surrogate markers for atherosclerosis.

The necessity for thyroid hormone replacements therapy for SCH is not supported by the present study results. The signs and symptoms of hypothyroidism are bradycardia, mild hypertension and hyperlipidemia, which might accelerate atherosclerosis in subjects with hypothyroidism.[16] We examined the association between SCH and these symptoms or signs and did not observe an association between SCH and blood pressure or serum lipid levels. The association between lipid profile and SCH has been previously reported in Norway and Australia[6,7] and the different results might relate to differences in genetic background, life style and BMI. In the Tromsø study,[7] the subjects were obese and those with SCH were even more obese than normal subjects. The difference in BMI between those and our investigations might induce different patterns of lipid metabolism. We did not observe any significant association between SCH and the symptoms or signs associated with hypothyroidism. Moreover, we did not find an association between SCH and IMT, as a surrogate marker for atherosclerosis, and did not find an association between SCN and past history of atherosclerotic disease. These results do not support the need for treatment of SCH in Japanese subjects. However, our investigation was a cross-sectional study, so the duration of SCH was not considered in the analysis. Our results do not completely deny that subjects with long-term SCH have increased risk of atherosclerosis.

In conclusion, we examined the association between subclinical thyroid dysfunction and various factors in a general population. We only found an association between glucose levels and subclinical thyroid dysfunction. The differences in serum glucose levels among the thyroid states (SCH, subclinical hyperthyroidism, and normal thyroid) were too small to lead to a recommendation for treatment of subclinical thyroid dysfunction and thus do not indicate a need for treatment of subclinical thyroid dysfunction in Japanese subjects.

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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	ttgcccctctgc[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]tttccagggggc		rs2239393
971G>A			Intron 3	0.995	0.005	agggtgggggcc[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

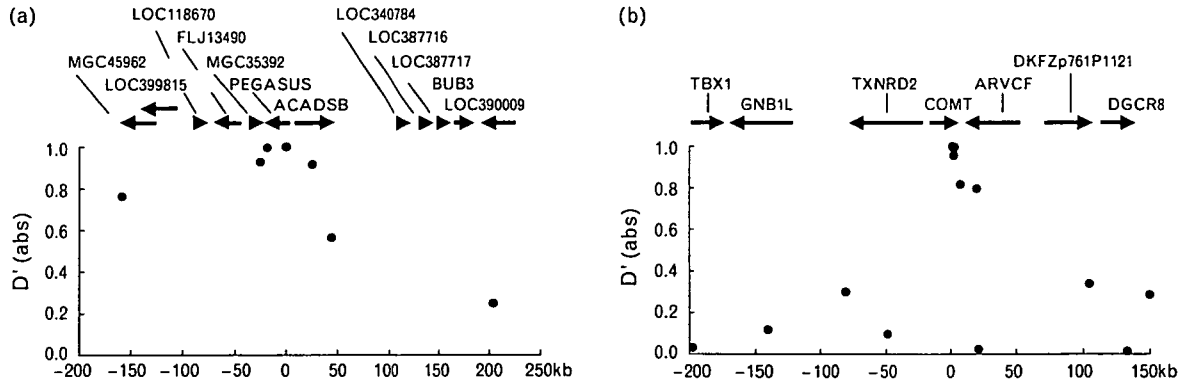
We previously identified differentially expressed genes in the kidneys of salt-loaded Dahl-S and Lewis rats [4].

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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REGULAR ARTICLE

Genotypes of vitamin K epoxide reductase, γ-glutamyl carboxylase, and cytochrome P450 2C9 as determinants of daily warfarin dose in Japanese patients

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Abstract The dose required for the anticoagulant effect of warfarin exhibits large inter-individual variations. This study sought to determine the contribution of four genes, vitamin K epoxide reductase (*VKORC1*), γ-glutamyl carboxylase (*GGCX*), calumenin (*CALU*), and cytochrome P450 2C9 (*CYP2C9*) to the warfarin maintenance dose required in Japanese patients following ischemic stroke. We recruited 93 patients on stable anticoagulation with a target International Normalized Ratio (INR) of 1.6–2.6. We genotyped eleven representative single nucleotide polymorphisms (SNPs) in the three genes involved in vitamin K cycle and the 42613A>C SNP in *CYP2C9*, known as *CYP2C9*3*, and then examined an association of these genotypes with warfarin maintenance doses (mean±SD=2.96±1.06 mg/day). We found an association of effective warfarin dose with the −1639G>A (p=0.004) and 3730G>A genotypes (p=0.006) in *VKORC1*, the 8016G>A genotype in *GGCX* (p=0.022), and the 42613A>C genotype in *CYP2C9* (p=0.015). The model using the multiple regression analysis including age, sex, weight, and three genetic polymorphisms accounted for 33.3% of total variations in warfarin dose. The contribution to inter-individual variation in warfarin dose was 5.9% for *VKORC1* −1639G>A, 5.2% for *CYP2C9*

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42613A>C, and 4.6% for *GGCX* 8016G>A. In addition to polymorphisms in *VKORC1* and *CYP2C9*, we identified *GGCX* 8016G>A, resulting in the missense mutation R325Q, as a genetic determinant of warfarin maintenance dose in Japanese patients.

Warfarin is the most widely prescribed anticoagulant for long-term prevention of thromboembolic events. The dose of warfarin required to achieve target levels of anticoagulation varies dependent on dietary intake and individual variations in pharmacokinetics. Management of warfarin therapy is difficult because of significant inter-individual and intra-individual variability and the narrow therapeutic range. The effectiveness and safety of warfarin must be monitored by serial determinations of prothrombin time using the standardized international normalized ratio (INR).

Warfarin exerts an anticoagulant effect by interfering with the regeneration of reduced vitamin K from the epoxide form, which is required for the enzymatic activity of vitamin K epoxide reductase subunit 1 (VKORC1) [1,2]. γ-Carboxylation of a wide variety of proteins, including numbers of factors in the clotting cascade, is catalyzed by γ-glutamyl carboxylase (GGCX), a vitamin K-dependent enzyme. This reaction incorporates a carbon dioxide molecule into specific glutamic acid residues with the help of the reduced form of vitamin K and oxygen, generating γ-carboxylglutamic acid and vitamin K 2,3-epoxide. When reduced vitamin K cannot be regenerated, the biosynthesis of vitamin K-dependent coagulation/anticoagulation factors, including prothrombin, factors VII, IX, and X, and proteins C and S, is suppressed. The endoplasmic reticulum resident protein calumenin (CALU) associates with γ-glutamyl carboxylase, inhibiting its activity [3]. Recent studies on the genetic aspects of the inter-individual variability of warfarin have demonstrated that single nucleotide polymorphisms (SNPs) in the *VKORC1* gene influence warfarin responses [4–15]. Haplotype analysis demonstrated that individuals who can be controlled by the low dose of warfarin showed the low hepatic expression of *VKORC1* mRNA [6].

The inter-individual variability of warfarin can also be explained by the genetic variability of the warfarin metabolizing enzyme, *CYP2C9*. The missense mutations R144C and I359L in the *CYP2C9* gene known as *CYP2C9*2* and *CYP2C9*3* are known to associate with warfarin dose [16]. These two genetic variations exhibited ethnic specificity. Asian population does not have the *CYP2C9*2* allele but carries the *CYP2C9*3* allele [17].

In this study, we investigated the influence of SNPs in four genes controlling γ-carboxylation (*VKORC1*, *GGCX*, *CALU*, and *CYP2C9*) on the inter-individual variability of warfarin dose requirements in Japanese patients. We identified SNPs in *VKORC1*, *GGCX*, and *CYP2C9* associated with the inter-individual differences in warfarin dosage.

Materials and methods

Subjects

The study population consisted of 93 unrelated Japanese patients admitted to the Cerebrovascular Division of the National Cardiovascular Center between November 2003 and March 2004. The patients had all experienced an ischemic stroke within the 7 days prior to admission. Stroke subtype consisted of cardioembolic infarction ($n=48$) and the embolic infarction of unknown origin with non-valvular atrial fibrillation ($n=45$). Anticoagulation of all patients was stably controlled with a target INR of 1.6–2.6 for the prevention of stroke recurrence [18,19]. Inclusion criteria were a confirmed date of initial exposure to warfarin, and current anticoagulation therapy. Data collection consisted of inpatient and outpatient medical records. The anticoagulant database was used to obtain information on daily warfarin doses. This study was approved by the Ethical Review Committee of the National Cardiovascular Center. All patients who participated in the study provided written informed consent for genetic analysis.

DNA analyses

We previously performed DNA sequence analyses of 3 genes (*VKORC1*, *GGCX*, and *CALU*) involved in vitamin K cycling in 96 Japanese stroke patients; that study identified genetic polymorphisms and pair-wise linkage disequilibrium (LD) [20]. Using the minor allele frequency (over 4%), LD (r^2 more than 0.5), and possible functional change (missense mutation) as guidance, we selected nine representative SNPs for genotyping: 523G>A, 1338A>G (H68R), and 3730G>A in *VKORC1*, 412G>A, 8016G>A (R325Q), and 8445C>T in *GGCX*, and 11G>A (R4Q), 344G>A, and 20943T>A in *CALU*. In *CYP2C9*, only the 42613A>C (I359L) SNP,

known as the *CYP2C9*3* genotype, was analyzed. In addition, recent studies have demonstrated the significant association of the *VKORC1* polymorphisms −1639G>A and 1173C>T with warf polymorphisms. We adopted the numbering standards of the Nomenclature Working Group, wherein the A of the initiator Met codon (ATG) is denoted nucleotide +1 [21].

The genotypes of the 12 SNPs in our subjects were identified by the TaqMan-PCR system. TaqMan genotyping methodology has been described previously [22]. The PCR primers and probes used for the TaqMan system are available on request.

Statistical analysis

The significance level for all statistical tests was set at $P<0.05$. Pair-wise LD between two polymorphisms was evaluated by r^2 using SNPAlyze v4.0 software (DYNACOM, Kanagawa, Japan). Statistical analyses were performed using JMP v 5.1 software and the SAS release 8.2 (SAS Institute Inc., Cary, NC). Associations between genotypes and warfarin daily doses were examined by one-way analysis of variance or univariate regression analysis. In addition, the relative contributions of age, sex, weight, and selected genetic variations to inter-individual variations in warfarin dose were estimated by using the multiple regression analysis. An index P_i, for estimating the relative contribution of a specific independent variable, x_i, was employed and given by

$$P_i = R^2 - R^2_{-i},$$

where R was the multiple correlation coefficient from the model with all of the selected independent variables (x_1, x_2,..., x_p) and R^2_{-i} was that of the model excluding x_i from the independent variables.

Results

We analyzed the frequency of 11 SNPs in three genes involved in the vitamin K cycle and one polymorphism in *CYP2C9* 42613A>C (*CYP2C9*3*) in 93 stroke patients under stable anticoagulation with warfarin. Characteristics of the patients are summarized in Table 1. The mean±SD daily warfarin dose was 2.96±1.06 mg/day (1.00–5.50 mg/day).

Table 1 Characteristics of patients

Number	93
Number of men (%)	66 (71.0)
Age (years)	68.1±10.6
Weight (kg)	59.8±9.7
Warfarin dose (mg/day)	2.96±1.06
Warfarin dose range (mg/day)	1.00–5.50

Age, weight, and warfarin dose are shown as mean±SD.

Table 2 Differences in daily warfarin dose for each genotype of the *VKORC1*, *GGCX*, and *CYP2C9* genes

Gene	SNP	Genotype	*n*	Mean±SD (mg/day)	*P*
VKORC1	−1639 G>A*	AA	79	2.83±1.00	
		GA	14	3.70±1.11	0.004
		GG	0	–	
VKORC1	1173 C>T*	TT	79	2.83±1.00	
		CT	14	3.70±1.11	0.004
		CC	0	–	
VKORC1	3730 G>A*	GG	79	2.84±1.00	
		GA	14	3.68±1.12	0.006
		AA	0	–	
GGCX	8016 G>A (R325Q)	GG	48	3.25±1.19	
		GA	39	2.63±0.77	0.022
		AA	6	2.79±1.07	
CYP2C9	42613 A>C (CYP2C9*3) (I359L)	AA	83	3.06±1.05	
		AC	9	2.17±0.84	0.015
		CC	0	–	

P values were calculated by one-way ANOVA. *These SNPs were in linkage disequilibrium. Rieder et al. reported that the hepatic expression levels of *VKORC1* mRNA were significantly decreased in the carriers with the *VKORC1* −1639A allele [6]. As for the *GGCX* R325Q mutation, there were no available data on its function. CYP2C9 mutant carrying the missense mutation, I359L (*CYP2C9*3*), showed a markedly high *Km* for the 7-hydroxylation of *S*-warfarin [28].

We examined the association of the genotype data with maintenance warfarin doses by one-way analysis of variance (ANOVA). Of the 12 SNPs examined, five SNPs, −1639G>A, 1173C>T, and 3730G>A in *VKORC1*, 8016G>A (R375Q) in *GGCX*, and *CYP2C9*3* exhibited a significant association with daily warfarin dose (Table 2). The *VKORC1* 1338G>A allele could not be evaluated due to the low minor allele frequency. None of the other SNPs demonstrated a significant association with warfarin dosage.

The mean warfarin dose was higher ($p=0.004$) in patients with the *VKORC1* −1639GA or 1173CT genotypes (3.70 mg/day) than in those with the −1639AA or 1173TT genotypes (2.83 mg/day). The mean warfarin dose was higher ($p=0.006$) in patients with the *VKORC1* 3730GA genotype (3.68 mg/day) than in those with the 3730GG genotype (2.84 mg/day). For *CYP2C9*, the mean warfarin dose was higher ($p=0.015$) in patients with the *CYP2C9*1*1* (*CYP2C9* 42613AA) genotype (3.06 mg/day) than in those with the **1*3* (42613AC) genotype (2.17 mg/day).

A significant association was observed between warfarin dosage and the 8016G>A SNP of *GGCX*. The mean warfarin dose was higher ($p=0.022$) among patients with the *GGCX* 8016GG genotype (3.25 mg/day) than in those with the GA (2.84 mg/day) or AA (2.79 mg/day) genotypes. The *GGCX* 8016G>A SNP,

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.
*: $P<0.01$, **: $0.01 \le P<0.05$.

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.

Acknowledgments

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REGULAR ARTICLE

Genotypes of vitamin K epoxide reductase, γ-glutamyl carboxylase, and cytochrome P450 2C9 as determinants of daily warfarin dose in Japanese patients

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Abstract The dose required for the anticoagulant effect of warfarin exhibits large inter-individual variations. This study sought to determine the contribution of four genes, vitamin K epoxide reductase (*VKORC1*), γ-glutamyl carboxylase (*GGCX*), calumenin (*CALU*), and cytochrome P450 2C9 (*CYP2C9*) to the warfarin maintenance dose required in Japanese patients following ischemic stroke. We recruited 93 patients on stable anticoagulation with a target International Normalized Ratio (INR) of 1.6–2.6. We genotyped eleven representative single nucleotide polymorphisms (SNPs) in the three genes involved in vitamin K cycle and the 42613A>C SNP in *CYP2C9*, known as *CYP2C9*3*, and then examined an association of these genotypes with warfarin maintenance doses (mean±SD=2.96±1.06 mg/day). We found an association of effective warfarin dose with the −1639G>A (p=0.004) and 3730G>A genotypes (p=0.006) in *VKORC1*, the 8016G>A genotype in *GGCX* (p=0.022), and the 42613A>C genotype in *CYP2C9* (p=0.015). The model using the multiple regression analysis including age, sex, weight, and three genetic polymorphisms accounted for 33.3% of total variations in warfarin dose. The contribution to inter-individual variation in warfarin dose was 5.9% for *VKORC1* −1639G>A, 5.2% for *CYP2C9*

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42613A>C, and 4.6% for *GGCX* 8016G>A. In addition to polymorphisms in *VKORC1* and *CYP2C9*, we identified *GGCX* 8016G>A, resulting in the missense mutation R325Q, as a genetic determinant of warfarin maintenance dose in Japanese patients.

Warfarin is the most widely prescribed anticoagulant for long-term prevention of thromboembolic events. The dose of warfarin required to achieve target levels of anticoagulation varies dependent on dietary intake and individual variations in pharmacokinetics. Management of warfarin therapy is difficult because of significant inter-individual and intra-individual variability and the narrow therapeutic range. The effectiveness and safety of warfarin must be monitored by serial determinations of prothrombin time using the standardized international normalized ratio (INR).

Warfarin exerts an anticoagulant effect by interfering with the regeneration of reduced vitamin K from the epoxide form, which is required for the enzymatic activity of vitamin K epoxide reductase subunit 1 (VKORC1) [1,2]. γ-Carboxylation of a wide variety of proteins, including numbers of factors in the clotting cascade, is catalyzed by γ-glutamyl carboxylase (GGCX), a vitamin K-dependent enzyme. This reaction incorporates a carbon dioxide molecule into specific glutamic acid residues with the help of the reduced form of vitamin K and oxygen, generating γ-carboxylglutamic acid and vitamin K 2,3-epoxide. When reduced vitamin K cannot be regenerated, the biosynthesis of vitamin K-dependent coagulation/anticoagulation factors, including prothrombin, factors VII, IX, and X, and proteins C and S, is suppressed. The endoplasmic reticulum resident protein calumenin (CALU) associates with γ-glutamyl carboxylase, inhibiting its activity [3]. Recent studies on the genetic aspects of the inter-individual variability of warfarin have demonstrated that single nucleotide polymorphisms (SNPs) in the *VKORC1* gene influence warfarin responses [4–15]. Haplotype analysis demonstrated that individuals who can be controlled by the low dose of warfarin showed the low hepatic expression of *VKORC1* mRNA [6].

The inter-individual variability of warfarin can also be explained by the genetic variability of the warfarin metabolizing enzyme, *CYP2C9*. The missense mutations R144C and I359L in the *CYP2C9* gene known as *CYP2C9*2* and *CYP2C9*3* are known to associate with warfarin dose [16]. These two genetic variations exhibited ethnic specificity. Asian population does not have the *CYP2C9*2* allele but carries the *CYP2C9*3* allele [17].

In this study, we investigated the influence of SNPs in four genes controlling γ-carboxylation (*VKORC1*, *GGCX*, *CALU*, and *CYP2C9*) on the inter-individual variability of warfarin dose requirements in Japanese patients. We identified SNPs in *VKORC1*, *GGCX*, and *CYP2C9* associated with the inter-individual differences in warfarin dosage.

Materials and methods

Subjects

The study population consisted of 93 unrelated Japanese patients admitted to the Cerebrovascular Division of the National Cardiovascular Center between November 2003 and March 2004. The patients had all experienced an ischemic stroke within the 7 days prior to admission. Stroke subtype consisted of cardioembolic infarction ($n=48$) and the embolic infarction of unknown origin with non-valvular atrial fibrillation ($n=45$). Anticoagulation of all patients was stably controlled with a target INR of 1.6–2.6 for the prevention of stroke recurrence [18,19]. Inclusion criteria were a confirmed date of initial exposure to warfarin, and current anticoagulation therapy. Data collection consisted of inpatient and outpatient medical records. The anticoagulant database was used to obtain information on daily warfarin doses. This study was approved by the Ethical Review Committee of the National Cardiovascular Center. All patients who participated in the study provided written informed consent for genetic analysis.

DNA analyses

We previously performed DNA sequence analyses of 3 genes (*VKORC1*, *GGCX*, and *CALU*) involved in vitamin K cycling in 96 Japanese stroke patients; that study identified genetic polymorphisms and pair-wise linkage disequilibrium (LD) [20]. Using the minor allele frequency (over 4%), LD (r^2 more than 0.5), and possible functional change (missense mutation) as guidance, we selected nine representative SNPs for genotyping: 523G>A, 1338A>G (H68R), and 3730G>A in *VKORC1*, 412G>A, 8016G>A (R325Q), and 8445C>T in *GGCX*, and 11G>A (R4Q), 344G>A, and 20943T>A in *CALU*. In *CYP2C9*, only the 42613A>C (I359L) SNP,

known as the *CYP2C9*3* genotype, was analyzed. In addition, recent studies have demonstrated the significant association of the *VKORC1* polymorphisms −1639G>A and 1173C>T with warf polymorphisms. We adopted the numbering standards of the Nomenclature Working Group, wherein the A of the initiator Met codon (ATG) is denoted nucleotide +1 [21].

The genotypes of the 12 SNPs in our subjects were identified by the TaqMan-PCR system. TaqMan genotyping methodology has been described previously [22]. The PCR primers and probes used for the TaqMan system are available on request.

Statistical analysis

The significance level for all statistical tests was set at $P<0.05$. Pair-wise LD between two polymorphisms was evaluated by r^2 using SNPAlyze v4.0 software (DYNACOM, Kanagawa, Japan). Statistical analyses were performed using JMP v 5.1 software and the SAS release 8.2 (SAS Institute Inc., Cary, NC). Associations between genotypes and warfarin daily doses were examined by one-way analysis of variance or univariate regression analysis. In addition, the relative contributions of age, sex, weight, and selected genetic variations to inter-individual variations in warfarin dose were estimated by using the multiple regression analysis. An index P_i, for estimating the relative contribution of a specific independent variable, x_i, was employed and given by

$$P_i = R^2 - R^2_{-i},$$

where R was the multiple correlation coefficient from the model with all of the selected independent variables (x_1, x_2,..., x_p) and R^2_{-i} was that of the model excluding x_i from the independent variables.

Results

We analyzed the frequency of 11 SNPs in three genes involved in the vitamin K cycle and one polymorphism in *CYP2C9* 42613A>C (*CYP2C9*3*) in 93 stroke patients under stable anticoagulation with warfarin. Characteristics of the patients are summarized in Table 1. The mean±SD daily warfarin dose was 2.96±1.06 mg/day (1.00–5.50 mg/day).

Table 1 Characteristics of patients

Number	93
Number of men (%)	66 (71.0)
Age (years)	68.1±10.6
Weight (kg)	59.8±9.7
Warfarin dose (mg/day)	2.96±1.06
Warfarin dose range (mg/day)	1.00–5.50

Age, weight, and warfarin dose are shown as mean±SD.

Table 2 Differences in daily warfarin dose for each genotype of the *VKORC1*, *GGCX*, and *CYP2C9* genes

Gene	SNP	Genotype	*n*	Mean±SD (mg/day)	*P*
VKORC1	−1639 G>A*	AA	79	2.83±1.00	
		GA	14	3.70±1.11	0.004
		GG	0	–	
VKORC1	1173 C>T*	TT	79	2.83±1.00	
		CT	14	3.70±1.11	0.004
		CC	0	–	
VKORC1	3730 G>A*	GG	79	2.84±1.00	
		GA	14	3.68±1.12	0.006
		AA	0	–	
GGCX	8016 G>A (R325Q)	GG	48	3.25±1.19	
		GA	39	2.63±0.77	0.022
		AA	6	2.79±1.07	
CYP2C9	42613 A>C (CYP2C9*3) (I359L)	AA	83	3.06±1.05	
		AC	9	2.17±0.84	0.015
		CC	0	–	

P values were calculated by one-way ANOVA. *These SNPs were in linkage disequilibrium. Rieder et al. reported that the hepatic expression levels of *VKORC1* mRNA were significantly decreased in the carriers with the *VKORC1* −1639A allele [6]. As for the *GGCX* R325Q mutation, there were no available data on its function. CYP2C9 mutant carrying the missense mutation, I359L (*CYP2C9*3*), showed a markedly high *Km* for the 7-hydroxylation of *S*-warfarin [28].

We examined the association of the genotype data with maintenance warfarin doses by one-way analysis of variance (ANOVA). Of the 12 SNPs examined, five SNPs, −1639G>A, 1173C>T, and 3730G>A in *VKORC1*, 8016G>A (R375Q) in *GGCX*, and *CYP2C9*3* exhibited a significant association with daily warfarin dose (Table 2). The *VKORC1* 1338G>A allele could not be evaluated due to the low minor allele frequency. None of the other SNPs demonstrated a significant association with warfarin dosage.

The mean warfarin dose was higher ($p=0.004$) in patients with the *VKORC1* −1639GA or 1173CT genotypes (3.70 mg/day) than in those with the −1639AA or 1173TT genotypes (2.83 mg/day). The mean warfarin dose was higher ($p=0.006$) in patients with the *VKORC1* 3730GA genotype (3.68 mg/day) than in those with the 3730GG genotype (2.84 mg/day). For *CYP2C9*, the mean warfarin dose was higher ($p=0.015$) in patients with the *CYP2C9*1*1* (*CYP2C9* 42613AA) genotype (3.06 mg/day) than in those with the **1*3* (42613AC) genotype (2.17 mg/day).

A significant association was observed between warfarin dosage and the 8016G>A SNP of *GGCX*. The mean warfarin dose was higher ($p=0.022$) among patients with the *GGCX* 8016GG genotype (3.25 mg/day) than in those with the GA (2.84 mg/day) or AA (2.79 mg/day) genotypes. The *GGCX* 8016G>A SNP,

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 β†	$P_i \times 100$
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≤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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