EPIDEMIOLOGY

Epidemiological investigations identified a strong association of TTP with ticlopidine (Table 1). The first cases of ticlopidine-associated TTP were identified in 1991 at an apheresis center in Paris.[11] In 1998, a survey of apheresis centers supplemented by FDA adverse event reports identified 60 cases of ticlopidine-associated TTP; one-third had died from the TTP.[12] Most patients had received between 2–12 weeks of ticlopidine.[12,13] Subsequently, after the introduction of coronary artery stent procedures, additional ticlopidine-associated TTP cases were identified at interventional cardiology laboratories and therapeutic plasma exchange (TPE) centers.[13,14] Two surveys of interventional cardiology laboratories that had placed coronary artery stents in 8000 and 45,000 persons identified rates of TTP after ticlopidine administration of 1 in 1600 and 1 in 5000 patients, respectively.[15,16] These findings placed ticlopidine as the drug with the highest reported rate of TTP.

Recent investigations evaluated the association of clopidogrel with TTP. The first two cases were identified by the directors of apheresis centers in 1998, shortly after the drug received FDA approval.[17] In 2000, eleven cases of TTP after administration of clopidogrel were identified at apheresis centers in six cities.[17] By 2004, 37 cases of

Table 1 | Comparison of basic science, epidemiological, clinical, and pharmacovigilance findings for ticlopidine- versus clopidogrel-associated TTP

Category	Ticlopidine-associated TTP	Clopidogrel-associated TTP
Basic science[20]		
Probable underlying pathophysiology	Antibody to ADAMTS13 and microvascular endothelial cell damage	Microvascular endothelial cell damage
High molecular weight vWF identified during the acute TTP phase	Yes	Yes
ADAMTS13 deficiency during the acute TTP phase	Yes	No
Functional IgG inhibitors to ADAMTS13 identified during acute phase	Yes	No
Clinical[20]		
Usual time period for onset	Two to 12 weeks after drug initiation	Within 2 weeks of drug initiation
Renal insufficiency	Mild to none	Severe
Thrombocytopenia	Severe	Mild
Survival after plasma exchange	>90%, usually within days of initiation of plasma exchange	70%, often takes several weeks of plasma exchange
Survival without plasma exchange	30%	70%
Spontaneous relapse	Occasional	Infrequent
Likelihood of relapse occurring with exposure to the other thienopyridine	High	Low
Epidemiological		
Epidemiological studies identifying cases of TTP after thienopyridine administration	Surveys of directors of interventional cardiology laboratories as well as directors of therapeutic plasma exchange centers (n=33 cases)[26,29]	Surveys of directors of therapeutic plasma exchange centers (n=13 cases)[5,31]
Estimated incidence based on information included in the FDA-approved package insert	0.01–0.02%[37]	0.0001%[18] (threefold greater than estimated incidence of idiopathic TTP)
Population-based case-control studies	None	Recent initiation of anti-platelet agents (clopidogrel, aspirin, or dipyridamole) is associated with 19.8-fold increased risk of developing TTP (n=86 cases; 177 age- and gender-matched controls)[41]
Pharmacovigilance		
Number of thienopyridine-associated TTP cases identified in the first year of marketing of the relevant drug	4 (year=1991)[11]	2 (year=1999)[38,39]
Number of cases included in the largest case series	98 patients[14]	50 patients[3]
Year of FDA approval	1991 (current sales are $100,000)[9]	1998 (current sales are $7.3 billion)[9]
Time from FDA approval to identification of first cases	0 years (4 cases) (1991)	1 year (2 cases) (1999)
Time from FDA approval to reporting of first case series	7 years (1998)	1.5 years (2000)
Rank in FDA MedWatch database in association with drug-associated TTP reports (1998–2006)	First—overall (first in the years 1998 and 1999)	Second—overall (first since 2000)
Advisories from the FDA	Package insert warning (1995)[40] Black box warning (1998)[37]	Package insert warning (2000)[18]
'Dear Doctor' warnings describing drug-associated TTP mailed by the pharmaceutical supplier	1998	2000

ADAMTS13, a disintegrin and metalloprotease, with thrombospondin-1-like domains; FDA, Food and Drug Administration; TTP, thrombotic thrombocytopenic purpura.

clopidogrel-associated TTP had been reported to the FDA.[3] The pharmaceutical supplier reported an estimated incidence rate of 12 TTP cases per million clopidogrel-treated patients,[18] three times the background rate for TTP in the general population.[19]

CLINICAL FEATURES

Thienopyridine-associated TTP is characterized by two clinical syndromes (Figure 1a). Most cases of ticlopidine-associated TTP and a minority of clopidogrel-associated TTP cases present with severe thrombocytopenia, microangiopathic hemolytic anemia, markedly elevated serum levels of lactate dehydrogenase, and normal renal function; and they occur between 2 and 12 weeks after initiation of thienopyridine therapy. Most cases of clopidogrel-associated TTP and a minority of ticlopidine-associated TTP cases present with mild thrombocytopenia, microangiopathic hemolytic anemia, mildly elevated serum levels of lactate dehydrogenase, and marked renal insufficiency. Onset usually occurs within 2 weeks of thienopyridine initiation.[3,17,20] Both syndromes differ from those reported for other drug-associated TTP syndromes.[2] Thrombotic microangiopathy associated with the calcineurin inhibitors gemcitabine and mitomycin-C is dose dependent, occurs after several weeks or months of use, and is attributed to the cumulative toxic effects on vascular endothelium.[2] Renal dysfunction is generally present, with many patients requiring hemodialysis. TPE is not effective.[2] Thrombotic microangiopathy developing after organ transplantation is associated with calcineurin inhibitors. The most common treatment strategy is discontinuation of the drug.[2] Although quinine-associated TTP/HUS is antibody mediated, the antibodies are directed against granulocytes, lymphocytes, endothelial cells, or platelet glycoprotein Ib/IX or IIb/IIIa complexes.[21] The syndrome can occur after ingestion of a single tablet of quinine in previously exposed persons, and is characterized by neurologic complications, thrombocytopenia, and hemolysis. Renal failure is absent occasionally. Treatment includes discontinuation of quinine, TPE, and hemodialysis.

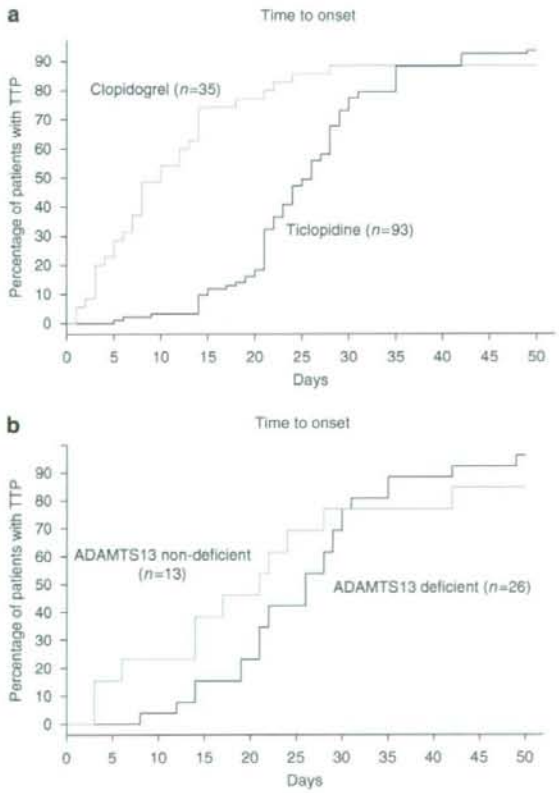

Figure 1 | Duration of thienopyridine exposure prior to TTP onset. (**a**) Thienopyridine-associated thrombotic thrombocytopenic purpura (TTP) onset: ticlopidine versus clopidogrel ($P = 0.0016$). (**b**) Thienopyridine-associated TTP onset: ADAMTS13 (a disintegrin and metalloprotease, with thrombospondin-1-like domains) deficient (<15%) versus near-normal levels (>15%) of ADAMTS13 activity ($P > 0.05$). This figure has previously been published in Bennett *et al.*[20]

PATHOPHYSIOLOGY

For most patients with ticlopidine-associated TTP and a minority of patients with clopidogrel-associated TTP, *in vitro* assessments of plasma ADAMTS13 activity show severely diminished activity at the time of TTP onset.[20] Onset of TTP occurs between 2 and 12 weeks after thienopyridine initiation (Figure 1b). Reduced *in vitro* ADAMTS13 activity correlates with deficient ADAMTS13 activity near the surface of stimulated endothelial cells that secrete ULVWF multimers. Plasma from six of seven patients with ticlopidine-associated TTP and from two of eleven patients with clopidogrel-associated TTP contained inhibitors to the ADAMTS13 metalloprotease.[17,22] Failure to process ULVWF multimers seems to lead to the binding of ULVWF to platelets, systemic platelet aggregation, and TTP.[23] After TPE and thienopyridine discontinuation, most patients with ADAMTS13 deficiency, anti-ADAMTS13 autoantibodies, and thienopyridine-associated TTP recover. Plasma exchange may lead to removal of ULVWF multimers, removal of autoantibodies to ADAMTS13, and replacement of the ADAMTS13 with that present in fresh frozen plasma. Clinical findings also require stimulation of endothelial cells to secrete ULVWF. Such a double-insult model is exemplified by the ADAMTS13 knockout mouse, which requires endothelial cell stimulation to evoke a TTP-like microvascular thrombosis.[24] In genetically predisposed individuals, thienopyridine may stimulate an autoimmune anti-ADAMTS13 antibody response and microvascular endothelial injury. Ticlopidine and clopidogrel are protein-bound in plasma and can function as haptens capable of eliciting IgE and IgG antibody formation.[25] However, they do not directly bind to ADAMTS13 and stimulate production of antibodies that inhibit ADAMTS13

enzyme activity. Anti-ADAMTS13 antibodies generated in a fraction of thienopyridine-treated patients do not require the presence of the drug (or metabolite).[17,22,26] Thienopyridine/anti-ADAMTS13 antibodies are analogous to warm autoantibodies against red blood cell antigens that emerge in a subset of patients treated with the antihypertensive agent, α-methyldopa.[27] Binding of thienopyridines to $P2Y_{12}$ molecules on different cell types may, in a fraction of exposed individuals, initiate anomalous intracellular signaling patterns or provoke antibody production against the haptenic thienopyridine–$P2Y_{12}$ protein complex on cell surfaces. Malfunction or injury to lymphocytes, CD34+ stem cells, or endothelial cells may result.

For most clopidogrel-associated and a minority of ticlopidine-associated TTP patients, the syndrome is characterized by mild thrombocytopenia, microangiopathic hemolytic anemia, and marked renal insufficiency.[20,28] Onset of TTP is generally within 2 weeks of thienopyridine initiation (Figure 1a). Most cases have ULVWF in their plasma and near-normal levels of plasma ADAMTS13 metalloprotease activity during the acute phase of the syndrome, suggesting endothelial cell injury or stimulation with release of ULVWF.[20] Thienopyridines may bind to $P2Y_{12}$ receptors (with or without anti-thienopyridine antibodies) on CD34+ stem cells, altering cell proliferation and differentiation. Interaction of thienopyridines with endothelial cells has been shown to result in nitric oxide and possibly prostacyclin (PGI_2) generation.[29,30] At least some thienopyridine binding to human endothelial cells is likely to be through the $P2Y_{12}$ receptors on these cells. In a few thienopyridine-treated patients, the endothelial cell response to the thienopyridine (with or without antibody) attachment may be a combination of cell injury, excessive secretion of ULVWF multimeric strings, or apoptosis.[23]

THERAPY

All patients who develop thienopyridine-associated TTP should have prompt plasma exchange.[1,3,12,17,31,32] Plasma exchange is continued until the goals of resolution of neurologic symptoms, improvement of LDH to near normal, and achieving and maintaining for 2–3 days a platelet count of 150,000/mm^3 are achieved.[33] After this, plasma exchange may be either discontinued or reduced in frequency.[32] Among thienopyridine-associated TTP patients who have antibody-mediated ADAMTS13 deficiency, a few days of plasma exchange are required. Although patients generally recover without permanent organ damage, a spontaneous relapse occurs occasionally. Among thienopyridine-associated TTP patients without autoantibodies against ADAMTS13, several weeks of plasma exchange are often required and spontaneous relapses are rare.[3,14,17,20] One report describes a patient with a drug-eluting coronary artery stent who developed TTP within days of clopidogrel initiation. After the TTP resolved with TPE and clopidogrel discontinuation, the patient was re-challenged with ticlopidine and did not experience a TTP relapse.[34]

CONCLUSIONS

Thienopyridine-associated TTP has served as the focus of intensive scientific investigation over the past two decades.[12,14,15,17,20,22,23,26,35,36] As with idiopathic TTP, most thienopyridine-associated TTP cases are associated with autoantibodies that inhibit the plasma metalloprotease, ADAMTS13. For these individuals, TTP onset usually occurs within 2–12 weeks after initiation of ticlopidine (rarely clopidogrel) and resolves rapidly with TPE. For a minority of cases of thienopyridine-associated TTP, autoantibodies directed against ADAMTS13 metalloprotease have not been implicated. For these persons, the syndrome occurs within 2 weeks of initiating clopidogrel (rarely ticlopidine) and is less responsive to TPE. Prominent warnings in FDA-approved labels for ticlopidine and clopidogrel describe clinical findings, and the importance of timely initiation of plasma exchange for patient who develop this toxicity. The clinical, laboratory, and epidemiological evaluation and related pharmaceutical safety interventions for thienopyridine-associated TTP can serve as a template for future efforts to investigate and protect patients against harm from other severe adverse drug reactions.

DISCLOSURE

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BRIEF COMMUNICATION

No association between vitamin K epoxide reductase complex subunit 1-like 1 (*VKORC1L1*) and the variability of warfarin dose requirement in a Japanese patient population

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Introduction

Warfarin therapy management is challenging due to the greater than 10-fold interindividual variability in the therapeutic dose [1–4]. Haplotypes in the gene of vitamin K epoxide reductase complex (*VKORC1*), encoding vitamin K epoxide reductase which is a target enzyme of warfarin, have been linked to the effective maintenance dose of warfarin [5–8]. Vitamin K epoxide reductase complex subunit 1-like 1 (*VKORC1L1*) is a paralogous gene of *VKORC1* and shares about 50% amino acid identity with VKORC1 protein [9,10]. The active sites of VKORC1 are predicted to reside at the thioredoxin-like Cys132-X-X-Cys135 center embedded in the transmembrane region, and the mutagenesis at one of Cys residues lost their activity [11,12]. A recent study suggested that the complex's protein disulfide isomerase subunit provides electrons for the reduction of the Cys132-X-X-Cys135 center in VKORC1 [13]. The Cys132-X-X-Cys135 center in VKORC1 is perfectly conserved in *VKORC1L1* [9,10], suggesting the functional importance of the VKORC1L1 protein as a reductase for vitamin K recycling. However, the function of VKORC1L1 is totally unknown, including whether or not the *VKORC1L1* genotypes are associated with the variability of the effective warfarin dose [14].

The International HapMap Project has deposited the patterns of more than 1 million single nucleotide polymorphisms (SNPs) in the genome sequences drawn from four diverse human populations including Japanese, with the data now available worldwide [15]. This data offer new tools for identifying phenotype-related genes and disease-causing genes in humans.

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Table 1 Characteristics of patients administered warfarin maintenance dose

Number	87
Male/Female	62/25
Age (years)	67.60±10.60
Weight (kg)	60.20±9.40
Warfarin dose (mg/day)	3.00±1.03
Warfarin dose range (mg/day)	1.25–5.50

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

In the present study, we constructed haplotype blocks for the *VKORC1L1* gene in a Japanese population using HapMap data and investigated the influence of 8 SNPs in the haplotype blocks on the interindividual variability of warfarin dose. Our data showed that none of the SNPs and haplotypes in the *VKORC1L1* gene showed an association with the variability of the daily warfarin maintenance dose.

Materials and methods

Haplotype block construction and selection of tag SNPs

The *VKORC1L1* gene is located on chromosome 7q11.21 and ranges from 64,975,692 to 65,057,237 bp on the NCBI Build 36 assembly. HapMap data regarding the NCBI Build 36 assembly covering 200 kb length centered on the *VKORC1L1* gene for 45 normal Japanese singletons were downloaded. Haplotype blocks were constructed using Haploview version 3.32 software (http://www.broad.mit.edu/mpg/haploview/index.php) [16]. Strong linkage disequilibrium between a pair of SNPs was defined by r^2 ranging from 0.7 to 0.98. A block was created if 95% of the informative comparisons were in strong linkage disequilibrium. The block-by-block tag SNPs were selected based on Paul de Bakker's Tagger [17] with an LOD score cutoff of 3.0 and r^2 a threshold of 0.80. Frequencies of the constructed haplotype blocks and selected tag SNPs were re-confirmed using SNPAlyze v4.0 software (DYNACOM, Kanagawa, Japan).

Genotyping of selected tag SNPs in patients with warfarin dose

The study population consisted of 87 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. Anticoagulation of all patients was stably controlled with a target international normalized ratio 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. The patient group had been previously studied for the association of four genes–*VKORC1*, γ-glutamyl carboxylase, calumenin, and *CYP2C9*–with the warfarin maintenance dose [20]. In the present study, we

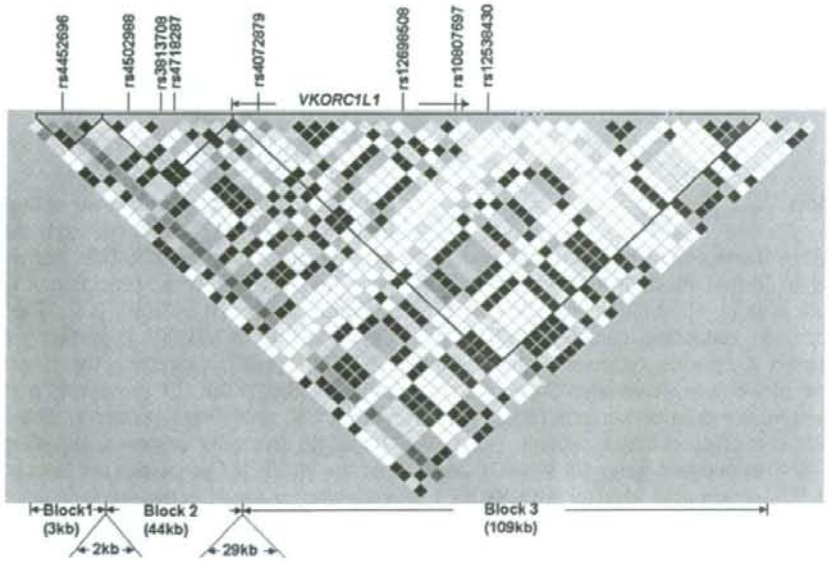

Figure 1 Haploview analysis of linkage disequilibrium structure and haplotype blocks centered on *VKORC1L1*. Pairwise linkage disequilibrium in r^2 was derived from 45 Japanese individuals (HapMap Data phase II, on NCBI Build 36 assembly). Shading represents the magnitude and significance of linkage disequilibrium, with the black-to-white gradient reflecting higher to lower linkage disequilibrium values. Three haplotype blocks were constructed covering a 200 kb length centered on the *VKORC1L1* gene. Based on the strength of linkage disequilibrium among SNP pairs, one SNP tagging the 3-kb block 1, three SNPs tagging the 44-kb block 2, and 4 SNPs tagging the 109-kb block 3 were identified. The interval length is 2 kb between blocks 1 and 2, and 29 kb between blocks 2 and 3. *VKORC1L1* is partly encompassed by block 3.

Table 2 Differences in daily warfarin dose for each SNP of the *VKORC1L1* gene

rs number	Position in reference sequence (bp)	SNP location	Genotype	Allele frequency	*n*	Mean±SD (mg/day)	*p*
rs4452696	64924147	upstream	AA	A/G	39	3.00±1.05	0.17
			AG	0.71/0.29	41	3.12±1.05	
			GG		7	2.32±0.61	
rs4502988	64935181	upstream	GG	G/A	63	2.99±1.03	0.87
			GA	0.89/0.11	24	3.03±1.07	
			AA		0	–	
rs3813708[a]	64943067	upstream	GG	G/T	60	3.07±1.05	0.44
			GT	0.83/0.17	26	2.89±0.99	
			TT		0	–	
rs4718287[b]	64944462	upstream	CC	C/G	20	3.05±1.18	0.70
			CG	0.49/0.51	40	2.90±0.95	
			GG		27	3.11±1.07	
rs4072879	65011286	intron 1	TT	T/C	47	2.96±0.90	0.93
			TC	0.76/0.24	36	3.01±1.18	
			CC		3	3.17±1.53	
rs12698508[a]	65049393	intron 1	AA	A/T	60	3.07±1.05	0.46
			AT	0.83/0.17	25	2.89±1.01	
			TT		1	1.75	
rs10807697	65053605	intron 2	AA	A/G	2	2.63±0.53	0.82
			AG	0.13/0.87	24	3.07±1.60	
			GG		61	2.99±1.05	
rs12538430[b]	65057623	3′ UTR	CC	C/T	18	3.09±1.24	0.55
			CT	0.49/0.51	40	2.87±0.93	
			TT		29	3.13±1.05	

Positions of SNPs in reference sequence are derived from NCBI Mapview Build 36. 2.
P values were calculated by one-way analysis of variance.
UTR: untranslated region. Warfarin daily maintenance dose are shown as mean±SD.
[a] SNPs were in linkage disequilibrium with $r^2=1$.
[b] These SNPs were in linkage disequilibrium with $r^2=0.99$.

genotyped 8 SNPs in *VKORC1L1*—rs4452696, rs4502988, rs3813708, rs4718287, rs4072879, rs12698508, rs10807697, and rs12538430—in the same patient group using the TaqMan-PCR system as previously described [21]. The PCR primers and probes used for the TaqMan system are available on request. We also resequenced directly the exon 1 and promoter regions of *VKORC1L1*, which were not covered by the constructed haplotype blocks, in all the patients. 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.

Statistical methods

All SNPs identified were tested for deviations from the Hardy–Weinberg disequilibrium through the use of a chi-square test. Pair-wise linkage disequilibrium between two SNPs was evaluated by r^2. Individual diplotype was estimated by means of an expectation maximization algorithm using SNPAlyze v4.0 software. The association of the genotypes and the inferred haplotypes with daily warfarin doses was examined by one-way analysis of variance. Statistical analyses were performed using Prism4 (GraphPad, San Diego, CA) and JMP V 5.1 software and the SAS release 8.2 (SAS Institute Inc., Cary, NC). *P* values lower than 0.05 were considered to be statistically significant. The association of the genotypes with daily warfarin doses was reconfirmed by quantitative associ-

Table 3 Differences in daily warfarin dose for each haplotype of the *VKORC1L1* gene

Haplotype sequence[a]	Genotype	*n*	Mean±SD (mg/day)	*p*
AGGCTAGC	homo	6	3.04±0.49	0.49
	hetero	20	2.74±1.10	
	none	58	3.06±1.06	
AGGCCAGC	homo	3	3.17±1.53	0.95
	hetero	34	2.96±1.19	
	none	47	2.98±0.91	
AGGGTAGT	homo	4	3.06±1.16	0.14
	hetero	32	3.26±1.00	
	none	48	2.79±1.03	
GGTGTTGT	homo	1	1.75	
	hetero	22	2.77±1.06	0.30
	none	61	3.04±1.03	
GAGGTAAT	homo	0	–	
	hetero	24	3.03±1.07	0.79
	none	60	2.96±1.04	

[a]For each haplotype sequence, SNPs are listed in sequential order as rs4452696, rs4502988, rs3813708, rs4718287, rs4072879, rs12698508, rs10807697, and rs12538430.
P values were calculated by one-way analysis of variance.
Warfarin daily maintenance dose are shown as Mean±SD.

```
Hs_VKORC1       MGSTWGSPGWVRLA---LCLTGLVLSLYALHVKAARARDRDYRALCDVGTAISCSR 53
Hs_VKORC1L1  MAAPVLLRVSVPRWERVARYAVCAAGILLSIYAYHVEREKERDPEHRALCDLGPWVKCSA 60
Mm_VKORC1L1  MAAPVLLRVSVPRWERVARYAVCAAGILLSIYAYHVEREKERDPEHRALCDLGPWVKCSA 60
Bt_VKORC1L1  MAAPVLLRVSVPRWERVARYAVCAAGILLSIYAYHVEREKERDPEHRALXXXGYWF---- 56
Gg_VKORC1L1        VSVPRWERVARSAVCAAGILLSLYACHLEREKGR-----ALCDLSERENCSA 47
Tr_VKORC1L1  MAAPVL-RVSTPRWERIARVLVCLLGILLSLYAPHVEREHARDPSYKALCDVSSSISCSK 59

Hs_VKORC1    VFSSRWGRGPGLVEHVLGQDSILNQSNSIPGCIFYTLQLLLGCLRTRWASVLMLLSSLVS 113
Hs_VKORC1L1  ALASRWGRGPGLLGSIPGKDGVLNQPNSVPGLIFYILQLLLGMTASAVAALILMTSSIMS 120
Mm_VKORC1L1  ALASRWGRGPGLLGSIPGKDGVLNQPNSVPGLIFYILQLLLGMTASAVAALVLMTSSIVS 120
Bt_VKORC1L1  SLFYRWGRGPGLLGSIPGKDGVLNQPNSIPGLIFYILQLLLGLTASAVAALILMTSSIMS 116
Gg_VKORC1L1  AITSRWGRGPGLLGSIPGKDSAINQSNSVPGLVFYILQMLLGMTASAVAALILMTSSIVS 107
Tr_VKORC1L1  VPGSRWGRGPGLLGSIPGNDSALNQPNSVYGIVFYAFQLLLGMTVSAMAALILMTTSIMS 119

Hs_VKORC1    LAGSVYLAWILFFVLYDFCIVCITTYAINVSLMWLSFRKVQEPQGKAKRH       163
Hs_VKORC1L1  VVGSLYLAYILYFVLKEFCIICIVTYVLNFLLLIINYKRLVYLNEAWKRQLQPKQD 176
Mm_VKORC1L1  VVGSLYLAYILYFVLKEFCIICVTTYVLNFLLLIINYKRLVYLNEAWKRQLQPKED 176
Bt_VKORC1L1  VVGSLYLAYILYFVLKEFCIICVITYVLNFILLIINYKRLVYLNEAWKRQLQPKQD 172
Gg_VKORC1L1  VVGSLYLAYILYFVLKEFCIVCVITYLLNFILFIINYKRLVYLNEAWKRQLQPKQE 163
Tr_VKORC1L1  VVGSLYLGYILYFVLKDLCVICVTTYALNFILFVLNYKRLVYLNEAWKQKLQAKQD 175
```

Figure 2 Amino acid sequence alignment of VKORC1L1 proteins from different species and from human VKORC1 protein. The proteins are labeled by their gene symbols and a prefix indicating the species (Hs, *Homo sapiens*; Mm, *Mus musculus*; Bt, *Bos taurus*; Gg, *Gallus gallus*; Tr, *Takifugu rubripes*). MultAlin (http://bioinfo.genopole-toulouse.prd.fr/multalin/) was used to perform the sequence alignment. Hyphens represent the amino acid deletion in the sequences. The active site Cys residues in the Cys132-X-X-Cys135 center of VKORC1 protein are indicated by a black background. The Cys-X-X-Cys center is highly conserved in VKORC1L1 proteins from various species.

ation test using the PLINK v 0.99p software (http://pngu.mgh.harvard.edu/purcell/plink/).

Results

Patient characteristics are summarized in Table 1. A total of 78 SNPs in the region of 200 kb centered on *VKORC1L1* in 45 normal Japanese has been retrieved from the Phase II HapMap data. Three haplotype blocks were constructed using 41 SNPs on Haploview, as shown in Fig. 1. The interval length between block 1 and 2 was 2 kb and between block 2 and 3 was 29 kb. Block 2 covered the putative promoter region of *VKORC1L1* (except for the 1.4 kb length of sequence in the upstream of exon 1), and block 3 included the region from intron 1 to exon 3 of *VKORC1L1*. Thus, three haplotype blocks covered the gene of *VKORC1L1* except for exon 1.

We identified 8 tag SNPs in three haplotype blocks, as shown in Fig. 1. One SNP, rs4452696, resided in block 1, three SNPs—rs4502988, rs3813708, and rs4718287—in block 2, and four SNPs—rs4072879, rs12698508, rs10807697, and rs12538430—were presented in block 3. Haplotype frequencies and selected tag SNPs were confirmed independently by means of Haplotype Inference analysis.

We genotyped 8 tag SNPs of *VKORC1L1* in 87 patients with warfarin dose. None of the SNPs showed genotype deviations from the Hardy–Weinberg equilibrium ($p>0.1$). Two pairs of SNPs (rs3813708 vs rs12698508, and rs4718287 vs rs12538430) were in tight linkage disequilibrium with r^2 values of more than 0.99, which were consistent with HapMap Phase II dataset. Comparisons of minor allele frequency between those found in the current study and those found in the HapMap dataset showed no significant differences ($p>0.2$).

None of the SNPs exhibited a significant association with the effective daily maintenance dose of warfarin (Table 2). These results were reconfirmed by the PLINK quantitative association test ($p>0.36$). Five haplotypes were estimated to show a frequency of more than 0.05. None of these haplotypes demonstrated a significant association with the variability of the daily warfarin maintenance dose (Table 3).

The exon 1 and part of the promoter regions of *VKORC1L1* were not covered by the constructed haplotype blocks, we sequenced these regions in all the patients, and a total of 9 SNPs were detected with minor allele frequency ranged from 0.05 to 0.15. No SNP could be detected in the coding region of exon 1. Association analysis showed that none of the detected SNPs had a significant association with the effective daily maintenance dose of warfarin ($p>0.22$, data not shown).

Discussion

The genes of *VKORC1* (encoding a warfarin target enzyme) and *CYP2C9* (encoding a warfarin metabolizing enzyme) are well established genetic factors for affecting inter-individual variability of the maintenance dose of warfarin [3]. VKORC1 protein

shows a reductase activity for conversion of vitamin K epoxide to vitamin K and vitamin K to vitamin KH2, which is essential for γ-glutamyl carboxylation of vitamin K dependent clotting factors [22]. *VKORC1L1* is a paralogous gene of *VKORC1*. Human VKORC1 and VKORC1L1 showed about 50% identity at the amino acid level, and the Cys-X-X-Cys center in VKORC1 is perfectly conserved in VKORC1L1. The amino acid sequence of VKORC1L1 among five different species showed 58% identity (102/176 residues) (Fig. 2).

In our previous study, we sequenced the entire coding regions of the *VKORC1* gene in order to identify the genetic variations and genotyped SNPs in relation to the daily warfarin maintenance dose in a Japanese population. We found that the *VKORC1* genotype can explain an approximately 0.9 mg daily interindividual difference in warfarin maintenance dose [20–23]. In the present study, we utilized the same patient group to evaluate the contribution of the tagging SNPs and the haplotypes of *VKORC1L1* for the interindividual variability of warfarin dose. However, no association of the *VKORC1L1* genotype with warfarin dose requirement was found. Two well known functional SNPs, *CYP2C9*3* and *VKORC1* 1173 C>T, might mask the effect of *VKORC1L1* genotype on the warfarin effective dose. To eliminate this possibility, we analyzed the association of the tagging SNPs and the haplotypes of *VKORC1L1* with interindividual variability of the daily warfarin maintenance dose in 67 patients who did not carry the variations of *CYP2C9*3* and *VKORC1* 1173 C>T. Unfortunately, significant association still could not be found ($p>0.13$, data not shown). These results suggested that *VKORC1L1* is not likely to be involved in the interindividual variability of the therapeutic dose of warfarin.

Tagging SNPs selected on the basis of HapMap data could be most effective only for the common potential functional sequence variations (more than 5% frequency) in the gene [24]. Resequencing the whole *VKORC1L1* gene would be imperative, and alternative ways for finding associations of rare variations (less than 5% frequency) with strong deleterious functional effects to the phenotype of warfarin dose would be necessary [25].

Summary

The *VKORC1* gene has been claimed to determine the interindividual variability in the maintenance dose of warfarin, but it is unknown whether the *VKORC1L1* gene, a paralogous gene of *VKORC1*, sharing about 50% amino acid identity with VKORC1 protein, also plays a role in the variability of warfarin dose. Our goal was to study the association of the *VKORC1L1* haplotypes with the warfarin maintenance dose. After constructing haplotype blocks covering a 200 kb length centered on the *VKORC1L1* gene using HapMap data in a Japanese population, we selected eight common tag SNPs in three haplotype blocks and genotyped them in 87 patients on stable anticoagulation with a target international normalized ratio of 1.6–2.6. None of the SNPs or haplotypes exhibited a significant association with the effective daily warfarin dose. Although the VKORC1L1 protein exhibits a significant amino acid identity with the VKORC1 protein, no association could be found between the *VKORC1L1* genotype and the variability of the warfarin dose in a Japanese patient population. The genotypes of this gene denied a role in the interindividual variability of the effects of warfarin. Further functional analysis of *VKORC1L1* is warranted.

Conflict of interest

We do not have any direct and indirect conflicts of interest.

Acknowledgements

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Original Article

Genetic Variations of *CYP2C9* in 724 Japanese Individuals and Their Impact on the Antihypertensive Effects of Losartan

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CYP2C9, a drug-metabolizing enzyme, converts the angiotensin II receptor blocker losartan to its active form, which is responsible for its antihypertensive effect. We resequenced *CYP2C9* in 724 Japanese individuals, including 39 hypertensive patients under treatment with losartan. Of two novel missense mutations identified, the Arg132Gln variant showed a fivefold lower intrinsic clearance toward diclofenac when expressed in a baculovirus-insect cell system, while the Arg335Gln variant had no substantial effect. Several known missense variations were also found, and approximately 7% of the Japanese individuals (53 out of 724) carried one of the deleterious alleles (*CYP2C9*3*, **13*, **14*, **30*, and Arg132Gln) as heterozygotes. After 3 months of losartan treatment, systolic blood pressure was not lowered in two patients with *CYP2C9*1/*30*, suggesting that they exhibited impaired *in vivo* CYP2C9 activity. *CYP2C9*30* might be associated with a diminished response to the antihypertensive effects of losartan. (*Hypertens Res* 2008; 31: 1549–1557)

***Key Words*: CYP2C9, single nucleotide polymorphism, hypertension, losartan**

Introduction

CYP2C9, a major isoform of the cytochrome P450 superfamily, accounts for approximately 20% of the total cytochrome P450 protein in liver microsomes and is responsible for the oxidative metabolism of up to 15% of drugs that undergo phase I metabolism (*1*, *2*). About 30 nonsynonymous variations of CYP2C9 have been identified. Of these, the effects of *CYP2C9*2* (Arg144Cys) and *CYP2C9*3* (Ile359Leu) have been well studied for their reduced metabolic activities towards substrates such as warfarin, tolbutamide, and losar-

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tan, both *in vitro* and *in vivo* (*3*, *4*). The allelic frequencies for these deleterious variations differ considerably among different ethnic populations. In Caucasian populations, the frequencies of *CYP2C9*2* and *CYP2C9*3* were 8–14% and 4–16%, respectively (*5*). In contrast, *CYP2C9*2* was not present in Asian populations, and *CYP2C9*3* was present in only 1–4% of Asian populations. Therefore, interethnic variability reported in the pharmacokinetics and pharmacodynamics of drugs, metabolized mainly by CYP2C9, could not be fully explained by the common variants alone. Recently, a number of novel nonsynonymous variations of CYP2C9 have been identified in different Asian populations (*6–11*). Functional analysis of these variations *in vitro* indicated the existence in Asians of new deleterious alleles of CYP2C9 that might have clinical relevance.

Losartan, the first selective angiotensin II receptor antagonist, was reported to significantly reduce the risk of cardiovascular endpoint outcomes compared with atenolol in high-risk hypertensive patients with left ventricular hypertrophy (*12*). Large interindividual variations in the efficacy and toxicity of losartan have been reported, and it has been suggested that they are genetically determined. A relationship was suggested between the polymorphism in the receptor gene, *AGT1R*, and its humoral and renal hemodynamic responses (*13*). However, losartan is oxidized primarily by CYP2C9 to an active carboxylic acid metabolite, E-3174, which has higher potency and a longer half-life than losartan and is therefore responsible for most of the antihypertensive effects (*14*, *15*). The effects of *CYP2C9*2* and *CYP2C9*3* on losartan oxidation have been extensively studied both *in vitro* and *in vivo*, consistently demonstrating the functional defect of the *CYP2C9*3* allele in decreasing the oxidation of losartan (*16–20*). However, the clinical relevance of genotypes of *CYP2C9* to the variable blood pressure–lowering responses to losartan in hypertensive patients has not been fully clarified. Furthermore, it remains unknown whether the other deleterious CYP2C9 alleles in Asians (*6–11*) might lead to the phenotypes of impaired therapeutic responses to this drug.

We studied several genes responsible for essential hypertension and interindividual differences in responses to warfarin and antihypertensive drugs (*21*, *22*). To identify the functional mutations, we resequenced some candidate genes including *WNK4*, *SCNN1B*, *SCNN1G*, *NR3C2*, and *RGS2* for hypertension (*23–26*) and *VKORC1*, *GGCX*, and *CALU* for warfarin (*22*, *27*). In the course of this resequencing, we noticed that the deleterious mutations are present more frequently than we expected, and the rare mutations with deleterious function would increase the total phenotype change.

In the present study, we resequenced the *CYP2C9* in 724 Japanese individuals. Two novel missense mutations were functionally analyzed in the baculovirus/insect cell expression system with diclofenac as a substrate. Furthermore, we assessed the blood pressure–lowering responses to losartan in hypertensive patients with the deleterious mutations in *CYP2C9*.

Methods

Subjects

Seven hundred twenty-four Japanese subjects in this study were enrolled for genetic sequencing of *CYP2C9*. The study subjects consisted of 312 patients with stroke and 412 patients with hypertension. Stroke patients (87 females and 225 males; average age: 65.36±11.87 years; body mass index: 23.28±3.01 kg/m^2) were admitted to the Cerebrovascular Division of the National Cardiovascular Center (*22*, *28*). They had all experienced an ischemic stroke within 7 d prior to admission. Hypertensive patients (196 females and 216 males; average age: 64.83±10.42 years; body mass index: 24.55±3.69 kg/m^2) were recruited from the outpatients clinic in the Division of Hypertension and Nephrology at the National Cardiovascular Center (*23–26*, *29*). Hypertension was defined as systolic blood pressure >140 mmHg, diastolic blood pressure >90 mmHg, or the current use of antihypertensive medication. Ninety-three percent of the study subjects (382 subjects) were diagnosed with essential hypertension, and the rest had secondary hypertension, including renal hypertension (10 subjects), renovascular hypertension (9 subjects), primary aldosteronism (7 subjects), and others (4 subjects).

Sixty-nine essential hypertensive patients (30 females and 39 males; average age: 64.36±9.34 years; body mass index: 22.65±7.84 kg/m^2) were taking one of three angiotensin II receptor blockers (losartan, candesartan, and valsartan) for treatment of hypertension. Among them, 39 patients had been receiving 50 mg/d of losartan for more than 3 months. We evaluated the patients' average resting blood pressure measured on three consecutive outpatient clinic visits, before and after losartan treatment.

The study was approved by the Ethics Review Committee of the National Cardiovascular Center, and only those subjects who provided written informed consent for genetic analyses were included in the study.

Resequencing of *CYP2C9* in 724 Japanese Subjects

Whole blood was collected from each participant, and genomic DNA was extracted from peripheral blood leukocyte. From each subject, 687 base pairs of the promoter region, all exons and intron-exon junctions, and the 3′-UTR of *CYP2C9* were amplified and sequenced directly on both strands using an ABI 3730 Automated Sequence Analyzer (Applied Biosystems, Foster City, USA), as described previously (*27*, *30*). Primers were designed to be specific to *CYP2C9*, with particular attention being paid to avoid amplification of sequences from homologous genes (*cf.* Online Table 1). The obtained sequences were examined for the presence of variations using Namihei software (Mitsui Knowl-

edge Industry Co., Ltd., Japan) and Sequencher software (Gene Codes Corporation, Ann Arbor, USA), followed by visual inspection. Novel nonsynonymous single nucleotide polymorphisms (SNPs) were confirmed by sequencing of PCR products generated from new genomic DNA amplifications. The genomic and cDNA sequences of *CYP2C9*, obtained from GenBank (NC_000010.8 and NM 000771.2, respectively), were used as reference sequences. The A of ATG of the initiator Met codon was denoted as nucleotide +1, and the initial Met residue was denoted as amino acid +1. The identified missense mutations were mapped in the human CYP2C9 crystal structure bound with warfarin (*31*) by the PyMOL v0.99 molecular visualization system (DeLano Scientific LLC, San Carlos, USA).

Cloning, Site-Directed Mutagenesis and Vector Constructions

A full-length human NADPH-cytochrome P450 oxidoreductase (OR) cDNA was isolated by PCR from human adult normal liver Quick-Clone cDNA (Clontech, Palo Alto, USA) with the forward primer, 5′-CACCAGTTTCATGATCAA CATGGG-3′, and the reverse primer, 5′-GCCCCTAGCTCC ACACGTCC-3′. The underlined sequence was introduced to the directional TOPO cloning system. The PCR products were cloned directly into the pcDNA3.1D/TOPO vector (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions (pcDNA3.1D/OR). Two single *CYP2C9* variations, 3573 G>A (Arg132Gln) and 42543 G>A (Arg335Gln), were introduced into the wild-type plasmid (pcDNA3.1D/ CYP2C9/Wild-type) as a template using a QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, USA). The primer sequences used for the construction of variant plasmids were as follows: 5′-CTCCCTCATGACGCTGCA GAATTTTGGGATGG-3′ (sense) and 5′-CCATCCCAA AATTCTGCAGCGTCATGAGGGAG-3′ (antisense) for pcDNA3.1D/CYP2C9/ Arg132Gln. 5′-TGATTGGCAGAA ACCAGAGCCCCTGCATGCA-3′ (sense) and 5′-TGCATG CAGGGGCTCTGGTTTCTGCCAATCA-3′ (antisense) for pcDNA3.1D/CYP2C9/ Arg335Gln.

The position of the exchanged nucleotide is underlined and in boldface. To ensure that no errors had been introduced during amplification, the entire cDNA regions were confirmed by sequencing the plasmid construct. Both OR and CYP2C9 wild-type or variant cDNAs were subcloned into the baculovirus transfer vector, pFastBac Dual (Invitrogen), 3′ of the P10 promoter, and the polyhedron promoter (polh), respectively (pFastBac Dual/P10.OR/ polh.CYP2C9). Recombinant baculoviruses carrying both CYP2C9 and OR cDNAs were produced according to the Bac-to-Bac Baculovirus Expression system protocol of Invitrogen.

Expression of Recombinant Proteins in Insect Cells and Preparation of Microsomal Fractions

For the expression of recombinant proteins using the baculovirus expression systems, adherent *Spodoptera frugiperda* (*Sf21*) insect cells (3.7×10^8 cells per 225 cm^2 flask) were infected with recombinant baculoviruses at a multiplicity of infection of 4 in supplemented form of Grace's Insect Medium (Invitrogen) with 10% fetal bovine serum and 10 μg/mL gentamycin. At 16–24 h post-infection, the culture media were supplemented with 0.2 mmol/L ferric citrate and 0.3 mmol/L δ-aminolevulinic acid, and the cells were harvested at 72-h post-infection. Microsomal fractions from *Sf21* cells were prepared as described previously (*11*).

Characterization of Protein Expression

The cytochrome P450 content in insect cell microsomes was measured by reduced CO-spectrum using the method of Omura and Sato (*32*). NADPH-cytochrome P450 OR activity in insect cell microsomes was measured using cytochrome C as a substrate as described by Phillips and Langdon (*33*). The molar amount of OR was calculated based on an assumed specific activity of 3.0 μmol cytochrome C reduced/min/nmol purified human OR (*34*). Western blotting of CYP2C9 and OR was performed using 2 μg of microsomal protein from insect cells as described previously (*11*). For immunostaining of OR, goat anti-rat OR antiserum (diluted 1:1,000; Daiichi Pure Chemical Co., Tokyo, Japan) and horseradish peroxidase–conjugated rabbit anti-goat IgG (diluted 1:20,000; Jackson ImmunoResearch Laboratories, West Grove, USA) were used as the first and second antibodies, respectively.

Assay for CYP2C9-Mediated Enzymatic Activity

CYP2C9 activities for the wild-type and two variants were assessed by diclofenac 4′-hydroxylation as described previously (*11*) except that the incubation mixture contained diclofenac (1.0–100 μmol/L), 5 pmol of P450 from insect microsomes, 10 pmol of purified cytochrome b5 (Oxford Biomedical Research, Oxford, UK), and an NADPH regenerating system (1.3 mmol/L $NADP^+$, 3.3 mmol/L glucose 6-phosphate, 3.3 mmol/L $MgCl_2$ and 0.4 unit/mL glucose-6-phosphate dehydrogenase), and the reactions were allowed to proceed for 10 min. The initial mobile phase of high-performance liquid chromatography consisted of 70% of a 30% acetonitrile solution containing 1 mmol/L perchloric acid (A) and 30% of methanol (B) and was delivered for 5 min, after which a 20 min linear gradient from 30% to 100% of B was formed at a flow rate of 1 mL/min. Under these conditions, the retention times of 4′-hydroxydiclofenac, 5-hydroxydiclofenac, and diclofenac were 14.2, 14.7, and 19.6 min, respectively.

Table 1. Genetic Variants in *CYP2C9* Identified in 724 Japanese Individuals

SNP position[a]	SNP position[b]	Location	Nomenclature[c]	Amino acid change	Number of subjects			Minor allele frequency	Flanking sequences (5′ to 3′)	rs ID No.	Reference
					Wild-type	Heterozygote	Homozygote				
−251 C>A[d]	−251	promoter			723	1	0	0.0007	ttattaccaata[C>A]ctaggctccaac		
−162 A>G	−162	promoter			723	1	0	0.0007	cattttatttttt[A>G]tctgtatcagtg		(27)
251 T>C	IVS1 + 83	Intron 1			716	7	1	0.0062	cctagaggtaca[T>C]gttacaagaggt	rs9332104	
3136 T>C[d]	IVS1 − 40	Intron 1			722	2	0	0.0014	aaatggacaaaa[T>C]agtaacttcgtt		
3154 T>C	IVS1 − 22	Intron 1			723	1	0	0.0007	cttcgtttgctg[T>C]tatctctgtcta		(11)
3235 G>A	228	Exon 2		Val76	706	18	0	0.0124	acccatagtggt[G>A]ctgcatggatat	rs17847036	
3276 T>C	269	Exon 2	*CYP2C9*13*	Leu90Pro	722	2	0	0.0014	ccctgattgatc[T>C]tggagaggagtt		(6)
3411 T>C	IVS2 + 73	Intron 2			712	11	1	0.0090	gacttacagagc[T>C]cctcgggcagag	rs9332120	
3451 G>A[d]	IVS2 − 59	Intron 2			723	1	0	0.0007	tggctgcccagt[G>A]tcagcttcctct		
3455 G>C[d]	IVS2 − 55	Intron 2			723	1	0	0.0007	tgcccagtgtca[G>C]cttcctctttct		
3488 G>T[d]	IVS2 − 22	Intron 2			723	1	0	0.0007	atctccctccta[G>T]tttcgtttctctt		
3514 T>C	336	Exon 3		Ile112	721	3	0	0.0021	tgttaggaat[T>C]gtttttcagca		(11)
3544 G>A[d]	366	Exon 3		Glu122	723	1	0	0.0007	gaaatggaagga[G>A]atccggcgtttc		
3552 G>A	374	Exon 3	*CYP2C9*14*	Arg125His	723	1	0	0.0007	aggagatccggc[G>A]tttctccctcat		(7)
3573 G>A[d]	395	Exon 3		Arg132Gln	723	1	0	0.0007	tcatgacgctgc[G>A]gaattttgggat		
3627 G>T	449	Exon 3	*CYP2C9*27*	Arg150Leu	721	3	0	0.0021	aagaggaagccc[G>T]ctgccttgtgga		(11)
9032 G>C	IVS3 − 65	Intron 3			592	126	6	0.0953	ctactattatct[G>C]ttaacaaataca	rs9332127	
10411 A>G[d]	IVS4 − 15	Intron 4			723	1	0	0.0007	atttaataaatt[A>G]ttgttttctctt		
33553 A>G[d]	951	Exon 6		Pro317	723	1	0	0.0007	gctgaagcaccc[A>G]gaggtcacaggt		
42543 G>A[d]	1004	Exon 7		Arg335Gln	722	2	0	0.0014	ttggcagaaacc[G>A]gagccccctgcat		
42614 A>C	1075	Exon 7	*CYP2C9*3*	Ile359Leu	677	47	0	0.0325	gtccagagatac[A>C]ttgaccttctcc	rs1057910	
42676 T>C	1137	Exon 7		Tyr379	714	10	0	0.0069	attcagaaacta[T>C]ctcattcccaag		(11)
47377 T>C[d]	1176	Exon 8		Thr392	723	1	0	0.0007	aatttccctgac[T>C]tctgtgctacat		
50298 A>T	1425	Exon 9		Gly475	678	46	0	0.0319	agttgtcaatgg[A>T]tttgcctctgtg	rs1057911	
50302 G>A	1429	Exon 9	*CYP2C9*30*	Ala477Thr	722	2	0	0.0014	gtcaatggattt[G>A]cctctgtgccgc		(11)
50369 C>T[d]	1496 (*23[e])	3′-UTR			723	1	0	0.0007	atggcctggctg[C>T]tgctgtgcagtc		
50378 A>G[d]	1505 (*32[e])	3′-UTR			722	2	0	0.0014	ctgctgctgtgc[A>G]gtccctgcagct		
50456 C>T[d]	1583 (*110[e])	3′-UTR			721	3	0	0.0021	cctgtcatctca[C>T]attttcccttcc		
50613 T>C[d]	1740 (*267[e])	3′-UTR			722	2	0	0.0014	ttgagttattaa[T>C]atgttattatta		
50614 AT>—	1741_1742 (*268_*269[e])	3′-UTR			721	3	0	0.0021	tgagttattaat[AT>—]gttattattaaa		(7)
50742 T>A	1835+34[f] (*396[e])	3′ flanking			686	38	0	0.0263	ttcttttatgca[T>A]aatgtaggtcag	rs9332245	

[a]The A of the ATG of the initiation Met codon is denoted as nucleotide +1. [b]From the translational initiation site or from the end of the nearest exon. [c]Nomenclature for *CYP2C9* allele cited from: http://www.cypalleles.ki.se/cyp2c9.htm [d]Novel mutations identified in this study. [e]The nucleotide following the translation termination codon TGA is numbered *1. [f]The first nucleotide downstream of the 3′-end of exon 9 is numbered +1.

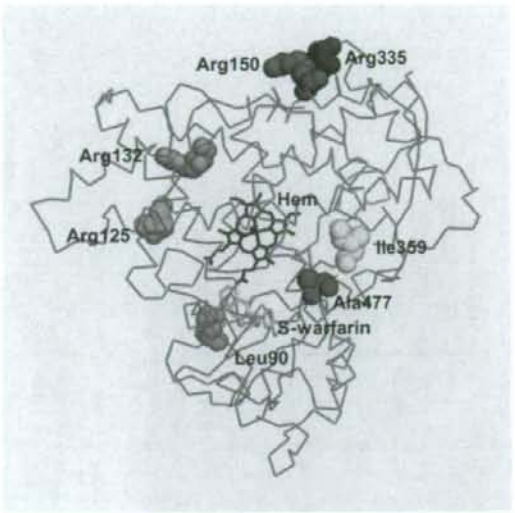

Fig. 1. *Mapping of identified missense variations on the crystal structure of human CYP2C9 protein bound with warfarin (PDB: 10G5). Hem and S-warfarin are shown by red and pink, respectively. The seven missense mutations identified in this study are presented by a space-filling model.*

Statistical Analysis

All SNPs identified were tested for deviations from the Hardy-Weinberg disequilibrium through the use of a χ^2 test. Pairwise linkage disequilibrium (LD) between two SNPs was evaluated by r^2 using SNPAlyze version 4.0 software (DYNACOM Co., Ltd., Mobara, Japan). Kinetic parameters K_m and V_{max} were estimated using a software program designed for non-linear regression analysis of a hyperbolic Michaelis-Menten equation (Prism v.3.0a, GraphPad Software, San Diego, USA). Kinetic data are presented as the mean±SD for three microsomal preparations derived from separate transfections for each variant and analyzed by one-way analysis of variance. Multiple comparisons were made with the Scheffe test.

Results

Resequence of *CYP2C9* in 724 Japanese Subjects

Upon sequencing the *CYP2C9* in 724 Japanese subjects, we identified a total of 31 genetic variations, including 15 novel ones (Table 1). All of the detected variations (except for the SNPs of 251 C>A in intron 1 and 3411 T>C in intron 2) were in Hardy-Weinberg equilibrium for two separate groups ($p \geq 0.81$ in stroke patients and $p \geq 0.82$ in hypertensive patients) and for all subjects ($p \geq 0.66$). Since we did not find any significant differences in frequencies between the stroke patients and the hypertensive patients ($p > 0.05$ by χ^2 test or Fisher's exact test), the data for all subjects were analyzed as one group.

Fourteen variations (seven missense and seven synonymous ones) were identified in the coding regions of CYP2C9. Two out of the seven missense mutations were novel, including Arg132Gln in one hypertensive patient and Arg335Gln in two stroke patients. The other five known missense mutations, Ile359Leu (*CYP2C9*3*), Leu90Pro (*CYP2C9*13*), Arg125His (*CYP2C9*14*), Arg150Leu (*CYP2C9*27*), and Ala447Thr (*CYP2C9*30*), were found in 47, 2, 1, 3, and 2 individuals, respectively. All the missense mutations were heterozygous, and there were no compound heterozygotes. The positions of seven missense mutations on the crystal structure of human CYP2C9 bound with warfarin are shown in Fig. 1.

Seven synonymous variations were identified, of which three novel ones (Glu122Glu; $n=1$, Pro317Pro; $n=1$, and Thr392Thr; $n=1$) were found as single heterozygotes. In the putative promoter region, two variants (−251 C>A and −162 A>G) (*35*) were detected, each in only one individual. A total of 15 variations were found in the intronic, 3′-UTR, and 3′-flanking regions. Five novel variations in introns 1, 2, and 4 and four novel variations in the 3′-UTR were identified with allele frequencies less than 0.01.

LD analysis showed that *CYP2C9*3* was in LD ($r^2 > 0.8$) with two variations, 50298 A>T (Gly475Gly) in exon 9 and 50742 T>A in the 3′-flanking region. LD ($r^2 = 0.7$) was also noted between two intronic variants, 251 T>C in intron 1 and 3411 T>C in intron 2.

Functional Characterization of Two Novel Missense Mutations

To functionally characterize the two novel missense mutations, Arg132Gln and Arg335Gln, the wild-type and two CYP2C9 variants were coexpressed with NADPH-cytochrome P450 OR in *Sf21* insect cells. The holo-CYP2C9 content was not significantly different between the wild-type and variants: 188.6±22.9 pmol/mg microsomal protein for wild-type, 192.3±14.5 pmol/mg microsomal protein for Arg132Gln, and 159.3±5.5 pmol/mg microsomal protein for Arg335Gln, as determined on three lots from independent expression experiments. Quantities of cytochrome P420 were negligible for all preparations (data not shown). Cytochrome C reductase activities varied slightly but were not significantly different among the preparations (632–808 nmol cytochrome C reduced/min/mg protein), and the mean OR/CYP2C9 molar ratios in microsomal fractions were calculated to be 1.2, 1.3, and 1.6 for wild-type, Arg132Gln, and Arg335Gln, respectively.

Immunoblot analyses of CYP2C9 and OR were performed using insect cell microsomes, and representative data from three independent preparations are shown in Fig. 2. Quantitative analysis revealed that neither apo-CYP2C9 nor OR pro-

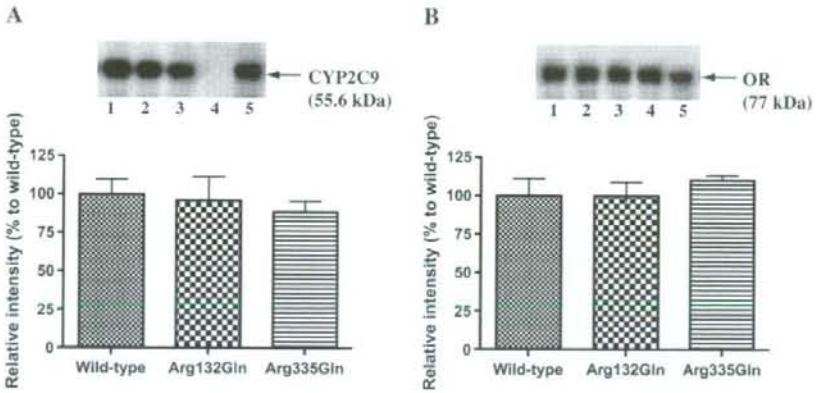

Fig. 2. *Expression of wild-type and two variants of CYP2C9 in insect cell microsomes. Representative Western blots of immunoreactive CYP2C9 (A) and OR (B) proteins (upper) are shown. Lanes 1–3: co-expressed microsomes containing wild-type, Arg132Gln, and Arg335Gln CYP2C9 each with OR; lane 4: microsomes containing solely OR; lane 5: commercially available co-expressed supersomes containing CYP2C9.1 and OR (BD Bioscience, San Jose, USA). Relative intensities of immunoreactive CYP2C9 (A) and OR (B) protein are shown in the lower panels. Each bar represents the mean±SD of three separate experiments.*

Table 2. Kinetic Parameters for Hydroxylation Activities of Wild-Type and Variant CYP2C9 against Diclofenac

Amino acid alteration	K_m (μmol/L)	V_{max} (pmol/min/pmol P450)	Clearance (V_{max}/K_m) (μL/min/pmol P450)
Wild-type	3.4±0.17	79.8±6.6	23.4±0.81
Arg132Gln	1.8±0.05**	7.8±0.4**	4.2±0.31**
Arg335Gln	3.0±0.10*	65.4±2.1*	22.0±0.06*

*$p<0.05$, **$p<0.0001$ *vs.* wild-type. One-way analysis of variance, post-hoc test: Scheffe. Data are represented by means±SD.

tein expression levels were significantly different among the wild-type and two variants ($p=0.77$ for CYP2C9, $p=0.64$ for OR). Catalytic activities of the wild-type and variant (Arg132Gln and Arg335Gln) proteins were assessed using diclofenac as a substrate. Diclofenac 4′-hydroxylation exhibited typical hyperbolic kinetic profiles in both the wild-type and variant proteins (data not shown). The kinetic parameters are summarized in Table 2. The Arg132Gln protein showed a 90% decrease in the V_{max} value and a partial decrease in the K_m value, resulting in fivefold lower intrinsic clearance relative to the wild-type (Table 2). A slight diminution in intrinsic clearance (6%) was observed for the Arg335Gln protein with slightly decreased K_m and V_{max} values (Table 2). The formation of 5-hydroxy diclofenac was observed in neither the wild-type nor variant (Arg132Gln and Arg335Gln) proteins (data not shown), suggesting that these substitutions do not alter the regioselectivity of diclofenac hydroxylation.

CYP2C9 Polymorphisms and the Effectiveness of Losartan in 39 Hypertensive Patients

Among 39 patients taking losartan, 34 patients carried the wild genotype of *CYP2C9*1/*1*, and the other 5 patients carried missense mutations, including *CYP2C9*1/*3* in 2 patients, *CYP2C9*1/*30* in 2 patients, and Arg132Gln mutation in one patient. The changes in systolic and diastolic blood pressure with respect to genotypes at 3 months of losartan treatment are presented in Table 3. Losartan obviously lowered systolic blood pressure in 2 patients with *CYP2C9*3* and in a patient with the Arg132Gln mutation. However, losartan was not effective in 2 patients with *CYP2C9*1/*30*.

Discussion

In the present study, the large-scale direct resequencing effort of the *CYP2C9* allowed us to detect 31 genetic variations in 724 Japanese individuals. We also obtained accurate frequencies of the known variations, *CYP2C9*3*, **13*, **14*, **27* and **30*, that are specific to Asians, except for **3*. As for the novel alleles, Arg132Gln and Arg335Gln, their effects on both protein expression levels and enzymatic activity were assessed using a baculovirus expression system.

The most frequently identified missense mutation in the present study was *CYP2C9*3* (Ile359Leu), with a frequency

Table 3. Patient Characteristics and Blood Pressure Response to Losartan with Respect to Genotypes: Essential Hypertensive Patients Taking Losartan

	CYP2C9 genotype					
	**1/*1*	**1/*3*		**1/*30*		Arg132Gln
Case number	34	2		2		1
Sex (male/female)	21/13	0/2		2/0		1/0
Age (years)	65.10±7.04	70	67	77	71	70
BMI (kg/m^2)	25.10±3.07	21.47	24.20	24.33	25.59	20.7
SBP						
At baseline (mmHg)	151.10±14.75	130[a]	156	155	172	157
At 3 month (mmHg)	142.80±16.23	119	141	151	173	128
Change (mmHg)	−8.70±14.35	−11	−15	−4	1	−29
DBP						
At baseline (mmHg)	88.80±9.26	71[a]	104	81	98	82
At 3 month (mmHg)	84.90±9.98	75	96	83	95	70
Change (mmHg)	−4.20±6.91	4	−8	2	−3	−12

Values are mean±SD. BMI, body mass index; SBP, DBP, systolic and diastolic blood pressures. [a]Office blood pressure in this patient with *CYP2C9 *1/*3* was 130/71 mmHg. Losartan was prescribed because this patient had higher home SBP (over 150 mmHg).

of 0.033, which was in good agreement with the previously published results in Japanese populations (*11*, *36*, *37*). The frequency of *CYP2C9*13* (Leu90Pro), 0.0014 in the present study, was comparable to that recently reported in a Japanese population (*11*) but much lower than those in previous studies of other Asian populations (*6*, *9*). *CYP2C9*13* was first identified in a Chinese individual who showed poor metabolizer phenotype for both lornoxicam and tolbutamide (*6*). Functional analysis of the *CYP2C9*13* protein showed decreased enzymatic activity for tolbutamide and diclofenac (*10*). Another recently published allele, *CYP2C9*14* (Arg125His), was detected in an individual in the present study. This allele was first identified in an Indian patient, and the variant protein exhibited 80–90% lower catalytic activity toward tolbutamide (*7*, *8*). *CYP2C9*27* (Arg150Leu) and **30* (Ala477Thr), both detected recently in a Japanese population (*11*), were also identified in 3 and 2 individuals in the present study, respectively. The *in vitro* study revealed that the *CYP2C9*30* protein had a twofold higher K_m value and a threefold lower V_{max} value than the wild-type towards diclofenac, whereas the catalytic activity of the *CYP2C9*27* protein was similar to the wild-type (*11*).

The novel Arg132Gln variant exhibited a 90% decrease in the V_{max} value toward diclofenac 4′-hydroxylation (Table 2). Arg132 is located in a loop region between the C and D helices (Fig. 1) and is highly conserved in the CYP2C family (http://drnelson.utmem.edu/humP450.aln.html). Arg133, the corresponding residue of CYP2B4, is suggested to play a prominent role in binding its redox partners, cytochrome b5 and P450 reductase (*38*). Accordingly, the loss of catalytic activity of the Arg132Gln variant might reflect the altered affinity of variant protein to these redox partners due to electrostatic changes as proposed for **2* (Arg144Cys), **14* (Arg125His), and **26* (Thr130Arg) (*8*, *11*, *39*).

The Arg335Gln variant showed a similar holo-CYP2C9 content to wild-type in insect cell microsomes. Furthermore, the intrinsic clearance of the Arg335Gln variant was only slightly lower than that of the wild-type. In contrast to Arg335Gln, a substitution in the same position, Arg335Trp (**11*), was reported to exhibit a threefold increase in K_m and more than a twofold decrease in the intrinsic clearance for tolbutamide when expressed in a bacterial cDNA expression system (*40*). In addition, catalytically active *CYP2C9*11* holo protein was expressed at a very low level due to its decreased stability in insect cells (*41*). To confirm whether or not the protein stability of the Arg335Gln variant might be influenced by the *in vitro* expression system used, the wild-type and variant proteins were expressed in a mammalian expression system using COS-1 cells. The protein expression level of Arg335Gln variant in COS-1 microsomes was decreased by only 30% compared with that of the wild-type (data not shown), indicating that the protein stability of the Arg335Gln product was not substantially different between mammalian expression systems and baculovirus/insect cell systems. Thus, the substituted residues (Trp *vs.* Gln) at this position might quite differently influence the stability of protein as well as catalytic activities.

Thirty-nine patients were taking losartan, which is known to exhibit considerable inter-individual variation in its antihypertensive effects. Losartan is primarily oxidized by CYP2C9 to an active carboxylic acid metabolite, E-3174 (*14–16*). CYP3A4 also plays a limited role in the metabolic activation of losartan *in vitro*; however, its significance *in vivo* has not been demonstrated (*3*, *15*, *16*). We evaluated the impact of *CYP2C9* variations on the antihypertensive effect of losartan based on the patients' average resting blood pressure measured before and three months after losartan treatment.

Two Japanese hypertensive patients carrying the *CYP2C9*3* heterozygous allele showed lowered systolic blood pressure by losartan (Table 3). This is in line with the previous report that no significant differences in the pharmacokinetics of losartan and E-3174 were observed between *CYP2C9*1/*3* and **1/*1* (*42*). Contrary to our result, a Danish prospective study of optimal monotherapy with losartan in type 1 diabetic patients with nephropathy showed that the reduction in systolic 24 h blood pressure was significantly greater in wild-type patients ($n=48$) than in *CYP2C9*3* carriers ($n=12$) (*43*). Furthermore, similar changes in diastolic and systolic 12 h blood pressures were also observed between *CYP2C9*1/*1* ($n=4$) and **1/*3* ($n=3$) Japanese patients (*20*). The role of heterozygous *CYP2C9*3* in the blood pressure–lowering response to losartan in hypertensive patients should be further studied in a large cohort of patients.

Inconsistent with our *in vitro* study, systolic blood pressure in a patient with Arg132Gln was obviously lowered by losartan (Table 3). For this variation, the substrate-dependent differences between diclofenac and losartan oxidation are unlikely because Arg132 might interact with redox partners but not with substrates as described above. However, the change in enzymatic activity toward losartan should be further analyzed.

However, losartan was not effective in 2 patients carrying the heterozygous *CYP2C9*30* (Ala477Thr) allele. A serious impact on the pharmacodynamics of losartan was not demonstrated statistically because of the small sample size of individuals with **30*. Ala477 is located in the substrate recognition site-6 region in the β2 sheet, which shows very strong hydrophobic interactions with the substrates (*44*), suggesting the importance of this residue in metabolic activity of CYP2C9 toward various substrates. Therefore, insufficient conversion of losartan to E-3174 by this defective mutation might be responsible for the therapeutic failure of these patients. Pharmacokinetic analysis of *CYP2C9*30* towards losartan would be necessary to further elucidate its clinical relevance.

In conclusion, multiple rare functional variations of *CYP2C9* were detected in a Japanese population. Approximately 7% of the Japanese individuals analyzed (53 of 724) carried one of the functionally deleterious alleles (*CYP2C9*3*, **13*, **14*, **30*, and Arg132Gln). In addition to *CYP2C9*3*, *CYP2C9*30* might also be used for determining inter-individual responses to losartan treatment in Japanese hypertensive patients.

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ADAMTS13 P475S polymorphism causes a lowered enzymatic activity and urea lability *in vitro*

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von Willebrand factor (VWF) is a plasma glycoprotein synthesized primarily in vascular endothelial cells and megakaryocytes [1]. VWF is released into plasma as ultra-large multimeric forms that are highly active in platelet aggregation. A plasma metalloprotease ADAMTS13 specifically cleaves the Tyr^{1605}-Met^{1606} peptidyl bond within the A2 domain of VWF [2]. Deficiency of the ADAMTS13 enzymatic activity, caused by genetic mutations or acquired autoantibodies against ADAMTS13, results in the accumulation of ultra-large VWF multimers in plasma that lead to the hyper-aggregation of platelets. This prothrombotic condition can cause thrombotic thrombocytopenic purpura (TTP) [3]. A number of non-synonymous mutations and polymorphisms of ADAMTS13 have been identified [4,5]. Among them, the P475S (c.1423C > T) polymorphism is noteworthy.

The allele frequency of ADAMTS13 P475S was 5.1% in Japanese subjects [6], 4.0% in Koreans [7], and 1.5% in Chinese [8], but absent in Caucasians [9]. The recombinant P475S mutant was normally secreted from cultured cells but showed greatly reduced enzymatic activity (~10%) in the VWF-multimer assay [6]. Recently, we have developed a quantitative ADAMTS13 activity assay using a synthetic fluorogenic substrate FRETS-VWF73 [10] and it is now widely utilized [11–14]. The assay can be performed in the absence of urea that is required for the VWF-multimer assay. In this study, we evaluated the activity of the P475S mutant using FRETS-VWF73 and found that the mutant exhibited the more profound loss of activity than the wild-type in the presence of urea.

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We have prepared four forms of recombinant ADAMTS13 using a transient expression system of HeLa cells: the full-length form (Met^{1}-Thr^{1427}) with the wild-type sequence (FL) or with the P475S polymorphism (FL-P475S), and the C-terminally-truncated forms (Met^{1}-Ala^{685}) with the wild-type sequence (MDTCS) or with the P475S polymorphism (MDTCS-P475S). FL and FL-P475S were tagged by the C-terminal FLAG sequence. MDTCS and MDTCS-P475S were tagged by the C-terminal 6xHis sequence. The culture media of cells expressing FL and FL-P475S were collected and concentrated 5-fold by centrifugal filtration (Ultrafree-MC; Millipore, Billerica, MA, USA). The media of cells expressing MDTCS and MDTCS-P475S were collected, and the recombinant proteins were purified by the Ni-NTA column chromatography. The relative amounts of the recombinant proteins were adjusted by their band intensities of Western blotting analysis using anti-FLAG or anti-6xHis antibodies, within the linear detection range. Enzymatic activity of each protein was measured by the FRETS-VWF73 assay [10] in the absence or presence of urea.

First, we compared the enzymatic activities between FL and FL-P475S in the absence of urea, and found that FL-P475S showed 71 ± 3.6% (n = 3) activity of FL. We previously reported that FL-P475S showed only approximately 10% activity of FL in the VWF-multimer assay [6]. This paradoxical observation led us to further study in order to understand the effect of P475S. We hypothesized that 1.5-M urea in the VWF-multimer assay [15] may affect the FL-P475S activity more severely than FL. Then, we added a series concentration of urea up to 2 M (final concentration) in the FRETS-VWF73 assay and measured the activity. Addition of urea inhibited the enzymatic activity of FL-P475S more severely than that of FL.