Table 3. Genotype Distribution Between Responders and Nonresponders Treated With L-Type CCBs

Gene	SNP	R=ΔDBP >10 mmHg					R=ΔSBP >20 mmHg				
		Genotype	R	NR	χ^2	P value	Genotype	R	NR	χ^2	P value
CACNA1C	527974G>A	GG	0	8	4.501	0.105	GG	0	8	4.418	0.110
		GA	23	39			GA	22	40		
		AA	33	58			AA	26	65		
		GG	0	8	4.490	0.034	GG	0	8	3.576	0.059
		GA+AA	56	97			GA+AA	48	105		
		GG+GA	23	47	0.202	0.653	GG+GA	22	48	0.154	0.694
		AA	33	58			AA	26	65		
		OR 1.163, 95%CI 0.603–2.242					OR 0.873, 95%CI 0.442–1.722				
CACNA1D	rs312481G>A	GG	51	80	5.291	0.071	GG	45	86	11.571	0.003
		GA	4	22			GA	1	25		
		AA	1	2			AA	2	1		
		GG	51	80	4.910	0.027	GG	45	86	6.516	0.011
		GA+AA	5	24			GA+AA	3	26		
		OR 0.327, 95%CI 0.117–0.911					OR 0.221, 95%CI 0.063–0.768				
		GG+GA	55	102	0.004	0.951	GG+GA	46	111	1.957	0.162
		AA	1	2			AA	2	1		
		OR 0.927, 95%CI 0.082–10.457					OR 4.826, 95%CI 0.427–54.544				
	rs3774426C>T	CC	48	70	6.705	0.035	CC	40	78	3.616	0.164
		CT	6	29			CT	6	29		
		TT	2	5			TT	2	5		
		CC	48	70	6.370	0.012	CC	40	78	3.253	0.071
		CT+TT	8	34			CT+TT	8	34		
		OR 0.343, 95%CI 0.146–0.805					OR 0.459, 95%CI 0.194–1.084				
		CC+CT	54	99	0.133	0.715	CC+CT	46	107	0.007	0.933
		TT	2	5			TT	2	5		
		OR 0.733, 95%CI 0.138–3.907					OR 0.930, 95%CI 0.174–4.972				

ΔDBP=DBP (before treatment)-DBP (after treatment); ΔSBP=SBP (before treatment)-SBP (after treatment). Other abbreviations see in Tables 1,2.

polymorphisms of *CACNA1C* and common SNPs of *CACNA1D* chosen from the db SNP database (http://www.ncbi.nlm.nih.gov/SNP/). For sequence-proven genetic polymorphisms, polymorphisms with a minor allele frequency greater than 5% (common polymorphism) were considered candidates for genotyping. We chose a representative common SNP for genotyping among SNPs showing strong LD with an r-square greater than 0.5. Because a missense mutation may cause a direct functional change of the α1c subunit, 2 missense mutations of *CACNA1C* with a minor allele frequency less than 5% were also subjected to genotype analysis. For genetic polymorphisms of *CACNA1D* chosen from the db SNP database, 5 common SNPs (rs219847 G>A, rs312481 G>A, rs3774414 C>T, rs3774425 G>A, rs3774426 C>T) with a minor allelic frequency greater than 5% were chosen for genotyping. There was no tight LD with an r-square greater than 0.5 among these 5 SNPs in *CACNA1D*. As a consequence, 11 SNPs for *CACNA1C* and 5 SNPs for *CACNA1D* in 161 Japanese patients with HT treated with L-type dCCBs were subjected to genotype analysis. We did not perform haplotype analysis because of the study design. We evaluated the synergistic effects of SNPs associated with the effect of CCBs.

Statistical Analysis

Values are expressed as means±SD. Hardy-Weinberg equilibrium was assessed with χ^2 analysis. The overall distribution of alleles was analyzed with χ^2 analysis. The distribution of genotypes between responders and nonresponders was analyzed with 2×2 contingency tables and a 2-sided Fisher exact probability test. The statistical significance was established at $P<0.05$. Comparison of BP reduction between allelic variants was performed with ANOVA followed by the Fisher protected least-significant difference test using Stat-View version 5.0 (SAS Institute Inc, Cary, NC, USA).

Results

Group Characteristics

Overall, both SBP and DBP were significantly reduced after treatment with L-type dCCBs (**Figure 1**). **Table 1** shows the characteristics of responders and nonresponders. When responder was defined as a SBP reduction >20 mmHg, 48 patients were defined as responders and 113 as nonresponders. When responder was defined as a DBP reduction >10 mmHg, 56 patients were responders and 105 were nonresponders. Neither sex nor body mass index showed a significant difference between responders and nonresponders. Average age and the percentage receiving monotherapy differed significantly between responders and nonresponders when responder was defined as a SBP reduction >20 mmHg. The BP before treatment with dCCBs was significantly higher in responders than in nonresponders. After treatment with dCCBs, the average BP in responders was markedly decreased; however, the average BP in nonresponders was significantly higher than that in responders. Heart rate did not differ significantly between responders and nonresponders before or after treatment with dCCBs. No significant difference in the types of L-type dCCB was found between responders and nonresponders.

Detection of Genetic Polymorphisms

First, we screened for genetic polymorphisms of *CACNA1C* in 48 randomly chosen patients with HT by means of direct sequencing. As shown in **Table 2**, we identified 2 missense mutations in *CACNA1C*. Three of 48 patients had a G-to-A substitution at nucleotide 632652 in

Table 4. Selected Genotype Interactions on the Effects of L-Type CCBs

Comparison	Positively-related polymorphisms			Number	ΔSBP	ΔDBP	P1	P2
	CACNA1C 527974G>A	*CACNA1D* rs312481G>A	*CACNA1D* rs3774426C>T					
2-way interaction								
1	AG+AA	GG	Any	124	15.2±21.1	9.9±9.9	0.0109	0.0007
	Any others			36	5.4±15.9	3.9±6.3		
2	AG+AA	Any	GG	112	13.6±22.3	10.1±10.2	0.5651	0.0031
	Any others			48	11.6±15.2	5.3±6.9		
3	Any	GG	GG	113	14.6±21.0	9.8±10.1	0.1098	0.0136
	Any others			46	8.9±18.7	5.7±7.3		
3-way interaction								
4	AG+AA	GG	GG	107	14.9±21.5	10.3±10.1	0.0801	0.0013
	Any others			52	8.9±17.6	5.2±7.2		

P1, comparison of ΔSBP between genotype groups; P2, comparison of ΔDBP between genotype groups. Other abbreviations see in Tables 1, 2.

exon 45, leading to an Arg-to-Gln substitution at position 1910 (R1910Q). One patient had a G-to-A substitution at nucleotide 635110 in exon 46, leading to a Gly-to-Ser substitution at position 2004 (G2004S). Both missense mutations were found in heterozygous form. In addition, we identified 5 synonymous variations (395458G>A in exon 4, 531910C>T in exon 17, 539757G>A in exon 19, 558409C>T in exon 29, 626151G>A in exon 43) encoded for A174 (minor allelic frequency, 0.052), for D812 (0.333), for A879 (0.010), for F1262 (0.415), and for T1787 (0.402). Thirty-one additional variations in the intron and 3'-untranslated regions were also detected. As described in the Methods section, we finally chose 11 genetic polymorphisms of *CACNA1C* and 5 common SNPs of *CACNA1D* for genotype analysis in 161 patients with EHT who were treated with L-type dCCBs (**Table 2**). We failed to genotype 638741-638742insT of CACNA1C because of incomplete discrimination of the genotyping signals. We did not identify 635110G>A (G2004S) of *CACNA1C* in the 161 samples. The allelic frequencies of another 8 SNPs of *CACNA1C* determined with genotyping were similar to those identified with direct sequencing.

Association Study for the Effect of L-Type dCCBs

The clinical characteristics of patients with the 632652G>A (R1910Q) mutation did not show any specific clinical features after treatment with L-type dCCBs (data not shown). Thus, 8 common SNPs of *CACNA1C* and 5 of *CACNA1D* subjected to genotype analysis were used to study their relationship to the effects of L-type dCCBs. Control for deviation from Hardy-Weinberg equilibrium yielded nonsignificant results in all SNPs examined in this study. On basis of a comparison of each allele frequency between responders and nonresponders, 1 of *CACNA1C*, 527974G>A, and 2 SNPs of *CACNA1D*, rs312481G>A and rs3774426C>T, showed significant correlations with the effects of L-type dCCBs (**Table 3**). When a response was defined as a DBP reduction >10 mmHg, the prevalence of *CACNA1C* 527974G>A differed significantly in the dominant model, in that *CACNA1D* rs3774426C>T differed in the additive and recessive models, and that of *CACNA1D* rs312481G>A differed only in the recessive model. When a response was defined as a SBP reduction >20 mmHg, the prevalence of *CACNA1D* rs312481G>A significantly differed in the additive and recessive models. *CACNA1C* 527974G>A and *CACNA1D* rs3774426C>T showed a marginal relation to the effects of L-type dCCBs.

Figure 1 show the comparison of BP in the dominant or recessive model in 3 SNPs that were significantly associated with the effect of L-type dCCBs shown in **Table 3**. The basal SBP and DBP were significantly reduced by treatment with L-type dCCBs in patients with GG carriers in *CACNA1D* rs312481G>A or CC carriers in rs3774426C>T, with GA+AA carriers in *CACNA1C* 527974G>A, and also with CT+TT carriers in *CACNA1D* rs3774426C>T. After treatment with dCCBs, DBP in patients with GG in rs312481G>A, with CC in rs3774426C>T, and with GA+AA in *CACNA1C* 527974G>A was significantly reduced when compared with patients with other allele carriers (P=0.0126 for rs312481G>A, 0.0283 for rs3774426C>T, and 0.0108 for 527974G>A) (**Figure 1**). Patients with GG carrier in rs312481G>A also showed a significant reduction in SBP after treatment with L-type dCCBs when compared with patients with GA+AA carrier (P=0.0101). Both SBP and DBP were significantly decreased by treatment with dCCBs in patients with GG carrier in *CACNA1D* rs312481G>A, but there was no significant reduction in BP in GA+AA in *CACNA1D* rs312481G>A. In contrast, significant differences in the antihypertensive effect on either SBP or DBP of treatment with dCCBs between alleles were not seen in *CACNA1D* rs3774426C>T or *CACNA1C* 527974G>A.

The genotype interactions on the effects of L-type dCCBs are shown in **Table 4**. When interactions between 2 polymorphisms were analyzed, a much greater reduction in DBP after treatment with dCCBs was observed for the simultaneous presence of *CACNA1C* 527974 GA+AA–*CACNA1D* rs312481 GG or *CACNA1C* 529874 GA+AA–*CACNA1D* rs3774426 CC. The 3-way interaction models also showed a much greater reduction in DBP for the simultaneous presence of *CACNA1C* 527974 GA+AA–*CACNA1D* rs312481 GG–*CACNA1D* rs3774426 CC.

Discussion

The present study has demonstrated that *CACNA1C* 527974G>A, *CACNA1D* rs312481G>A, and *CACNA1D* rs3774426C>T are associated with the antihypertensive effects of L-type dCCBs in Japanese patients with EHT. In particular, the greatest sensitivity to the effects of dCCBs was observed with *CACNA1D* rs312481G>A, which showed a significant association with the effects of L-type dCCBs in the reduction of both SBP and DBP. A patient with HT and GA+AA in *CACNA1D* rs312481G>A or with GG in *CACNA1C* 527974G>A is predicted to be a nonresponder to

L-type dCCBs (**Table 3**, **Figure 1**). In addition, there was a synergistic effect between the genetic polymorphisms of *CACNA1C* and *CACNA1D* on the lowering BP by L-type dCCBs (**Table 4**). The L-type α1c subunit plays a central role in regulating cardiac function and BP[22,23] and is a target of the L-type dCCBs widely used in the treatment of HT.[24] Therefore, we speculated that genetic polymorphisms of *CACNA1C* might be related to the effects of L-type dCCBs. In this study, we demonstrated that 527974G>A of *CACNA1C* has a significant association with the effects of L-type dCCBs. While we were preparing this report, Bremer et al reported that *CACNA1C* polymorphisms are associated with the efficacy of dCCBs in the treatment of HT in white subjects.[25] The results of both studies suggest that genetic polymorphisms of *CACNA1C* influence the effects of L-type dCCBs in patients with HT; however, how these genetic polymorphisms affect the effects of L-type dCCBs is still unknown. Because 527974G>A is located in intron 13, this SNP itself might not influence α1c function. Although we could not find functional polymorphisms linked with 527974G>A in our results or in HapMap data for Japanese, there may be functional polymorphisms in the promoter region (which we failed to sequence) or genes adjacent to *CACNA1C*. In addition, human *CACNA1C*, spanning >500kb, maps to chromosome 12p11.2 and undergoes extensive mRNA splicing, leading to numerous isoforms with different functions in altering electrophysiology properties,[26–28] affinity to DHPs,[29,30] and loss of channel functions.[31] Alternative splicing is regulated by multiple factors, including the 5' splice site, the 3' splice site, the branch site and the Py tract, as well as the intronic or exonic splicing enhancer and silencer.[31] Identifying genetic polymorphisms that affect splicing has proven difficult, as they can be located not just in the splice regions but anywhere in the large intron. Therefore, we could not rule out the possibility that 527974G>A, as well as polymorphisms linked with it in intron regions, might influence *CACNA1C* mRNA splicing.

The present study is the first to demonstrate that genetic polymorphisms of *CACNA1D* might be associated with the effects of L-type dCCBs in patients with EHT. Of the 3 SNPs that were identified to be associated with the effects of L-type dCCBs in the present study, *CACNA1D* rs312481G>A was the most strongly associated. Patients with GG homozygous for rs312481G>A were more sensitive to the effects of L-type dCCBs for reducing DBP and SBP than were patients with the GA+AA genotype. *CACNA1D* rs3774426C>T also showed a significant association with the effects of L-type dCCBs for reducing DBP. A previous study has shown that α1D does not mediate the contractility of ventricular muscle or aortic smooth muscle.[18] In addition, all L-type calcium channels studied to date are sensitive to L-type dCCBs. However, α1D-containing L-type calcium channels appear to be significantly less sensitive to L-type dCCBs.[19,32] Therefore, how the genetic polymorphisms of *CACNA1D* affect the L-type dCCBs reduction of BP would be very interesting to know. Importantly, recent studies have shown that the lower sensitivity of α1D-containing L-type calcium channels to L-type dCCBs becomes even more significant when membrane potentials are hyperpolarized and α1c-containing L-type calcium channels are not open. The α1D-containing L-type calcium-channel current that remains in the presence of DHPs takes on the profile of an inactivating current with barium as the charge carrier.[32] This is consistent with the state-dependent nature of the blockade by DHPs.[33,34] In the presence of L-type dCCBs, α1D-containing L-type calcium channels generate low-threshold, drug-resistant, inactivating currents that resemble the R-type current of many neurons or the T-type current of sinoatrial node cells and control physiological processes, such as diastolic depolarization in sinoatrial node cells and neurotransmitter release and neuronal excitability. Because the neuroendocrine system and pacemaking may play important roles in regulating BP, variations of *CACNA1D* may influence the effects of L-type dCCBs through a change in the sensitivity of α1D-containing L-type calcium channels to L-type dCCBs. *CACNA1D* rs312481G>A and rs3774426C>T are both in intron regions. We did not find functional polymorphisms linked with them in HapMap data for Japanese (data not shown). The α1D subunit also undergoes extensive mRNA splicing, which may lead to numerous isoforms with different functions.[35,36] Whether *CACNA1D* rs312481G>A and rs3774426C>T or polymorphisms linked with them in intron regions influence *CACNA1D* mRNA splicing needs to be clarified.

Our data also show a possible synergistic effect of genetic polymorphisms of *CACNA1C* and those of *CACNA1D* on L-type dCCBs treatment in patients with EHT. This result suggests that α1D-containing and α1c-containing L-type calcium channels might coordinate the regulation of BP under physiological conditions or the responsiveness to treatment with L-type dCCBs under pathological conditions. Further functional studies are needed to clarify this point.

There is a question as to whether the contributions of *CACNA1D* rs312481G>A and rs3774426C>T and of *CACNA1C* 527974G>A to the effects of L-type dCCBs are an L-type CCB-specific finding. We speculate that the contribution of these 3 SNPs to the antihypertensive effects of L-type dCCBs is in fact dCCB-specific, because these SNPs also showed a significant association with the effects of L-type dCCBs in a study of patients who received only L-type dCCB monotherapy, despite a small sample size (data not shown).

Study Limitations

The present study was retrospective design and had a small sample size. The study subjects included not only patients receiving monotherapy with L-type dCCBs, but also those receiving combined therapy with L-type dCCBs and other antihypertensive drugs. We do not believe that this issue greatly affects the relationship between the 3 SNPs and the effects of L-type dCCBs, because the percentages of patients receiving monotherapy with L-type dCCBs and of patients receiving different L-type dCCBs, such as amlodipine and nifedipine, did not differ significantly between each allele of these SNPs. In addition, the SNPs also showed a significant association with the effects of L-type dCCBs in a study that examined only patients who had received amlodipine therapy (data not shown). However, a large-scale, prospective, controlled study of L-type dCCBs is needed to confirm the importance of these SNPs in the antihypertensive effects of L-type dCCBs. Furthermore, the BP before treatment is an important factor in the effects of antihypertensive drugs. In the present study, both SBP and DBP before treatment with L-type dCCBs were significantly higher in responders than in nonresponders. However, the BP before treatment with L-type dCCBs did not differ significantly between dCCB-sensitive and dCCB-insensitive genotypes in *CACNA1D* rs312481G

>A and rs3774426C>T and in *CACNA1C* 527974G>A when a response was defined as a change in SBP>20 mmHg or in *CACNA1D* rs3774426C>T and *CACNA1C* 527974G>A when a response was defined as a change in DBP>10 mmHg (**Table 3**). In addition, age and aging may influence the effects of antihypertensive drugs because of higher SBP and slower metabolism of dCCBs (compared with younger patients)? However, there was no significant difference in the average age of patients with dCCB-sensitive or -insensitive genotypes. Finally, regarding the statistical approach, the Bonferroni method was not performed, although multiple SNPs were investigated in the present study. No SNPs were significantly associated with the effects of L-type dCCBs according to Bonferroni criteria (P=0.05/13 SNPs, P<0.005). Although this correlation might be considered weak for this type of genetic research, we consider these 3 SNPs to be prominent candidates related to the effectiveness of L-type dCCBs, because both *CACNA1C* and *CACNA1D* have been suggested to play important roles in the effectiveness of L-type dCCBs in patients with EHT, as mentioned earlier.

In summary, rs312481G>A and rs3774426C>T of *CACNA1D* and 527974G>A of *CACNA1C* are believed to be genetic polymorphisms that confer sensitivity to the antihypertensive effects of L-type dCCBs in patients with EHT. Because association studies are not consistently reproducible, as a result of false-positive and false-negative results;[37] the association of these polymorphisms with the effects of L-type dCCBs should be re-examined in other populations. These genetic polymorphisms may be useful for predicting the sensitivity of patients to treatment with L-type dCCBs and may lead to individualized therapies for HT based on genetic background.

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Original Article

Associations of Hypertension and Its Complications with Variations in the Xanthine Dehydrogenase Gene

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Hyperuricemia and oxidative stress participate in the pathophysiology of hypertension and its complications. Xanthine dehydrogenase (XDH) produces urate and, in its oxidase isoform, reactive oxygen species. Here we have studied whether or not the genetic variations in *XDH* could be implicated in hypertension and its complications. By sequencing the promoter region and all exons of *XDH* in 48 subjects, we identified three missense mutations (G172R, A932T, N1109T) in a heterozygous state in addition to 34 variations, including 15 common single nucleotide polymorphisms (SNPs). The three missense mutations and eight common SNPs (11488C>G, 37387A>G, 44408A>G, 46774G>A, 47686C>T, 49245A>T, 66292C>G, and 69901A>C) were genotyped in 953 hypertensive Japanese subjects and in 1,818 subjects from a general Japanese population. Four hypertensive patients with rare missense mutations (G172R or N1109T) in homozygous form had severe hypertension. Multivariate logistic regression analysis showed a significant association of three SNPs with hypertension in men: 47686C>T (exon 22, odds ratio [OR]: 1.52, p=0.047) and 69901A>C (intron 31, OR: 3.14, p=0.039) in the recessive model, and 67873A>C (N1109T) (exon 31, OR: 1.84, p=0.018) in the dominant model. After full adjustment for all confounding factors, only one polymorphism (69901A>C) was found to be associated with carotid atherosclerosis in the dominant model (p=0.028). Multiple logistic regression analysis showed that one SNP (66292C>G) was significantly associated with chronic kidney disease (CKD: estimated creatinine clearance <60 mL/min) in the recessive model (p=0.0006). Our results suggest that genetic variations in *XDH* contribute partly to hypertension and its complications, including atherosclerosis and CKD. (*Hypertens Res* 2008; 31: 931–940)

***Key Words*: xanthine dehydrogenase gene, missense mutation, single nucleotide polymorphism, hypertension, atherosclerosis, chronic kidney disease**

Introduction

Hypertension is one of the most common and important risk factors for stroke, coronary heart diseases (CHD), and chronic kidney disease (CKD). The major contribution to the etiology of this disorder is proposed to come from the combined effects of genes that modify the response of blood pressure to environmental stresses, including diet and environmental susceptibility genes (*1*). This multifactoral trait increases the

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affected individuals' risks of stroke, CHD, and CKD, and is one of the leading causes of morbidity and mortality in adults (*2*). The population-wide application of preventative measures and analyses of candidate genes to predict modifiable risks, in addition to developing new treatments for hypertension and its complications, are thus very worthwhile (*3*, *4*).

Xanthine oxidoreductase (XOR), best known as the rate-limiting enzyme of the purine degradation pathway, converts hypoxanthine to xanthine and xanthine to uric acid (UA) *via* its two interconvertible isoforms, xanthine dehydrogenase (XDH) and xanthine oxidase (XO); in its oxidase isoform, XOR produces reactive oxygen species (ROS) (*5*). Hyperuricemia is commonly seen in hypertensive patients (*6*). Several large epidemiologic studies have identified an association between increased serum UA levels and cardiovascular risk in the general population (*7–10*), among patients with hypertension (*11*, *12*), between increased serum UA levels and renal failure in the general population (*13*, *14*), and among patients with hypertension (*15*). ROS plays critical roles in the pathogenesis of a number of cardiovascular diseases, including atherosclerosis, hypertension, diabetes mellitus, and heart failure (*16*). They have also been implicated as important mediators of the progression of renal injury in different animal models of hypertension (*17–20*). The conversion of XDH to XO and increased XO activity have been reported in some pathological conditions, including hypertension (*21–23*) and atherosclerosis (*24*). Importantly, treatments with XO inhibitors were recently reported to normalize ROS levels in microvessels from rats fed a high-salt diet (*25*) and to promote endothelial-dependent relaxation in arteries from SHR (*26*). These findings suggest that XO is an important source of ROS in patients with hypertension. Therefore, the XDH gene is suspected to be associated with constitutional susceptibility to hypertension and its complications.

So far, there are no reports about the relation between variations in human XDH gene (*XDH*) and hypertension and its complications. Human *XDH*, located on chromosome 2 at p23.1 (*27*), consists of 36 exons that encode a 1,333-amino acid protein. The aim of the present study was to screen for possible genetic variations in the promoter and all exon regions of *XDH* in 48 patients with hypertension. By genotyping the missense mutations and common single nucleotide polymorphisms (SNPs) in a large hypertensive population and the general population, we further assessed the role of these genetic variations in hypertension and clarified the contributions of common SNPs to hypertension and its complications, including atherosclerosis and CKD.

Methods

Hypertensive Population

The characteristics of the hypertensive population analyzed in the present study are summarized in Table 1. A total of 953 hypertensive subjects (522 men and 431 woman, average age: 65.1±10.5 years old) were recruited from the Division of Hypertension and Nephrology at the National Cardiovascular Center, as reported previously (*28*, *29*). Briefly, 92% of study subjects (880 subjects) were diagnosed with essential hypertension, and the rest had secondary hypertension. The hypertension criteria were a systolic blood pressure (SBP) above 140 mmHg and/or a diastolic blood pressure (DBP) above 90 mmHg, or the use of antihypertensive agents. Hyperlipidemia was defined by a total cholesterol level ≥220 mg/dL or the taking of antihyperlipidemia medication. Diabetes mellitus was defined by a fasting plasma glucose level ≥126 mg/dL, nonfasting plasma glucose ≥200 mg/dL, HbA1c ≥6.5%, or the taking of antidiabetic medication. Smoking was defined as current smoking. Total cholesterol, high-density lipoprotein (HDL) cholesterol, triglyceride, and low-density lipoprotein (LDL) cholesterol levels were measured as previously described (*30*). Study subjects underwent routine laboratory tests, including examinations of electrolytes, renal function, blood glucose, HbA1c, plasma renin activity (PRA), and plasma aldosterone concentration (PAC) by radioimmunoassay.

Table 1. Characteristics of Patients with Hypertension

Number	953
Age, years	65.1±10.5
Gender (male/female)	522/431
Body mass index, kg/m^2	24.2±3.3
Systolic blood pressure, mmHg	145.5±19.2
Diastolic blood pressure, mmHg	84.8±13.4
Essential hypertension	880
Secondary hypertension	72
Renal hypertension	36
Renovascular hypertension	23
Primary aldosteronism	11
Hypothyroid-induced hypertension	2
Ischemic heart disease	102
Stroke	145

Values are expressed as mean±SD.

Evaluation of Atherosclerosis and CKD in the Hypertensive Population

Carotid ultrasonography was used to measure mean intima-medial thickness (IMT) using ultrasonography (SSA-390A; Toshiba, Tokyo, Japan) as previously described (*31*). IMT above 1.0 mm in either the left or right common carotid artery defined with the presence of an atherosclerotic lesion. We also assessed arterial stiffness using brachial-ankle–pulse wave velocity (ba-PWV) measured by form ABI (Omron Health Care, Kyoto, Japan) as described in a previous report (*32*). Estimated creatinine clearance (Ccr) determined with the Cockcroft-Gault formula (*33*) was used for the evaluation of CKD. We defined CKD as Ccr <60 mL/min according to

the guidelines of the National Kidney Foundation (*34*).

Screening of Genetic Variations in *XDH*

We sequenced the promoter region and all exons of *XDH* in 48 randomly chosen patients with hypertension. Blood samples were obtained from all hypertensive patients, and genomic DNA was isolated from peripheral blood leukocytes using an NA-3000 nucleic acid isolation system (Kurabo, Osaka, Japan) (*35*). All exons with their flanking sequences and 1 kb of the promoter region were directly sequenced with an ABI PRISM 3700 DNA analyzer (Applied Biosystems, Foster City, USA) as described previously (*36*) using 38 sets of primers. Information on the primers and PCR conditions is available on request. The sequences obtained were examined for the presence of variations using Sequencher software (Gene Codes, Ann Arbor, USA), followed by visual inspection.

Genotyping of Missense Mutations and Common SNPs in Hypertensive Subjects and the General Population

Three missense mutations and eight common SNPs with a minor allelic frequency of greater than 10% were genotyped in 953 hypertensive patients and in 1,818 subjects (835 men and 983 women) participating in the Suita Study. We chose just one common SNP for genotyping among SNPs that show strong linkage disequilibrium (LD) with an r^2 above 0.5. The sample selection and study design of the Suita Study were described previously (*37*). Briefly, the subjects visited the National Cardiovascular Center every 2 years for general health checkups. In addition to a routine blood examination that included lipid profiles, glucose levels, blood pressure, and anthropometric measurements, a physician or nurse administered questionnaires covering the subject's personal history of cardiovascular diseases, including angina pectoris, myocardial infarction, and/or stroke. Nondrinkers were those who had had no drink in the past month. Current drinkers were those who were drinking at least 30 mL of ethanol per day, and past drinkers were those who used to drink that much in the past but not in the present. Subjects were regarded as having a disease if they were currently taking antihypertensive, antihyperlipidemic, or antidiabetic medication. Seven-hundred and ninety-five subjects were diagnosed as having hypertension. All of the participants were Japanese. The characteristics of the subjects in the Suita Study are summarized in Table 2.

The TaqMan-PCR (Roche Molecular Systems, Pleasanton, USA) method was used for genotyping (*35*). The sequences of PCR primers and probes for the TaqMan-PCR method are available on request. All of the participants in the genetic analysis in the present study gave their written informed consent. All clinical data, as well as the results of sequencing and genotyping, were anonymous. The study protocol was approved by the Ethics Review Committee of the National Cardiovascular Center, Japan.

Table 2. Baseline Characteristics of Subjects in Suita Study

	Women (n=983)	Men (n=835)
Age, years	63.3±11.0	66.3±11.1*
Systolic blood pressure, mmHg	128.0±19.7	131.8±19.4*
Diastolic blood pressure, mmHg	76.5±9.8	79.7±10.7*
Body mass index, kg/m^2	22.3±3.2	23.3±2.9*
Total cholesterol, mg/dL	215.6±30.6*	197.9±30.3
HDL-cholesterol, mg/dL	64.5±15.3*	55.0±14.1
Current smokers, %	6.3	30.2*
Current drinkers, %	29.6	67.2*
Present illness, %		
Hypertension	38.0	47.3*
Hyperlipidemia	54.4*	27.8
Diabetes mellitus	5.2	12.8*

*p<0.05 *vs.* women or men. HDL, high-density lipoprotein.

Statistical Analysis

Values are expressed as means±SD. The distribution of patient characteristics between men and women in the general population and in the hypertensive population was analyzed with Student's t-test or χ^2 analysis.

The associations of genetic models with blood pressures were tested with a logistic regression analysis considering potential confounding risk variables, including age, body mass index (BMI), present illness (hyperlipidemia and diabetes mellitus), lifestyle (current smoking and drinking), and antihypertensive medication by sex. For multivariate risk predictors, the adjusted odds ratios (ORs) were given with 95% confidence intervals. The relationship between genotype and risk of hypertension was expressed in terms of ORs adjusted for possible confounding factors, including age, BMI, present illness (hyperlipidemia and diabetes mellitus), and lifestyle (current smoking and drinking) by sex. The relationship between genotype and risk of atherosclerosis or CKD in hypertensive patients was expressed in terms of ORs adjusted for possible confounding factors, including age, sex, BMI, LDL cholesterol, HbA1c, SBP, and DBP for atherosclerosis; and age, BMI, SBP, DBP, and diabetes mellitus for CKD. For each pair of SNPs, the pairwise LD parameters, D' and r^2, were calculated on the basis of the genotyping data using SNPAlyze version 3.1 Pro (Dynacom, Mobara, Japan). All analyses were performed with SAS statistical software release 8.2 (SAS Institute, Cary, USA) or JMP statistical software version 4.0 (SAS Institute). Statistical significance was established at p<0.05.

Table 3. Sequence Variations in the Promoter Region and All Exons in *XDH* Identified in 48 Japanese Patients with Hypertension and/or Renal Failure

SNP (allele 1>allele 2)	LD	Amino acid substitution	Region	Allele frequency		Flanking sequence	Genotyping
				Allele 1	Allele 2		
8787C>T	a		intron2	0.979	0.021	gagtgggagtga[c/t]ggagaaggggg	
11451G>T	a		intron2	0.968	0.032	gcccacagctct[g/t]cccaggcatttc	
11488C>G	b, c		intron2	0.862	0.138	cagactcctctc[c/g]ctgagttcattc	done
26245G>A			intron6	0.958	0.042	ggcaggcaggat[g/a]ccctgctgttg	
26390G>A	b, c, d	Gly172Arg	exon7	0.906	0.094	ggatgctgtgga[g/a]gagatgggaata	done
26479T>A			intron7	0.958	0.042	gcctgggggtaa[t/a]ctgagacttaga	
26504C>T	e		intron7	0.625	0.375	ggagtcagtgca[c/t]gagctccatgtc	
26832G>A	b, c, d	Glu209Glu	exon8	0.915	0.085	tccaacccagga[g/a]cccatttttccc	
28272G>A			intron9	0.989	0.011	gccagggaggct[g/a]ccctggggctgc	
30863C>T	c, d	Val279Val	exon10	0.936	0.064	tcctatgattgt[c/t]tgcccagcctgg	
31503G>T			intron10	0.989	0.011	gtgattccgaac[g/t]tgcgttcccagg	
34636G>A			intron13	0.917	0.083	tttctccccatg[g/a]ggggttcccagc	
37387A>G	f, g		intron14	0.181	0.819	tttgcagcccct[a/g]cagagcaaggtg	done
39048A>G	h		intron15	0.604	0.396	ccctgggcacac[a/g]gctctacacaaa	
44408A>G	i		intron19	0.875	0.125	tggaaaggttat[a/g]catttgcatgga	done
44426G>A			intron19	0.990	0.010	gcatggattatg[g/a]ccatcatccagt	
46476T>C			intron20	0.979	0.021	acttcaagtctg[t/c]atgtgaagcata	
46748G>C	h		intron21	0.660	0.340	ggggtggccctg[g/c]tttgcaaattaa	
46774G>A	e		intron21	0.638	0.362	ttcaagagatat[g/a]cattgaaccctg	done
47686C>T	h	Ile737Ile	exon22	0.670	0.330	ggagatatacat[c/t]ggtggccaagag	done
47804G>A			intron22	0.989	0.011	acccaggtagat[g/a]ccttttgggtca	
47879A>G	e		intron22	0.638	0.362	catgtgggaaat[a/g]ggaagagggaga	
49096G>A	i		intron23	0.875	0.125	gaaggctcacag[g/a]cttctaacactg	
49245A>T	f, g, j		intron24	0.125	0.875	tggggcgggatg[a/t]gccattttgtga	done
50298C>T	g, j		intron24	0.146	0.854	accttttttca[c/t]gggatgatgtgg	
50391T>C			intron24	0.917	0.083	aaacgggactta[t/c]gataaatccctc	
64606G>A		Ala932Thr	exon26	0.990	0.010	atgagtgaagtt[g/a]cagtgacctgtg	done
65050–65051insC	k		intron27	0.135	0.865	tctgctgacccc[-/c]atataggaagct	
65747T>C	k	Phe1010Phe	exon28	0.135	0.865	tggaataagctt[t/c]acagttccttt	
66292C>G	k		intron28	0.135	0.865	tctggcatcctt[c/g]tctttccctagg	done
67157A>G	k		intron30	0.128	0.872	tgtaaggagccc[a/g]tgggatcccgca	
67873A>C		Asn1109Thr	exon31	0.969	0.031	acaagaagaaga[a/c]tcccagtggctc	done
69901A>C			intron31	0.795	0.205	aaacctcacttc[a/c]cctgcctgatgg	done
73380C>T			intron34	0.938	0.063	agacttggccac[c/t]gatgcaccccat	
74894G>A	l		intron34	0.968	0.032	acattccaggcc[g/a]cgctgcagttgg	
75121G>A		Glu1239Glu	exon35	0.989	0.011	catccccattga[g/a]ttcagggtgtcc	
78750G>C	l	3'UTR	exon37	0.969	0.031	tgctgcctttgg[g/c]cttccatggagc	

The A of the ATG of the initiator Met codon is denoted nucleotide +1, as recommended by the Nomenclature Working Group (*Hum Mut* 1998; **11**: 1–3). The nucleotide sequence (GenBank Accession ID: NT_022184.14) was used as a reference sequence. The apparent linkage disequilibrium (LD), defined by r^2 more than 0.5, was indicated by a in the LD column. *XDH*, xanthine dehydrogenase gene; SNP, single nucleotide polymorphism; UTR, untranslated region.

Results

Identification of Genetic Variations in *XDH*

As shown in Table 3, we identified 3 missense mutations in *XDH*. Nine of the 48 individuals had a G-to-A substitution at nucleotide 26390 in exon 7, leading to an amino acid substitution from Gly to Arg at position 172 (G172R). One individual had a G-to-A substitution at nucleotide 64606 in exon 26, leading to a change from Ala to Thr at position 932 (A932T). Three of the 48 individuals had an A-to-C substitution at nucleotide 67873 in exon 31, leading to the substitution of Asn with Thr at position 1109 (N1109T). These missense

Table 4. Clinical Profiles of Four Hypertensive Patients with Two Rare Missense Mutations in Homozygous Form in *XDH*

	Case			
	1	2	3	4
SNP	26390G>A	67873A>C	67873A>C	67873A>C
(Amino acid change)	(G172R)	(N1109T)	(N1109T)	(N1109T)
Age, years old	79	70	74	67
Sex	male	male	female	female
Body mass index, kg/m^2	21.01	23.43	23.68	21.91
Diagnosis	EHT, HL	EHT, HL, HU	EHT	EHT, HL
Hypertension duration, years	5	23	22	2
Hypertension family history	mother	unknown	mother, brother	unknown
CV complications	no	no	stroke	no
Systolic blood pressure, mmHg	144	138	168	170
Diastolic blood pressure, mmHg	70	90	100	96
Medication	ARB, BB, DU	CCB, ARB, HUD	CCB, ACEI	CCB
Na^+, mEq/L	141	141	139	139
K^+, mEq/L	4.8	3.8	3.9	4.1
Cl^-, mEq/L	108	105	104	108
Creatinine, mg/dL	1	0.8	0.5	0.6
Ccr, mL/min	50.8	73.2	84.2	68.9
UA, mg/dL	5.1	4.2	4.8	4.8
Overt proteinuria	yes	no	no	no
PRA, ng/mL/h	0.1	1.3	1.3	0.4
PAC, ng/dL	9.7	35.4	19.8	4.6
FBS, mg/dL	96	115	82	92
HbA1c, %	5	5.9	4.9	5.7
ba-PWV, cm/s	2,189	no data	1,710	1,734
Average IMT, mm	1.0	no data	0.7	1.0

XDH, xanthine dehydrogenase gene; EHT, essential hypertension; HL, hyperlipidemia; HU, hyperuricemia; CV, cardiovascular; ARB, angiotension II receptor blocker; BB, β-adrenergic blocker; DU, diuretics; CCB, calcium channel blocker; HUD, antihyperuricemic drug; ACEI, angiotensin II converting enzyme inhibitor; SNP, single nucleotide polymorphism; Ccr, creatinine clearance; UA, uric acid; PRA, plasma renin activity; PAC, plasma aldosterone conc.; FBS, fasting blood sugar; ba-PWV, brachial-ankle pulse wave velocity; IMT, intima-media thickness. Normal values: body mass index, between >18.5 and <25.0 kg/m^2; SBP, <140 mmHg; DBP, <90 mmHg; Na^+, 136 to 146 mEq/L; K^+, 3.6 to 4.9 mEq/L; Cl^-, 99 to 109 mEq/L; creatinine, 0.6 to 1.1 mg/dL; Ccr, <60 mL/min; UA, 3.6–7.0 mg/dL; PRA, 0.2 to 2.7 ng/mL/h; PAC, 2 to 13 ng/dL; FBS, <126 mg/dL; HbA1c, <6.5%; ba-PWV, <1,400 cm/s; average IMT, <1.0 mm.

mutations were all found in heterozygous form. In addition, we identified five synonymous variations (26382G>A in exon 8, 80868C>T in exon 10, 47686C>T in exon 22, 65747T>C in exon 28, and 75121G>A in exon 35) encoded for E209 (minor allelic frequency, 0.085), for V279 (0.064), for I787 (0.33), for F1010 (0.135), and for E1239 (0.011), respectively. Twenty-nine additional variations in the introns and a 3′-untranslated region were also detected. Among all the variations, there were 15 common polymorphisms with a minor allelic frequency of over 0.1 (11488C>G, 26504C>T, 37387A>G, 39048A>G, 44408A>G, 46748G>C, 46774G>A, 47879A>G, 49096G>A, 49245A>T, 50298C>T, 65050–65051 ins C, 66292C>G, 67157A>G, and 69901A>C).

Characteristics of Hypertensive Subjects with Missense Mutations in Homozygous Form

After genotyping the three missense mutations in 953 patients with hypertension, including secondary hypertension, we found one subject with G172R and three with N1109T in homozygous form. The characteristics of these four patients with rare missense mutations in the homozygous form are shown in Table 4. All four had resistant hypertension despite antihypertensive drug therapy. One of the patients with N1109T (patient 2) had hyperuricemia and was taking allopurinol. The patient with G172R (patient 1) and the two others with N1109T (patients 2 and 4) had hyperlipidemia. Patients 1 and 4 had low PRA levels (0.1 and 0.4 ng/mL/h, respectively) and high average IMT values (1.0 mm for both). Patient 1 had low Ccr (50.8 mL/min) and overt proteinurea. Three of the four patients had high ba-PWV values: no data

Table 5. Comparison of Hypertension Prevalence by Genotypes of Three Polymorphisms of *XDH* in a Japanese General Population by Sex

SNP	Genotype group	Women		Men	
		Odds ratio (95% CI)	p*	Odds ratio (95% CI)	p*
47686C>T	CC	1		1	
[CC/CT/TT=815/819/244]	CT+TT	0.90 (0.68–1.20)	0.469	1.10 (0.83–1.46)	0.521
	CC+CT	1		1	
	TT	1.04 (0.67–1.62)	0.861	1.52 (1.01–2.29)	0.047
67873A>C	AA	1		1	
[AA/AC/CC=1,720/154/5]	AC+CC	0.97 (0.58–1.61)	0.906	1.84 (1.11–3.06)	0.018
	AA+AC	1		1	
	CC	0.62 (0.05–7.33)	0.704	3.98 (0.20–80.72)	0.368
69901A>C	AA	1		1	
[AA/AC/CC=1,372/463/42]	AC+CC	1.30 (0.95–1.78)	0.099	1.11 (0.80–1.53)	0.530
	AA+AC	1		1	
	CC	0.96 (0.40–2.35)	0.936	3.14 (1.06–9.27)	0.039

*Conditional logistic analysis, adjusted for age, body mass index, present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking) for hypertension. *XDH*, xanthine dehydrogenase gene; SNP, single nucleotide polymorphism; CI, confidence interval; [ ], sample numbers of three kinds of genotypes.

on ba-PWV were available for patient 2.

Associations of 11 Variations with Hypertension in the General Population

Three missense mutations (G172R, A932T, and N1109T) and eight common SNPs (11488C>G, 37387A>G, 44408A>G, 46774G>A, 47686C>T, 49245A>T, 66292C>G, and 69901A>C) were used for the association studies in the case-control setting for men and woman separately. Adjusted for age, BMI, present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking), a logistic regression analysis of the case-control study showed that three of the eight SNPs were significantly associated with hypertension in men: TT *vs.* CC+CT for 47686C>T (exon 22, OR: 1.52, $p=0.045$) and CC *vs.* AC+AA for 69901A>C (intron 31, OR: 3.14, $p=0.039$) in the recessive model, and AC+CC *vs.* AA for 67873A>C (N1109T) (exon 31, OR: 1.84, $p=0.018$) in the dominant model (Table 5).

SBP was 2.44 mmHg higher in women with the AC+CC genotype of the positively associated SNP 69901A>C in *XDH* than in women with the AA genotype ($p=0.037$). Although there was no significant difference in SBP or DBP between the AC+CC and AA genotypes of 69901A>C in men, DBP was 4.18 mmHg higher in men with the CC genotype of 69901A>C than in men with the AA+AC genotype ($p=0.088$). DBP was 2.75 mmHg higher in men with the AC+CC genotype of the positively associated SNP 67873A>C than in men with the AA genotype ($p=0.021$) (Table 6).

Regarding the three missense mutations, there were 6 subjects with a homozygote allele in *XDH* G172R and 5 subjects with one in N1109T, but no subjects with one in A932T. The subjects with a homozygote allele in G172 and N1109T did not have any specific clinical characteristics (data not shown).

Association of 11 Variations with Carotid Atherosclerosis in Hypertensive Subjects

Three missense mutations (G172R, A932T, and N1109T) and eight common SNPs (11488C>G, 37387A>G, 44408A>G, 46774G>A, 47686C>T, 49245A>T, 66292C>G, and 69901A>C) were tested for associations with carotid atherosclerosis in patients with essential hypertension. After the full adjustment for all confounding factors (age, BMI, SBP, DBP, current smoking status, alcohol consumption, and presence of diabetes mellitus and dyslipidemia), only one polymorphism (69901A>C) was found to be independently associated with carotid atherosclerosis in the dominant model ($\chi^2=4.82$, $p=0.028$). Other factors—age ($\chi^2=67.70$, $p<0.001$), SBP ($\chi^2=15.11$, $p<0.001$), and DBP ($\chi^2=4.28$, $p=0.039$)—were related to carotid atherosclerosis. We compared IMT and ba-PWV values among the alleles in *XDH* 69901A>C. There were no significant differences between alleles in either IMT or ba-PWV. However, ba-PWV values tended to differ significantly (AA: 1,794, AC: 1,825, CC: 2,024 cm/s, $p=0.075$) in *XDH* 69901A>C. These findings may indicate that hypertensive patients with the CC of *XDH* 69901A>C are more susceptible to atherosclerosis than those with the A allele.

Associations of 11 Variations with Chronic Kidney Disease in Hypertensive Subjects

We divided the essential hypertensive patients into two groups using a cutoff estimate of Ccr 60 mL/min. The CKD group (Ccr <60 mL/min) showed significantly higher age

Table 6. Multivariate-Adjusted Blood Pressure Levels on Genotypes of Three SNPs of *XDH* by Sex

SNP	Genotype group	Women				Men			
		SBP, mmHg	p*	DBP, mmHg	p*	SBP, mmHg	p*	DBP, mmHg	p*
47686C>T	CC	127.60±0.79	0.752	79.69±0.45	0.630	131.73±0.89	0.976	79.58±0.51	0.707
	CT+TT	127.93±0.69		76.40±0.39		131.76±0.78		79.83±0.45	
	CC+CT	127.84±0.56	0.782	76.69±0.31	0.138	131.68±0.63	0.779	79.60±0.36	0.393
	TT	127.40±1.50		75.34±0.85		132.16±1.57		80.44±0.90	
67873A>C	AA	127.87±0.54	0.538	76.48±0.31	0.546	131.50±0.61	0.178	79.48±0.35	0.021
	AC+CC	126.70±1.83		77.13±1.04		134.30±1.99		82.23±1.14	
	AA+AC	127.82±0.52	0.108	76.55±0.30	0.375	131.77±0.59	0.441	79.73±0.34	0.425
	CC	112.54±9.48		71.76±5.38		122.35±12.19		74.14±7.00	
69901A>C	AA	127.10±0.61	0.037	76.33±0.35	0.290	131.23±0.68	0.136	79.37±0.39	0.079
	AC+CC	129.54±0.99		77.03±0.56		133.23±1.15		80.72±0.66	
	AA+AC	127.87±0.53	0.253	76.58±0.30	0.289	131.72±0.59	0.689	79.64±0.34	0.088
	CC	124.04±3.30		74.57±1.87		133.43±4.23		83.82±2.42	

Data are mean±SD. *Conditional logistic analysis, adjusted for age, body mass index, present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking) for hypertension. SNP, single nucleotide polymorphism; *XDH*, xanthine dehydrogenase gene; SBP, systolic blood pressure; DBP, diastolic blood pressure.

Table 7. Comparison of Chronic Kidney Disease Prevalence by Genotypes of 66292 C>G in *XDH* in Hypertensives by Sex

Genotype group	Men [CC/CG/GG=11/123/363]		Women [CC/CG/GG=11/83/315]	
	Odds ratio (95% CI)	p*	Odds ratio (95% CI)	p*
CG+GG	1	0.5545	1	0.1093
CC	1.51 (0.369–5.924)		3.48 (0.725–16.412)	
GG	1	0.0006	1	0.5617
CC+CG	2.36 (1.348–3.850)		1.18 (0.663–2.084)	

*Multiple logistic regression analysis, adjusted for age, body mass index, diabetes mellitus, systolic blood pressure, and diastolic blood pressure. *XDH*, xanthine dehydrogenase gene; [ ], sample numbers of three kinds of genotypes; CI, confidence interval.

($p<0.001$), lower BMI ($p<0.001$), and lower DBP ($p<0.001$) than the non-CKD group.

As shown in Table 7, after adjustment for age, BMI, SBP, DBP, and the number of patients that suffer from diabetes mellitus, logistic regression analysis showed that one SNP (66292C>G) of the 11 variations was strongly associated with chronic kidney disease in the recessive model in men (OR=2.36, $p=0.0006$). This significant association was still positive after a Bonferroni correction ($p=0.0006<0.05/11$). However, there was no significant difference in Ccr value between GG and CC+CG in *XDH* 66292C>G in male hypertensive patients (GG: 84.73±39.14 *vs.* CC+CG: 80.32±73.26 mL/min, $p=0.384$).

Discussion

The present study is the first to examine the relationships between genetic variations in *XDH* and hypertension or its complications in human. After the screening for possible genetic variations in the promoter and all exon regions of *XDH* in 48 patients with hypertension, 11 variations, including 3 missense mutations and 8 common SNPs, were genotyped and used to assess the roles of these genetic changes in hypertension in a large population of hypertensive subjects and in a general population. The 4 hypertensive patients with a rare missense mutation (G172R or N1109T) in homozygous form had hypertension. More importantly, 67873A>C (N1109T) also showed a positive association with hypertension in men in a multivariable logistic analysis. In addition, DBP was 2.75 mmHg higher in men with the AC+CC genotype of 67873A>C than in men with the AA genotype ($p=0.021$). This indicates that 67873A>C may be a functional risk factor for hypertension in males. Another two SNPs, 47686C>T in the exon region and 69901A>C in the intron region, were also found to be significantly related to hypertension in men. Furthermore, SBP was 2.44 mmHg higher in women with the AC+CC genotype of 69901A>C than those with the AA genotype ($p=0.037$). Since a significant association was obtained in the multivariable analysis with adjustment for confounding risk factors, including age, BMI, present illness (hyperlipidemia and diabetes mellitus), and lifestyle (current smoking and drinking) by sex, these

three SNPs appear to be independent risk factors for hypertension. The C allele of 69901A>C was associated with greater susceptibility in male subjects. In females, there was a significant association between 69901A>C and blood pressure. Although there was no significant difference in SBP or DBP between the AC+CC and AA genotypes of 69901A>C in men, DBP was 4.18 mmHg higher in men with the CC genotype of 69901A>C than in men with the AA+AC genotype ($p=0.088$). Taking these findings together, we speculate that, among males, those with 67873A>C (N1109T) were most susceptible to hypertension.

This is also the first report to show a positive relationship between SNPs of *XDH* and CKD in hypertensive patients. It is well reported that age, sex, blood pressure, BMI, and diabetes mellitus are all factors in renal dysfunction (*38–41*). Our results also showed that age, DBP, and BMI differed significantly between hypertensive patients with Ccr <60 mL/min and those with Ccr ≥60 mL/min. But no significant difference in SBP or the number of diabetes mellitus patients was found with or without CKD in these hypertensive subjects. After adjustment for age, sex, BMI, SBP, DBP, and the number of patients having diabetes mellitus, the logistic regression analysis showed that only one SNP (66292C>G) was strongly associated with CKD in hypertensive patients. This indicates that 66292C>G may be an independent risk factor for CKD in hypertensive patients.

SNP 69901A>C was found to be significantly associated with carotid atherosclerosis in hypertensive patients in our study. Although we did not find a significant difference between genotypes in any of the various atherosclerotic variables, hypertensive patients with the A allele of 69901A>C tend to be more susceptible to atherosclerosis than those with the C allele.

How the SNPs of *XDH* influence the pathogenesis of hypertension and its complications, including atherosclerosis and CKD, remains unclear. Among the four SNPs that showed a positive association with hypertension or with atherosclerosis and CKD in hypertensive patients, 67873A>C and 47686C>T are in exon regions, and 69901A>C and 66292C>G are in intron regions. 67873A>C causes a missense mutation in exon 31, leading to an amino acid substitution from Asn to Thr at position 1109. But 47686C>T does not result in a change in amino acids. In addition, the three missense mutations, 26390G>A (G172R), 64606G>A (A932T), and 67873A>C (N1109T), occurred in highly conserved residues among different species, all resulting in a hydrophilic amino acid substitution, which may influence reactive centers of enzymes. The XDH protein consists of three functional subunit domains, each of which binds a different cofactor, from amino acids 1 to 165 for binding $2Fe_2S_2$, from 226 to 531 for binding flavin adenine dinucleotide, and from 590 to 1332 for binding molybdoptern (Mo-Co) (*5*). The missense mutation G172R is not in the predicted functional domain, but A932T and N1109T are in the domain for binding molybdoptern. A932T and N1109T are not in the domain for binding flavin adenine dinucleotide, which is thought to play a major role in the conversion of XDH to XO and which increases ROS production in some pathological conditions, including hypertension and atherosclerosis (*5*). However, it is important to note a recent report that XOR has both inorganic nitrate reductase and nitrite reductase activity at its Mo-Co site (*42*, *43*). This implies that an amino acid mutation at the Mo-Co site may influence nitric oxide production and modulate ROS production. Those four hypertensive patients with A932T and N1109T in the homozygous form all had high blood pressure, N1109T showed significant associations with hypertension and blood pressure, and the Mo-Co–binding site is the most conserved region of *XDH* among human, rat, and mouse (*44*). This strongly indicates that the mutations A932T and N1109T may be functional risk factors for hypertension. Further *in vivo* and *in vitro* studies are needed to clarify this point.

Both 69901A>C and 66292C>G SNPs are in intron regions, while 47686C>T is a synonymous variation and, as such, is probably not functional. These SNPs are considered preferable as genetic markers. Human *XDH* is located on chromosome 2 at p23.1. Recently, Angius *et al.* reported strong evidence that a 0.54-cM region of chromosome 2 (2p 26.5–27.1) harbors a locus-affecting risk of hypertension in an isolated Sardinian population (*45*). In addition, a number of regions of chromosome 2 (57–59, 86, 103, and 96–115 cM) have been found likely to harbor blood-pressure–modifying loci (*45–48*). More importantly, our group recently reported some hypertension-susceptibility genes at 2p24–p25 and a positive relationship between hypertension and SNPs of the Na^+/Ca^{2+} exchanger 1 gene, which is located at 2p22–p23, in a general Japanese population (*49*, *50*). Expanded genotyping and a detailed cross-study of candidate genes are necessary.

In summary, in human *XDH*, we found three SNPs, 47686C>T, 67873A>C, and 69901A>C, that are significantly associated with hypertension. Another SNP, 66292C>G, was significantly associated with CKD, and 69901A>C also showed a positive relation to carotid atherosclerosis in hypertensive patients. These SNPs may be independent risk factors for hypertension or CKD and carotid atherosclerosis in hypertensive patients. There was a limitation in this study owing to its cross-sectional design. Prospective studies investigating the relationships between these SNPs and the development of hypertension, CKD, and atherosclerosis over a long term are necessary. These gene polymorphisms in *XDH* may be useful for predicting and preventing hypertension and its complications in future individualized treatment.

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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	catttttattttt[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.

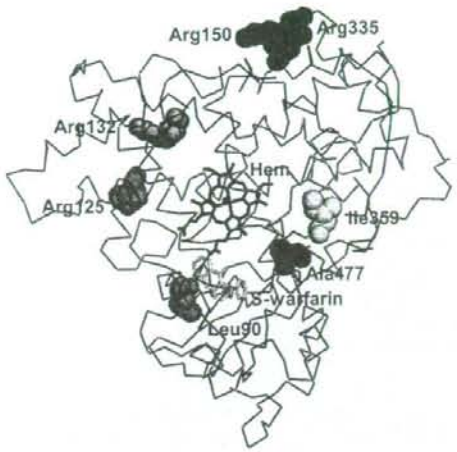

Fig. 1. *Mapping of identified missense variations on the crystal structure of human CYP2C9 protein bound with warfarin (PDB: 10G5). Hem and S-warfarin are shown by red and pink, respectively. The seven missense mutations identified in this study are presented by a space-filling model.*

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-