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′-CTCCCTCATGACGCTGC**A** GAATTTTGGGATGG-3′ (sense) and 5′-CCATCCCAA AATTC**T**GCAGCGTCATGAGGGAG-3′ (antisense) for pcDNA3.1D/CYP2C9/ Arg132Gln. 5′-TGATTGGCAGAA ACC**A**GAGCCCCTGCATGCA-3′ (sense) and 5′-TGCATG CAGGGGCTC**T**GGTTTCTGCCAATCA-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	cattttatttt[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]gttttcagca		(*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]gagcccctgcat		
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.

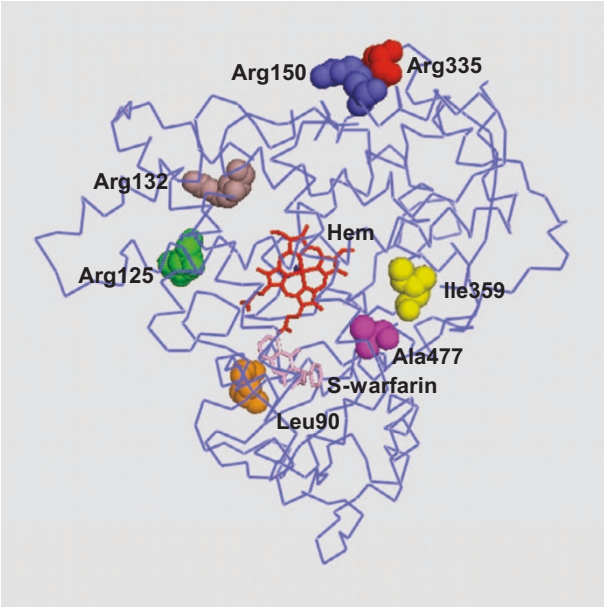

Fig. 1. *Mapping of identified missense variations on the crystal structure of human CYP2C9 protein bound with warfarin (PDB: 10G5). Hem and S-warfarin are shown by red and pink, respectively. The seven missense mutations identified in this study are presented by a space-filling model.*

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-

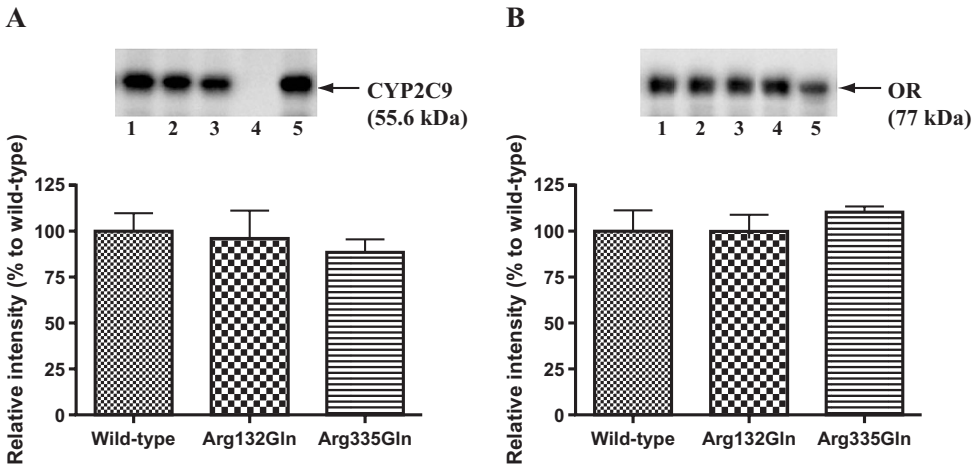

Fig. 2. *Expression of wild-type and two variants of CYP2C9 in insect cell microsomes. Representative Western blots of immunoreactive CYP2C9 (A) and OR (B) proteins (upper) are shown. Lanes 1–3: co-expressed microsomes containing wild-type, Arg132Gln, and Arg335Gln CYP2C9 each with OR; lane 4: microsomes 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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