Table 1. Primer sequences used for the analysis of human *SLC22A1*.

	Amplified and sequenced region	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Amplified length (bp)
1st PCR	Exons 1 to 2	TGACCATTTGGAGTTTAACG	ACCCTCCCCACACTCTTTTA	11,721
	Exons 3 to 8	CTGGCAGAAGTTCCTATGTT	TGGTTGTGTGAAGGTACTTA	12,919
	Exons 9 to 11	GTCTTGATCTCCTGACCTCG	ACATCCTCCCTTATGCTTTA	6,652
2nd PCR	Exon 1	CTGAACTTCAATTCTCTTCG	CCCACGAACTGCACAATAAA	808
	Exon 2	CCAGGGATACCGAGTTTGAT	ATGCAGCTTGGACTCTGAAA	440
	Exon 3	GCTCAGACTCCTCTTCAGAC	AAAGAGAGGAGGCCATTCTA	531
	Exon 4	AAGGAGAAATGGGAGACACA	GCGTTATGCATGTGGACACC	493
	Exon 5	CACAGAAGGAAGGCTACATA	GGGAGAGCATCAGCCACACT	573
	Exon 6	AGTTGCCTCCTGCTGATTTA	GGTGATCTCCCAAAGGTAAC	569
	Exon 7	GCTCTAGGGCATTCTAAACC	GGCCCCTCAATTTAAAATCT	489
	Exon 8	CCCCTGTTCAATGGAGTCTT	GCTATAATTATGGGTTGACC	521
	Exon 9	TTCACTCTAGCCTGTTACCT	CCTAGGAATGGATTCTTATC	539
	Exon 10	TGTACCCCAACAACAAATCC	CAGTAGCTATCATGGGTTTT	575
	Exon 11	AAAAACAGGCTATAAGCTCG	TCTACTGATCTTCAAACGCC	555
Sequencing	Exon 1	AATTCTCTTCGGGCTTAGAC	CTGCACAATAAACATAGCCC	
	Exon 2	GGATACCGAGTTTGATGAAC	TTGGACTCTGAAACACACCT	
	Exon 3	TGGGCACTGCTGTCCTGATA	CTGCCCTAGTCACATAAATA	
	Exon 4	GACACACAAGAGAGAAGCCT	CTTTGGAAGACGGCCTGTAG	
	Exon 5	GGCTCCCTTTTGGTCTATAA	TGCTTCACACCCATGACAAG	
	Exon 6	TAGGAAGGGGTATCTCACAT	AAGGGAGAAACAAAAAGAGC	
	Exon 7	TCTCTGACTCATGCCTTTGA	CCTCATCTTTGTTCTCATTC	
	Exon 8	ATATGTCATCGTCAACTCCC	AATGCCACTCAATGTCCAAA	
	Exon 9	GCCTGTTACCTCCTCTCAAT	TGAAGCAAGACAGAATAAGC	
	Exon 10	CATTTCCCCAGTTATCCTAT	ATAGCAGTTCTGGGAGTAAT	
	Exon 11	CAGGCTGTAGTTTGCTATGC	TAAGTACCCGATACCAATAG	

4-phenylpyridinium uptake.[9] Five variant proteins showed reduced or diminished activities. As for the Japanese population, four nonsynonymous variations (Phe160Leu, Pro283Leu, Arg287Gly, and Pro341Leu) were found from 48 subjects.[10] Recently, two independent groups reported significantly reduced (Pro341Leu) and abrogated (Pro283Leu and Arg287Gly) transport activities using tetraethylammonium as a substrate.[11,12]

In this study, we searched for novel SNPs by sequencing all the exons and the surrounding introns of *SLC22A1* from 116 Japanese individuals. Seven novel variations, including one novel nonsynonymous SNP located in exon 1, were identified.

Materials and Methods

Human genomic DNA samples: All of the 116 patients participating in this study were administered cationic antiarrhythmic drugs at the National Cardiovascular Center. The ethical review boards of the National Cardiovascular Center and the National Institute of Health Sciences approved this study. Written informed consent was obtained from all participating subjects. Genomic DNA extracted from blood leukocytes was used as template in the polymerase chain reaction (PCR).

PCR conditions for DNA sequencing: The genomic and cDNA sequences of *SLC22A1* obtained from GenBank (NT_007422.12 and NM_003057.2, respectively) were used as the reference sequences in this study. First, the entire *SLC22A1* gene was divided into three regions (from exon 1 to 2, from exon 3 to 8, and from exon 9 to 11), and each region was amplified from 50 ng of genomic DNA using 1.25 units of *Z*-Taq (Takara Shuzo, Tokyo, Japan) with 0.2 μM primers listed in **Table 1** (1st PCR). The first PCR conditions were 30 cycles of 98°C for 5 sec, 55°C for 5 sec, and 72°C for 190 sec. Next, each exon was amplified by *Ex*-Taq (0.625 units) (Takara Shuzo) with an appropriate set of *SLC22A1*-specific primers (0.2 μM) designed in the introns, as listed in **Table 1** (2nd PCR). The second round PCR conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 1 min, and 72°C for 2 min, and then a final extension for 7 min at 72°C. The PCR products were then treated with a PCR Product Pre-Sequencing Kit (USB Co., Cleveland, OH, USA) and directly sequenced on both strands using an ABI BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with the sequencing primers listed in **Table 1** (Sequencing). The excess dye was removed by a DyeEx96 kit (Qiagen, Hilden, Germany). The eluates were analyzed on an ABI Prism 3700 DNA Analyzer (Applied Biosystems). All the novel SNPs were confirmed by repeated sequencing of amplified products from the 1st PCR.

Table 2. Summary of variations of the *SLC22A1* gene detected in a Japanese population.

SNP ID			Location	Position		Nucleotide change and flanking sequence (5′ to 3′)	Amino acid change	Frequency
This Study	NCBI (dbSNP)	JSNP		NT_007422.12	From the translational initiation site or from the nearest exon			
MPJ6_OC1001[a]			Exon 1 (5′-UTR)	2830215	−94[b]	ACTGATTTCAAA**C/A**CACTCCTTTTCA		0.004
MPJ6_OC1002	rs2297373	IMS-JST051906	Exon 1	2830431	123[b]	GGGCATCGTCTT**C/G**CTGGGTTTCACA	Phe41Leu	0.004
MPJ6_OC1003	rs1867351	IMS-JST051907, ssj0008475	Exon 1	2830464	156[b]	CCACTGCCAGAG**T/C**CCTGGGGTGGCT	Ser52Ser	0.444
MPJ6_OC1004[a]			Exon 1	2830658	350[b]	GGAGCCACCTGC**C/T**GCTGGGTCCCTG	Pro117Leu	0.004
MPJ6_OC1005	rs4646272	ssj0005309	Intron 1	2838434	IVS1−43	ACTCACACATGG**T/G**TCTGTGCTTTTC		0.629
MPJ6_OC1006[a]			Intron 1	2838442	IVS1−35	ATGGTTCTGTGC**T/C**TTTCGTCCTCCT		0.082
MPJ6_OC1007	rs683369	ssj0008476	Exon 2	2838545	480[b]	TGCGGGCTTCTT**C/G**TTTGGCTCTCTC	Phe160Leu	0.086
MPJ6_OC1008	rs4646273	ssj0005310	Intron 2	2838677	IVS2+97	ACATGACCAGTT**G/A**GAATTAACTGCA		0.457
MPJ6_OC1009	rs3737088	IMS-JST082069	Intron 2	2840506	IVS2−99	AGGACAGTGGTG**C/T**GGTGGCCTCCGA		0.017
MPJ6_OC1010[a]			Exon 3	2840650	561[b]	GCTGGTCAACGC**G/A**GTGTCGGGCGTG	Ala187Ala	0.009
MPJ6_OC1011	rs4646276	ssj0005318	Intron 4	2844495	IVS4−98	TACAGCCCCAAC**G/A**TGGGGAGGGCAG		0.470
MPJ6_OC1012	rs2282142	IMS-JST031025, ssj0008479	Intron 5	2844856	IVS5−61	ACCTCCCCAGGG**G/A**CTCCCAGGTGGC		0.168
MPJ6_OC1013	rs2282143	IMS-JST031026, ssj0008480	Exon 6	2844984	1022[b]	TGTTCCGCACGC**C/T**GCGCCTGAGGAA	Pro341Leu	0.168
MPJ6_OC1014[a]			Intron 6	2845098	IVS6+75	ATAGATTAGAGA**C/G**AGTGGAATACTC		0.022
MPJ6_OC1015	rs628031		Exon 7	2848186	1222[b]	TACCCCATGGCC**A/G**TGTCAAATTTGT	Met408Val	0.810
MPJ6_OC1016	rs4646281	ssj0005324	Intron 7	2848247_2848254	IVS7+7_14	CACCTGGTAAGT**TGGTAAGT**/−TGTCTGCTTTCA		0.810
MPJ6_OC1017[a]			Intron 8	2852130	IVS8+108	CAAGCATTGCTC**A/G**GTTTGGACATTG		0.004
MPJ6_OC1018	rs2297374	IMS-JST051908, ssj0008483	Intron 9	2863326	IVS9+43	TCTTTTGCAGCT**C/T**GGCAGTGGGCTC		0.302
MPJ6_OC1019	rs622591	IMS-JST070846, ssj0008484	Intron 10	2866868	IVS10−21	TTTTAACTCCAA**C/T**TTTTAATTTTGT		0.591
MPJ6_OC1020[a]			Exon 11 (3′-UTR)	2866961_2866963	1671_1673[b]	CACCTGAGAGAG**ATG**/−TTTTGCGGCGATG		0.004

[a] Seven novel variations detected in our study.

[b] A of the translation initiation codon ATG is numbered +1.

Results and Discussion

We sequenced all the *SLC22A1* exons (exons 1～11) and their flanking regions from 116 Japanese arrhythmic patients, and found 20 variations, including 7 novel ones (**Table 2**). The novel variations were −94C>A in the 5′-untranslated region (UTR) of exon 1 (A of the translation start codon is numbered +1 in the cDNA sequence; MPJ6_OC1001), 350C>T in exon 1 (Pro117Leu, MPJ6_OC1004), IVS1−35T>C in intron 1 (35 nucleotides upstream from exon 2; MPJ6_OC1006), 561G>A in exon 3 (Ala187Ala, MPJ6_OC1010), IVS6+75C>G in intron 6 (75 nucleotides downstream from exon 6; MPJ6_OC1014), IVS8+108A>G in intron 8 (MPJ6_OC1017), and 1671_1673delATG in the 3′-UTR of exon 11 (MPJ6_OC1020). The frequencies were 0.082 for IVS1 −35T>C, 0.022 for IVS6+75C>G, 0.009 for 561G>A, and 0.004 for the other 4 variations. We also detected the four previously reported nonsynonymous variations, 123C>G (Phe41Leu), 480C>G (Phe160Leu), 1022C>T (Pro341Leu), and 1222A>G (Met408Val) at frequencies of 0.004, 0.086, 0.168 and 0.810, respectively. The frequency (0.168) for 1022C>T (Pro341Leu) was almost the same as that reported in the JSNP database (0.163). The frequencies of the other nonsynonymous SNPs have not been reported for the Japanese population.

Previously, 25 polymorphisms were identified from 57 Caucasian subjects.[8] Among them, eight variations, 156T>C, IVS1−43T>G, 480C>G (Phe160Leu), IVS2+97G>A, 1222A>G (Met408Val), IVS7+7_14delTGGTAAGT, IVS9+43C>T, and IVS10−21C>T, were also detected in our study. Most of their frequencies were different between the Caucasians and Japanese. Over two-fold differences were observed in IVS1−43T>G (0.085 for Caucasians and 0.629 for Japanese), 480C>G (Phe160Leu) (0.216 for Caucasians and 0.086 for Japanese), IVS2+97G>A (0.052 for Caucasians and 0.457 for Japanese), and IVS10−21C>T (0.228 for Caucasians and 0.591 for Japanese). These SNPs, especially IVS1−43T>G and IVS2+97G>A, may be ethnic-specific. Shu *et al.* detected 15 nonsynonymous polymorphisms from 5 different ethnic groups, African-Americans (100 subjects), European-Americans (100 subjects), Asian-Americans (30 subjects), Mexican-Americans (10 subjects), and Pacific-Islanders (7 subjects).[9] Regarding Asian-Americans, they identified 480C>G (Phe160Leu), 1022C>T (Pro341Leu), and 1222A>G (Met480Val), which were also detected in our study. The frequencies of these SNPs were almost the same in the two studies, except for 480C>G (Phe160Leu) (0.017 by their study and 0.086 by ours). This discrepancy may be caused by differences in sample sizes between these studies.

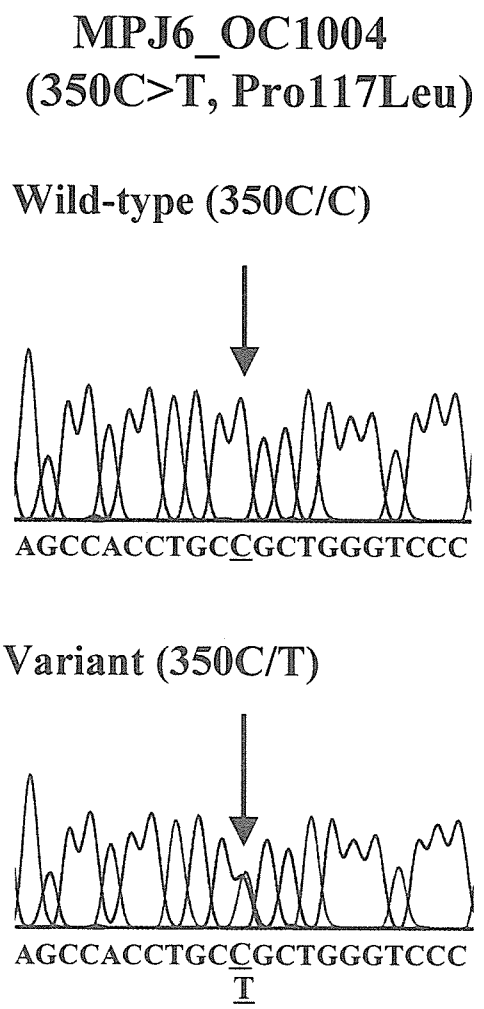

Fig. 1. Electropherograms (sense strands) for the novel nonsynonymous SNP of *SLC22A1*, MPJ6_OC1004 (wild-type 350C/C; variant 350C/T). Arrows indicate the position of the nucleotide change.

One novel nonsynonymous SNP, 350C>T (Pro117Leu), was identified in one heterozygous subject. The electropherograms for this SNP are shown in **Fig. 1**. This SNP is located in exon 1, coding a long extracellular loop between the first and second transmembrane domains. The functional effect of this amino acid substitution on transport activity is currently unknown. This proline residue is conserved among the mammalian OCTs (OCT1, OCT2, and OCT3).[2] The effects of some amino acid substitutions on the OCT1 structure have been evaluated[9,11] by using amino acid scoring systems, especially Grantham and BLOSUM62 values.[13,14] For example, it was shown that the variants with reduced or diminished activity (Pro283Leu, Arg287Gly, and Pro341Leu) gave much higher Grantham values (98, 125, and 98, respectively) indicative of larger chemical changes and more negative BLOSUM62 values (−3, −2, and −3, respectively) showing evolutionally unfavorable changes than those

(22 and 0, respectively) for the variant Phe160Leu with unchanged function.[11] Since the calculated Grantham and BLOSUM62 values for Pro117Leu are 98 and −3, respectively, it is possible that the Leu117 variant shows altered function. Thus, further studies are necessary for this SNP.

Another exonic SNP, 561G>A, resulted in the silent variation Ala187Ala. The other 5 novel SNPs were located in the 5′-UTR region, introns, or 3′-UTR region. The biological significance of these 5 SNPs also remains to be evaluated.

In conclusion, we identified 20 genetic variations including 7 novel ones in *SLC22A1* from Japanese subjects. The SNP 350C>T resulted in an amino acid substitution (Pro117Leu). The present results would be useful for haplotype analysis and pharmacogenetic studies on OCT1.

Acknowledgments: We thank Ms. Chie Knudsen for her secretarial assistance.

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SNP Communication

Genetic Variations of the AHR Gene Encoding Aryl Hydrocarbon Receptor in a Japanese Population

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Full text of this paper is available at http://www.jssx.org

Summary: Aryl hydrocarbon receptor (AhR), encoded by the *AHR* gene, is a transcriptional factor that induces various drug metabolizing enzymes in response to diverse endogenous and exogenous ligands. In order to identify genetic variations of the *AHR* gene, genomic DNA from 242 Japanese individuals was sequenced. We identified 32 single nucleotide variations, including 25 novel ones [7 were in the coding exons, 7 in the introns, 1 in the 5′-untranslated region (UTR), 5 in the 3′-UTR, 2 in the 5′-flanking region, and 3 in the 3′-flanking region] and a GGGGC repeat polymorphism (a novel microsatellite marker) in the promoter region. The novel nonsynonymous variations were 50A>C (Lys17Thr), 1202A>G (Lys401Arg), 1459A>G (Asn487Asp), and 1541T>C (Ile514Thr). The allele frequencies were 0.010 for 1459A>G (Asn487Asp) and 0.002 for the other 3 variations. Also detected in this analysis was the known nonsynonymous single nucleotide polymorphism 1661G>A (Arg554Lys) at a 0.444 frequency.

Key words: *AHR*; genetic variation; nonsynonymous alteration; microsatellite marker

Introduction

Aryl hydrocarbon receptor (AhR), encoded by the *AHR* gene, is a member of the basic-loop-helix/Per-Arnt-Sim family of transcriptional factors.[1] *AHR* mRNA is dominantly expressed in the placenta, lung, heart, pancreas, and liver.[2] Under resting conditions, AhR exists as a cytosolic complex with Hsp90, the co-chaperone p23, and the immunophilin-like protein

On May 20, 2004, these variations were not found in the Japanese Single Nucleotide Polymorphisms (JSNP) (http://snp.ims.u-tokyo.ac.jp/), dbSNP in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP/), or PharmGKB (http://www.pharmgkb.org/do/) databases.

This study was supported in part by the Program for Promotion of Fundamental Studies in Health Sciences (MPJ-1, -3 and -6) of the Pharmaceuticals and Medical Devices Agency (PMDA) of Japan.

Received; May 24, 2004, Accepted; July 5, 2004

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XAP2. Upon binding a ligand, AhR translocates into the nucleus, followed by the replacement of its associated molecule with Arnt, and binds to the xenobiotic responsive elements (XRE) found in the regulatory elements of diverse genes. For example, AhR ligands activate the transcription of drug metabolizing enzymes *CYP1A1*, *CYP1A2*, *CYP1B1*, *UGT1A1*, and *UGT1A6* through XREs in the enhancer regions of these genes.[3-8]

AhR has been reported to be activated by various exogenous aromatic hydrocarbons: e.g., 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and 3-methylcholanthrene.[9] Recently, several endogenous ligands were also identified, such as tryptophan derivatives (e.g., indirubin) and arachidonic acid metabolites (e.g., lipoxin A4).[9,10]

The human *AHR* gene is located on chromosome 7p15 and consists of 12 exons, including a non-coding exon (exon 12).[11] Over 30-fold interindividual differences in CYP1A1 inducibility by aromatic hydrocarbons have been reported in mitogen activated lymphocytes.[12,13] Furthermore, it was suggested that the 7p15 region was involved in such interindividual differences.[11] From these findings, it is possible that differences in AhR transcriptional activity caused by genetic polymorphisms in the *AHR* gene might affect the inducibility of target genes.

Several genetic polymorphisms have been reported in the *AHR* gene.[14] The most common single nucleotide polymorphism (SNP) is 1661G>A (Arg554Lys), which was first found by polymerase chain reaction (PCR)-single strand conformational polymorphism analysis, followed by direct sequencing of these products from 25 Japanese subjects.[13] The functional effect of this variation was marginal in *in vitro* TCDD-induced *CYP1A1* mRNA expression.[15] However, another study suggested that 3-methylcholanthrene-induced CYP1A1 activity in lymphocytes was significantly higher in 554Lys-positive Caucasian subjects than in 554Lys-negative ones.[16] Furthermore, the less frequent variation found in African populations, Val570Ile, is linked with Arg554Lys, and this haplotype shows abrogated TCDD-induced *CYP1A1* mRNA expression.[17] Thus, it is suggested that the genetic polymorphisms in *AHR* at least partly contribute to the interindividual differences in AhR transcriptional activity. However, there has been no comprehensive sequence analysis of *AHR* for the Japanese population.

In this study, all the exons and surrounding introns of *AHR* were sequenced for 242 Japanese subjects. Sequence analysis revealed the identification of 33 genetic variations, including 26 novel ones.

Materials and Methods

Human DNA samples: DNA was extracted from the blood leukocytes of 118 Japanese cancer patients (with lung, stomach and colon cancers) administered irinotecan, and 76 Japanese arrhythmic patients administered mexiletine. Written informed consent was obtained from all participating patients. Forty-eight DNA samples from Epstein-Barr virus-transformed lymphoblastoid cells were also used. They were prepared from healthy Japanese volunteers at the Tokyo Women's Medical University under the auspices of the Pharma SNP Consortium (Tokyo, Japan). Informed consent was also obtained from all healthy subjects. The ethical review boards of the National Cancer Center, the National Cardiovascular Center, the National Institute of Health Sciences, the Pharma SNP Consortium, and the Tokyo Women's Medical University approved this study.

Polymerase chain reaction (PCR) conditions and DNA sequencing: First, the entire *AHR* gene was amplified by three mixed primer sets (Mix 1, Mix 2, and Mix 3 in the "1st PCR" section) shown in **Table 1**. Amplification was performed from 100 ng of genomic DNA using 1.25 units of Ex-Taq (Takara Bio. Inc., Shiga, Japan) with 0.2 μM of the primer sets. Since the exon 1 region is highly GC-rich, this exon was amplified by using the GC-buffer Kit (for LA-Taq, Takara Bio. Inc.). The first PCR conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 60°C for 1 min, and 72°C for 2 min, and then a final extension at 72°C for 7 min. Then, each exon was amplified by Ex-Taq (0.625 units) with a set of primers (0.2 μM) listed in the "2nd PCR" section of **Table 1** (primers were designed in the intronic regions). The second-round PCR conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 60°C for 1 min, and 72°C for 2 min, and then a final extension at 72°C for 7 min. Thereafter, the PCR products were treated with a PCR Product Pre-Sequencing Kit (USB Co., Cleveland, OH, USA) and directly sequenced on both strands using an ABI BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with the primers listed in the "Sequencing" section of **Table 1**. The excess dye was removed by a DyeEx96 kit (Qiagen, Hilden, Germany). The eluates were analyzed on an ABI Prism 3730 DNA Analyzer (Applied Biosystems). For exons 2, 3, and exons 5 through 9, the primer sets for the 2nd PCR were also utilized for sequencing. As for exons 1 (with the 5′-flanking region), 10 and 11, more than 2 primer sets were utilized for sequencing since these regions were long. The 3′ end (about 180 bases) of exon 11 and the 5′ end (about 200 bases) of exon 12 [both are in the 3′-untranslated region (UTR)] were excluded from the current analysis because of the presence of successive thymine or adenine nucleotides in these regions, respectively. All the detected variations were confirmed by repeating the PCR from the genomic DNA and sequenc-

Table 1. Primer sequences utilized for the analysis of human *AHR*

		Amplified and sequenced region	Forward primer (5′ to 3′)	Position of the forward primer*	Reverse primer (5′ to 3′)	Position of the reverse primer*	Amplified length (bp)
1st PCR	Mix 1	Exon 1	GTCTCTCAAACAGGTGAAGT	16633629	AGGAGATTTCAAGACAGGTT	16634962	1,334
	Mix 2	Exon 2	CACTGTGCTACAAATGCTTG	16644944	CTGTTGGGTGAATAAAACTG	16645590	647
		Exon 4 to 6	CAGGAGTGTATGTTTTGGCT	16662888	GGGAATAGTTCTCTGCTGAA	16666264	3,377
		Exon 10	GTGTCAGGTAGGGATGTAAC	16674009	CTGGAAAAAGTACAGGCTTG	16675682	1,674
	Mix 3	Exon 3	GCTGACAACTTGACTAAACC	16657542	TTACGGGACCACTGTCGCAT	16658119	578
		Exon 7 to 9	TGACCATTAGGAACAAGGAG	16668945	AATCACTGCTGGGTTTAGAG	16671399	2,455
		Exon 11 to 12	GTTTACAACTCTCAGGGGTA	16678086	GAATACTGGTAAGTCTTCAG	16681215	3,130
2nd PCR		Exon 1	GTCTCTCAAACAGGTGAAGT	16633629	AGGAGATTTCAAGACAGGTT	16634962	1,334
		Exon 2	CACTGTGCTACAAATGCTTG	16644944	CTGTTGGGTGAATAAAACTG	16645590	647
		Exon 3	GCTGACAACTTGACTAAACC	16657542	TTACGGGACCACTGTCGCAT	16658119	578
		Exon 4	CAGGAGTGTATGTTTTGGCT	16662888	TATCCTCTGCTATCCATAAG	16663445	558
		Exon 5	GCAAGCACCCACTAATCTAA	16665133	ACTCTGTTGCCTGAAACTCA	16665570	438
		Exon 6	GTATTCAGAACACAGACTCC	16665921	GGGAATAGTTCTCTGCTGAA	16666264	344
		Exon 7	TGACCATTAGGAACAAGGAG	16668945	AGGCTACACTGGAAGAATGT	16669531	587
		Exon 8	TCTGTTACCCATACATCTGC	16669881	GAACAAAGAGTGTAACCCTG	16670432	552
		Exon 9	CCAGAACTATGTCACAAGAG	16670829	AATCACTGCTGGGTTTAGAG	16671399	571
		Exon 10	GTGTCAGGTAGGGATGTAAC	16674009	CTGGAAAAAGTACAGGCTTG	16675682	1,674
		Exon 11	GTTTACAACTCTCAGGGGTA	16678086	GGACAGTAAAGTTGGTAGGG	16679865	1,780
		Exon 12	TGCTTACCTACTTTCTTCAG	16679041	GAATACTGGTAAGTCTTCAG	16681215	2,175
Sequencing		Exon 1	GTCTCTCAAACAGGTGAAGT	16633629	GCCGTCTATTTAGAATCCTG	16634194	
			GGTCGGGGGTGCTCCTGCTA	16633879	CGGTGTAGGCTGGGACCACT	16634481	
			CAGGATTCTAAATAGACGGC	16634175	GCTGTCAACAAATCAGGACC	16634906	
		Exon 4	TGTTTTGGCTGTGTTTGTGA	16662898	TATCCTCTGCTATCCATAAG	16663445	
		Exon 10	TGTCAGGTAGGGATGTAACC	16674010	GTATCATTTCCCATCGGTGC	16674621	
			TCTCTATCCTGCTTCAAGTA	16674505	TAGGGGAACTCTTGACTGAT	16675161	
			TGTGTCAGAAGATGAAGCAC	16675014	TGAATGCTGTAGATAACCGA	16675614	
		Exon 11	GTTTACAACTCTCAGGGGTA	16678086	GGAGGAAAGCACTGAGATTA	16678866	
			CTACAGTCAAGATAGAAAGG	16678538	TTCAACATAAGGCACATAGC	16679152	
			TGCTTACCTACTTTCTTCAG	16679041	ACACATAGTTTCCACTGTTC	16679506	
			GAACAGTGGAAACTATGTGT	16679487	GGACAGTAAAGTTGGTAGGG	16679865	
		Exon 12	ATAAAAATGGCTTCGGACAA	16680646	TAAATCCCACAATGTAGCAG	16681116	

* The position of the 5′ end of each primer on NT_007819.14.

Table 2. Summary of *AHR* variations detected in a Japanese population.

SNP ID			Reference	Location	Position		Nucleotide change and flanking sequences (5′ to 3′)	Amino acid change	Number of subjects			
This Study	NCBI (dbSNP)	JSNP			NT_007819.14	From the translational initiation site or from the nearest exon			Wild-type	Hetero-zygote	Homo-zygote	Frequency
MPJ6_AHR001[b]	rs11330131		20)	5′-flanking	16633733_16633741	−808_−800[c]	ATGGCTACCGGCG/−GGGGGGGG CGTCCTTACGTC		0	2	240	0.996
MPJ6_AHR002[a,b]				5′-flanking	16633740	−801[c]	CCGGCGGGGGGGG/AGCGTCCTTACGT		241	1	0	0.002
MPJ6_AHR003[a]				5′-flanking	16633775	−766[c]	CACGTGCCGGGAT/CGAGGGTGGGGCC		241	1	0	0.002
MPJ6_AHR004	rs10249788		20)	5′-flanking	16633799	−742[c]	CCTCAAGGAAGAC/TGGAATGGAATCC		114	107	21	0.308
MPJ6_AHR005[a]				5′-flanking	16633861_16633870	−680_−671[c]	CGGGATCTGGGC(GGGGC)nCGGTGAGGGGT[e]					
MPJ6_AHR006[a,b]				Exon 1 (5′-UTR)	16633910	−631[c]	ATTCAGCCGGTGC/TGCGCGGCGGCGG		241	1	0	0.002
MPJ6_AHR007	rs7796976		20)	Exon 1 (5′-UTR)	16634082	−459[c]	CACCCTGGATTTG/AGGAAGTCCCGGG		101	111	30	0.353
MPJ6_AHR008[a,b]				Exon 1	16634590	50[c]	GCAAGCGGCGGAA/CGCCGGTGCAGAA	Lys17Thr	241	1	0	0.002
MPJ6_AHR009			13)	Exon 2	16645278	132[c]	AGACCGACTTAAT/CACAGAGTTGGAC	Ala44Ala	229	12	1	0.029
MPJ6_AHR010[a]				Intron 3	16657928	IVS3+45	AGGACAGTTGTAA/GATGGAAAATGAA		241	1	0	0.002
MPJ6_AHR011[a]				Intron 4	16663417	IVS4+293	AAATTATTTGCTA/CTCAAATAACTTA		241	1	0	0.002
MPJ6_AHR012[a]				Intron 5	16665372	IVS5+21	TGATGGTACAAAA/CAATAGTGTTGGT		241	1	0	0.002
MPJ6_AHR013[a]				Intron 6	16669025	IVS6−163	GATTTAATGGGCG/ATCCCATGGAGAG		239	3	0	0.006
MPJ6_AHR014	rs2074113	IMS-JST000840		Intron 7	16669423	IVS7+33	TTTATTTTATTGG/TATGTACATTATG		70	129	43	0.444
MPJ6_AHR015[a,b]				Intron 7	16669983	IVS7−180	AAATTTGCATCA/CTAAGCAAAGAAG		241	1	0	0.002
MPJ6_AHR016[a,b]				Intron 8	16670310	IVS8+38	GTCAGAAGAAAAC/TGGCATATACTGT		241	1	0	0.002
MPJ6_AHR017[a,b]				Intron 8	16670885	IVS8−36	TATATTGATTTGG/TGGGTTTGATAAT		241	1	0	0.002
MPJ6_AHR018[a,b]				Exon 10	16674303	1202[c]	AACGAAATACGAA/GGTTGCCTTTTAT	Lys401Arg	241	1	0	0.002
MPJ6_AHR019[a]				Exon 10	16674307	1206[c]	AAATACGAAGTTG/ACCTTTTATGTTT	Leu402Leu	241	1	0	0.002
MPJ6_AHR020[a]				Exon 10	16674560	1459[c]	AACAACTTTTTCA/GACGAATCTATGA	Asn487Asp	237	5	0	0.010
MPJ6_AHR021[a,b]				Exon 10	16674642	1541[c]	AACATGAGCAAAT/CTGACCAGCCTCA	Ile514Thr	241	1	0	0.002
MPJ6_AHR022	rs2066853		13)	Exon 10	16674762	1661[c]	TTGAAGACATCAG/AACACATGCAGAA	Arg554Lys	70	129	43	0.444
MPJ6_AHR023[a,b]				Exon 10	16674835	1734[c]	CATTGACTTAACG/AGATGAAATCCTG	Thr578Thr	241	1	0	0.002
MPJ6_AHR024[a,b]				Exon 10	16674850	1749[c]	TGAAATCCTGACG/ATATGTCCAAGAT	Thr583Thr	241	1	0	0.002
MPJ6_AHR025[a]				Exon 11 (3′-UTR)	16678583	2790[c]	GTGGTGAGGTACC/TGTCTACATTTCA		241	1	0	0.002
MPJ6_AHR026[a]				Exon 11 (3′-UTR)	16678728	2935[c]	TTTGAGCTACTGG/CATTCTTATTAGT		241	1	0	0.002
MPJ6_AHR027[a]				Exon 11 (3′-UTR)	16679080	3287[c]	GCAAATAATGATC/TGAAAAAATAATT		241	1	0	0.002
MPJ6_AHR028[a]				Exon 11 (3′-UTR)	16679087	3294[c]	ATGATCGAAAAAA/GTAATTATTTATT		236	6	0	0.012
MPJ6_AHR029[a]				Exon 11 (3′-UTR)	16679593	3800[c]	ATGGTGCATTGTA/TTAGATATAATGA		240	2	0	0.004
MPJ6_AHR030[a]				3′-flanking	16681128	+84[d]	ATTTCTAGATGAT/CGTGCACATCTAA		239	3	0	0.006
MPJ6_AHR031[a,b]				3′-flanking	16681156	+112[d]	TATGGATGTGTCT/CAATTTAGTCTTT		241	1	0	0.002
MPJ6_AHR032	rs11400459			3′-flanking	16681158_16681159	+114_115[d]	GGATGTGTCTAA−/TTTTAGTCTTTTC		98	112	32	0.364
MPJ6_AHR033[a]				3′-flanking	16681171	+127[d]	TTTAGTCTTTTCC/ATGTACCAGGTTT		241	1	0	0.002

[a] Novel variations detected in our study.
[b] Detected only from the cancer patients.
[c] A of the translation initiation codon ATG is numbered +1.
[d] The nucleotide number from the end of exon 12.
[e] Microsatellite; n=2>n=4, 5 and 6.

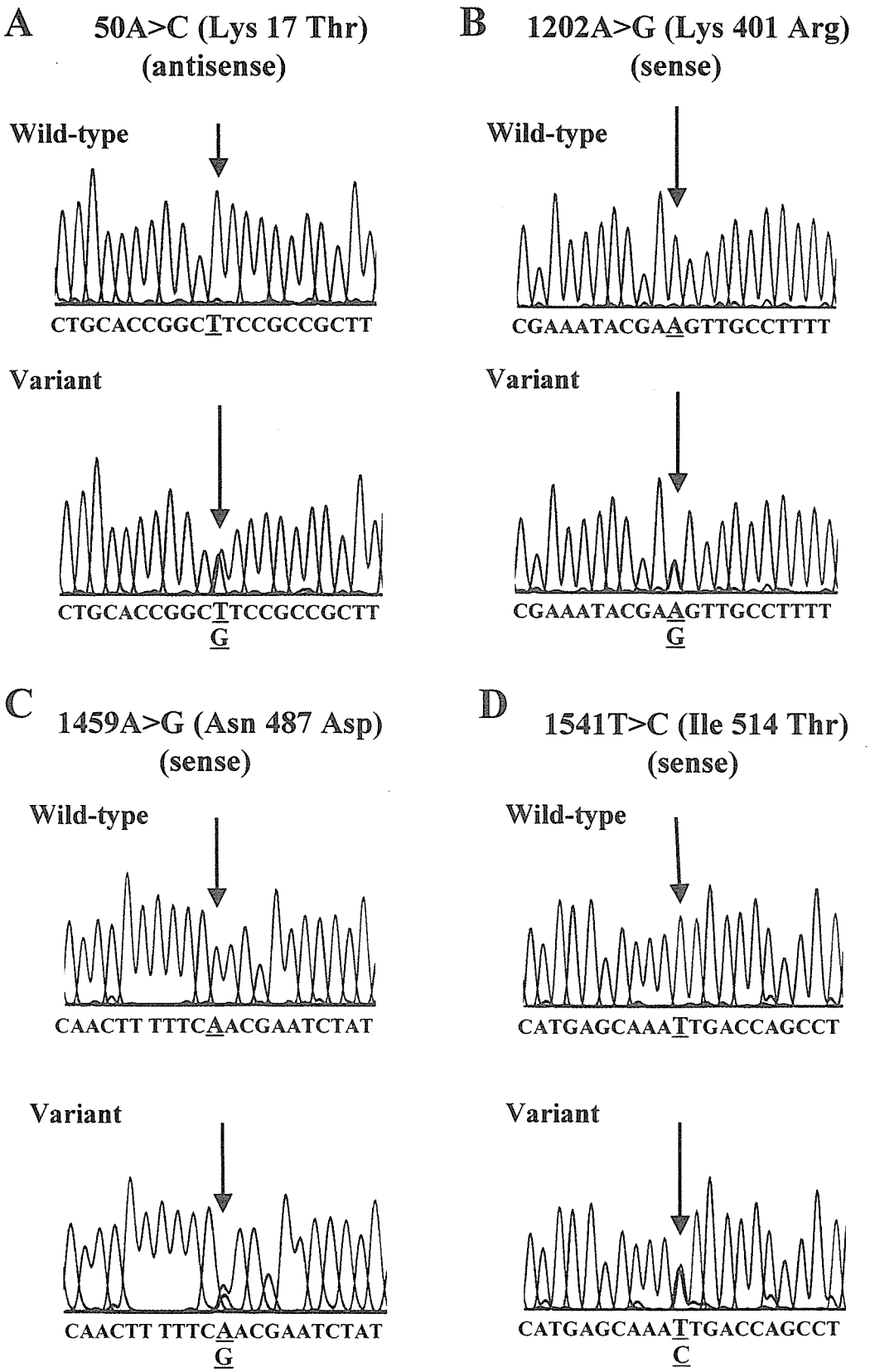

Fig. 1. Electropherograms for the novel nonsynonymous variations of *AHR*. (A) MPJ6_AHR008 (wild-type 50A/A; variant 50A/C). (B) MPJ6_AHR018 (wild-type 1202A/A; variant 1202A/G). (C) MPJ6_AHR020 (wild-type 1459A/A; variant 1459A/G). (D) MPJ6_AHR021 (wild-type 1541T/T; variant 1541T/C). Arrows indicate the variant nucleotide positions.

ing the newly generated PCR products. Furthermore, the rare variations found in only one subject were confirmed by sequencing the PCR fragments produced by amplification with a high fidelity DNA polymerase KOD-Plus- (TOYOBO, Tokyo, Japan). All variable sites in all samples were clearly visible in the electropherograms.

Results and Discussion

NT_007819.14 (Genbank Accession number) was utilized as the reference sequence of *AHR*. Thirty-three genetic variations were identified, including 25 novel single nucleotide variations and 1 novel microsatellite marker, from 242 Japanese individuals (see **Table 2**). Of the 32 single nucleotide variations, 9 were in the coding exons, 8 in the introns, 2 in the 5′-UTR, 5 in the 3′-UTR, 4 in the 5′-flanking region, and 4 in the 3′-flanking region. One novel microsatellite marker was found in the 5′-flanking region. All 32 single nucleotide variations were in Hardy-Weinberg equilibrium.

In the coding region, we detected the known nonsynonymous SNP 1661G>A (Arg554Lys) with a frequency of 0.444, which was similar to the previously reported frequency of 0.43.[13] This SNP was perfectly linked to IVS7+33G>T. SNP 1661G>A (Arg554Lys) was the most frequent in the coding exons, and the other variations were relatively rare (below 0.03). Of these, four novel nonsynonymous variations, 50A>C (Lys17Thr), 1202A>G (Lys401Arg), 1459A>G (Asn487Asp), and 1541T>C (Ile514Thr) were found. Variation 50A>C (Lys17Thr) was detected in a patient with colon cancer. Variation 1202A>G (Lys401Arg) or 1541T>C (Ile514Thr) was found in a patient with small cell lung cancer. Variation 1459A>G (Asn487Asp) was detected from 2 cancer patients (colon and non-small cell lung cancers) and 3 healthy volunteers. All these novel variations were detected as heterozygotes. The electropherograms of these variations are shown in **Fig. 1**. The allele frequencies were 0.010 for 1459A>G (Asn487Asp) and 0.002 for the other variations. Lys-17 is located within the nuclear localization signal sequence. Though a previous mutational study showed no apparent effect of the Lys17Ala mutation on nuclear localization,[18] the effect of the Lys-to-Thr substitution on receptor localization is unknown. Successive deletions of the C-terminal domain showed that the region containing Lys-401 and Asn-487 was important for both ligand binding and ligand-independent binding to DNA, and also that the region including Ile-514 was required for ligand-dependent binding to DNA.[19] Thus, these amino acid alterations might influence AhR function. Among 242 Japanese samples analyzed, we did not detect the low-activity variation 1708G>A (Val570Ile) found in Africans.[17]

In the 5′-flanking region, −808_−800G_9>G_8 and −801G>A were found in 9 successive G repeats within the GC-rich region, which has been recently characterized as the element important for *AHR* basal expression.[20] Notably, the 8 G repeat was dominant (allele frequency of 0.996) in the Japanese population, and the 10 G repeat, which is dominant in Caucasians (0.741), was not found, while the 9 G repeat is described in the reference sequence NT_007819.14. However, the 8 G and 9 G repeats have been reported not to affect *AHR* mRNA expression levels, compared to the 10 G repeat.[20]

AhR is one of the key regulators for many drug metabolizing enzymes. In addition, this receptor has also been suggested to be involved in the induction of various genes related to cell cycle control.[21] Our findings provide fundamental and useful information for genotyping *AHR* in the Japanese, and could be utilized for haplotype determination.

Acknowledgments: We thank Ms. Chie Knudsen for her secretarial assistance.

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0090-9556/04/3209-1048–1054$20.00
DRUG METABOLISM AND DISPOSITION

DMD 32:1048–1054, 2004

Vol. 32, No. 9
1387/1168772
Printed in U.S.A.

SINGLE NUCLEOTIDE POLYMORPHISMS AND HAPLOTYPE FREQUENCIES OF *UGT2B4* AND *UGT2B7* IN A JAPANESE POPULATION

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Received January 12, 2004; accepted May 18, 2004

This article is available online at http://dmd.aspetjournals.org

ABSTRACT:

Both UDP-glucuronosyltransferase 2B4 (UGT2B4) and UGT2B7 are expressed mainly in the human liver and have several overlapping substrates; e.g., catechol estrogens, bile acids, codeine, and carvedilol. To identify novel single nucleotide polymorphisms (SNPs) and haplotypes in a Japanese population, the enhancer/promoter regions, all the exons, and the surrounding intronic regions of *UGT2B4* and *UGT2B7* were sequenced from 136 Japanese individuals. We found 16 and 21 polymorphisms, including 10 and 4 novel ones in *UGT2B4* and *UGT2B7*, respectively. The novel nonsynonymous SNPs were 1364A>G (K455R) and 1531T>C (C511R) in *UGT2B4* and 1192G>A (D398N) in *UGT2B7*. From linkage disequilibrium analysis, several SNPs in *UGT2B7* were found to be highly linked with each other. No close linkage between the SNPs in *UGT2B4* and *UGT2B7* was observed, indicating that each gene is located within an independent haplotype block. Thus, haplotype analysis was separately performed for the two genes. In *UGT2B4*, we unambiguously determined 8 haplotypes and inferred an additional 12 haplotypes using an expectation-maximization-based program. In *UGT2B7*, five haplotypes were unambiguously assigned and an additional eight haplotypes were inferred. The haplotype structure of *UGT2B7* was more diverse than that of *UGT2B4* in terms of the number of frequent SNPs. In addition, ethnic differences in the *UGT2B42 and *UGT2B7**2 haplotypes between the Japanese and the Caucasian and/or African populations were found. Our findings provide fundamental and useful information for genotyping *UGT2B4* and *UGT2B7* in the Japanese, and probably other populations.**

The glucuronidation reaction catalyzed by UDP-glucuronosyltransferases (UGTs) is responsible for clearance of endogenous substrates including bilirubin, bile acids, steroid hormones and thyroid hormones, and xenobiotics, such as clinical drugs and environmental pollutants (Tukey and Strassburg, 2000). Based on homology, the UGTs are classified into two major families, UGT1 and UGT2. The UGT2 family is further divided into two subfamilies, UGT2A and UGT2B (Mackenzie et al., 1997). To date, seven active UGT2B enzymes (UGT2B4, UGT2B7, UGT2B10, UGT2B11, UGT2B15, UGT2B17, and UGT2B28) have been found in humans (Jackson et al., 1987; Ritter et al., 1990; Jin et al., 1993; Beaulieu et al., 1996, 1998; Lévesque et al., 1999, 2001; Turgeon et al., 2000). In addition, numerous homologous pseudogenes have also been discovered, which are clustered with the *UGT2B* gene region on chromosome 4 (4q13) (Monaghan et al., 1994; Turgeon et al., 2000).

This study was supported in part by the Program for Promotion of Fundamental Studies in Health Sciences (MPJ-3 and -6) of the Organization for Pharmaceutical Safety and Research of Japan.

UGT2B4 and UGT2B7 are highly homologous (85.6%) and expressed mainly in the liver. They glucuronidate catechol estrogens, bile acids, codeine, and 3′-azido-3′-dideoxythymidine with overlapping substrate specificities (Pillot et al., 1993; Lévesque et al., 1999; Turgeon et al., 2001; Court et al., 2003; Mackenzie et al., 2003). Our previous study also suggested that both UGT2B4 and UGT2B7 were involved in the glucuronidation of the β-adrenoceptor antagonist, carvedilol (Ohno et al., 2004)

About 7-fold interindividual differences were reported in hepatic mRNA expression levels of both *UGT2B4* and *UGT2B7* (Congiu et al., 2002). Furthermore, the morphine 3-*O*-glucuronidation activity, which is mainly mediated by UGT2B7, varied about 3-fold in the liver microsomes from 20 individuals (Fisher et al., 2000). These differences could be caused in part by polymorphisms in these genes. A common polymorphism in the *UGT2B7* gene (H268Y, *UGT2B7**2) has been found in Caucasians and Asians (Bhasker et al., 2000). More recently, Hirota et al. (2003) found a novel single nucleotide polymorphism (SNP) in *UGT2B7*, 211G>T (A71S), by polymerase chain reaction (PCR)-single-strand conformational polymorphisms analysis

ABBREVIATIONS: UGT, UDP-glucuronosyltransferase; SNP, single nucleotide polymorphism; LD, linkage disequilibrium; PCR, polymerase chain reaction.

TABLE 1

Primers for amplification and sequencing of UGT2B4 *and* UGT2B7

Gene	Step	Direction	Site	Primer Name	Sequence (5′ to 3′)
UGT2B4	First Amplification	Forward		UGT2B4ZF	ATTGACTCTTACGGTAGAAGCAC
		Reverse		UGT2B4ZR	GTAGAAACCAGGTAGGAGTTGC
	Second Amplification	Forward	PPRE[a]	UGT2B4proF1	GATAAAATCAGTAGGAGTTGGAAA
		Reverse	PPRE[a]	UGT2B4proR1	GTGTTGATTCTTACAACCATCAT
		Forward	Exon1	UGT2B4Ex1-1stF	TAAACGCACTTGTGTAAACAGG
		Reverse	Exon1	UGT2B4Ex1-1stR	AAATACCCCACTACCCTGACTT
		Forward	Exon2	UGT2B4Ex2-1stF	CTTCTTTCAGAAAATTACCTAGTTT
		Reverse	Exon2	UGT2B4Ex2-1stR	TGCTTTACTCTTCCCACTTCC
		Forward	Exon3	UGT2B4Ex3F	ACAATTCTTTCACAGTGCTTGC
		Reverse	Exon3	UGT2B4Ex3R	ACCAATGAAAAACACCTTCTGT
		Forward	Exon4	UGT2B4Ex4newF	ACATCAGTCTGAGTAGTTCTTGTT
		Reverse	Exon4	UGT2B4Ex4newR	CTCTGACTCTGATTGTAAAGAATG
		Forward	Exon5	UGT2B4Ex5F	ATCTAGGAAACACCGTCACATT
		Reverse	Exon5	UGT2B4Ex5R	AATAACCACTCAAAAATAAAAGCA
		Forward	Exon6	UGT2B4Ex6F	ATCTCCTGACCTCGTGATCC
		Reverse	Exon6	UGT2B4Ex6R	TGTCACAAGAAGAAAGGAATCTC
	Sequencing	Forward	PPRE	UGT2B4proF1	GATAAAATCAGTAGGAGTTGGAAA
		Reverse	PPRE	UGT2B4proRseq1	AAACAAAAGTAAACCAAGCCTGT
		Forward	Exon1	UGT2B4Ex1-1stF	TAAACGCACTTGTGTAAACAGG
		Forward	Exon1	UGT2B4Ex1-Fseq2	GATGAACTTGTCCAGAGAGGTC
		Forward	Exon1	UGT2B4Ex1-Fseq3	AGATGCTGTTTTCCCCTTTG
		Reverse	Exon1	UGT2B4Ex1-Rseq1	TAAGAGTAGATGGGCTGTTGG
		Reverse	Exon1	UGT2B4Ex1Rseq2-3	ACAACAGGCACATAGGAAGG
		Reverse	Exon1	UGT2B4Ex1-1stR	AAATACCCCACTACCCTGACTT
		Forward	Exon2	UGT2B4Ex2-Fseq1	TTTTTTTCCCCCATCAGGA
		Reverse	Exon2	UGT2B4Ex2-1stR	TGCTTTACTCTTCCCACTTCC
		Forward	Exon3	UGT2B4Ex3F	ACAATTCTTTCACAGTGCTTGC
		Reverse	Exon3	UGT2B4Ex3R	ACCAATGAAAAACACCTTCTGT
		Forward	Exon4	UGT2B4Ex4newF	ACATCAGTCTGAGTAGTTCTTGTT
		Reverse	Exon4	UGT2B4Ex4newR	CTCTGACTCTGATTGTAAAGAATG
		Reverse	Exon4	UGT2B4Ex4Rseq-he	TTTCATTTTATTTTTAAGTTTTTCC
		Forward	Exon5	UGT2B4Ex5F	ATCTAGGAAACACCGTCACATT
		Reverse	Exon5	UGT2B4Ex5R	AATAACCACTCAAAAATAAAAGCA
		Forward	Exon6	UGT2B4Ex6Fseq	GCTGGGATTGCAGGTGTG
		Reverse	Exon6	UGT2B4Ex6R	TGTCACAAGAAGAAAGGAATCTC
UGT2B7	First Amplification	Forward		UGT2B7ZF2	ATCTTGATTTCATAACTGTCATT
		Reverse		UGT2B7ZtaqR	GTAGTGTTTTCTTCATTGCCAC
	Second Amplification	Forward	Promoter	UGT2B7proF1	AAGTTTACAAAAATATGTGGACC
		Reverse	Promoter	UGT2B7proR2	TATTAGCAAAATTACTGAAGTCC
		Forward	Exon1	UGT2B7Ex1F1st2seq1	AAGGGTTACATTTTAACTTCTTGG
		Reverse	Exon1	UGT2B7Ex1R1st1seq3	ATTCACTTACCAAAACCCCACT
		Forward	Exon2	UGT2B7Ex2-1stF2	GATATTTGCCTACATTTTTGCC
		Reverse	Exon2	UGT2B7Ex2-1stR2	CCCTTTGTAAATATTATTTGATTGG
		Forward	Exon3	UGT2B7Ex3Fseq1	TCCAATAATTCCTCAAAATACTG
		Reverse	Exon3	UGT2B7Ex3-Rseq2	CTCATAGTTTCTCCAAGATTATCC
		Forward	Exon4	UGT2B7Ex4-1stF3	GCCACACGTAGGTTTTCTTTT
		Reverse	Exon4	UGT2B7Ex4Rseq1	ATAAAGATTCCCCCGATTCAGA
		Forward	Exon5	UGT2B7Ex5-1stF3	ACCGTATAGCCTTCAGTTACATAC
		Reverse	Exon5	UGT2B7Ex5-1stR4	AGAAAATGGTTATACTCTGAGGTG
		Forward	Exon6	UGT2B7Ex6F	TTCTTTAACTCGGTGTCTGAGGG
		Reverse	Exon6	UGT2B7Ex6R	TGGAATAAACTGAAGTAGTCTCAC
	Sequencing	Forward	Promoter	UGT2B7proF1	AAGTTTACAAAAATATGTGGACC
		Reverse	Promoter	UGT2B7proR2	TATTAGCAAAATTACTGAAGTCC
		Forward	Exon1	UGT2B7Ex1seqF1-4	TTTATCTTTGGACATAACCATGA
		Forward	Exon1	UGT2B7Ex1seqF2	GACTGTACTGGCATCTTCAGC
		Forward	Exon1	UGT2B7Ex1seqF3	ATGCTATTTTTCCCTGTAGTGAG
		Reverse	Exon1	UGT2B7Ex1Rseq1-2	TGACCATCTCTTAATCTGTTGC
		Reverse	Exon1	UGT2B7Ex1seqR2	CGTAGGAAGGAGGGAAAATAA
		Reverse	Exon1	UGT2B7Ex1R1st1seq3	ATTCACTTACCAAAACCCCACT
		Forward	Exon2	UGT2B7Ex2Fseq2	TTTTTTCTATTCCTGTCAGGAAG
		Reverse	Exon2	UGT2B7Ex2-Rseq2	ACTCATAAAACTCATATACGTGTGA
		Forward	Exon3	UGT2B7Ex3Fseq1	TCCAATAATTCCTCAAAATACTG
		Reverse	Exon3	UGT2B7Ex3-Rseq2	CTCATAGTTTCTCCAAGATTATCC
		Forward	Exon4	UGT2B7Ex4-1stF3	GCCACACGTAGGTTTTCTTTT
		Reverse	Exon4	UGT2B7Ex4Rseq1	ATAAAGATTCCCCCGATTCAGA
		Reverse	Exon4	UGT2B7Ex4heteroR	TTCTCCCTTAAGACTGGAAAATC
		Forward	Exon5	UGT2B7Ex5-1stF3	ACCGTATAGCCTTCAGTTACATAC
		Reverse	Exon5	UGT2B7Ex5seqR2	ATAAAAGCAGATTTCAGATTGGT
		Forward	Exon6	UGT2B7Ex6F	TTCTTTAACTCGGTGTCTGAGGG
		Reverse	Exon6	UGT2B7Ex6Rseq1	GTCTCACCTATCAGGTTTTCCA

[a] Peroxisome proliferator-activated receptor responsive element (PPRE) was used for the direct amplification of genomic DNA.

TABLE 2

UGT2B4 SNPs and haplotypes in a Japanese population

Exon / Intron			enhancer	promoter	Intron 2	Intron 3	Intron 4	Intron 4	Intron 4	Intron 4	Intron 4	Exon 5	Intron 5	Intron 5	Exon 6	Exon 6	Exon 6	Exon 6	Number of subject	
Position (cDNA)[a]			-1255	-162	IVS2+44	IVS3-13_6	IVS4+61	IVS4+109_114	IVS4+145	IVS4+146	IVS4+161_162	1212	IVS5-83	IVS5-52	1364	1374 T>A	1375 C>A	1531		
Amino acid change			A>C	T>G	C>T	delT	T>C[d]	delATAAAA	C>T	G>A	insTGATAA	A>T Δ404Δ	G>C	C>T	A>G K455R	D458E	R459R	T>C C511R		
Reference			rs 6821129[b]	rs 941389[b]	Novel	Novel	rs 1826690[b]	Novel	Novel	Novel	Novel	Novel	rs 4415025[b]	Novel	Novel	Lévesque *et al.* (1999)	Lévesque *et al.* (1999)	Novel		
Allele specificity															*455R	*2		*511R		
Diplotype[c]	*1/*1	*1a/*1b																	32	133
		*1a/*1a																	27	
		*1b/*1b																	14	
		*1b/*1c																	7	
		*1a/*1c																	7	
		*1a/*1d																	5	
		*1b/*1d																	4	
		*1a/*1f																	4	
		*1b/*1g?																	3	
		*1a/*1c																	3	
		*1b/*1c																	2	
		*1b/*1f																	2	
		*1a/*1j																	2	
		*1a/*1k																	2	
		*1b/*1i																	1	
		*1a/*1h																	1	
		*1a/*1i																	1	
		*1a/*1g																	1	
		*1a/*1l																	1	
		*1a/*1m?																	1	
		*1g/*1h?																	1	
		*1a/*1o?																	1	
		*1c/*1c																	1	
		*1c/*1d																	1	
		*1c/*1e																	1	
		*1c/*1j?																	1	
		*1c/*1h																	1	
		*1c/*1l																	1	
		*1c/*1n?																	1	
		*1e/*1h																	1	
		*1e/*1i																	1	
		*1a/*1p?																	1	
		*1a/*1q?																	1	
	*1/*2	*1a/*2a?																	1	1
	*1/*455R	*1a/*455R																	1	1
	*1/*511R	*1a/*511R?																	1	1
SNP frequency			0.151	0.169	0.004	0.033	0.434	0.004	0.015	0.044	0.511	0.052	0.055	0.004	0.004	0.004	0.004	0.004		

			Frequency	
Haplotype	*1	*1a	0.441	0.989
		*1b	0.290	
		*1c	0.081	
		*1d	0.037	
		*1e	0.029	
		*1f	0.022	
		*1g	0.018	
		*1h	0.015	
		*1i	0.011	
		*1j	0.011	
		*1k	0.007	
		*1l	0.007	
		*1m?	0.004	
		*1n?	0.004	
		*1o?	0.004	
		*1p?	0.004	
		*1q?	0.004	
	*2	*2a?	0.004	0.004
	*455R	*455R	0.004	0.004
	*511R	*511R?	0.004	0.004

[a] Position (cDNA-based): A of the translational start codon is numbered 1. NT_077444.2 was used as the reference sequence.
[b] dbSNP number in the National Center for Biotechnology Information.
[c] White, wild type; gray, heterozygote; black, homozygote. The haplotypes were described as a number plus a small alphabetical letter.
[d] C in the reference sequence (NT_077444.2).

and subsequent sequencing of genomic DNA from 46 Japanese individuals (Hirota et al., 2003). As for *UGT2B4*, a T-to-A transversion at nucleotide 1374 has been found in Caucasians and Africans, but not in Asians, which leads to an amino acid change at codon 458 from aspartic acid to glutamic acid (*UGT2B4*2*) (Lévesque et al., 1999; Lampe et al., 2000; Riedy et al., 2000). However, there has been no report on comprehensive sequencing or haplotype analysis of the *UGT2B4* and *UGT2B7* genes in a Japanese population.

To identify novel SNPs and to reveal haplotype structures in the Japanese, the known enhancer/promoter regions, all the exons, and the surrounding intronic regions of *UGT2B4* and *UGT2B7* were sequenced from 136 Japanese individuals. The enhancer/promoter regions surveyed were −1400 to −1110 upstream of the translational initiation codon, which included the peroxisome proliferator-activated receptor responsive element in *UGT2B4*, and 360 base pairs upstream of the *UGT2B7* initiation codon (Carrier et al., 2000; Ishii et al., 2000; Barbier et al., 2003a,b). We found 16 and 21 genetic polymorphisms in *UGT2B4* and *UGT2B7*, respectively, performed linkage disequilibrium (LD) analysis, and estimated their respective haplotypes.

Materials and Methods

Patients. The 136 Japanese subjects were arrhythmic patients who were administered beta-blockers. Genomic DNA was extracted directly from blood leukocytes. The ethics committees of the National Cardiovascular Center and the National Institute of Health Sciences approved this study. Written informed consent was obtained from all patients.

PCR Conditions for DNA Sequencing. First, the entire *UGT2B4* (except for the enhancer regions amplified with the UGT2B4proF1-R1 primers) and *UGT2B7* genes were amplified from genomic DNA (200 ng) using 2.5 units of Z-*Taq* (Takara Bio Inc., Shiga, Japan) with a 0.2 μM concentration of the first amplification primers ("First Amplification" in Table 1). The PCR was performed as follows: 30 cycles of 98°C for 5 s, 55°C for 5 s, and 72°C for 190 s. Then, each region/exon was amplified by Ex-*Taq* (0.625 unit) (Takara Bio Inc.) using the first PCR products as templates with the second amplification primers (0.2 μM) that were designed in the introns ("Second Amplification" in Table 1). The second round of PCR was 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min, and then a final extension for 5 min at 72°C. These PCR products were then purified using a PCR Product PreSequencing Kit (USB, Cleveland, OH) and directly sequenced using an ABI Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and the primers listed in Table 1 ("Sequencing"). The excess dye

was removed by a DyeEx96 kit (QIAGEN, Hilden, Germany), and the eluates were analyzed on an ABI Prism 3700 DNA Analyzer (Applied Biosystems). All the SNPs were confirmed by repeating the PCR from genomic DNA and sequencing these newly generated PCR products.

LD and Haplotype Analysis. LD analysis was carried out using the SNPAlyze software (version 3.1) (Dynacom Co. Ltd., Yokohama, Japan), and a pairwise two-dimensional map between SNPs was obtained for the chi square and rho square values. Some of the haplotypes were unambiguous, with homozygous SNPs at all sites or a heterozygous SNP at only one site. Separately, the diplotype configurations (combinations of haplotypes) were inferred by an expectation-maximization-based program, LDSUPPORT, which determines the posterior probability distribution of the diplotype configuration for each subject based on the estimated haplotype frequencies (Kitamura et al., 2002). The diplotype configurations of the subjects had a probability (certainty) over 0.93 for 129 subjects in *UGT2B4* and over 0.99 for all 136 subjects in *UGT2B7*. The haplotypes inferred in only 1 of the 272 total chromosomes are described as the haplotype name with a question mark, since the predictability for these rare haplotypes is known to be low in some cases.

Results

***UGT2B4* and *UGT2B7* Polymorphisms Detected in a Japanese Population.** First, the enhancer/promoter regions, all exons, and the surrounding intronic regions of *UGT2B4* and *UGT2B7* were sequenced from 136 Japanese subjects. For the reference sequences, NT_077444.2 and NT_030640.1 (GenBank accession numbers) were utilized for *UGT2B4* and *UGT2B7*, respectively.

In *UGT2B4*, 16 polymorphisms, including 10 novel ones (two nonsynonymous SNPs, one synonymous SNP, four intronic SNPs, one insertion, and two deletions in the introns) were detected (see Table 2). All the allele frequencies were in Hardy-Weinberg equilibrium. No SNP was found within the known peroxisome proliferator-activated receptor-α and farnesoid X receptor-binding DR-1 site (peroxisome proliferator-activated receptor responsive element). Two novel transitions found in exon 6, A-to-G at position 1364 and T-to-C at position 1531, were nonsynonymous with amino acid changes, K455R and C511R, respectively (Fig. 1, A–D). The known nonsynonymous SNP, 1374T>A (D458E, *UGT2B4**2), was also found in one subject as heterozygous. The frequency of *2 was low compared with those of Caucasians and Africans (Lampe et al., 2000; Riedy et al., 2000).

As for *UGT2B7*, 21 polymorphisms were detected in this study. Among them, four polymorphisms were novel (Table 3): 1192G>A (D398N) in exon 5 (Fig. 1, E and F), 915G>A (V305V) in exon 3, IVS4 + 154_155insA, and IVS4 + 185C>A in intron 4. All the allele frequencies were in Hardy-Weinberg equilibrium. Also, the known nonsynonymous SNPs, 802C>T (H268Y, *UGT2B7**2) and 211G>T (A71S), with frequencies of 0.254 and 0.173, respectively, were detected. These frequencies were similar to those seen in a previous report for a Japanese population (Hirota et al., 2003) but were different from Caucasian frequencies (Bhasker et al., 2000; Holthe et al., 2003). Also, the SNPs −327G>A, −161C>T, −125T>C, 372A>G (R124R), 1059G>C (L353L), and 1062C>T (Y354Y), which have been reported by Hirota et al. (2003), were found.

LD Analysis. Using the SNPs detected, LD analysis was performed

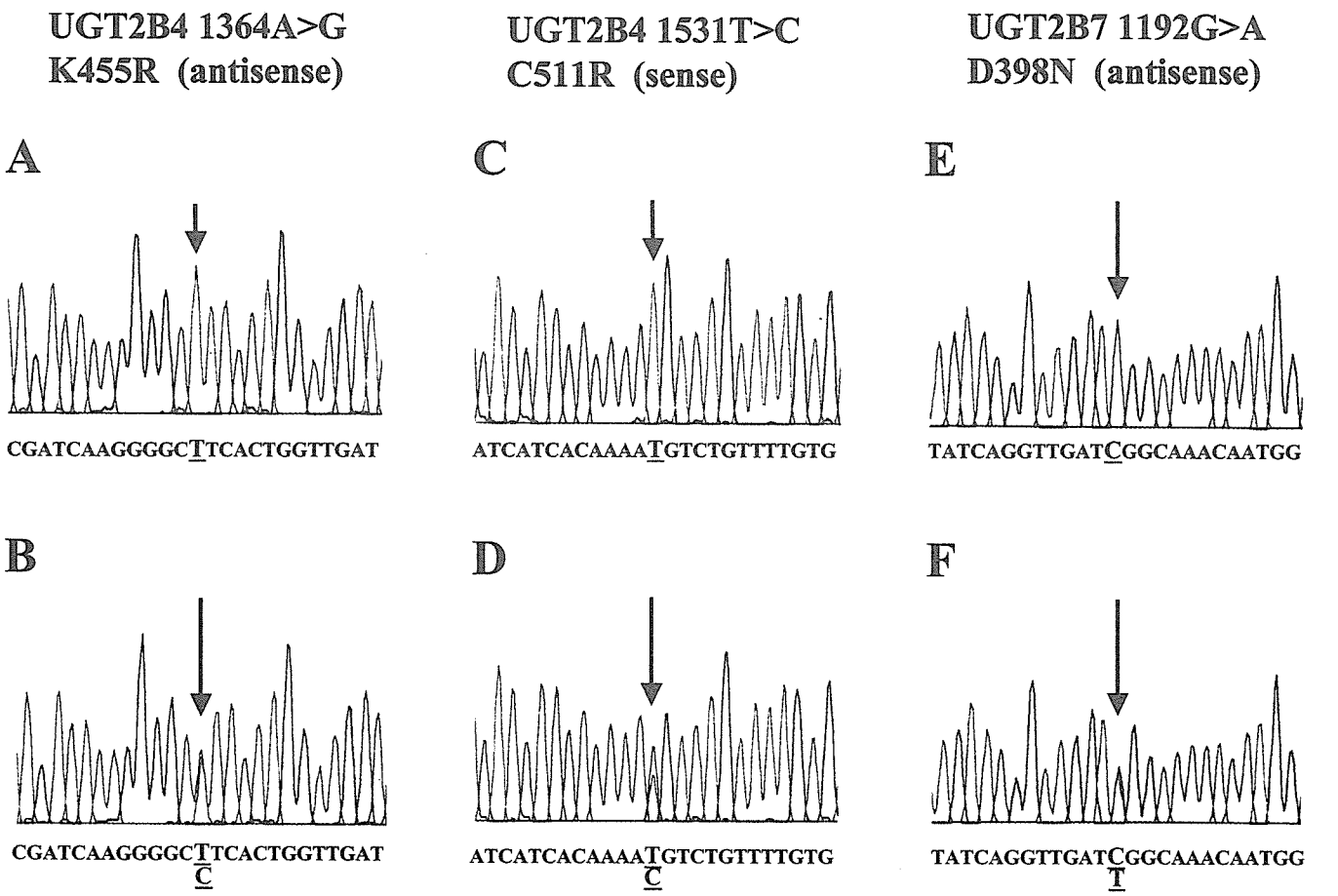

FIG. 1. Electropherograms for the novel nonsynonymous SNPs *UGT2B4* 1364A>G (A and B), *UGT2B4* 1531T>C (C and D), and *UGT2B7* 1192G>A (E and F). A, Wild-type (WT)/WT (A/A). B, WT/variant (VT) (A/G). C, WT/WT (T/T). D, WT/VT (T/C). E, WT/WT (G/G). F, WT/VT (G/A). Arrows indicate the variant nucleotide positions.

TABLE 3

UGT2B7 SNPs and haplotypes in a Japanese population

Exon / Intron			promoter	promoter	promoter	Exon 1	Exon 1	Exon 1	Exon 2	Exon 2	Intron 2	Intron 2	Exon 3	Intron 3	Intron 3	Exon 4	Exon 4	Intron 4	Intron 4	Intron 4	Intron 4	Intron 4	Exon 5	Number of subject	
Position (cDNA)[a]			-327 G>A	-161 C>T	-125 T>C	211 G>T	372 A>G	735 A>G	801 T>A	802 C>T	IVS2+11 5 A>G	IVS2+148 A>G	915 G>A	IVS3-116 A>G	IVS3-114 G>A	1059 G>C	1062 C>T	IVS4+64 T>A	IVS4+154 155 insA	IVS4+185 C>A	IVS4-154 G>C	IVS4-129 T>C	1192 G>A		
Amino acid change						A71S	R124R	T245T	P267P	H268Y			V305V			I353L	Y354Y						D398N		
Reference			Hirota et al. (2003)	Hirota et al. (2003)	Hirota et al. (2003)	Hirota et al. (2003)	Hirota et al. (2003)	Holthe et al. (2003)	Bhaskar et al. (2000)	Bhasker et al. (2000)	rs 7441750[b]	rs 7441774[b]	Novel	rs 5013211[b]	rs 4257713[b]	Hirota et al. (2003)	Hirota et al. (2003)	rs 4337789[b]	Novel	Novel	rs 4588522[b]	rs 4364327[b]	Novel		
Allele specificity						*71S				*2													*398N		
Diplotype[c]	*1 *1	*1a *1a																						20	44
		*1a *1b																						10	
		*1a *1c																						7	
		*1c *1d																						3	
		*1a *1d																						2	
		*1b *1b																						1	
		*1b *1d																						1	
	*1 *2	*1a *2a																						24	45
		*1a *2b																						7	
		*1c *2a																						6	
		*1b *2a																						3	
		*1a *2d?																						1	
		*1a *2c?																						1	
		*1d *2a																						1	
		*1f *2b?																						1	
		*1g *2a?																						1	
	*1 *71S	*1a *71S																						13	22
		*1b *71S																						5	
		*1c *71S																						3	
		*1e *71S?																						1	
	*2 *2	*2a *2a																						5	6
		*2a *2b																						1	
	*2 *71S	*2a *71S																						11	12
		*2b *71S																						1	
	*71S *71S	*71S *71S																						6	6
	*71S *398N	*71S *398N?																						1	1
SNP frequency			0.254	0.254	0.077	0.173	0.199	0.077	0.254	0.254	0.254	0.254	0.004	0.257	0.217	0.257	0.074	0.257	0.257	0.390	0.257	0.257	0.004		

			Frequency	
Haplotype	*1	*1a	0.386	0.570
		*1b	0.077	
		*1c	0.070	
		*1d	0.026	
		*1e?	0.004	
		*1f?	0.004	
		*1g?	0.004	
	*2	*2a	0.210	0.254
		*2b	0.037	
		*2c?	0.004	
		*2d?	0.004	
	*71S	*71S	0.173	0.173
	*398N	*398N?	0.004	0.004

[a] Position (cDNA-based): A of the translational start codon is numbered 1. NT 030640.1 was used as the reference sequence.
[b] dbSNP number in the National Center for Biotechnology Information.
[c] White, wild type; gray, heterozygote alteration; black, homozygote. The haplotypes were described as a number plus a small alphabetical letter.

and the pairwise values for rho square and chi square were obtained. Since the data for chi square and rho square were almost equivalent, the data for rho square are depicted in Fig. 2. In *UGT2B4*, a perfect linkage was seen among IVS4 + 109_114delATAAAA, IVS5-52C>T, 1374T>A (D458E), and 1375C>A (ρ^2 = 1.00). Close associations were found between −1255A>C and −162T>G (ρ^2 = 0.87) and between IVS4 + 61T>C and IVS4 + 161_162insT-GATAA (ρ^2 = 0.80). A weak association between IVS3-13_6delT and IVS5-83G>C (ρ^2 = 0.49) was also found. The other associations were much lower (below 0.2 as rho square values).

In contrast to *UGT2B4*, strong LDs were observed in multiple SNPs within *UGT2B7*. The associations among −327G>A, −161C>T, 801T>A, 802C>T (H268Y), IVS2 + 115A>G, and IVS2 + 148A>G, and among IVS3-116A>G, 1059G>C, IVS4 + 64T>A, IVS4 + 154_155insA, IVS4-154G>C, and IVS4-129T>C were prominent, and both of the combinations showed perfect linkages (ρ^2 = 1.00). Furthermore, these two groups of linkages were also strongly associated with each other at ρ^2 values over 0.94, and IVS3-114G>A was often associated with these 12 variations (ρ^2 = 0.78 or higher). Strong LDs were also seen between 735A>G and 1062C>T (ρ^2 = 0.95), and between 211G>T (A71S) and 372A>G (ρ^2 = 0.84).

UGT2B4 and *UGT2B7* are separated by approximately 360 kilobases on chromosome 4. Strong linkages were not found between the SNPs of *UGT2B4* and those of *UGT2B7* (Fig. 2). Thus, our results suggest that the two genes are not within the same haplotype block.

Haplotype Analysis. Since no strong linkage was observed between *UGT2B4* and *UGT2B7*, haplotype analysis was performed separately.

As for *UGT2B4*, a group of haplotypes without amino acid changes was defined as **1*, and the group bearing the nonsynonymous D458E (*2 allele) was named the *2 haplotypes. Eight haplotypes were first unambiguously assigned by the presence of homozygous SNPs at all sites (**1a*, **1b*, and **1c*) or a heterozygous SNP at only one site [**1d*, **1f*, **1h*, **1j*, and **455R* (the haplotype bearing 455R was tentatively named **455R*)] (Table 2). Separately, we estimated the diplotype configuration (a combination of haplotypes) for each subject by LDSUPPORT software. The diplotype configurations of all the subjects had a probability (certainty) greater than 0.93, except for seven subjects with the maximum probability of 0.51 or higher. In Table 2, the diplotypes for these seven subjects were also described as diplotypes with a question mark. The additionally inferred haplotypes were 12 haplotypes [**1e*, **1g*, **1i*, **1k*-**1q*, **2a*, and **511R* (the haplotype bearing 511R was tentatively named **511R*)] (Table 2). The most frequent haplotype was **1a* (frequency: 0.441), followed by **1b* (0.290), **1c* (0.081), **1d* (0.037), **1e* (0.029), and **1f* (0.022). The frequencies of the other haplotypes were less than 0.02. Thus, the *UGT2B4*1* haplotypes mainly consist of **1a* and **1b* (total frequency, 0.731).

Regarding *UGT2B7*, the haplotypes without amino acid changes were defined as **1* haplotypes, and the haplotypes bearing H268Y (**2* allele) were named **2*. Five haplotypes were first unambiguously assigned by homozygous SNPs at all sites [**1a*, **1b*, **2a*, and **71S* (the haplotype bearing 71S was tentatively named **71S*)] or a heterozygous SNP at only one site (**2b*) (Table 3). Estimation by LDSUPPORT software inferred all the diplotype configurations with a probability (certainty) greater than 0.99. Eight additionally inferred haplotypes were **1c* to **1g*, **2c*, **2d*, and **398N* (the haplotype bearing 398N was tentatively named **398N*) (Table 3). The most frequent haplotype was **1a* (frequency, 0.386), followed by **2a* (0.210) and **71S* (0.173). The frequencies of the other haplotypes were less than 0.1. Our **2a*, **2b*, and **2d* haplotypes correspond to the Norwegian haplotype A; **1a*, **1b*, and **1e* to haplotype B; and **1c* to haplotype C (Holthe et al., 2003). These data demonstrated that the haplotype structure of *UGT2B7* is more diverse than that of

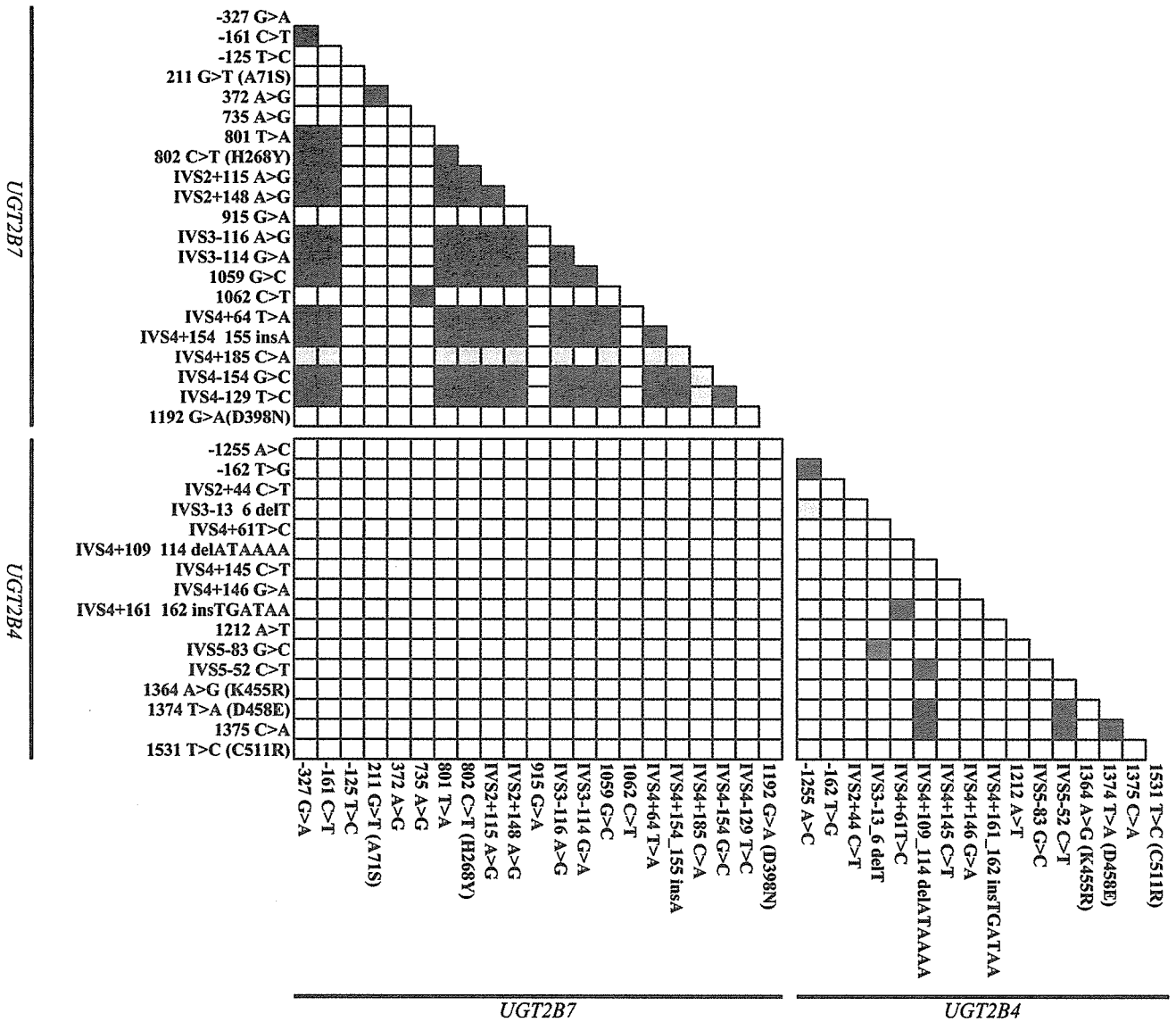

FIG. 2. LD analysis of *UGT2B4* and *UGT2B7* SNPs. Pairwise LD is expressed as ρ^2 (from 0 to 1) by a 10-graded blue color. The denser color represents the higher linkage. Since *UGT2B7* is located upstream of *UGT2B4* (Riedy et al., 2000), the SNPs in *UGT2B7* are described first.

UGT2B4 in terms of the number of frequent SNPs, although their substrate specificities and gene structures are similar.

Discussion

In this study, the genomic DNA from 136 Japanese subjects was sequenced, and 16 and 21 polymorphisms in *UGT2B4* and *UGT2B7*, respectively, were found.

As for *UGT2B4*, *UGT2B4*2* (1374T>A, D458E) is thought to be fairly common in Caucasian and African populations, with frequencies of approximately 0.2 and 0.15, respectively (Lampe et al., 2000; Riedy et al., 2000). This SNP was detected in one subject as a heterozygote in this study, but this SNP is still rare in the Japanese (allele frequency, 0.004). We found two novel nonsynonymous SNPs in *UGT2B4*, 1364A>G (K455R) and 1531T>C (C511R). Their positions are located in the latter half (UDP-glucuronic acid binding) domain and cytosolic domain, respectively. The cysteine at position 511 in UGT2B4 is highly conserved in human UGTs. It has been suggested that cytosolic cysteine residues (507, 511, and 514 in rat UGT1A6) are important for UGT1A6 enzymatic activity (Ikushiro et al., 2002). Furthermore, the mutant truncated at amino acid 512 of UGT2B1 (C513 corresponds to C511 in UGT2B4) was reduced to approximately 40% of the activity of the enzyme truncated at the 514-residue (Meech et al., 1996). Thus, the C511R substitution might alter enzymatic activity, although the functional significance remains to be determined.

The *UGT2B4* haplotype structure is relatively simple. *UGT2B4*2*, **455R*, and **511R* were each found in only one subject. Most haplotypes were rare except for the two major haplotypes, **1a* and **1b*. Furthermore, we found no polymorphisms in the reported *UGT2B4* enhancer region.

As for *UGT2B7*, *UGT2B7*2* (802C>T, H268Y) was shown to be perfectly linked with −327G>A, −161C>T, 801T>A, IVS2 + 115A>G, and IVS2 + 148A>G, and closely linked with IVS3-116A>G, 1059G>C, IVS4 + 64T>A, IVS4 + 154_155insA, IVS4-154G>C, and IVS4-129T>C. Furthermore, on the basis of previous studies (Hirota et al., 2003; Holthe et al., 2003), −1302G>A, −1295C>T, −1111C>T, and −899A>G may also be associated with this haplotype group (*2). Holthe et al. (2003) identified three haplotypes using SNPs detected in Norwegians: haplotype A (**2a*, **2b*, and **2d* in this study), haplotype B (**1a*, **1b*, and **1e* in this study), and haplotype C (**1c* in this study). Their frequencies were 0.56, 0.33, and 0.11 for

haplotypes A, B, and C, respectively. These frequencies were different from those in the Japanese determined in this study: 0.25, 0.47, and 0.07 for haplotype A, B, and C, respectively. Thus, the **1* and **2* haplotype distributions of *UGT2B7* are suggested to be different between Caucasians and Asians ($P < 0.01$ by the χ^2 test), and the frequency of the **2* haplotypes in the Japanese was much lower than that in Norwegians (Holthe et al., 2003). Although no remarkable functional difference was observed between the **1* and **2* haplotypes in several reports (Bhasker et al., 2000; Holthe et al., 2002, 2003; Court et al., 2003), it was recently reported that *UGT2B7*2* showed a significantly higher morphine-6-*O*-glucuronide/morphine ratio than that with *UGT2B7*1* (Sawyer et al., 2003). Thus, it is possible that the difference in the *UGT2B7*2* frequencies might lead to ethnic differences in morphine metabolism and disposition.

SNP 211G>T (A71S) in *UGT2B7* was recently reported in the Japanese at a frequency of 0.185 (Hirota et al., 2003), which was similar to our data (0.173). Codon 71 is located within the N-terminal (substrate binding) domain, and A71S causes a change from a lipophilic side chain to a hydrophilic one. This SNP has not been reported in other ethnic groups and is always associated with 372A>G in the Japanese (this haplotype was named **71S* in this study). SNP 372A>G alone was found at a frequency of 0.03 without association with 211G>T (**71S*) in Norwegians (Holthe et al., 2003)

One novel nonsynonymous SNP, 1192G>A (D398N), was detected in this study. D398 is located in the latter half (UDP-glucuronic acid binding) domain. This acidic amino acid is highly conserved in mammalian UGTs. In *UGT1A6*, D394 (corresponding to D398 in *UGT2B7*) and D397 (corresponding to D401 in *UGT2B7*) are the most probable sites for interactions with a uridinyl moiety (Radominska-Pandya et al., 1999). Thus, alteration from an acidic amino acid (D) to a neutral amino acid (N) might influence the binding of UDP-glucuronic acid. In fact, we have preliminary findings that the variant enzyme with the *UGT2B7*398N* (but not **71S*) haplotype has reduced glucuronidation activity compared with the wild-type enzyme (with**1a* haplotype) toward 7-hydroxy-4-trifluoromethylcoumarin (50 μM) in vitro (Jinno et al., unpublished data).

A SNP in the *UGT2B7* promoter region, −125T>C, which is located in the canonical binding site for the octamer transcription factor-1 (Carrier et al., 2000), was shown to be the binding site for nuclear proteins by the DNase I footprint assay (Ishii et al., 2000). Because only the **1b* haplotype has this −125T>C SNP, it would be interesting to determine whether the expression of UGT2B7 was different between the subjects with **1b* and the other **1* haplotypes.

Finally, the 20 and 13 haplotypes in *UGT2B4* and *UGT2B7*, respectively, estimated in this study provide fundamental information for genotyping *UGT2B4* and *UGT2B7* in the Japanese and would be useful for studies on the association between the haplotypes and pharmacokinetic or clinical parameters.

Acknowledgments. We thank Chie Knudsen for secretarial assistance.

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Celiprolol, A Vasodilatory β-Blocker, Inhibits Pressure Overload–Induced Cardiac Hypertrophy and Prevents the Transition to Heart Failure via Nitric Oxide–Dependent Mechanisms in Mice

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Background—The blockade of β-adrenergic receptors reduces both mortality and morbidity in patients with chronic heart failure, but the cellular mechanism remains unclear. Celiprolol, a selective β_1-blocker, was reported to stimulate the expression of endothelial NO synthase (eNOS) in the heart, and NO levels have been demonstrated to be related to myocardial hypertrophy and heart failure. Thus, we aimed to clarify whether celiprolol attenuates both myocardial hypertrophy and heart failure via the NO-signal pathway.

Methods and Results—In rat neonatal cardiac myocytes, celiprolol inhibited protein synthesis stimulated by either isoproterenol or phenylephrine, which was partially suppressed by N^G-nitro-L-arginine methyl ester (L-NAME). Four weeks after transverse aortic constriction (TAC) in C57BL/6 male mice, the ratio of heart weight to body weight (mg/g) (8.70±0.42 in TAC, 6.61±0.44 with celiprolol 100 mg · kg^{-1} · d^{-1} PO, $P<0.01$) and the ratio of lung weight to body weight (mg/g) (10.27±1.08 in TAC, 7.11±0.70 with celiprolol 100 mg · kg^{-1} · d^{-1} PO, $P<0.05$) were lower and LV fractional shortening was higher in the celiprolol-treated groups than in the TAC group. All of these improvements were blunted by L-NAME. Celiprolol treatment significantly increased myocardial eNOS and activated phosphorylation of eNOS. Myocardial mRNA levels of natriuretic peptide precursor type B and protein inhibitor of NO synthase, which were increased in the TAC mice, were decreased in the celiprolol-treated mice.

Conclusions—These findings indicated that celiprolol attenuates cardiac myocyte hypertrophy both in vitro and in vivo and halts the process leading from hypertrophy to heart failure. These effects are mediated by a selective β_1-adrenergic receptor blockade and NO-dependent pathway. (***Circulation.* 2004;110:692-699.**)

Key Words: receptors, adrenergic, beta ■ heart failure ■ hypertrophy ■ nitric oxide

Accumulated evidence shows that stimulation of β-adrenergic receptors (ARs) can induce cardiac myocyte hypertrophy. Indeed, a nonselective β-AR agonist, isoproterenol, is frequently used as a pharmacological inducer of cardiac hypertrophy.[1] Animal studies and clinical trials have shown that β-blockers can attenuate ventricular hypertrophy.[2,3] However, the mechanism is not completely understood.

In recent years, nitric oxide (NO) has been demonstrated to be effective in antihypertrophy and inhibiting cardiac remodeling.[4,5] Augmented endothelial NO synthase (eNOS) signaling by some drugs, such as ACE inhibitors,[6] statins,[7,8] and estrogens,[9] has been reported by our and other laboratories to be associated with improvement of cardiac remodeling. Intriguingly, β-blockers with vasodilating properties, such as nebivolol and carvedilol, have also been reported to augment NO release from endothelial cells.[10] Recent studies in our laboratory demonstrated that celiprolol, a selective β_1-blocker with vasodilating properties, increased NO production in canine myocardial ischemia,[11] and we also reported that inhibition of eNOS can induce myocardial hypertrophy in rats.[12] However, very few studies were designed to explore the relation between eNOS signaling pathway and the inhibitory effect of β-blockers on cardiac remodeling. Thus, we

Received September 4, 2003; de novo received March 21, 2004; revision received May 6, 2004; accepted May 11, 2004.

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Circulation **is available at http://www.circulationaha.org** **DOI: 10.1161/01.CIR.0000137831.08683.E1**

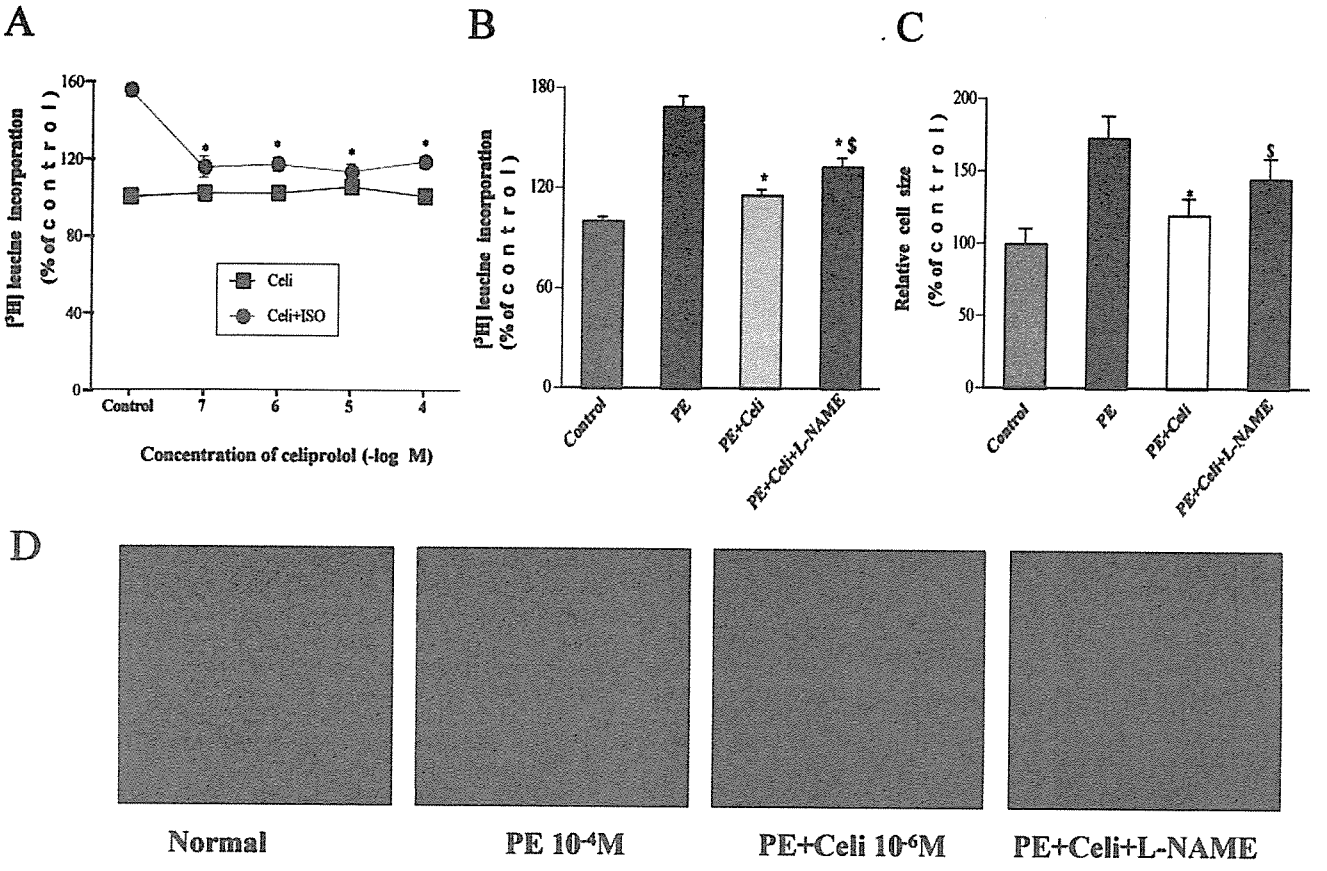

Figure 1. Results of cardiac myocyte culture. A, Protein synthesis was inhibited by celiprolol (celi) at concentrations ranging from 10^{-4} to 10^{-7} mol/L in a dose-independent manner, and this concentration range did not affect normal myocytes. *$P<0.01$ vs Control. B, Celiprolol (10^{-6} mol/L) inhibited protein synthesis stimulated by PE (10^{-4} mol/L), and this effect was partially abolished by cotreatment with L-NAME (10^{-6} mol/L). *$P<0.01$ vs PE, \$$P<0.05$ vs PE+Celi. C, Cell size was calculated from 200 cells in every treatment group. Increase in cell size caused by PE was reduced by treatment with celiprolol (10^{-6} mol/L), and L-NAME diminished effect of celiprolol. *$P<0.01$ vs PE, \$$P<0.05$ vs PE+Celi. D, Representative images of cardiac myocytes stained with rhodamine phalloidin and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). Concentrations for all agents are same as in B.

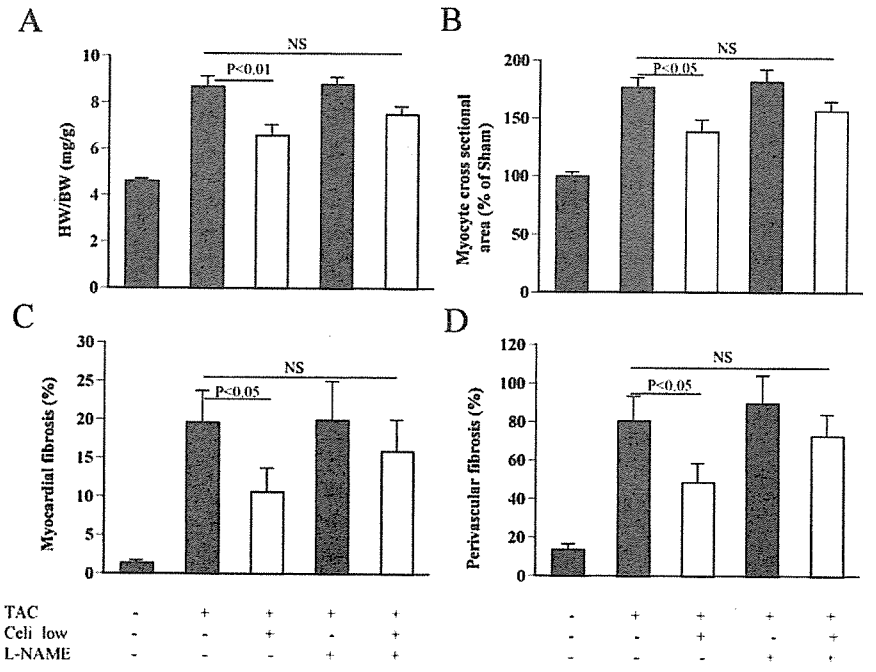

Figure 2. Celiprolol improves heart remodeling. HW/BW ratio (A) and myocyte cross-sectional area (B) were decreased significantly in TAC mice treated with celiprolol 100 mg · kg^{-1} · d^{-1} (Celi low) or 200 mg · kg^{-1} · d^{-1} (Celi high) in comparison with untreated TAC mice. L-NAME (100 mg · kg^{-1} · d^{-1}) alone did not increase degree of myocyte hypertrophy under conditions of pressure overload. However, it partially abolished antihypertrophic effect of celiprolol (100 mg · kg^{-1} · d^{-1}). Similar results on myocardial fibrosis (C) and perivascular fibrosis (D) were also noted. Numbers of mice in Sham, TAC, TAC+Celi low, TAC+L-NAME, and TAC+Celi+L-NAME groups are 10, 19, 11, 6, and 5, respectively.