

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

This Study	NCBI (dbSNP)	JSNP	Location	Position		From the translational initiation site or from the nearest exon	Nucleotide change and flanking sequence (5' to 3')	Amino acid change	Frequency
				NT_007422.12					
MPJ6_OC1001 ^a			Exon 1 (5'-UTR)	2830215		-94 ^b	ACTGATTTCAAAC/ACACTCCTTTTCA		0.004
MPJ6_OC1002	rs2297373	IMS-JST051906	Exon 1	2830431		123 ^b	GGGCATCGTCTTC/GCTGGGTTTCACA	Phe41Leu	0.004
MPJ6_OC1003	rs1867351	IMS-JST051907, ssj0008475	Exon 1	2830464		156 ^b	CCACTGCCAGAGT/CCCTGGGGTGGCT	Ser52Ser	0.444
MPJ6_OC1004 ^a			Exon 1	2830658		350 ^b	GGAGCCACCTGCC/TGCTGGGTCCCTG	Pro117Leu	0.004
MPJ6_OC1005	rs4646272	ssj0005309	Intron 1	2838434		IVS1 - 43	ACTCACACATGGT/GTCTGTGCTTTTC		0.629
MPJ6_OC1006 ^a			Intron 1	2838442		IVS1 - 35	ATGGTTCTGTGCT/CTTTGCTCCTCT		0.082
MPJ6_OC1007	rs683369	ssj0008476	Exon 2	2838545		480 ^b	TGGGGTCTTTC/GTTTGGCTCTCT	Phe160Leu	0.086
MPJ6_OC1008	rs4646273	ssj0005310	Intron 2	2838677		IVS2 + 97	ACATGACCAATTG/AGAAATTAAGTCA		0.457
MPJ6_OC1009	rs3737088	IMS-JST082069	Intron 2	2840506		IVS2 - 99	AGGACAGTGTGC/TGGTGGCTCCGA		0.017
MPJ6_OC1010 ^a			Exon 3	2840650		561 ^b	GCTGGTCAACCCG/AGTGTGGGGCGTG	Ala187Ala	0.009
MPJ6_OC1011	rs4646276	ssj0005318	Intron 4	2844495		IVS4 - 98	TACAGCCCAACG/ATGGGAGGGCAG		0.470
MPJ6_OC1012	rs2282142	IMS-JST031025, ssj0008479	Intron 5	2844856		IVS5 - 61	ACCTCCCAAGGG/ACTCCAGGTGGC		0.168
MPJ6_OC1013	rs2282143	IMS-JST031026, ssj0008480	Exon 6	2844984		1022 ^b	TGTTCCGCACGCC/TGCGCTGAGGAA	Pro341Leu	0.168
MPJ6_OC1014 ^a			Intron 6	2845098		IVS6 + 75	ATAGATTAGAGAC/GAGTGGAACTACT		0.022
MPJ6_OC1015	rs628031		Exon 7	2848186		1222 ^b	TACCCATGGCCA/GTGTCAAATTTGT	Met408Val	0.810
MPJ6_OC1016	rs4646281	ssj0005324	Intron 7	2848247_2848254		IVS7 + 7_14	CACCTGGTAAGTGGTAAGT/-TGICTGCTTTCA		0.810
MPJ6_OC1017 ^a			Intron 8	2852130		IVS8 + 108	CAAGCATTGTCA/GGTTTGGACATTG		0.004
MPJ6_OC1018	rs2297374	IMS-JST051908, ssj0008483	Intron 9	2863326		IVS9 + 43	TCTTTTGCAGCTC/TGGCAGTGGGCTC		0.302
MPJ6_OC1019	rs622591	IMS-JST070846, ssj0008484	Intron 10	2866868		IVS10 - 21	TTTTAACTCCAAC/TTTTAAATTTGT		0.591
MPJ6_OC1020 ^a			Exon 11 (3'-UTR)	2866961_2866963		1671_1673 ^b	CACCTGAGAGAGATG/-TTTTGCGGGCATG		0.004

^a Seven novel variations detected in our study.

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

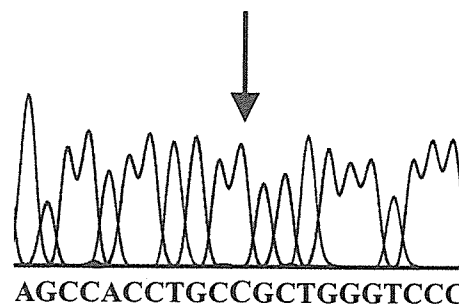
Results and Discussion

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_14delITGGTAAGT, 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.

MPJ6_OC1004 (350C>T, Pro117Leu)

Wild-type (350C/C)



Variant (350C/T)

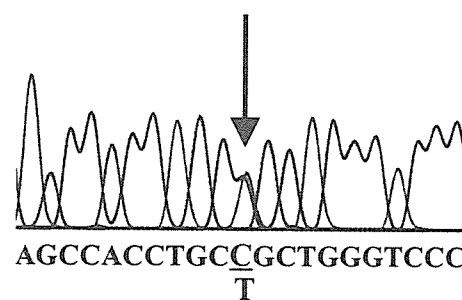


Fig. 1. Electropherograms (sense strands) for the novel nonsynonymous SNP of *SLC22A1*, MPJ6_OC1004 (wild-type 350C/C; variant 350C/T). Arrows indicate the position of the nucleotide change.

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.

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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

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.

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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

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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 μ M 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 μ M) 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	GTCTCTCAAAACAGGTGAAGT	16633629	AGGAGATTTCAAGACAGGTT	16634962	1,334
	Mix 2	CACTGTGCTACAAAATGCTTG CAGGAGTGTATGTTTGGCT GTGTCAGGTAGGATGTAAC	16644944 16662888 16674009	CTGTTGGGTGAATAAAAACCTG GGGAATAGTTCCTGCTGAA CTGGAAAAAGTACAGGCTTG	16645590 1666264 16675682	647 3,377 1,674
	Mix 3	GCTGACAAACTTGACTAAACC TGACCATTAGGAACAAGGAG GTTTACAACCTCAGGGGTA	16657542 16668945 16678086	TTACGGGACCACTGTCCGCAT AATCACTGCTGGGTTTAGAG GAATACTGGTAAGTCTTCAG	16658119 16671399 16681215	578 2,455 3,130
2nd PCR	Exon 1	GTCTCTCAAAACAGGTGAAGT	16633629	AGGAGATTTCAAGACAGGTT	16634962	1,334
	Exon 2	CACTGTGCTACAAAATGCTTG	16644944	CTGTTGGGTGAATAAAAACCTG	16645590	647
	Exon 3	GCTGACAACTTGACTAAACC	16657542	TTACGGGACCACTGTCCGCAT	16658119	578
	Exon 4	CAGGAGTGTATGTTTGGCT	16662888	TATCCTCTGCTATCCATAAG	16663445	558
	Exon 5	GCAAGCACCCACTAATCTAA	16665133	ACTCTGTTGCCGAAACTCA	16665570	438
	Exon 6	GATTTCAGAACACAGACTCC	16665921	GGGAATAGTTCCTGCTGAA	16666264	344
	Exon 7	TGACCATTAGGAACAAGGAG	16668945	AGGCTACACTGGAAGAATGT	16669531	587
	Exon 8	TCTGTTACCCATACATCTGC	16669881	GAACAAAGAGTGTAAACCCTG	16670432	552
	Exon 9	CCAGAACTATGTCACAAGAG	16670829	AATCACTGCTGGGTTTAGAG	16671399	571
	Exon 10	GTGTCAGGTAGGATGTAAC	16674009	CTGGAAAAAGTACAGGCTTG	16675682	1,674
	Exon 11	GTTTACAACCTCAGGGGTA	16678086	GGACAGTAAAGTTGGTAGGG	16679865	1,780
	Exon 12	TGCTTACCTACTTCTTCAG	16679041	GAATACTGGTAAGTCTTCAG	16681215	2,175
Sequencing	Exon 1	GTCTCTCAAAACAGGTGAAGT GGTCGGGGGTGCTCCTGCTA CAGGATCTAAATAGACGGC	16633629 16633879 16634175	GCCGTCATTTAGAAATCCTG CGGTGTAGGCTGGGACCACT GCTGTCAACAAATCAGGACC	16634194 16634481 16634906	
	Exon 4	TGTTTTGGGTGTTTGTGA TGTCAGGTAGGATGTAACC	16662898 16674010	TATCCTCTGCTATCCATAAG GTATCATTTCCCATCGGTGC	16663445 16674621	
	Exon 10	TCCTATCCTGCTTCAAGTA TGTCAGAAAGATGAAGCAC	16674505 16675014	TAGGGGAACCTTTGACTGAT TGAATGCTGTAGATAACCGA	16675161 16675614	
	Exon 11	GTTTACAACCTCAGGGGTA CTACAGTCAAGATAGAAAAGG	16678086 16678538	GGAGAAAGCACTGAGATTA TTCAACATAAGGCACATAGC	16678866 16679152	
	Exon 12	TGCTTACCTACTTCTTCAG GAACAGTGGAAAATATGTT ATAAAAATGGCTTCGGACAA	16679041 16679487 16680646	ACACATAGTTTCCACTGTTT GGACAGTAAAGTTGGTAGGG TAAATCCCACAATGTAGCAG	16679506 16679865 16681116	

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

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This Study	NCBI (dbSNP)	JSNP	Reference	Location	NT_007819.14	Position		Nucleotide change and flanking sequences (5' to 3')	Amino acid change	Number of subjects		
						From the translational initiation site or from the nearest exon	From the translational initiation site or from the nearest exon			Wild-type	Hetero-zygote	Homo-zygote
MPJ6_AHR001 ^b	rs11330131		20)	5'-flanking	16633733_16633741	-808_ -800 ^c	ATGGCTACCGCG/-GGGGGGGGG CGTCCCTTACGTC		0	2	240	0.996
MPJ6_AHR002 ^{a,b}				5'-flanking	16633740	-801 ^c	CCGGGGGGGGG/AGCGTCTTACGT		241	1	0	0.002
MPJ6_AHR003 ^a				5'-flanking	16633775	-766 ^c	CACGTCCGGGAT/CGAGGTGGGCC		241	1	0	0.002
MPJ6_AHR004	rs10249788		20)	5'-flanking	16633799	-742 ^c	CCTAAGGAAGAC/TGGAAATGGAATCC		114	107	21	0.308
MPJ6_AHR005 ^a				5'-flanking	16633861_16633870	-680_ -671 ^c	CGGATCTGGGC(GGGG)CGGTGAGGGGT ^c					
MPJ6_AHR006 ^{a,b}				Exon 1 (5'-UTR)	16633910	-631 ^c	ATTCACCGGTGC/TGCCGCGCGGGG		241	1	0	0.002
MPJ6_AHR007	rs7796976		20)	Exon 1 (5'-UTR)	16634082	-459 ^c	CACCCTGGATTG/AGGAAGTCCCGG		101	111	30	0.353
MPJ6_AHR008 ^{a,b}				Exon 1	16634590	50 ^c	GCAAGCGCGGA/CGCCGGTGCAGAA	Lys17Thr	241	1	0	0.002
MPJ6_AHR009			13)	Exon 2	16645278	132 ^c	AGCCGACTTAAT/CACAGATTGGAC	Ala44Ala	229	12	1	0.029
MPJ6_AHR010 ^a				Intron 3	16657928	IVS3+45	AGACAGCTTAA/TGATGAAATGAA		241	1	0	0.002
MPJ6_AHR011 ^a				Intron 4	16663417	IVS4+293	AAATATTGCTA/CTCAATAACTTA		241	1	0	0.002
MPJ6_AHR012 ^a				Intron 5	16665372	IVS5+21	TGATGTACAAA/CAATAGTGTGGT		241	1	0	0.002
MPJ6_AHR013 ^a				Intron 6	16669025	IVS6+163	GATTAATGGCG/ATCCATGGAG		239	3	0	0.006
MPJ6_AHR014	rs2074113	IMS-JST000840		Intron 7	16669423	IVS7+33	TTTATTTATGG/TAATACATATG		70	129	43	0.444
MPJ6_AHR015 ^{a,b}				Intron 7	16669983	IVS7-180	AAATTTGCATCA/CTAAGCAAAAG		241	1	0	0.002
MPJ6_AHR016 ^{a,b}				Intron 8	16670310	IVS8+38	GTCAGAAGAAAC/TGGCATACTGT		241	1	0	0.002
MPJ6_AHR017 ^{a,b}				Intron 8	16670885	IVS8-36	TATATTGATTGG/TGGGTTTGATAAT		241	1	0	0.002
MPJ6_AHR018 ^{a,b}				Exon 10	16674303	1202 ^c	AACGAAATACGAA/GGTTGGCTTTAT	Lys401Arg	241	1	0	0.002
MPJ6_AHR019 ^a				Exon 10	16674307	1206 ^c	AAATACGAAATTG/ACCTTTATGTTT	Leu402Leu	241	1	0	0.002
MPJ6_AHR020 ^a				Exon 10	16674560	1459 ^c	AACAATTTTCA/GACGAATCTATGA	Asn487Asp	237	5	0	0.010
MPJ6_AHR021 ^{a,b}				Exon 10	16674642	1541 ^c	AACATGAGCAAAT/CTGACCACTCA	Ile514Thr	241	1	0	0.002
MPJ6_AHR022	rs2066853		13)	Exon 10	16674762	1661 ^c	TTGAAGACATCAG/AACACATGCAGA	Arg554Lys	70	129	43	0.444
MPJ6_AHR023 ^{a,b}				Exon 10	16674835	1734 ^c	CAITGACTTAACG/AGATGAAATCCTG	Thr578Thr	241	1	0	0.002
MPJ6_AHR024 ^{a,b}				Exon 10	16674850	1749 ^c	TGAAATCCCTGACG/ATATGTCCAAGAT	Thr583Thr	241	1	0	0.002
MPJ6_AHR025 ^a				Exon 11 (3'-UTR)	16678583	2790 ^c	GTGGTGAGGTACC/TGCTACATTTCA		241	1	0	0.002
MPJ6_AHR026 ^a				Exon 11 (3'-UTR)	16678728	2935 ^c	TTTGAGTACTGG/CATCTTTATTAGT		241	1	0	0.002
MPJ6_AHR027 ^a				Exon 11 (3'-UTR)	16679087	3287 ^c	GCAAATAATGATC/TGAAAATAAAT		241	1	0	0.002
MPJ6_AHR028 ^a				Exon 11 (3'-UTR)	16679087	3294 ^c	ATGATCGAAAAA/GTAAATTTATT		236	6	0	0.012
MPJ6_AHR029 ^a				Exon 11 (3'-UTR)	16679593	3800 ^c	ATGGTCATGTA/TTAGATAATAATGA		240	2	0	0.004
MPJ6_AHR030 ^a				3'-flanking	16681128	+84 ^d	ATTCTAGATGAT/CGTGCACATCAA		239	3	0	0.006
MPJ6_AHR031 ^{a,b}				3'-flanking	16681156	+112 ^d	TATGATGTGTCT/GAATTTAGTCTT		241	1	0	0.002
MPJ6_AHR032	rs11400459			3'-flanking	16681158_16681159	+114_115 ^d	GGATGTGCTAA - TTTTAGTCTTTC		98	112	32	0.364
MPJ6_AHR033 ^a				3'-flanking	16681171	+127 ^d	TTTAGTCTTTC/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.

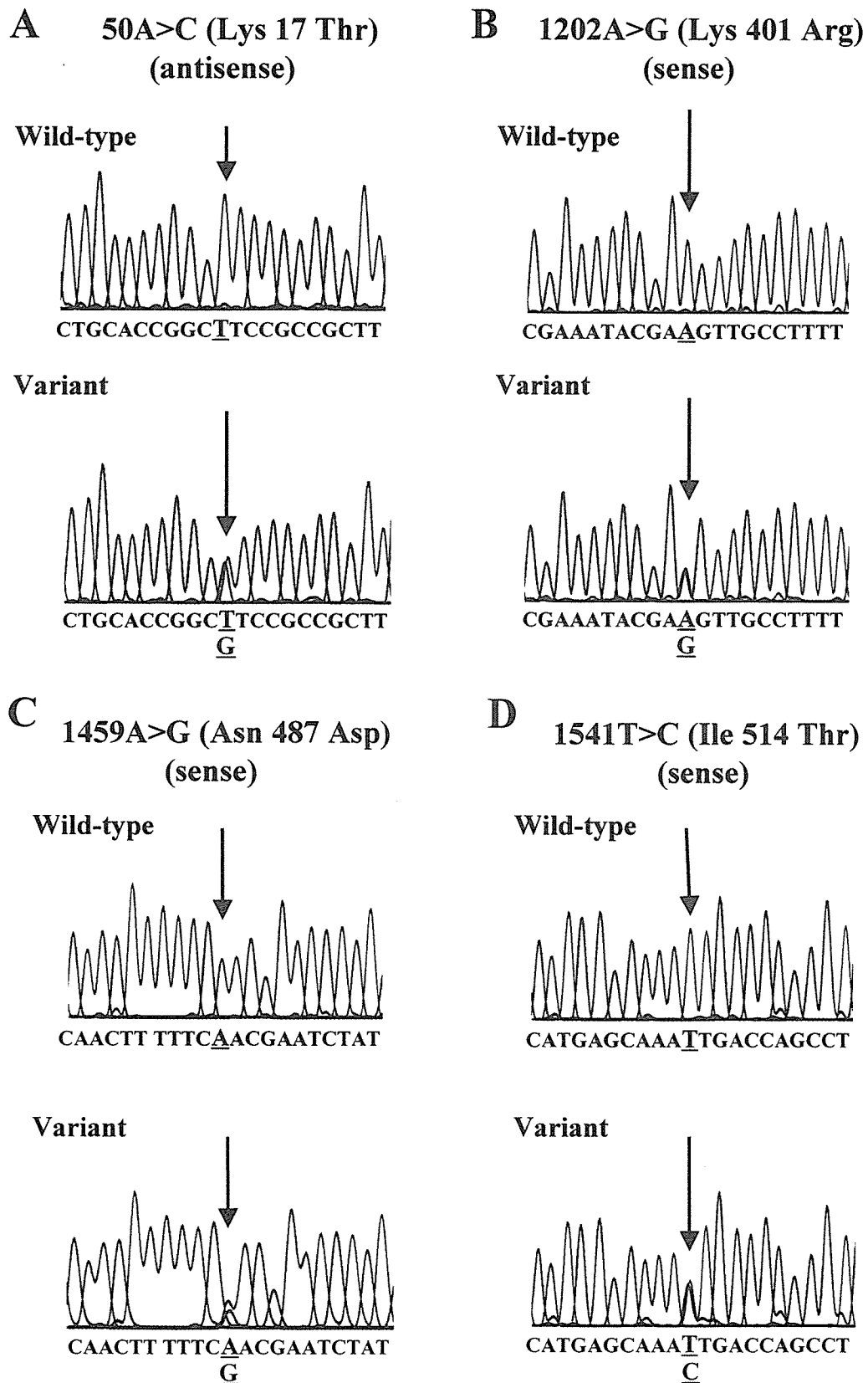


Fig. 1. Electropherograms for the novel nonsynonymous variations of *AHR*. (A) MPJ6_AHR008 (wild-type 50A/A; variant 50A/C). (B) MPJ6_AHR018 (wild-type 1202A/A; variant 1202A/G). (C) MPJ6_AHR020 (wild-type 1459A/A; variant 1459A/G). (D) MPJ6_AHR021 (wild-type 1541T/T; variant 1541T/C). Arrows indicate 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 non-synonymous 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₋-800G₉>G₈ 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.

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

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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 non-synonymous 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 *UGT2B4*2* 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).

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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	GATAAAATCAGTAGGAGTTGAAA	
		Reverse	PPRE ^a	UGT2B4proR1	GTGTTGATTCTTACAACCATCAT	
		Forward	Exon1	UGT2B4Ex1-1stF	TAAACGCACCTGTGTAAACAGG	
		Reverse	Exon1	UGT2B4Ex1-1stR	AAATACCCCACTACCTGACTT	
		Forward	Exon2	UGT2B4Ex2-1stF	CTTCTTTACAGAAAATTAC2AGTTT	
		Reverse	Exon2	UGT2B4Ex2-1stR	TGCTTTACTCTTCCCCTTCC	
		Forward	Exon3	UGT2B4Ex3F	ACAATTCCTTACAGTGCTTGC	
		Reverse	Exon3	UGT2B4Ex3R	ACCAATGAAAAACACCTTCTGT	
		Forward	Exon4	UGT2B4Ex4newF	ACATCAGTCTGAGTAGTTCTTGTT	
		Reverse	Exon4	UGT2B4Ex4newR	CTCTGACTCTGATTGTAAAGAATG	
		Forward	Exon5	UGT2B4Ex5F	ATCTAGGAAACACCGTCACATT	
		Reverse	Exon5	UGT2B4Ex5R	AATAACCACTCAAAAATAAAAAGCA	
		Forward	Exon6	UGT2B4Ex6F	ATCTCCTGACCTCGTGATCC	
		Reverse	Exon6	UGT2B4Ex6R	TGTCACAAGAAGAAAGGAATCTC	
		Sequencing	Forward	PPRE	UGT2B4proF1	GATAAAATCAGTAGGAGTTGAAA
			Reverse	PPRE	UGT2B4proRseq1	AAACAAAAGTAAACCAAGCCTGT
			Forward	Exon1	UGT2B4Ex1-1stF	TAAACGCACCTGTGTAAACAGG
			Forward	Exon1	UGT2B4Ex1-Fseq2	GATGAACCTGTCCAGAGAGGTC
			Forward	Exon1	UGT2B4Ex1-Fseq3	AGATGCTGTTTTCCCTTTG
			Reverse	Exon1	UGT2B4Ex1-Rseq1	TAAGAGTAGATGGGCTGTTGG
	Reverse		Exon1	UGT2B4Ex1Rseq2-3	ACAACAGGCACATAGGAAGG	
	Reverse		Exon1	UGT2B4Ex1-1stR	AAATACCCCACTACCTGACTT	
	Forward		Exon2	UGT2B4Ex2-Fseq1	TTTTTTTCCCCCATCAGGA	
	Reverse		Exon2	UGT2B4Ex2-1stR	TGCTTTACTCTTCCCCTTCC	
	Forward		Exon3	UGT2B4Ex3F	ACAATTCCTTACAGTGCTTGC	
	Reverse		Exon3	UGT2B4Ex3R	ACCAATGAAAAACACCTTCTGT	
	Forward		Exon4	UGT2B4Ex4newF	ACATCAGTCTGAGTAGTTCTTGTT	
	Reverse		Exon4	UGT2B4Ex4newR	CTCTGACTCTGATTGTAAAGAATG	
	Reverse		Exon4	UGT2B4Ex4Rseq-he	TTTCATTTTATTTTAAAGTTTTTC	
	Forward		Exon5	UGT2B4Ex5F	ATCTAGGAAACACCGTCACATT	
	Reverse		Exon5	UGT2B4Ex5R	AATAACCACTCAAAAATAAAAAGCA	
	Forward		Exon6	UGT2B4Ex6Fseq	GCTGGGATTGCAGGTGTG	
	Reverse		Exon6	UGT2B4Ex6R	TGTCACAAGAAGAAAGGAATCTC	
	UGT2B7		First Amplification	Forward		UGT2B7ZF2
		Reverse			UGT2B7ZtaqR	GTAGTGTTTTCTTCATTGCCAC
		Second Amplification	Forward	Promoter	UGT2B7proF1	AAGTTTACAAAAATATGTGGACC
			Reverse	Promoter	UGT2B7proR2	TATTAGCAAAATTACTGAAGTCC
			Forward	Exon1	UGT2B7Ex1F1st2seq1	AAGGGTTACATTTTAACTTCTGG
			Reverse	Exon1	UGT2B7Ex1R1st1seq3	ATTCACCTACCAAAACCCCACT
			Forward	Exon2	UGT2B7Ex2-1stF2	GATATTTGCCACATTTTTGCC
			Reverse	Exon2	UGT2B7Ex2-1stR2	CCCTTTGTAAATATTTTATTGG
Forward			Exon3	UGT2B7Ex3Fseq1	TCCAATAATTCCTCAAAATACTG	
Reverse			Exon3	UGT2B7Ex3-Rseq2	CTCATAGTTTCTCCAAGATTATCC	
Forward			Exon4	UGT2B7Ex4-1stF3	GCCACACGTAGGTTTTCTTTT	
Reverse			Exon4	UGT2B7Ex4Rseq1	ATAAAGATTCCCCGATTTCAGA	
Forward			Exon5	UGT2B7Ex5-1stF3	ACCGTATAGCCTTCAGTTACATAC	
Reverse			Exon5	UGT2B7Ex5-1stR4	AGAAAAATGGTTACTCTGAGGTT	
Forward			Exon6	UGT2B7Ex6F	TTCTTTAACTCGGTGCTGAGGG	
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	ATTCACCTACCAAAACCCCACT	
		Forward	Exon2	UGT2B7Ex2Fseq2	TTTTTTCTATTCTGTCCAGGAAG	
		Reverse	Exon2	UGT2B7Ex2-Rseq2	ACTCATAAAACTCATATACGTGTGA	
		Forward	Exon3	UGT2B7Ex3Fseq1	TCCAATAATTCCTCAAAATACTG	
		Reverse	Exon3	UGT2B7Ex3-Rseq2	CTCATAGTTTCTCCAAGATTATCC	
		Forward	Exon4	UGT2B7Ex4-1stF3	GCCACACGTAGGTTTTCTTTT	
		Reverse	Exon4	UGT2B7Ex4Rseq1	ATAAAGATTCCCCGATTTCAGA	
		Reverse	Exon4	UGT2B7Ex4heteroR	TTCTCCCTTAAAGACTGGAAAATC	
		Forward	Exon5	UGT2B7Ex5-1stF3	ACCGTATAGCCTTCAGTTACATAC	
Reverse		Exon5	UGT2B7Ex5seqR2	ATAAAGCAGATTTCAGATTGGT		
Forward		Exon6	UGT2B7Ex6F	TTCTTTAACTCGGTGCTGAGGG		
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 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 T>C	
Amino acid change			A>C	T>G	C>T	delT	T>C ^d	delATAAAAA	C>T	G>A	insTGATAA	A>T	G>C	C>T	A>G				
Reference		rs	rs	Novel	Novel	rs	Novel	Novel	Novel	Novel	Novel	A4043	Novel	rs	Novel	Novel	Novel		
Allele specificity		6821129 ^b	941389 ^b			1876690 ^b						4415025 ^b		K455R	D458E	R459R	C511R		
														*J55R	Lévesque et al. (1999)	Lévesque et al. (1999)	Novel	*S11R	
Diplotype	*I/*I																	32	
	*Ia/*Ib																	27	
	*Ia/*Ia																	14	
	*Ib/*Ib																	7	
	*Ib/*Ic																	7	
	*Ia/*Ic																	5	
	*Ib/*Id																	4	
	*Ia/*Id																	4	
	*Ia/*Ij																	3	
	*Ib/*Ij																	3	
	*Ia/*Ic																	2	
	*Ib/*Ic																	2	
	*Ia/*Ij																	2	
	*Ia/*Ij																	2	
	*Ia/*Ih																	1	
	*Ib/*Ii																	1	
	*Ia/*Ii																	1	
	*Ia/*Ig																	1	
	*Ia/*Ii																	1	
	*Ia/*Im?																	1	
	*Ib/*Ih?																	1	
	*Ia/*Ia?																	1	
	*Ic/*Ic																	1	
	*Ic/*Id																	1	
	*Ic/*Ic																	1	
	*Ic/*Ij?																	1	
	*Ic/*Ih																	1	
	*Ic/*Ii																	1	
	*Ic/*Im?																	1	
	*Ic/*Ih																	1	
	*Ic/*Ii																	1	
	*Ia/*Ip?																	1	
	*Ia/*Iq?																	1	
	*I/*2	*Ia/*2a?																1	
	*I/*J55R	*Ia/*J55R																1	
	*I/*S11R	*Ia/*S11R?																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	1
	Haplotype	*I																	Frequencies
		*Ia																	0.441
		*Ib																	0.290
		*Ic																	0.081
		*Id																	0.037
*Ie																		0.029	
*If																		0.022	
*Ig																		0.018	
*Ih																		0.015	
*Ii																		0.011	
*Ij																		0.011	
*Ik																		0.007	
*Il																		0.007	
*Im?																		0.004	
*In?																		0.004	
*Ip?																		0.004	
*Io?																		0.004	
*I?																	0.004		
*2	*2a?																0.004		
*J55R	*J55R																0.004		
*S11R	*S11R?																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 *UGT2B4*proF1-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 μ M 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 μ M) 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 Pre-Sequencing 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

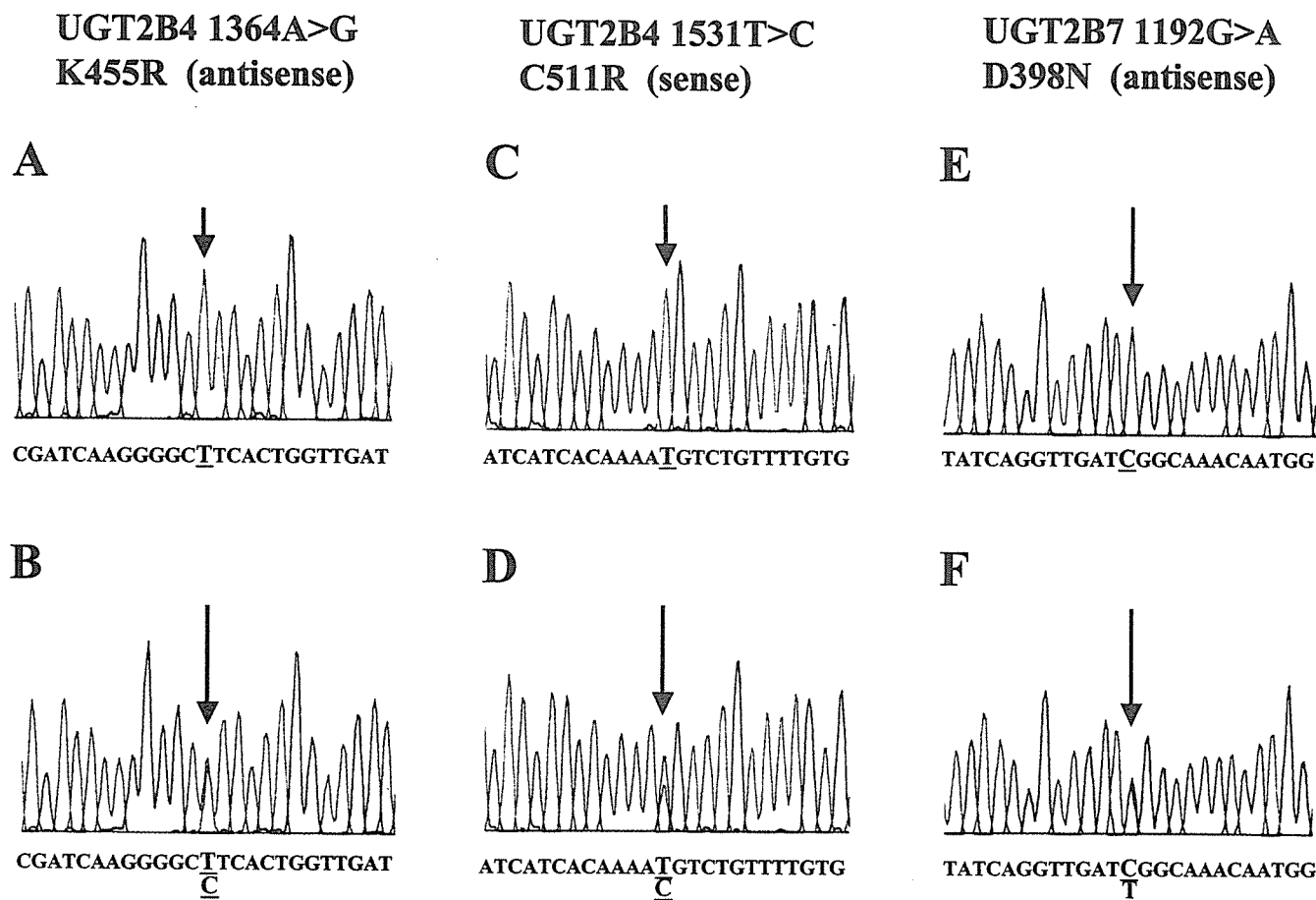


FIG. 1. Electropherograms for the novel nonsynonymous SNPs *UGT2B4* 1364A>G (A and B), *UGT2B4* 1531T>C (C and D), and *UGT2B7* 1192G>A (E and F). A, Wild-type (WT)/WT (A/A). B, WT/variant (VT) (A/G). C, WT/WT (T/T). D, WT/VT (T/C). E, WT/WT (G/G). F, WT/VT (G/A). Arrows indicate the variant nucleotide 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	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+154 185 insA	IVS4+185 C>A	IVS4-154 G>C	IVS4-129 T>C	1192 G>A		
Amino acid change					A71S	R124R	T245T	P267P	I1268Y	rs	rs	V305V			I353L	V354V						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)	Bhaskar et al. (2000)	7441750 ^b	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																			
DiploTYPE	*1 *1	*1a *1a																					20	
		*1a *1b																						10
		*1a *1c																						7
		*1c *1d																						3
	*1 *2	*1a *1d																						2
		*1b *1b																						1
		*1b *1d																						1
		*1a *2a																						24
		*1a *2b																						7
		*1c *2a																						6
	*1 *71S	*1b *2a																						3
		*1a *2d?																						1
		*1a *2c?																						1
		*1d *2a																						1
	*2 *2	*1e *2a																						1
		*1f *2a																						1
		*1g *2a																						1
	*2 *71S	*1a *71S																						13
		*1b *71S																						5
		*1c *71S																						3
	*2 *71S	*1e *71S?																						1
		*2a *2a																						5
		*2a *2b																						1
	*71S *71S	*2a *71S																						11
		*2b *71S																						1
		*71S *71S																						6
	*398N	*71S *398N?																						1
		*398N																						1
		*398N																						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	0.604	

^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 ($\rho^2 = 1.00$). Close associations were found between -1255A>C and -162T>G ($\rho^2 = 0.87$) and between IVS4 + 61T>C and IVS4 + 161_162insT-GATAA ($\rho^2 = 0.80$). A weak association between IVS3-13_6delT and IVS5-83G>C ($\rho^2 = 0.49$) 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 ($\rho^2 = 1.00$). 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 ($\rho^2 = 0.78$ or higher). Strong LDs were also seen between 735A>G and 1062C>T ($\rho^2 = 0.95$), and between 211G>T (A71S) and 372A>G ($\rho^2 = 0.84$).

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

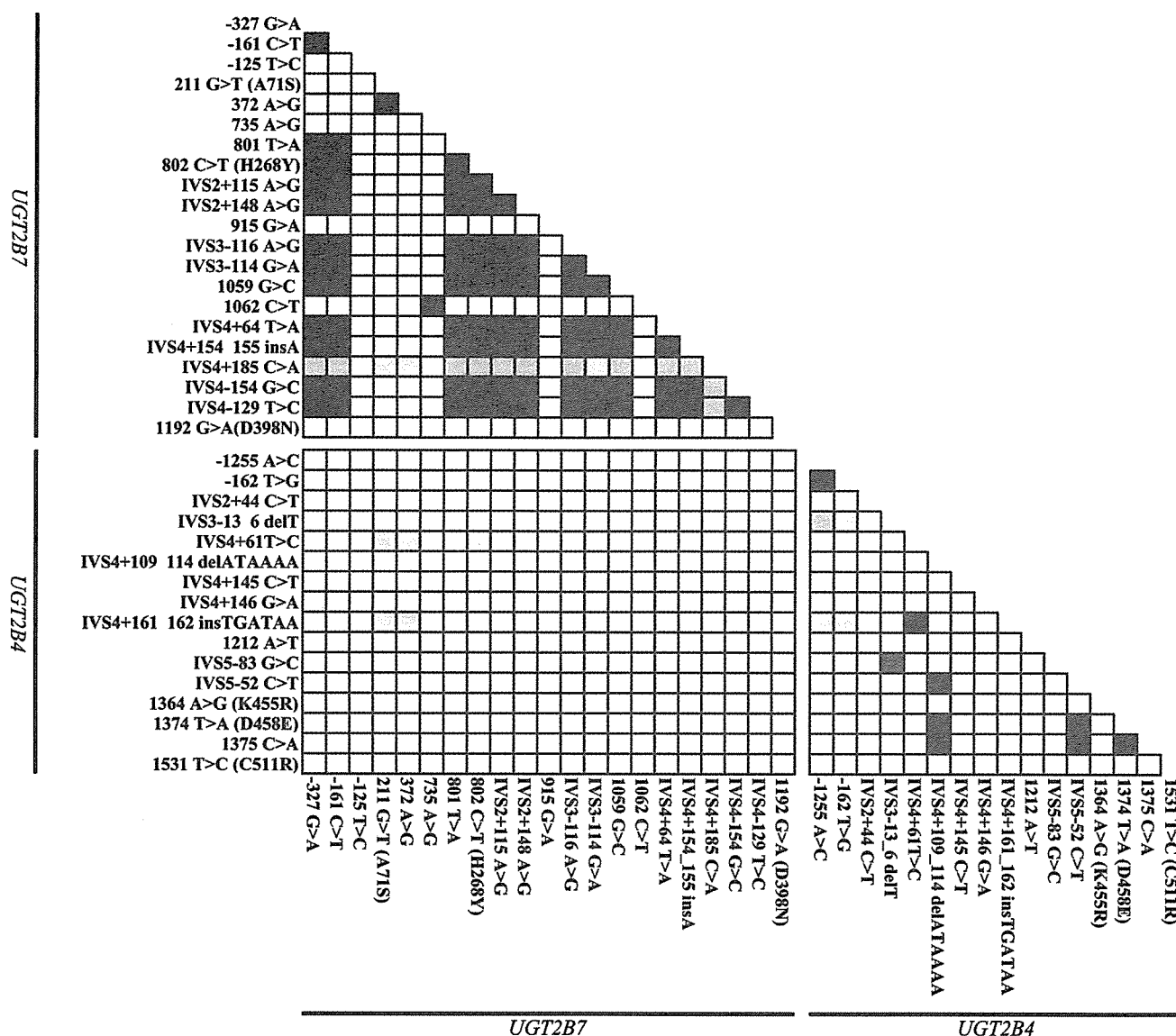


Fig. 2. LD analysis of *UGT2B4* and *UGT2B7* SNPs. Pairwise LD is expressed as r^2 (from 0 to 1) by a 10-graded blue color. The denser color represents the higher linkage. Since *UGT2B7* is located upstream of *UGT2B4* (Riedy et al., 2000), the SNPs in *UGT2B7* are described first.

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 μ M) 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.

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Heart Failure

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 $100 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ 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 $100 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ 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

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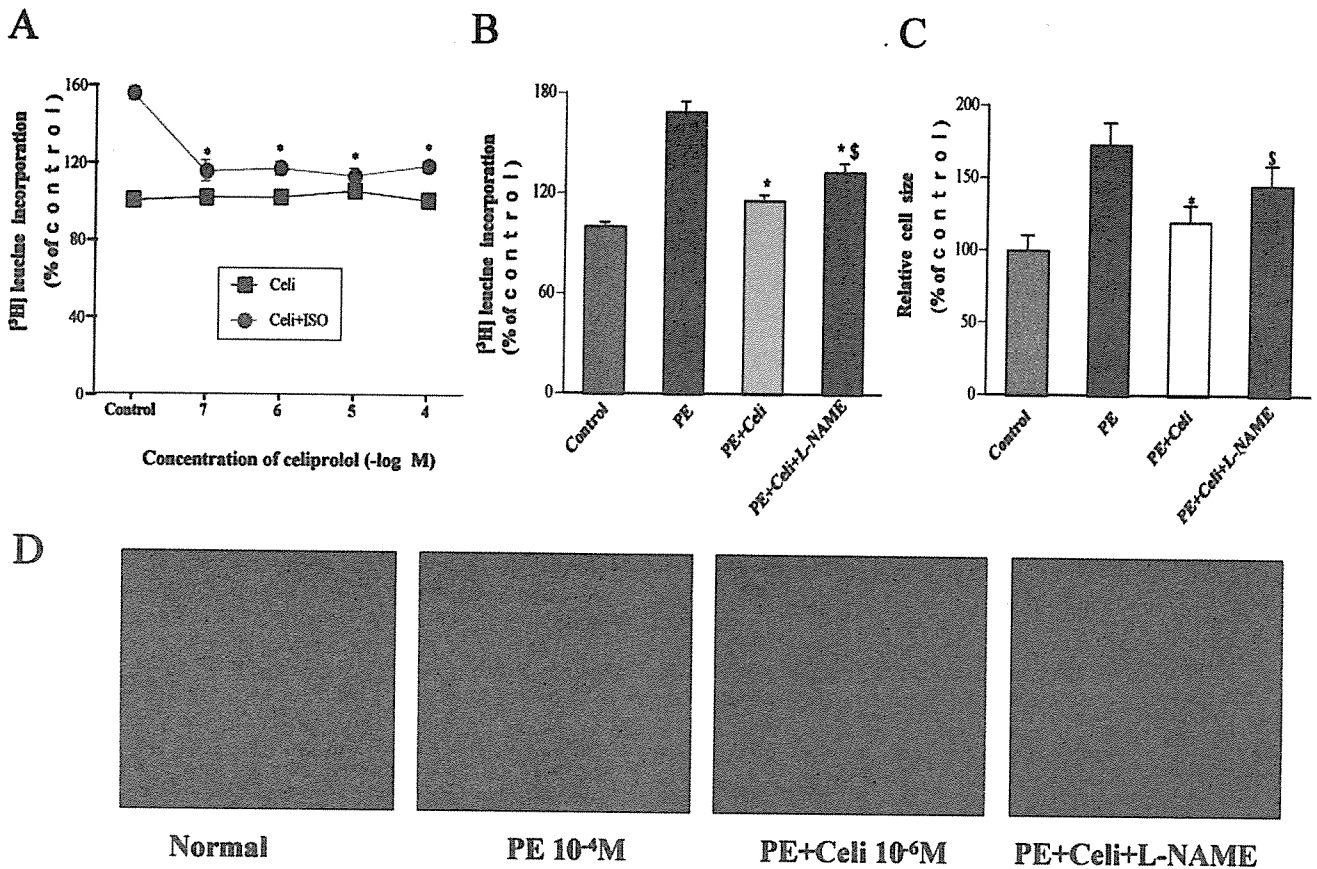


Figure 1. Results of cardiac myocyte culture. A, Protein synthesis was inhibited by celiprolol (celi) at concentrations ranging from 10^{-4} to 10^{-7} mol/L in a dose-independent manner, and this concentration range did not affect normal myocytes. $*P < 0.01$ vs Control. B, Celiprolol (10^{-6} mol/L) 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.

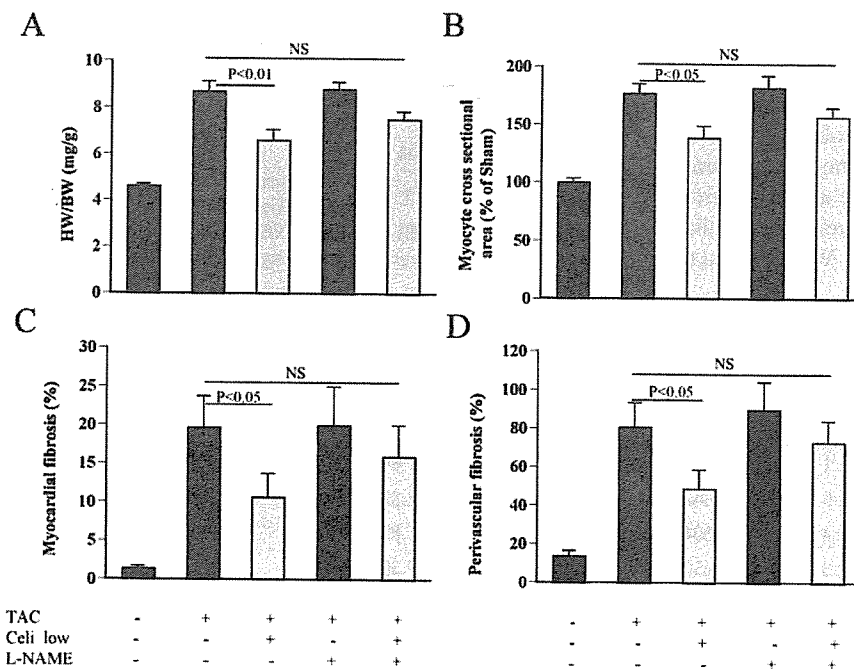


Figure 2. Celiprolol improves heart remodeling. HW/BW ratio (A) and myocyte cross-sectional area (B) were decreased significantly in TAC mice treated with celiprolol $100 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ (Celi low) or $200 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ (Celi high) in comparison with untreated TAC mice. L-NAME ($100 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) alone did not increase degree of myocyte hypertrophy under conditions of pressure overload. However, it partially abolished antihypertrophic effect of celiprolol ($100 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$). 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.

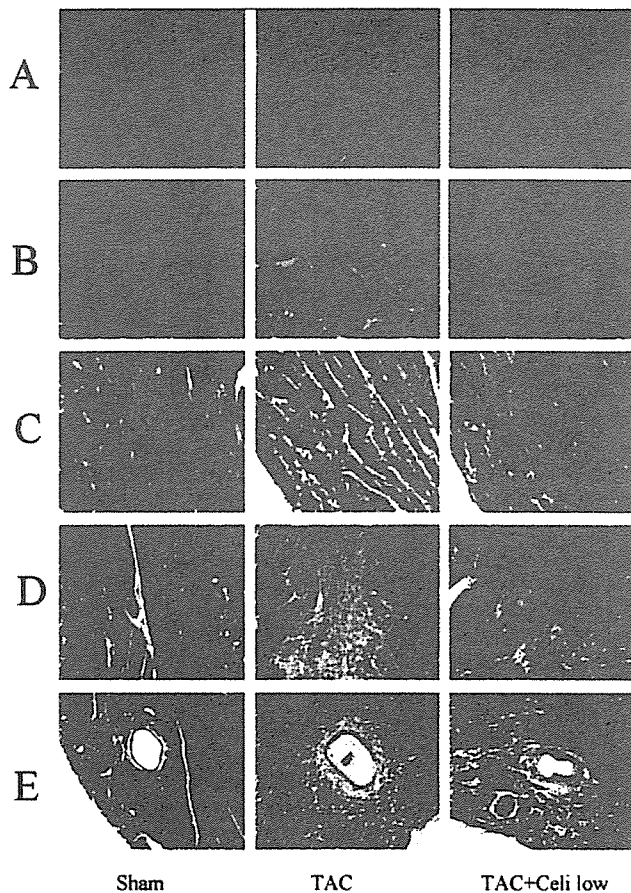


Figure 3. Representative pictures of cardiac histology. Row A, representative images of whole-heart cross section obtained by microscopic analysis (hematoxylin-eosin stain). LV wall thickness was markedly decreased in celiprolol ($100 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$)-treated compared with TAC mice. Row B, cardiac myocyte cross surface (hematoxylin-eosin stain; magnification $\times 400$; scar = $20 \mu\text{m}$). Row C, long-axis view of cardiac myocyte (hematoxylin-eosin stain, $\times 200$). Myocardial fibrosis (row D) and perivascular fibrosis (row E) were stained with azan-Mallory stain ($\times 100$).

hypothesized that celiprolol may inhibit cardiac remodeling via the NO signal pathway. To test this idea, we evaluated the effects of long-term treatment with celiprolol on cardiac hypertrophy and heart function in a murine model of ventricular pressure overload induced by transverse aortic constriction (TAC). We examined plasma NO levels, myocardial eNOS protein levels, and its phosphorylation. We also tried to

confirm whether celiprolol-induced attenuation of cardiac hypertrophy is blunted by *N*^G-nitro-L-arginine methyl ester (L-NAME). Moreover, to delineate the role of NO in cardiac hypertrophy and failure, we measured cardiac hypertrophy, pulmonary congestion and plasma NO levels in a time course and analyzed the correlation among them.

Methods

Cell Culture

Rat neonatal ventricular myocytes were isolated and cultured as we described previously.¹³ Cardiomyocytes were exposed to either phenylephrine (PE) (10^{-4} mol/L), or isoproterenol (10^{-6} mol/L or other concentrations) for 24 hours in the presence or absence of celiprolol (Nippon Shinyaku Co Ltd), and the extent of increase in [³H]leucine uptake was examined. To test whether the inhibitory effect of celiprolol on protein synthesis induced by PE was mediated via release of NO, we used L-NAME in an attempt to suppress this effect.

TAC Model and Protocols

All procedures were in accordance with institutional guidelines for animal research. Mice (C57BL/6, male, 8 to 9 weeks old, weighing 19 to 23 g) were anesthetized with a mixture of xylazine (5 mg/kg) and ketamine (25 mg/kg, intraperitoneal injection). The TAC model was created as we described previously.^{14,15}

We treated the mice with saline (TAC group) or celiprolol at $100 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ (PO, Celi low group) and $200 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ (PO, Celi high group). L-NAME at $100 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ in drinking water and L-NAME plus celiprolol $100 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ PO were also used. No difference was found among all the experimental groups in age and body weight before surgery. Two to 3 mice in each group were used to measure the transstenosis pressure gradient. Tail-cuff blood pressure and heart rate were measured (BP-98A, Softron). Mice were euthanized at different time points after TAC, and morphometric analysis was performed. Cell surface area and myocardial and perivascular fibrosis measurements were performed using 3 hearts in each group as described previously.^{15,16}

Echocardiographic Assessment

Transthoracic echocardiography was performed with a Sonos 4500 and a 15-6 L MHz transducer (Philips). Mice were lightly anesthetized with 2.5% Avertin (0.06 mL/10 g) and fixed. When they had recovered consciousness (≈ 10 minutes), good 2D short-axis views of the left ventricle (LV) were obtained for guided M-mode measurements of the LV posterior wall thickness, LV end-diastolic diameter, and LV end-systolic diameter.

NO Measurement

The plasma levels of nitrite and nitrate (NO_x) were measured as previously described.¹⁷ Cardiac myocardial protein level of eNOS, inducible NOS (iNOS), neuronal NOS (nNOS), and eNOS phosphorylated activity were checked by use of Western blot analy-

Hemodynamic and Echocardiographic Results

Group	Body Weight, g	Transstenosis Gradient Pressure, mm Hg (n=2-3 in each group)	Heart Rate, bpm	Tail-Cuff Systolic Blood Pressure, mm Hg	LV End-Systolic Dimension, mm	LV End-Diastolic Dimension, mm	LV Diastolic Posterior Wall Thickness, mm
Sham (10)	24.0±0.3*	5±1	640±21	116±2.9*	1.64±0.04*	3.07±0.06*	0.65±0.02*
TAC (8)	22.6±0.5	55±5	647±11	100±3.7	2.32±0.24	3.37±0.17	1.01±0.06
TAC+Celi (11)	22.4±0.7	52±4	604±17*	108±3.1	1.83±0.1*	3.04±0.11*	0.79±0.03*
TAC+L-NAME (6)	21.2±0.6	50±3	652±11	108±3.5	2.30±0.21	3.31±0.20	0.99±0.05
TAC+Celi+L-NAME (5)	21.9±0.5	54±3	616±13*	106±2.1	1.95±0.2	3.15±0.12	0.93±0.02

TAC indicates transverse aortic constriction; Celi, celiprolol $100 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ PO. The number of mice in each group is indicated in parentheses.

* $P < 0.05$ vs TAC.