3, and 90% of those in block 6, respectively. Two SNPs, rs212528 and rs212526, in block 3 had an r^2 of 0.031 and LOD score of 0.43, and rs2038090 and rs2038089 in block 6 had an r^2 of 0.163 and LOD score of 2.33.

In this study, the rs212528-T>C polymorphism in *ECE1* in

women was identified as the SNP conferring susceptibility for hypertension and blood pressure change. It is well known that the incidence of coronary artery disease shows a gender difference that may in part be related to the female sex hormones estrogen and progesterone. The literature provides evidence that estrogen inhibits EDN1 production (*26*). Furthermore, estrogen inhibits ECE-1 mRNA expression (*27*). These findings may explain the gender difference of *ECE1* polymorphisms for hypertension. The mean age of women in our population was 63.3 years. Despite the relatively advanced age of this population, we identified a contribution of the rs212528 polymorphism to hypertension and blood pressure change, while haplotypes containing the rs212528-C allele were not clearly associated with normotension or hypertension. The association might have been stronger if we had used a younger female population.

Another polymorphism, rs212526-C>T in intron 6, was associated with a blood pressure change in women and men. The mean DBP of the 996 women with the CC+CT genotype was 6.57 mmHg higher than that of the 18 women with the TT genotype (p=0.003), and the SBP change also showed the same trend—that is, women with the CC+CT genotype had higher blood pressure than women with the TT genotype (p=0.044) (Table 4). However, in men, the opposite trend was seen. The mean DBP of the 842 men with the CC+CT genotype was 4.52 mmHg lower than that of the 24 men with the TT genotype (p=0.030). Haplotype H5 containing the rs212528-T allele was significantly more prevalent in the normotensive group. This association also suggested that the T-allele of rs212528 was involved in blood pressure in women (Tables 3–5). Thus, the significance of rs212526 on blood pressure change should be evaluated using other population.

The association of SNP with hypertension and blood pressure change is at best marginally significant given the number of tests performed. All the p-values were more than 0.007. However, rs212528 is present in the *ECE1* gene, which encodes the endothelin-converting enzyme. In addition, this SNP showed a positive association with both hypertension and blood pressure change. Thus, we regarded this SNP as a hypertension candidate. SNP and blood pressure/hypertension described in the present study needs to be confirmed by another set of studies.

In the hypertensive population, we sequenced the coding region of the EDN1 polypeptide and its flanking region in 942 Japanese hypertensives and identified one novel missense mutation, G36R, that was not present in the EDN1 polypeptide but was present in the preproEDN-1 region (Fig. 1). At present, the effect of G36R mutation on the EDN1 function is not clear, because it was located far from the scissile site, the R52–C53 bond, by the furin-like enzyme. From the evolutionary point of view, G36 was conserved in humans, chimpanzees, cows, and dogs, but mice and rats have Val and chickens have Ala. The arginine residue at position 36 was not found in preproEDN1 in any species. To reveal the functional effect of this missense mutation on the processing of preproEDN1, an expression study of the mutant preproEDN1 is needed.

We have hypothesized that rare nonsynomymous mutations in candidate genes could collectively contribute to complex traits. In this model, the extensive sequence-based approaches focusing on identification of these mutations is necessary. So far, we have sequenced several hypertension candidate genes to evaluate whether rare variants could contribute to the etiology of hypertension. At present, however, whether rare variants contribute to hypertension is not clear due to the lack of *in vitro* or *in vivo* expression studies of the mutant protein (*14*, *15*, *17*). The exception was the nonsense mutation identified in the *RGS2* gene, which has been clearly shown to produce the defective protein (*16*). In this study, we identified one missense mutation, G36R, in preproEDN1. The further collection of such missense mutations in hypertension candidate genes could lead to an enhanced understanding of the etiology of essential hypertension.

In summary, we revealed that the rs212528 polymorphism in *ECE1* was associated with hypertension and blood pressure change. In earlier reports, the Lys198Asn polymorphism in *EDN1* showed a positive association with blood pressure elevation in overweight people (*3–5*). Thus, endothelin family gene polymorphisms might play an important role in the etiology of essential hypertension.

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References

1. Yanagisawa M, Kurihara H, Kimura S, *et al*: A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 1988; **332**: 411–415.
2. Miyauchi T, Masaki T: Pathophysiology of endothelin in the cardiovascular system. *Annu Rev Physiol* 1999; **61**: 391–415.
3. Tiret L, Poirier O, Hallet V, *et al*: The Lys198Asn polymorphism in the endothelin-1 gene is associated with blood pressure in overweight people. *Hypertension* 1999; **33**: 1169–1174.
4. Asai T, Ohkubo T, Katsuya T, *et al*: Endothelin-1 gene variant associates with blood pressure in obese Japanese subjects: the Ohasama Study. *Hypertension* 2001; **38**: 1321–1324.
5. Jin JJ, Nakura J, Wu Z, *et al*: Association of endothelin-1 gene variant with hypertension. *Hypertension* 2003; **41**: 163–167.
6. Tanaka C, Kamide K, Takiuchi S, *et al*: Evaluation of the Lys198Asn and −134delA genetic polymorphisms of the endothelin-1 gene. *Hypertens Res* 2004; **27**: 367–371.

7. Funalot B, Courbon D, Brousseau T, *et al*: Genes encoding endothelin-converting enzyme-1 and endothelin-1 interact to influence blood pressure in women: the EVA study. *J Hypertens* 2004; **22**: 739–743.
8. Funke-Kaiser H, Reichenberger F, Kopke K, *et al*: Differential binding of transcription factor E2F-2 to the endothelin-converting enzyme-1b promoter affects blood pressure regulation. *Hum Mol Genet* 2003; **12**: 423–433.
9. Risch N, Merikangas K: The future of genetic studies of complex human diseases. *Science* 1996; **273**: 1516–1517.
10. Collins FS, Guyer MS, Charkravarti A: Variations on a theme: cataloging human DNA sequence variation. *Science* 1997; **278**: 1580–1581.
11. Lander ES: The new genomics: global views of biology. *Science* 1996; **274**: 536–539.
12. Cohen JC, Kiss RS, Pertsemlidis A, *et al*: Multiple rare alleles contribute to low plasma levels of HDL cholesterol. *Science* 2004; **305**: 869–872.
13. Frikke-Schmidt R, Nordestgaard BG, Jensen GB, *et al*: Genetic variation in ABC transporter A1 contributes to HDL cholesterol in the general population. *J Clin Invest* 2004; **114**: 1343–1353.
14. Kamide K, Tanaka C, Takiuchi S, *et al*: Six missense mutations of the epithelial sodium channel β and γ subunits in Japanese hypertensives. *Hypertens Res* 2004; **27**: 333–338.
15. Kamide K, Takiuchi S, Tanaka C, *et al*: Three novel missense mutations of *WNK4*, a kinase mutated in inherited hypertension, in Japanese hypertensives: implication of clinical phenotypes. *Am J Hypertens* 2004; **17**: 446–449.
16. Yang J, Kamide K, Kokubo Y, *et al*: Genetic variations of regulator of G-protein signaling 2 in hypertensive patients and in the general population. *J Hypertens* 2005; **23**: 1497–1505.
17. Kamide K, Yang J, Kokubo Y, *et al*: A novel missense mutation, F826Y, in the mineralocorticoid receptor gene in Japanese hypertensives: implication of clinical phenotypes. *Hypertens Res* 2005; **28**: 703–709.
18. Vanhoutte PM, Feletou M, Taddei S: Endothelium-dependent contractions in hypertension. *Br J Pharmacol* 2005; **144**: 449–458.
19. Mannami T, Baba S, Ogata J: Strong and significant relationships between aggregation of major coronary risk factors and the acceleration of carotid atherosclerosis in the general population of a Japanese city: the Suita Study. *Arch Intern Med* 2000; **160**: 2297–2303.
20. Kokubo Y, Inamoto N, Tomoike H, *et al*: Association of genetic polymorphisms of sodium-calcium exchanger 1 gene, *NCX1*, with hypertension in a Japanese general population. *Hypertens Res* 2004; **27**: 697–702.
21. Haga H, Yamada R, Ohnishi Y, *et al*: Gene-based SNP discovery as part of the Japanese Millennium Genome Project: identification of 190,562 genetic variations in the human genome. Single-nucleotide polymorphism. *J Hum Genet* 2002; **47**: 605–610.
22. Hirakawa M, Tanaka T, Hashimoto Y, *et al*: JSNP: a database of common gene variations in the Japanese population. *Nucleic Acids Res* 2002; **30**: 158–162.
23. Okuda T, Fujioka Y, Kamide K, *et al*: Verification of 525 coding SNPs in 179 hypertension candidate genes in the Japanese population: identification of 159 SNPs in 93 genes. *J Hum Genet* 2002; **47**: 387–394.
24. Matayoshi T, Kamide K, Takiuchi S, *et al*: The thiazide-sensitive Na^+-Cl^- cotransporter gene, *C1784T*, and adrenergic receptor-β3 gene, *T727C*, may be gene polymorphisms susceptible to the antihypertensive effect of thiazide diuretics. *Hypertens Res* 2004; **27**: 821–833.
25. Kokubo Y, Kamide K, Inamoto N, *et al*: Identification of 108 SNPs in *TSC*, *WNK1*, and *WNK4* and their association with hypertension in a Japanese general population. *J Hum Genet* 2004; **49**: 507–515.
26. Webb CM, Ghatei MA, McNeill JG, *et al*: 17β-Estradiol decreases endothelin-1 levels in the coronary circulation of postmenopausal women with coronary artery disease. *Circulation* 2000; **102**: 1617–1622.
27. Rodrigo MC, Martin DS and Eyster KM: Vascular ECE-1 mRNA expression decreases in response to estrogens. *Life Sci* 2003; **73**: 2973–2983.
28. Antonarakis SE, Nomenclature Working Group: Recommendations for a nomenclature system for human gene mutations. *Hum Mut* 1998; **11**: 1–3.

nature genetics

A nonsynonymous SNP in *PRKCH* (protein kinase C η) increases the risk of cerebral infarction

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Cerebral infarction is the most common type of stroke and often causes long-term disability. To investigate the genetic contribution to cerebral infarction, we conducted a case-control study using 52,608 gene-based tag SNPs selected from the JSNP database. Here we report that a nonsynonymous SNP in a member of protein kinase C (PKC) family, *PRKCH*, was significantly associated with lacunar infarction in two independent Japanese samples ($P = 5.1 \times 10^{-7}$, crude odds ratio of 1.40). This SNP is likely to affect PKC activity. Furthermore, a 14-year follow-up cohort study in Hisayama (Fukuoka, Japan) supported involvement of this SNP in the development of cerebral infarction ($P = 0.03$, age- and sex-adjusted hazard ratio of 2.83). We also found that PKCη was expressed mainly in vascular endothelial cells and foamy macrophages in human atherosclerotic lesions, and its expression increased as the lesion type progressed. Our results support a role for *PRKCH* in the pathogenesis of cerebral infarction.

Stroke is a major cause of long-term disabilities, leading to very serious public health issues. Once a stroke has occurred, most affected individuals suffer from disability, cognitive dysfunction and other complications and have a higher risk of death[1]. In Japan, stroke mortality rate has decreased significantly in the last three decades, but the incidence of stroke has remained high in recent years, especially in the elderly[2]. As the proportion of elderly individuals is increasing rapidly worldwide, primary prevention of stroke is becoming an important medical and social issue requiring urgent attention.

Cerebral infarction is the most common form of stroke and is classified into the following subtypes based on clinical and neuroimaging data: lacunar infarction due to presumed arteriosclerosis of small penetrating arteries, atherothrombotic infarction due to atherosclerosis involving the external and major intracranial arteries, cardioembolic infarction due to a cardiac source of the embolus and undetermined subtype[3]. Twin- and family-based studies indicate a

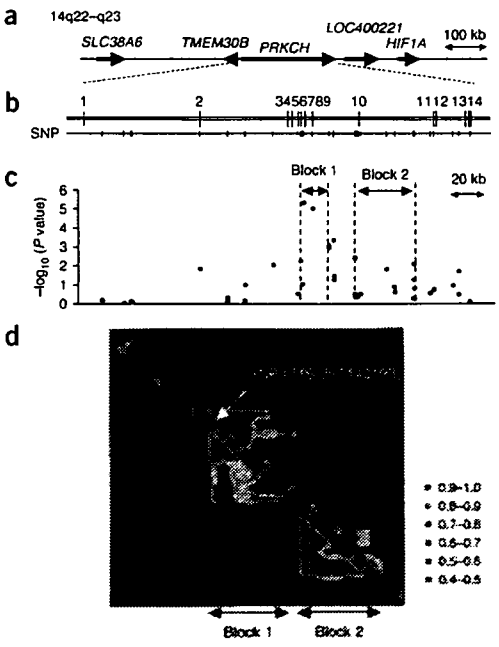

Figure 1 Genomic structure, case-control association results and linkage disequilibrium map of the *PRKCH* locus. (a) Genomic structure around *PRKCH*. (b) Exon-intron structure of *PRKCH*. Genotyped SNPs in *PRKCH* are indicated below the gene (vertical line). (c) Case-control association study results for lacunar infarction. The $-\log_{10}$-transformed *P* values for an allele frequency model are plotted on the *y* axis. (d) Pairwise linkage disequilibrium map between SNPs, as measured by D′ (lower left) and Δ (upper right).

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