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REVIEW ARTICLE

Warfarin dose and the pharmacogenomics of *CYP2C9* and *VKORC1* — Rationale and perspectives ☆

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Abstract Warfarin is the most widely prescribed oral anticoagulant, but there is greater than 10-fold interindividual variability in the dose required to attain a therapeutic response. Information from pharmacogenomics, the study of the interaction of an individual's genotype and drug response, can help optimize drug efficacy while minimizing adverse drug reactions. Pharmacogenetic analysis of two genes, the warfarin metabolic enzyme *CYP2C9* and warfarin target enzyme, vitamin K epoxide reductase complex 1 *VKORC1*, confirmed their influence on warfarin maintenance dose. Possession of *CYP2C9**2* or *CYP2C9**3* variant alleles, which result in decreased enzyme activity, is associated with a significant decrease in the mean warfarin dose. Several single nucleotide polymorphisms (SNPs) in *VKORC1* are associated with warfarin dose across the normal dose range. Haplotypes based on these SNPs explain a large fraction of the interindividual variation in warfarin dose, and *VKORC1* has an approximately three-fold greater effect than *CYP2C9*. Algorithms incorporating genetic (*CYP2C9* and *VKORC1*), demographic, and clinical factors to estimate the warfarin dosage, could potentially minimize the risk of over dose during warfarin induction.
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Introduction

Genetic polymorphisms can affect an individual's response to pharmacologic agents, and the study of these interactions is pharmacogenomics. Pharmacogenomic information may allow predictions about effective drug dose and therapeutic and toxic effects to be made prior to drug administration [1]. Most current pharmacogenomic information is based on association studies examining polymorphisms in genes encoding drug-metabolizing enzymes, transporters, receptors, and proteins involved in drug-signaling pathways. In current clinical practice, pharmacogenomic testing is performed for only a few drugs, and an important potential candidate is warfarin.

Warfarin, a derivative of coumarin, is a commonly prescribed oral anticoagulant for the treatment and prevention of thrombotic diseases, including myocardial infarction, ischemic stroke, venous thrombosis, and following heart valve replacement and atrial fibrillation [2]. Recently, oral anticoagulation therapy was confirmed to be superior to clopidogrel plus aspirin for prevention of vascular events in patients with atrial fibrillation at high risk of stroke [3]. However, warfarin has a narrow therapeutic range and a given dose has a large interindividual variation. An insufficient dose may fail to prevent thromboembolism, while an overdose increases the risk of bleeding. The degree of anticoagulation achieved in each patient is followed by obtaining the prothrombin time expressed as the international normalized ratio (PT–INR).

Warfarin therapy management is challenging for several reasons including the need to determine a safe and effective maintenance dose during the early phase of therapy and the fact that maintenance doses must be adjusted to compensate for changes in patients' weight, diet, disease state, concomitant use of other medications, and genetic factors. Traditional warfarin induction algorithms rely on trial-and-error dosing after an initial warfarin dose of 5 mg or 10 mg in Caucasians and 3.5 mg in Asian, rather than being tailored to individual genetic and clinical factors [4–7]. It usually takes not less than several weeks to obtain the stable warfarin control. The alternative to these algorithms incorporates pharmacogenomic, demographic, and clinical factors to more accurately estimate the warfarin dose a priori, potentially decreasing the risk of over dose during therapy induction and minimizing the warfarin induction period [8]. In particular, increasing evidence suggests that genetic variation in *CYP2C9* and *VKORC1* greatly influences effective warfarin dose. In this review, we discuss the implications of variability in *CYP2C9* and *VKORC1* with respect to warfarin dose and its clinical efficacy. Additionally, we describe novel algorithms incorporating genetic and clinical factors to predict effective warfarin doses and the risk of side effects.

Mechanisms of warfarin anticoagulation

Warfarin is a specific inhibitor of the vitamin K epoxide reductase (VKOR) encoded by the vitamin K epoxide reductase complex subunit 1 (*VKORC1*) gene [9,10]. Warfarin exerts its anticoagulant effects by preventing the ability of *VKORC1* to regenerate reduced vitamin K from its epoxide form [11]. Reduced vitamin K is an essential cofactor for γ-glutamylcarboxylase (GGCX), the enzyme catalyzing the post-translationalγ-glutamyl carboxylation

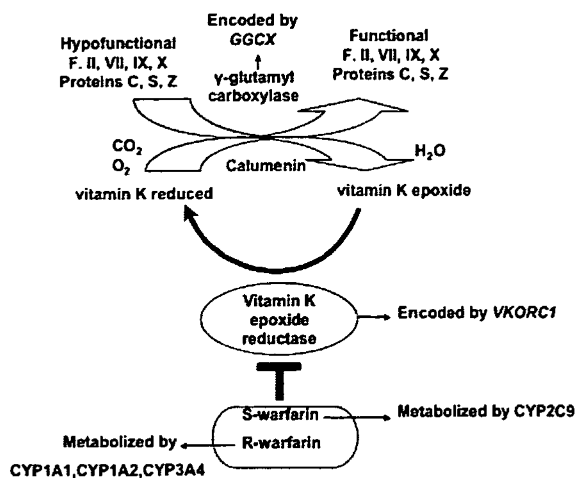

Figure 1 Pathway of warfarin metabolism.

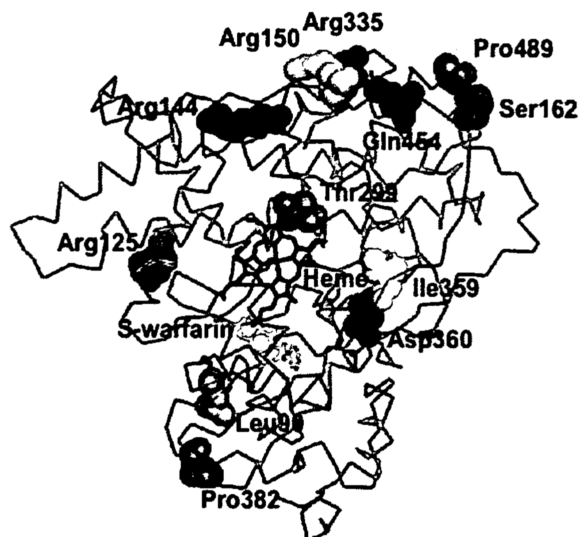

Figure 2 Missense mutations with functional effects mapped in the crystal structure of human *CYP2C9* protein bound with warfarin (PDB: 1OG5). *S*-warfarin and heme are shown in the skeleton model with pink and red, respectively. Amino acid residues are shown in the sphere mode with colors.

of the vitamin K-dependent clotting factors, II (prothrombin), VII, IX and X (Fig. 1). Thus, warfarin prevents the functional maturation of vitamin K-dependent clotting factors, leading to reduced coagulation [12,13]. Patients with congenital deficiencies in *GGCX* and *VKORC1* have disordered hemostasis, and these conditions are known as combined deficiency of vitamin K-dependent clotting factors type 1 and 2, respectively [9,14]. Functional abnormalities in *VKORC1* also confer resistance to coumarin-type anticoagulant drugs (warfarin resistance) [9].

Table 1 Nonsynonymous mutations in *CYP2C9* with functional effects

Alleles	Nucleotide change in cDNA	Amino acid change	Enzymatic activity	References
*CYP2C9*2*	430C>T	Arg144Cys	Decrease: an approximately 50% decrease of the maximum rate of metabolism (*V*max) and 30–50% lower turnover (kcat) of *S*-warfarin	[22]
*CYP2C9*3*	1075A>C	Ile359Leu	Decrease: a markedly higher *K*m and lower intrinsic clearance with an approximately 90% decrease of *S*-warfarin	[23]
*CYP2C9*4*	1076T>C	Ile359Thr	Decrease: 72–81% reduction of intrinsic clearance of diclofenac	[28,29]
*CYP2C9*5*	1080C>G	Asp360Glu	Decrease: intrinsic clearance of warfarin approximately 10% of wild type	[30]
*CYP2C9*6*	del818A	Frame shift	Null	[31]
*CYP2C9*8*	449G>A	Arg150His	Increase: more than two-fold increase in the intrinsic clearance of tolbutamide	[32]
*CYP2C9*11*	1003C>T	Arg335Trp	Decrease: a three-fold increase in the *K*m and more than a two-fold decrease in the intrinsic clearance of tolbutamide	[32,33]
*CYP2C9*12*	1465C>T	Pro489Ser	Decrease: a modest decrease in the *V*max and the intrinsic clearance of tolbutamide	[32]
*CYP2C9*13*	269T>C	Leu90Pro	Decrease: decreased activity toward all studied CYP2C9 substrates	[34–36]
*CYP2C9*14*	374G>A	Arg125His	Decrease: 80–90% lower catalytic activity toward tolbutamide	[37,38]
*CYP2C9*15*	485C>A	Ser162X	Null	[37,38]
*CYP2C9*16*	895A>G	Thr299Ala	Decrease: 80–90% lower catalytic activity toward tolbutamide	[37,38]
*CYP2C9*17*	1144C>T	Pro382Ser	Decrease: modest 30 to 40% decreases in caltalytic activity toward tolbutamide	[37,38]
*CYP2C9*19*	1362G>C	Gln454His	Decrease: modest 30 to 40% decreases in caltalytic activity toward tolbutamide	[37,38]

Nonsynonymous mutations with functional activity are listed. Those that functional activity has not been examined were not listed.

Genetic polymorphisms in *CYP2C9* relevant to warfarin metabolism

Warfarin metabolism by cytochrome *P450*, CYPs

Warfarin is a racemic mixture of *R*- and *S*-enantiomers [2], and these differ both in their potency and metabolism. *S*-warfarin is a five-fold more potent vitamin K antagonist than *R*-warfarin [2]. Under steady state conditions, *S*-warfarin accounts for 60–70% of the anticoagulation response, with the *R*-enantiomer accounting for 30–40% [15]. *S*-warfarin is metabolized primarily by *CYP2C9*, but *R*-warfarin is metabolized by *CYP3A4*, *1A2* and *1A1* [16]. Genetic variations in *CYP2C9*, *3A4*, *1A2* and *1A1* can potentially lead to the interindividual variation in effective warfarin dose [17,18], and the most extensively studied isomer among the four is *CYP2C9*. To date, more than 50 variants in *CYP2C9* have been described, and two variants, *CYP2C9**2* and *CYP2C9**3*, have been examined with respect to warfarin dosing.

Metabolic activity of *CYP2C9**2* and *CYP2C9**3* proteins

The human *CYP2C9* gene is approximately 55-kb long and located on chromosome 10q24.2 [19,20]. The most common allele is designated *CYP2C9**1*, and it is considered the wild-type genotype. Approximately 24 nonsynonymous variations in *CYP2C9* have been identified [21], and the functional consequences of *CYP2C9**2* (Arg144Cys) and *CYP2C9**3* (Ile359Leu) are well defined. The maximum rate of metabolism (*V*max) of the *CYP2C9**2* protein is approximately 50% that of the wild-type protein, and the turnover (kcat) is reduced by 30 to 50%. The *CYP2C9**3* protein has a markedly higher *K*m and lower intrinsic clearance leading to an approximately 90% decrease in *S*-warfarin 7-hydroxylation [22–24].

CYP2C9 genotype and adverse bleeding events

Most clinical studies examining warfarin pharmacogenomics assessed differences in the mean daily warfarin dose and susceptibility to bleeding. A direct association between *CYP2C9* genotype and anticoagulation status or bleeding was first reported by Higashi et al. [25]. Subsequently, a systematic meta-analysis showed that patients with either the *CYP2C9**2* or *CYP2C9**3* variant required a lower warfarin maintenance dose, and this was especially pronounced for patients with *CYP2C9**3* (a 30% dose reduction) [26]. However, the risk of bleeding for patients with the *CYP2C9**2* and/or *CYP2C9**3* alleles is approximately doubled. Patients with *CYP2C9**2* and/or *CYP2C9**3* metabolize warfarin more slowly than wild-type patients, and a traditional warfarin dose would more likely lead to overdose and bleeding in these individuals [8]. Patients with the *CYP2C9* variants, particularly the *CYP2C9**3* allele or a combination of *CYP2C9**2* and *CYP2C9**3*, may have elevated PT–INRs, require longer to achieve a stable warfarin dose, and have a higher risk of serious or life threatening bleeding events during the induction or dose-titration period of warfarin therapy. However, there was no association between these variants and either PT–INR stability or risk of excessive anticoagulation during long-term treatment [27].

Potential relevance of deleterious mutations in *CYP2C9* to warfarin

Rare missense mutations in *CYP2C9* may affect enzyme function and warfarin clearance [28–38], and these mutations are summarized in Table 1. Missense mutations with functional effects were mapped in the crystal structure of human *CYP2C9* bound with warfarin (Fig. 2) [39]. The population frequencies of these *CYP2C9* variants have not been studied thoroughly. The *CYP2C9**4* allele has only been found at very low frequencies in Asian individuals [28]. The *CYP2C9**5* and *CYP2C9**6* alleles have been identified in approximately no more than 1% of black individuals, and they are virtually absent in Caucasian and Asian populations [30,31,40,41]. The presences of other recently identified *CYP2C9* alleles need to be confirmed in different ethnic populations.

Genetic polymorphisms in *VKORC1* relevant to warfarin

Genetic mutations in *VKORC1* as combined deficiency of vitamin K-dependent clotting factors type 2

As mentioned above, VKOR is the target enzyme of warfarin. VKOR was first identified in 1974, but the gene encoding VKOR, *VKORC1*, was not identified until 2004 [9,10]. *VKORC1* is found on chromosome 16p11.2, and it is approximately 4-kb long. Congenital deficiency of *VKORC1* leads to a bleeding phenotype, named combined deficiency of vitamin K-dependent clotting factors type 2, and a missense mutation, Arg98Trp, has been identified in this patient [9]. Other *VKORC1* missense mutations, Val45Ala, Arg58Gly, and Leu128Arg, have also been identified in patients with warfarin resistance

[9,42,43]. These missense mutations could affect *VKORC1* enzyme function, leading to a global decrease in all vitamin K coagulation factors. Alternatively, these mutations could lead to warfarin non-responsiveness. However, several more common SNPs in *VKORC1* significantly affect warfarin maintenance dose, as described below.

Relationship of genetic polymorphisms in *VKORC1* and warfarin dose

Several genetic polymorphisms in *VKORC1* are associated with warfarin dose across the normal dose range [44–54]. Two common polymorphisms, 1173C>T in intron 1 and 3730G>A in the 3′-untranslated region (defined by the nucleotide position from the translation start site), affect the interindividual variability of warfarin dose [44]. Regardless of the presence of confounding variables, the mean warfarin dose was higher (6.2 mg/day) in patients with the *VKORC1* 1173CC genotype than those patients with the CT (4.8 mg/day; $p=0.002$) or TT genotype (3.5 mg/day; $p<0.001$).

Subsequent haplotype analysis established a significant contribution of *VKORC1* to interindividual variability of warfarin dose [45]. The 10 most common SNPs were used to construct five major haplotypes, and the relationship of these haplotypes to warfarin dose was examined in Caucasian patients. A low-dose haplotype group (A) and a high-dose haplotype group (B) were identified. The mean (± SE) warfarin maintenance dose differed significantly between the three combinations of haplotype group, with a dose of 2.7±0.2 mg/day for group A/A, 4.9±0.2 mg/day for group A/B, and 6.2± 0.3 mg/day for group B/B. Thus, *VKORC1* haplotype explained a large degree of the interindividual variations of warfarin dose.

Estimated contribution of *CYP2C9* and *VKORC1* genotypes in interindividual variability of warfarin dose

Since the cloning of *VKORC1*, several pharmacogenomic studies have examined the contribution of *VKORC1* genetic polymorphisms in the interindividual variability of warfarin responsiveness [44–51]. These studies suggest that variations in *CYP2C9* and *VKORC1* can potentially account for 5–22% and 6–37% of the interindividual variability of warfarin dose, respectively (Table 2). Taken together, these data indicate that the interindividual variability of warfarin dose can be partly explained by genetic polymorphisms in *VKORC1* and *CYP2C9*. Thus, when pharmacogenomic knowledge of *CYP2C9* and *VKORC1* is considered together with clinical factors, such as age, gender, body weight, height, concurrent medications, and indication for treatment, more than 33% of the variability in the warfarin dose can be predicted.

Function of *VKORC1* polymorphisms

A component of one of the examined haplotypes is the −1639G>A polymorphism in the *VKORC1* promoter. This polymorphism occurs in the second nucleotide of an E-box (CANNTG) and is predicted to alter the E-box consensus sequence with potential changes in the *VKORC1* promoter activity. When this was examined using a luciferase reporter assay, one study found that the promoter activity of the G allele variant was 44% higher compared with the A allele [52], but another group did not identify any differences in *VKORC1* promoter activity between these variants [46]. When *VKORC1* mRNA levels were examined in human liver tissue, *VKORC1* mRNA expression significantly correlated with haplotype group with expression in the B/B (high-dose) group about three times higher than the A/A (low-dose) group [45]. Thus, despite inconclusive *in vitro* data, *VKORC1* haplotype is associated with variable mRNA levels that can contribute to interindividual variability in warfarin dose.

VKORC1 genotype and adverse bleeding events

Genetic polymorphisms in *VKORC1* can clearly affect warfarin dose, but can polymorphisms affect the occurrence of adverse bleeding events? To address this question, a case-control study examined 110 patients with episodes of severe bleeding during warfarin therapy and 220 control patients without bleeding undergoing the same therapy. They specifically examined the *VKORC1* 1173C>T polymorphism, and carriers of at least one T allele had an increased risk of bleeding (crude odds ratio=1.7, 95% CI: 1.1–2.5) compared to individuals with the CC genotype [55]. In this study, phenprocoumon and acenocoumarol were used for anticoagulation. When analyzed separately, phenprocoumon seems to more strongly modify the bleeding risk of patients with the 1173C>T genotype (crude odds ratio=2.6, 95% CI: 1.2–5.7 for T-allele carriers), whereas genotype did not affect acenocoumarol users (crude odds ratio=1.2, 95% CI: 0.6–2.3).

Ethnicity and interindividual variation in warfarin dose

Ethnicity is an important factor contributing to the warfarin maintenance dose. The warfarin

Table 2 Estimated contribution of various factors for interindividual variation of warfarin dose

Variable	Estimated contribution [a]		Reference
VKORC1	14%		D'Andrea et al. [44]
CYP2C9	22%		
VKORC1	21%		Rieder et al. [45]
CYP2C9	6%		
VKORC1	37% [b]	30% [c]	Bodin et al. [46]
CYP2C9	14% [b]		
Body weight, *VKORC1*, *CYP2C9*	54% [b]	40% [c]	
Age	17%		Sconce et al. [47]
VKORC1	15%		
CYP2C9	18%		
Age, *VKORC1*, *CYP2C9*, height	54%		
VKORC1	30%		Wadelius et al. [48]
CYP2C9	12%		
Age, *VKORC1*, *CYP2C9*, *GGCX*, body weight, interacting drugs, indication for treatment	56%		
Age	21.5%		Veenstra et al. [49]
Gender	0.4%		
VKORC1	31.0%		
CYP2C9	7.9%		
Age, gender, *VKORC1*, *CYP2C9*	60.8%		
Age	1.7%		Kimura et al. [50]
Gender	8.1%		
Body Weight	7.8%		
VKORC1	5.9%		
CYP2C9	4.6%		
GGCX	5.2%		
Age, gender, body weight, *VKORC1*, *CYP2C9*, *GGCX*	33.3%		

a Estimated contribution of variables is denoted as R^2 (coefficient of determination), calculated from multivariate linear regression models.

b Decrease in factor VII in healthy individuals.

c PT–INR change in healthy individuals.

maintenance dose in Asian patients was approximately 30–40% less than that of Caucasian patients [37,50,51,56], and these differences are, in part, attributable to genetic differences in *CYP2C9* and *VKORC1*.

Ethnic differences in allelic frequencies of *CYP2C9*2* and *CYP2C9*3*

The allelic frequencies of *CYP2C9*2* and *CYP2C9*3* are considerably different between ethnic populations. In Caucasians, the allelic frequencies of *CYP2C9*2* and *CYP2C9*3* are approximately 8% to 20% and 6% to 10%, respectively [40,57–59]. These deleterious variants are less prevalent in Asian and African-American populations. *CYP2C9*2* is not present in Asian populations, and only approximately 2–4% of African-American populations carry the *CYP2C9*2* allele. *CYP2C9*3* is present in 1–4% of Asians and 1–2% of African-Americans [40,60]. The clinical effects of this polymorphism have been widely documented *in vivo* [23,60–63].

Ethnic differences in *VKORC1* variants

The frequencies of different *VKORC1* alleles in Asian, African-American and Caucasian subjects are listed in Table 3. The frequency of the AA genotype of the −1639G>A variant in Japanese (83%) was much higher than that in Caucasians (14%) [53], but it is comparable to Chinese (82%) [52]. The *VKORC1* haplotype group A related to low warfarin dose was highest in Asian populations (89%), while haplotype group B was highest in Caucasian populations (58%) [45]. One studied examined the combination of *CYP2C9*2* and *CYP2C9*3* frequencies and *VKORC1* haplotype in 556 unrelated healthy individuals from different ethnic backgrounds, and the Asian population had the highest frequency (86%) of the "low dose" genotype [64]. African-Americans had the lowest frequency (22%) of the "low dose" phenotype, and these data are consistent with the observations that Asian patients require a lower average maintenance warfarin dose and African-Americans a higher average dose to obtain a therapeutic PT–INR. These results were also confirmed in a Hong Kong Chinese population [49].

Proposed pharmacogenomic algorithms for warfarin dose determination

A dosing algorithm was developed based on the study of 297 Caucasian warfarin-treated patients [47]. The formula predicts that dose = 0.628 − 0.0135 (age) − 0.240 (*CYP2C9*2*) − 0.370 (*CYP2C9*3*) − 0.241 (*VKORC1*) + 0.0162 (height), where age (year), *CYP2C9* (*2 *3) and *VKORC1* (−1639G>A) genotypes, and height (cm) allow the best estimate of warfarin maintenance dose. This formula accounted for nearly 55% of the variability in warfarin daily dose requirements in Caucasian. In this study, comorbid

Table 3 Common variant alleles and haplotype group frequencies of *VKORC1* in Asian, African and Caucasian individuals

	Frequency (%)		
	African	Asian	Caucasian
−1639G>A	–	82–83	14
1173C>T	9	89	42
1542G>C	25	91	37
3730G>A	49	13	45
Haplotype group A (low dose)	14–23	85–89	37–42
Haplotype group B (high dose)	49	10–14	57–58

Taken from the references of Yuan et al. [52], Mushiroda et al. [53], Rieder et al. [45], Marsh et al. [64], and Veenstra et al. [49].
Sequence number is defined by the nucleotide position from the translational start site ATG.
Haplotype groups A and B are based on classifications from Reider et al. [45] where haplotype A represents individuals at risk for excessive anticoagulation with standard warfarin dosing, and haplotype B represents individuals at risk for subtherapeutic anticoagulation from standard warfarin dosing.

conditions and concurrent medication were exclusion criteria, so that their contributions to warfarin dose could not be determined. This dosing algorithm was validated in an unrelated cohort of patients on warfarin chronic therapy.

VKORC1 (1173C>T) and *CYP2C9* (*2/*3/*11) genotypes, age and weight were identified as independent covariates contributing to interindividual variability in warfarin dose in different ethnic groups [51]. In this study, 70% of Caucasian, 83% of African-American and 20% of Japanese patients carried the *CYP2C9* and *VKORC1* genetic factors respectively, resulting in the observed wide interindividual variation in warfarin dose. The final regression equation for estimating maintenance doses of warfarin was as follows: for patients with homozygous wild-type genotype for both *CYP2C9* and *VKORC1*: maintenance dose (mg) = 6.6 − 0.035 × (age, year) + 0.031 × (body weight, kg); for those either heterozygous or homozygous for variants of *CYP2C9*, the maintenance dose was further reduced by 1.3 and 2.9 mg, respectively, from those predicted by the respective equations. Based on the standardized partial regression coefficients, genotypes of *CYP2C9* and *VKORC1* were the principal covariates contributing equally to interindividual variability in warfarin dose requirements. Collectively, the identified covariates accounted for 57% of the overall variability in the daily dose of warfarin.

An alternative warfarin-dosing algorithm was developed by studying 828 Japanese warfarin-treated patients [53]. Patients were classified into three groups according to *CYP2C9* (*1/*3) and *VKORC1* (intron 1–136T>C, same as 1173T>C) genotype, and this was referred to as the "warfarin-response index" [53]. The median warfarin daily dose varied significantly in the three index groups, with the lowest median dose being 2.0 mg/day for the *CYP2C9*3/*3* and *VKORC1* 1173T/T group, and highest dose of 3.5 mg/day for the *CYP2C9*1/*1* and *VKORC1* 1173C/C group ($p = 4.4 \times 10^{-13}$).

Contribution of other genes to warfarin interindividual variability

Despite our current knowledge of phamacogenomic and clinical factors, the source of more than 40% of the variability in warfarin dose remains unclear. Additional genetic factors, including multidrug resistance 1 (*MDR1*) [65], genes encoding vitamin K-dependent clotting factors [66], *GGCX* encoding γ-glutamyl carboxylase in the vitamin K cycle (Fig. 1) [48,50], the γ-glutamyl carboxylase inhibitory protein calumenin (Fig. 1) [67], apolipoprotein E [68], candidate genes encoding microsomal epoxide hydrolase (*mEH*) [69], and possible genes encoding additional components of the vitamin K epoxide reductase complex [9], might be responsible for the observed interindividual variability in warfarin dose requirements.

Perspective

We have greatly increased our knowledge of the factors contributing to the interindividual variability of warfarin dose. The relationship between genetic variations in *CYP2C9* and *VKORC1* and therapeutic warfarin dose is biologically and statistically compelling. Use of new warfarin-dosing algorithms will not eliminate the need for PT–INR monitoring, but these algorithms may prevent bleeding caused by excessive warfarin initiation. However, current evidence does not indicate widespread genotyping of *CYP2C9* and *VKORC1* for a variety of reasons.

The utility of pre-prescription *CYP2C9* and *VKORC1* genotyping and the proposed pharmacogenomic algorithms have not yet been established in prospective randomized clinical trials. Comparisons between patients treated based on genotype information and patients treated with only conventional empirical therapy are needed before a widespread genotyping should be performed. The hypothesis that pharmacogenomic based dosing will reduce the risk of bleeding during warfarin induction should be tested prospectively.

A cost-benefit analysis of pre-prescription *CYP2C9* and *VKORC1* genotyping during warfarin treatment should be performed. Genotyping large numbers of patients to identify the small minority with a markedly increased risk of adverse effects may not be cost-effective. However, for patients

treated with warfarin, even a small reduction in the risk of major hemorrhage during induction could make genotyping cost-effective because of the devastating clinical and economic consequences of a major bleeding event [8].

In conclusion, a warfarin-dosing regimen using clinical data and pharmacogenomic information of *CYP2C9* and *VKORC1* genotype could benefit patients treated with warfarin, but treatment algorithms incorporating pharmacogenomic data must be evaluated prospectively in a randomized controlled clinical trial before incorporating into routine clinical practice. Additionally, the prospective validation of a pharmacogenomics dosing model would benefit from a platform that could quickly and economically genotype individuals.

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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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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.

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.

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.

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: Allele 1	Allele frequency: Allele 2	Flanking sequence	Taqman	dbSNP ID
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	aggtgggggggcc[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	GACTGCCCCCCC[-/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: Odds ratio (95% confidence interval)*	Women: *P* value	Men: Odds ratio (95% confidence interval)*	Men: *P* value
ACADSB	−512A>G[b] (0.738/0.262)	AA	1	0.0163	1	0.3832
		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] (0.738/0.262)	GG	1	0.0171	1	0.3785
		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] (0.703/0.297)	GG	1	0.2791	1	0.0122
		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] (0.704/0.296)	CC	1	0.3097	1	0.0116
		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] (0.695/0.305)	GG	1	0.1522	1	0.0742
		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 AG + GG	128.77 ± 0.69 126.48 ± 0.80	0.0302	2.29
ACADSB	−254G>A[a]	G/A (0.738/0.262)	Women	SBP	GG GA + AA	128.82 ± 0.69 126.47 ± 0.79	0.0264	2.35
ACADSB	38G>A (Arg13Lys)	G/A (0.878/0.122)	Women	DBP	GG + GA AA	76.46 ± 0.30 82.37 ± 2.59	0.0235	5.91

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

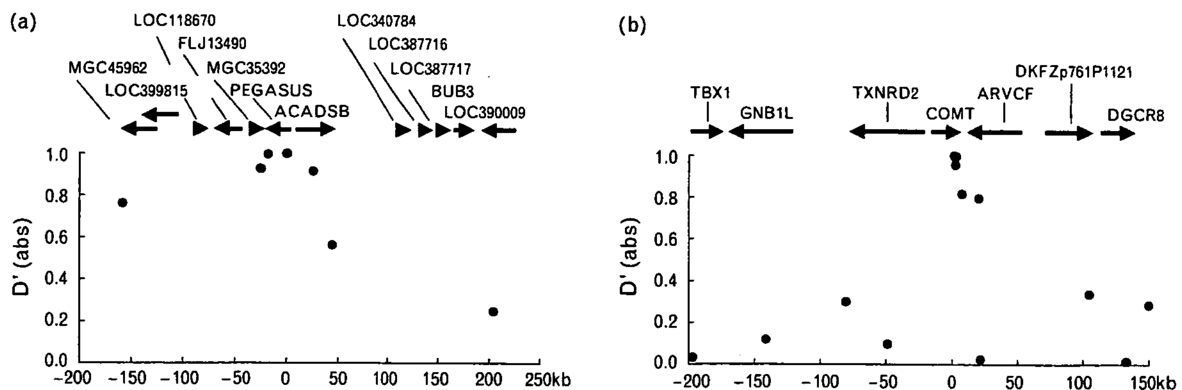
We previously identified differentially expressed genes in the kidneys of salt-loaded Dahl-S and Lewis rats [4].

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

Gene	Haplotype		Men (%) HT (812 alleles)	Men (%) NT (902 alleles)	Men χ^2	Men *P*	Women (%) HT (772 alleles)	Women (%) NT (1242 alleles)	Women χ^2	Women *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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ADAMTS13 assays and ADAMTS13-deficient mice

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Purpose of review

Thrombotic thrombocytopenic purpura can be induced by acquired or congenital deficiency of the plasma von Willebrand factor-cleaving protease, ADAMTS13. Measurement of ADAMTS13 activity is important for the diagnosis and treatment of microangiopathies including thrombotic thrombocytopenic purpura. Phenotypic analysis of mice lacking the Adamts13 gene is valuable for understanding the pathogenesis of microangiopathies.

Recent findings

The minimum substrate for ADAMTS13 activity was identified as 73 amino acid residues in the A2 domain of von Willebrand factor, called VWF73. Several new assays have been developed using this sequence. The VWF73-based assays are rapid, quantitative, and easy to handle, and are well correlated with the measures from previous assays. Mice lacking the Adamts13 gene were produced. The mice were viable and fertile. They showed a prothrombotic state but no symptoms of spontaneous thrombocytopenia, hemolytic anemia, or microvascular thrombosis were observed.

Summary

VWF73-based ADAMTS13 assays will significantly facilitate the accurate diagnosis of microangiopathies and contribute to the improved clinical treatment of these diseases. Accumulated clinical information on patients with ADAMTS13 deficiency and mice lacking the Adamts13 gene indicates that additional environmental or genetic susceptibility factors are required to trigger thrombotic thrombocytopenic purpura.

Keywords

ADAMTS13, microangiopathy, thrombotic thrombocytopenic purpura, von Willebrand factor

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Abbreviations

CUB	complement components C1r/C1s, Uegf (epidermal growth factor-related sea urchin protein), and bone morphogenetic protein-1
HUS	hemolytic uremic syndrome
TSP-1	thrombospondin type-1
TTP	thrombotic thrombocytopenic purpura
ULVWF	ultralarge von Willebrand factor
VWF	von Willebrand factor

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Introduction

Thrombotic thrombocytopenic purpura (TTP) is characterized by thrombocytopenia and microangiopathic hemolytic anemia accompanied by variable-penetrance of neurologic dysfunction, renal failure, and fever. In the microvasculature of patients with TTP, systemic platelet thrombi are developed, largely resulting from the accumulation of ultralarge von Willebrand factor (ULVWF) multimers [1]. ULVWF can be accumulated by acquired or congenital deficiency of the von Willebrand factor (VWF)-cleaving protease, ADAMTS13 (a disintegrin-like and metalloprotease with thrombospondin type 1 motif, 13) [2,3••]. TTP caused by congenital deficiency of ADAMTS13 is also called Upshaw–Schulman syndrome.

Since the cloning of its cDNA in 2001, this new antithrombotic factor has been intensively studied [4–6,7,8••, 9••,10•]. Here we summarize the recent progress on ADAMTS13, focusing on assays for ADAMTS13 and mice lacking the Adamts13 gene.

Genetic mutations in congenital ADAMTS13 deficiency

ADAMTS13 consists of 1427 amino acid residues with a calculated molecular mass of 145 kDa. It is composed of multiple discrete domains, as shown in Fig. 1 [4–6,7•,8••, 9••]. Unlike other ADAMTS family members, the ADAMTS13 sequence has a short pro-sequence and two C-terminal CUB [complement components C1r/C1s, Uegf (epidermal growth factor-related sea urchin protein), and bone morphogenetic protein-1] domains. The human ADAMTS13 gene comprises 29 exons, encompassing 37 kb on chromosome 9q34. It is expressed mainly in the liver; primarily in stellate cells [11,12]. Platelets and endothelial cells also express ADAMTS13 [13,14,15•,16••]. The CUB domains are required for apical sorting of ADAMTS13 in endothelial cells [16••].