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G．　知的所有権の取得状況

1．特許取得

二次無効発現と相関する遺伝子多型につき、特許出願を予定している（平成 20 年 5 月頃出願予定）。

2．実用新案登録

なし

3．その他

なし

別添４

II. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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III. 研究成果の刊行物・別刷

Drug Metab. Pharmacokinet. 20 (4): *SNP27* (300)-*SNP34* (307) (2005).

SNP Communications

Genetic Variations and Haplotypes of CYP2C19 in a Japanese Population

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Full text of this paper is available at http://www.jstage.jst.go.jp/browse/dmpk

Summary: Forty-eight single nucleotide variations, including 27 novel ones, were found in the 5′-regulatory region, all of the exons and their surrounding introns of *CYP2C19* in 253 Japanese subjects (134 diabetic patients and 119 healthy volunteers). Identified novel variations were as follows: −2772G>A, 2767_−2760delGGTGAACA, −2720T>C, −2547delG, −2545G>T, −2545_−2544 delGC, and −2040C>T in the enhancer region; −778C>T, −777G>A, −529G>C, −189C>A, and −185A>G in the promoter region; 151A>G (S51G), 481G>C (A161P), 986G>A (R329H), 1078G>A (D360N), and 1119C>T (D373D) in the exons, and IVS1+128T>A, IVS3+163G>A, IVS4+271A>G, IVS5−49A>G, IVS6−210C>T, IVS6−196T>A, IVS6−32T>A, IVS7+84G>A, IVS7−174C>T, and IVS8+64C>T in the introns. Since we found no significant differences in the variation frequencies between healthy volunteers and diabetic patients, the data for all subjects were treated as one group in further analysis. The allele frequencies were 0.265 for IVS6−196T>A, 0.045 for −2772G>A and −2720T>C, 0.024 for −2040C>T, 0.014 for IVS7−174C>T, 0.010 for −529G>C, 0.006 for IVS1+128T>A and 481G>C (A161P), 0.004 for −2767_−2760delGGTGAACA and IVS6−210C>T, and 0.002 for the other 17 variations. In addition, the two known nonsynonymous single nucleotide polymorphisms, 681G>A (splicing defect, **2* allele) and 636G>A (W212X; **3* allele) were detected at 0.267 and 0.128 frequencies, respectively. No variation was detected in the known binding sites for constitutive androstane receptor and glucocorticoid receptor. Linkage disequilibrium analysis showed several close linkages of variations throughout the gene. By using the variations, thirty-one haplotypes of *CYP2C19* and their frequencies were estimated. Our results would provide fundamental and useful information for genotyping *CYP2C19* in the Japanese and probably other Asian populations.

Key words: CYP2C19; genetic variation; amino acid alteration; haplotype

On June 13, 2005, these variations were not found on the homepage of the CYP Allele Nomenclature Committee (http://www.imm.ki.se/CYPallele/CYP2C19), 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/) database.

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Introduction

In human, cytochrome P450 2C (CYP2C) subfamily consists of four members: CYP2C8, CYP2C9, CYP2C18 and CYP2C19. Their genes are tandemly located on chromosome 10q24.[1] One of the subfamily members, CYP2C19 is a clinically important enzyme that metabolizes a wide variety of drugs, such as antiulcer drugs omeprazole and lansoprazole, an anticonvulsant *S*-mephenytoin, an antidiabetic drug tolbutamide, and an anxiolytic drug diazepam.[2,3] Metabolism of these drugs *in vivo* has been known to be polymorphic, and individuals can be divided into extensive metabolizers and poor metabolizers (PMs). PMs would face higher area under concentration-time curve (AUC) values of the drugs.[4-6] Regarding omeprazole, for example, the PMs show higher cure rates for *Helicobacter pyloli* infection and peptic ulcer because of the higher AUCs, which lead to increased gastric pH and thus are suggested to result in stable antibacterial activity of amoxicillin.[2,4,7]

Ethnic differences in the incidence of PMs among Caucasians (2–5%), Africans (*ca.* 6%) and Asians (13–23%) have been reported.[2,8] These differences are known to be attributed to the genetic polymorphisms of *CYP2C19* gene. de Morais *et al.* first reported the common single nucleotide polymorphisms (SNPs) 681G>A (splicing defect, *CYP2C19*2* allele) and 636G>A (W212X, **3* allele), the latter of which was found only in Japanese but not in Caucasian populations.[9,10] Recent studies showed that the **3* allele is also distributed in Chinese, Thai and Vietnamese with different frequencies.[11] *CYP2C19*2* and **3* generate the null-activity enzyme protein and have been considered to account for >99% of PM alleles in the Japanese population.[10,12] Recently, another minor allele, *CYP2C19*16* (1324C>T, R442C), was found in a Japanese subject who had received mephobarbital (at 0.006 frequency in Japanese population).[13] As for other Asian populations, the two defective alleles *CYP2C19*4* (1A>G, no protein) and **5* (1297C>T, R433W) were also found below 0.005 frequencies in Chinese.[14,15] However, the other alleles (*CYP2C19*6-*15*) have not been detected in Asians.

Recently, the transcriptional regulatory regions of the *CYP2C19* gene were analyzed. Reporter assay with up to 1.8 kb upstream of the 5′-flanking region showed that there were potentially negative and positive elements between 650 to 453 bases and between 224 to 17 bases, respectively, upstream of the translational start site.[16] Furthermore, another report revealed enhancer elements for constitutive androstane receptor (−1891 to −1876 bases from the translational start site) and glucocorticoid receptor (−1750 to −1736).[17]

While the effects of *CYP2C19* polymorphisms have been extensively studied on the **2* and **3* polymorphisms, a comprehensive search for genetic polymorphisms of *CYP2C19* in Asian populations, including the Japanese, is currently lacking. In this study, the 5′-regulatory region, all the exons and their surrounding introns of *CYP2C19* were sequenced in 253 Japanese subjects, and 27 novel variations, including four non-synonymous ones, were identified.

Materials and Methods

Human genomic DNA samples: DNA was extracted from the blood leukocytes of 134 Japanese diabetic patients who had received glimepiride. DNA was also extracted from Epstein-Barr virus-transformed lymphoblastoid cells, which were derived from blood samples collected from 119 healthy Japanese volunteers at the Tokyo Women's Medical University. The ethical review boards of the International Medical Center of Japan, the Nerima General Hospital, the Tokyo Women's Medical University, and the National Institute of Health Sciences approved this study. Written informed consent was obtained from all participating patients as well as all healthy subjects.

Polymerase chain reaction (PCR) conditions for DNA sequencing: First, the multiplex PCR was performed to amplify the entire *CYP2C19* gene by the two mixed primer sets (Mix 1 and Mix 2 in "1st PCR" 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 the 0.2 μM of the primers sets. 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 separately using one-fifth volume of the 1st PCR product as a template by Ex-Taq (0.625 units) (Takara Shuzo) with a set of primers (0.2 μM) listed in "2nd PCR" of **Table 1** (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 "Sequencing" 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). All the detected variations were confirmed by repeating the PCR from the genomic DNA and sequencing the newly generated PCR products.

Linkage disequilibrium (LD) and haplotype analysis: Hardy-Weinberg equilibrium and LD analysis was

Table 1. Primer sequences used for the analysis of the *CYP2C19* gene

		Amplified or sequenced region	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Amplified length (bp)
1st PCR	Mix 1	−3k to Exon 1	TTATTTGTTGCTAGGGCTCGTG	CTTACTGTTTACCCTCAGCC	3,281
		Exon 2 to 3	AGGTAGACACAAGAGTGCTGA	TTCTCTGGTGACATGTTCTGGA	1,250
	Mix 2	Exon 4 to 5	CCATTATTTAACCAGCTAGGC	TCCTATCCTGACATCCTTATTG	1,969
		Exon 6	CAAACTATACCAAACAGCAGGCTA	ACTCTACATAGCTTAAAGGGCTCA	6,557
		Exon 7	AATGCTGAAGTGGGTTGTTG	ACCCTGACAGAAATTCTAGCCC	1,272
		Exon 8 to 9	CCCACAACAGTCCCCGAA	CACAAAGGAAGGAAGGTCTAA	3,650
2nd PCR		−3K	TTATTTGTTGCTAGGGCTCGTG	ATCACATCCCGTCTCATAGAA	476
		−2K	CTCAACTTAGCAGAAGAGAGG	CTCATATCCCTTTGGAATCTCT	562
		−1K	AAGCCTTAGTTTCTCAAGCCC	CTTGTTCTCCTTCGTCCCAG	925
		Exon 1	AGAAGACCTCAGCTCAAATCC	CTTACTGTTTACCCTCAGCC	1,249
		Exon 2 to 3	AGGTAGACACAAGAGTGCTGA	TTCTCTGGTGACATGTTCTGGA	1,250
		Exon 4	CCATTATTTAACCAGCTAGGC	AGCCTTGTGAGTAATGGAAGA	727
		Exon 5	AGAAGTCATTTAACTGCTCTGG	TCCTATCCTGACATCCTTATTG	950
		Exon 6	CTCTCTCACCGCTCCTATTCA	GCTGGGATTACAGTGGTGTG	627
		Exon 7	GGTCTTGTTTCTTCATCTAGTCAG	ACCCTGACAGAAATTCTAGCCC	915
		Exon 8	CCCACAACAGTCCCCGAA	GAGGATGTATCACCAGCGGAG	580
		Exon 9	TTGTTTAGTTGCCTATCCATCC	CACAAAGGAAGGAAGGTCTAA	775
Sequencing		−3K	TTATTTGTTGCTAGGGCTCGTG	ATCACATCCCGTCTCATAGAA	
		−2K	CTCAACTTAGCAGAAGAGAGG	CTCATATCCCTTTGGAATCTCT	
		−1K	TGTCTTCTGTTCTCAAAGCATC	CTGAATATATACCACATTCATCC	
		Exon 1	AGGCTGCTGTATTTTTAGTAGG	GACACTGACAGACTGGAAAAGG	
		Exon 2	AGGTAGACACAAGAGTGCTGA	GAGAAACGAAACTAGGAGG	
		Exon 3	GTTTCAGCATCTGTCTTGG	TTCTCTGGTGACATGTTCTGGA	
		Exon 4	CCATTATTTAACCAGCTAGGC	AGCCTTGTGAGTAATGGAAGA	
		Exon 5	AGAGGCTGCTTGATAGAAAT	TCCTATCCTGACATCCTTATTG	
		Exon 6	CTCACCGCTCCTATTCAATATT	AGTGGTGTGCCACAATGC	
		Exon 7	GGTCTTGTTTCTTCATCTAGTCAG	ACCCTGACAGAAATTCTAGCCC	
		Exon 8	CCCACAACAGTCCCCGAA	GAGGATGTATCACCAGCGGAG	
		Exon 9	TTGTTTAGTTGCCTATCCATCC	CACAAAGGAAGGAAGGTCTAA	

performed by SNPAlyze software (Dynacom Co., Yokohama, Japan), and pairwise LD between variations was analyzed by rho square (r^2) values. Some of the haplotypes were unambiguous from subjects with homozygous variations at all sites or a heterozygous variation at only one site. Separately, the diplotype configurations (a combination of haplotypes) were inferred by LDSUPPORT software, which determines the posterior probability distribution of the diplotype configuration for each subject based on the estimated haplotype frequencies.[18] The diplotype configurations of all subjects had a probability (certainty) of more than 0.975 except for 5 subjects. The haplotypes inferred in single subjects are described with haplotype names and a question mark in **Table 3**, since the predictability for these very rare haplotypes is known to be low in some cases. The haplotypes detected in this study were tentatively named as the numbers plus small alphabetical letters, except for the four haplotypes with novel nonsynonymous variations (*CYP2C19*18-*21*), which were registered to the CYP Allele Nomenclature Committe and will be publicized on the committee's Web site.

Results and Discussion

The enhancer (from 2780 to 2350 bases and from 2090 to 1590 bases upstream of the translational start site) and promoter regions (up to 1020 bases upstream of the translational start site), all the 9 exons and their flanking introns of *CYP2C19* were sequenced in 253 Japanese subjects. Genbank accession number NT_030059.12 was utilized for the reference sequence. Forty-eight genetic variations, including 27 novel ones (7 were in the enhancer region, 5 in the promoter region, 5 in the exons and 10 in the introns), were detected (**Table 2**). Since we did not find any significant differences in the frequencies of these variations between healthy volunteers and diabetic patients (by χ^2 test or Fisher's exact test, $P>0.25$), the data for all subjects were analyzed as one group. All of the detected variations were found in Hardy-Weinberg equilibrium ($P \geqq 0.449$).

Four novel nonsynonymous variations, 151A>G (S51G), 481G>C (A161P), 986G>A (R329H) and 1078G>A (D360N), were found as individual heterozygotes at 0.002, 0.006, 0.002 and 0.002 frequencies, respectively (**Fig. 1**). Among them, A161 and D360 are

Table 2. Summary of *CYP2C19* variations detected in a Japanese population

SNP ID			Refe-rence	Location	Position		Nucleotide change and flanking sequences (5′ to 3′)	Amino acid change	Number of subjects			Frequency		
This Study	JSNP	dbSNP (NCBI)			NT_030059.12	From the translational initiation site or from the end of nearest exon			Wild-type	Hetero-zygote	Homo-zygote	Total (n = 253)	Healthy volunteers (n = 119)	Diabetic patients (n = 134)
MPJ6_2C19_001[a]				Enhancer	15268217	−2772	GCTCGTGAGGAAG/AAGAGGGTGAACA		230	23	0	0.045	0.055	0.037
MPJ6_2C19_002[a]				Enhancer	15268222_15268229	−2767_−2760	TGAGGAAGAGAGGGTGAACA/-GGTAGAGCACAG		251	2	0	0.004	0.000	0.007
MPJ6_2C19_003[a]				Enhancer	15268269	−2720	GTAAAGCTATTTT/CATATGATACTAC		230	23	0	0.045	0.055	0.037
MPJ6_2C19_004[a]				Enhancer	15268442	−2547	GGCTGTGTGTGGG/-TGCAGGGAAAGA		252	1	0	0.002	0.004	0.000
MPJ6_2C19_005[a]				Enhancer	15268444	−2545	CTGTGTGTGGGTG/TCAGGGAAAGAGG		252	1	0	0.002	0.004	0.000
MPJ6_2C19_006[a]				Enhancer	15268444_15268445	−2545_−2544	CTGTGTGTGGGTGC/-AGGGAAAGAGGC		252	1	0	0.002	0.004	0.000
MPJ6_2C19_007[a]				Enhancer	15268949	−2040	TAAAGAGAGCAAC/TCAAGCTTATCTT		241	12	0	0.024	0.017	0.030
MPJ6_2C19_008		rs11568732	16)	Promoter	15270100	−889	CAGAATAACTAAT/GGTTTGGAAGTTG		174	70	9	0.174	0.193	0.157
MPJ6_2C19_009		rs12248560	20)	Promoter	15270183	−806	CTGTTCTCAAAGC/TATCTCTGATGTA		249	4	0	0.008	0.008	0.007
MPJ6_2C19_010[a]				Promoter	15270211	−778	GATAATGCGCCAC/TGATGGGCATCAG		252	1	0	0.002	0.000	0.004
MPJ6_2C19_011[a]				Promoter	15270212	−777	ATAATGCGCCACG/AATGGGCATCAGA		252	1	0	0.002	0.004	0.000
MPJ6_2C19_012[a]				Promoter	15270460	−529	TTTCATGTTTAGG/CCTGCTGTATTTT		248	5	0	0.010	0.008	0.011
MPJ6_2C19_013[a]				Promoter	15270800	−189	GACGAAGGAGAAC/AAAGACCAAAGGA		252	1	0	0.002	0.000	0.004
MPJ6_2C19_014[a]				Promoter	15270804	−185	AAGGAGAACAAGA/GCCAAAGGACATT		252	1	0	0.002	0.000	0.004
MPJ6_2C19_015		rs4986894	16)	Promoter	15270891	−98	GATTGGCCACTTT/CATCCATCAAAGA		137	97	19	0.267	0.256	0.276
MPJ6_2C19_016		rs17885098	20)	Exon 1	15271087	99	CCCTCCTGGCCCC/TACTCCTCTCCCA	P33P	9	70	174	0.826	0.807	0.843
MPJ6_2C19_017[a]				Exon 1	15271139	151	ATTAAGGATGTCA/GGCAAATCCTTAA	S51G	252	1	0	0.002	0.000	0.004
MPJ6_2C19_018[a]				Intron 1	15271284	IVS1 + 128	TTGAAAGGCTTTT/AGTTGCCTTTTCC		250	3	0	0.006	0.008	0.004
MPJ6_2C19_019		rs17884832		Intron 1	15283001	IVS1 − 340	TTCAAATTTGGGT/GCTTCTGTATATT		174	70	9	0.174	0.193	0.157
MPJ6_2C19_020		rs7916649		Intron 1	15283110	IVS1 − 231	GTTGGTGTGAGGG/ATTAATTGTAATC		76	127	50	0.449	0.458	0.440
MPJ6_2C19_021		rs17878649		Intron 1	15283294	IVS1 − 47	GCTTAGTAAATGG/AACAAAACAGTGA		193	55	5	0.128	0.139	0.119
MPJ6_2C19_022		rs12769205	20)	Intron 2	15283650	IVS2 − 23	GATCTCCCTCCTA/GGTTTCGTTTCTC		137	97	19	0.267	0.256	0.276
MPJ6_2C19_023[a]				Exon 3	15283822	481	AGAAAAACCAAGG/CGTGGGTGAACAT	A161P	250	3	0	0.006	0.000	0.011
MPJ6_2C19_024[a]				Intron 3	15283985	IVS3 + 163	GAATTTGCACATG/ATTTGTGCTGTGT		252	1	0	0.002	0.004	0.000
MPJ6_2C19_025		rs17879992		Intron 3	15284154	IVS3 + 332	TTTTTCCCATTAT/CCTATTCCAGAAC		174	70	9	0.174	0.193	0.157
MPJ6_2C19_026		rs4986893	10)	Exon 4	15288936	636	AAGCACCCCCTGG/AATCCAGGTAAGG	W212X	193	55	5	0.128	0.139	0.119
MPJ6_2C19_027[a]				Intron 4	15289213	IVS4 + 271	TTTCTATAAAGTA/GCTTTGGTGACAG		252	1	0	0.002	0.004	0.000
MPJ6_2C19_028		rs7088784		Intron 4	15289899	IVS4 − 205	GAAATGATTATCA/GTCTTTGATTCTC		174	70	9	0.174	0.193	0.157
MPJ6_2C19_029		rs4244285	9)	Exon 5	15290142	681	TGATTATTTCCCG/AGGAACCCATAAC	splicing defect	137	97	19	0.267	0.256	0.276
MPJ6_2C19_030		rs12571421		Intron 5	15290508	IVS5 + 228	TAAATATAATCTA/GTCTGAACAATAA		137	97	19	0.267	0.256	0.276
MPJ6_2C19_031			20)	Intron 5	15328666	IVS5 − 113	TTTTTCTAGTACT/GATACTTTACAGT		230	23	0	0.045	0.055	0.037
MPJ6_2C19_032		rs4417205	20)	Intron 5	15328728	IVS5 − 51	ATTTACTGTCATC/GAAATATGCTGTT		140	94	19	0.261	0.248	0.272
MPJ6_2C19_033[a]				Intron 5	15328730	IVS5 − 49	TTACTGTCATCAA/GATATGCTGTTAA		252	1	0	0.002	0.000	0.004
MPJ6_2C19_034[a]				Intron 6	15350910	IVS6 − 210	CATATATGTGTAC/TAGATTTTTCTTA		251	2	0	0.004	0.000	0.007
MPJ6_2C19_035[a]				Intron 6	15350924	IVS6 − 196	GATTTTTCTTAAT/AGCCTAGCTTAAG		138	96	19	0.265	0.252	0.276
MPJ6_2C19_036[a]				Intron 6	15351088	IVS6 − 32	CCATTTCTCTCCT/ATTTCCATCAGTT		252	1	0	0.002	0.000	0.004
MPJ6_2C19_037[a]				Exon 7	15351144	986	AAGAGATTGAACG/ATGTCGTTGGCAG	R329H	252	1	0	0.002	0.000	0.004
MPJ6_2C19_038	IMS-JST111900	rs3758580	20)	Exon 7	15351148	990	GATTGAACGTGTC/TGTTGGCAGAAAC	V330V	138	96	19	0.265	0.252	0.276
MPJ6_2C19_039	IMS-JST111901	rs3758581	3)	Exon 7	15351149	991	ATTGAACGTGTCA/GTTGGCAGAAACC	I331V	0	23	230	0.955	0.945	0.963
MPJ6_2C19_040[a]				Exon 7	15351236	1078	CAGAGATACATCG/AACCTCATCCCCA	D360N	252	1	0	0.002	0.000	0.004
MPJ6_2C19_041[a]				Exon 7	15351277	1119	AGTGACCTGTGAC/TGTTAAATTCAGA	D373D	252	1	0	0.002	0.000	0.004
MPJ6_2C19_042[a]				Intron 7	15351391	IVS7 + 84	TCTACCATCACTG/AGGTGAGAGAAGT		252	1	0	0.002	0.004	0.000
MPJ6_2C19_043		rs17882222		Intron 7	15357999	IVS7 − 201	TCCTGATTGTGGG/ACATTTTAGCAAG		241	12	0	0.024	0.017	0.030
MPJ6_2C19_044[a]				Intron 7	15358026	IVS7 − 174	TATTGTCACTGGC/TCTTAAGCTCATG		246	7	0	0.014	0.017	0.030
MPJ6_2C19_045		rs4917623		Intron 7	15358094	IVS7 − 106	TCTTTGGAATGGT/CGTTTCATCATCT		56	126	71	0.530	0.529	0.530
MPJ6_2C19_046		rs17886522	20)	Exon 8	15358301	1251	GGATGAAGGTGGA/CAATTTTAAGAAA	G417G	193	55	5	0.128	0.139	0.119
MPJ6_2C19_047[a]				Intron 8	15358405	IVS8 + 64	GATCAGTTGGAAC/TTTACATGTGCCT		252	1	0	0.002	0.004	0.000
MPJ6_2C19_048		rs12268020		Intron 8	15360897	IVS8 − 119	ATCTACTCATCCC/TTCCTATGATTCA		250	3	0	0.006	0.004	0.007

[a]Novel variations detected in this study.

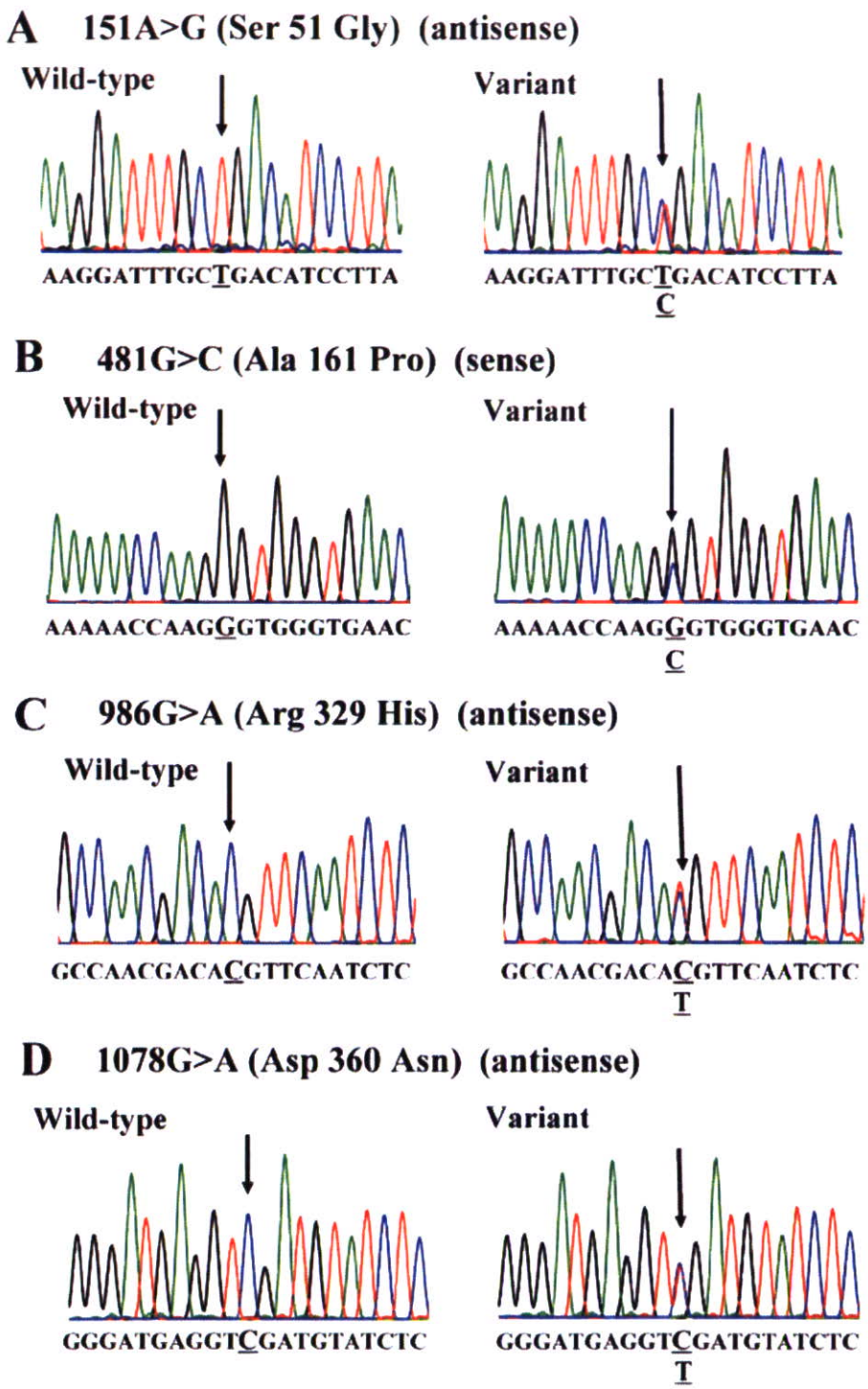


Fig. 1. Four novel nonsynonymous variations of human *CYP2C19*. (A) MPJ6_2C19_017 (wild-type, 151A/A; variant, 151A/G). (B) MPJ6_2C19_023 (wild-type, 481G/G; variant, 481G/C). (C) MPJ6_2C19_037 (wild-type, 986G/G; variant, 986G/A). (D) MPJ6_2C19_040 (wild-type, 1078G/G; variant, 1078G/A). Arrows indicate the positions of the nucleotide changes.

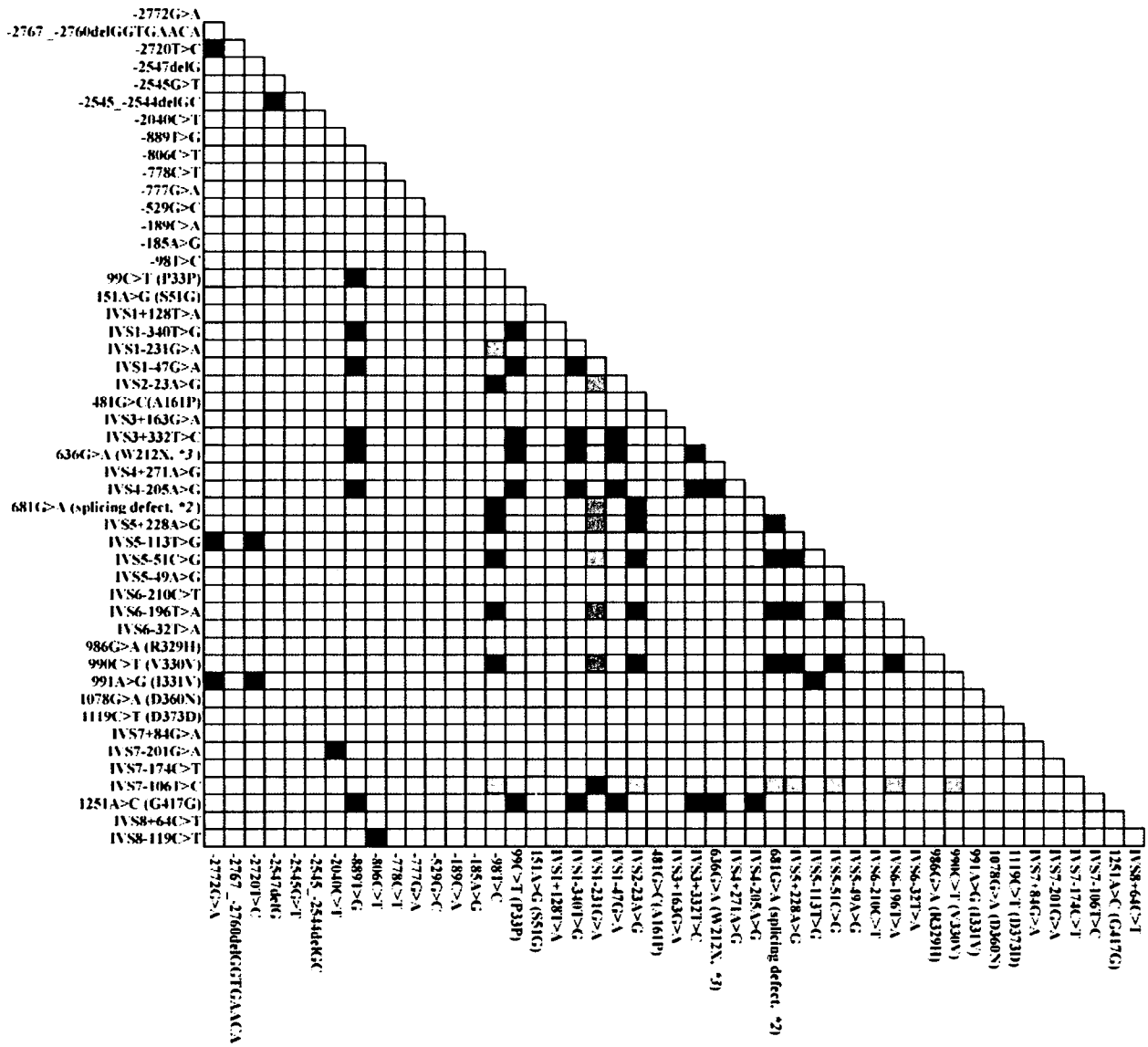


Fig. 2. Linkage disequilibrium (LD) analysis of *CYP2C19* genetic variations.
Pairwise LD is depicted using the r^2 values (from 0 to 1) by a 10-graded gray color. The denser color represents the higher linkage.

conserved in the human CYP2C subfamily.[19] In particular, D360 is located in the substrate recognition site 5.[19] It is possible that the substitution from an acidic (Asp) to a rather neutral (Asn) amino acid might affect the binding of substrates or catalytic activity of the enzyme. The other variations, S51, A161, and R329 are located in the A-helix, in the loop between the D-helix and the E-helix, and in the J-helix, respectively. Further functional analysis should be pursued for these four variations. Moreover, it is necessary to evaluate the real frequencies of the very rare variations found in only one subject (frequency: 0.002).

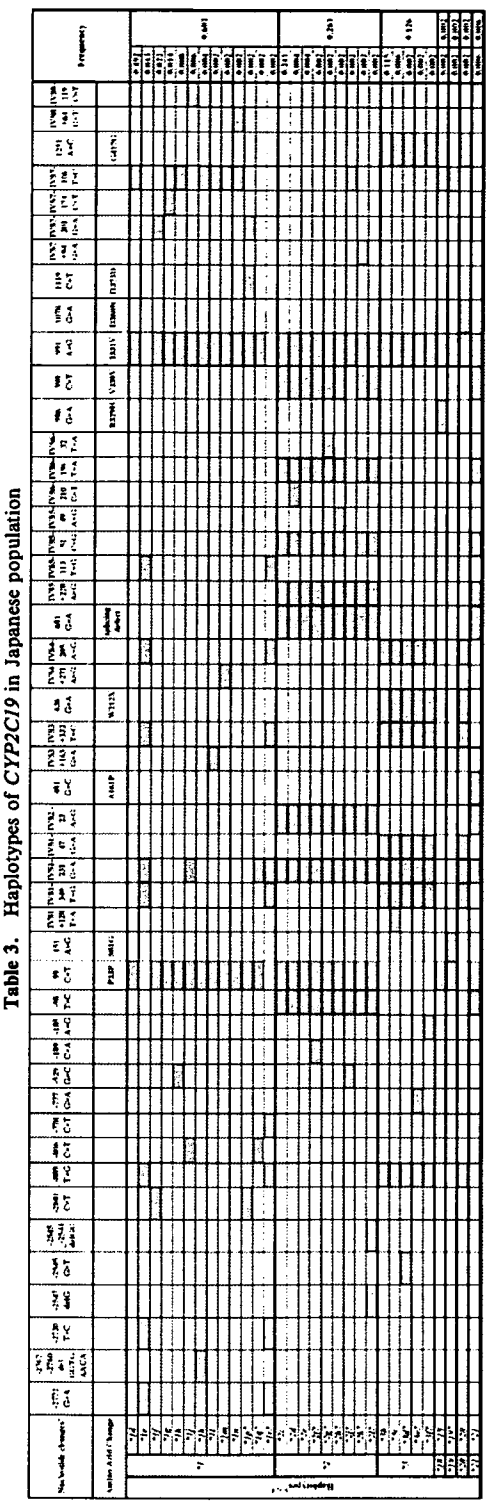
The two known nonsynonymous SNPs, 681G>A (splicing defect, **2* allele) and 636G>A (W212X; **3* allele) were detected at 0.267 and 0.128 frequencies, respectively. These frequencies were comparable to those of the Japanese in the previous reports.[8,11] The variation 991A>G (I331V, **1B* and **1C* alleles) was found with a 0.955 frequency, indicating that this SNP is rather common in the Japanese population. Other nonsynonymous variations including *CYP2C19*4*, **5* and **16* were not detected in this study. Thus, our results confirmed that, except for I331V which has no functional significance, nonsynonymous variations other than *CYP2C19*2* and **3* were very rare in the Japanese population.

Seven and five novel variations were detected in the enhancer and promoter regions, respectively. No variation was found in the reported regions as the binding sites for the constitutive androstane receptor and the glucocorticoid receptor.[17] However, −529G>C was present within the potential negative element (−650 to −453 bases from the translational start site) in the promoter region.[16] Transcriptional activity with this variation needs to be examined in the future.

Using the detected variations, linkage disequilibrium (LD) was analyzed (**Fig. 2**). Perfect linkage ($r^2 = 1$) was observed among −2772G>A, −2720T>C, IVS5-113T>G, and 991A>G (I331V), between −2547delG and −2545_-2544delGC, and between −2040C>T and IVS7-201G>A. Strong LDs were observed among −98T>C, IVS2-23A>G, 681G>A (splicing defect, **2* allele), IVS5+228A>G, IVS5-51C>G, IVS6-196T>A, and 990C>T (V330V) ($r^2 \geqq 0.96$), between IVS1-231G>A and IVS7-106T>C ($r^2 = 0.90$), between −806C>T and IVS8-119C>T ($r^2 = 0.75$), and among −889T>G, 99C>T (P33P), IVS1-340T>G, IVS1-47G>A, IVS3+332T>C, 636G>A (W212X, **3* allele), IVS4-205A>G, and 1251A>C (G417G) ($r^2 \geqq 0.70$). The r^2 values were below 0.45 for the other pairs of variations. Collectively, the LD was observed throughout the *CYP2C19* gene, suggesting that one LD block covers the entire analyzed region spanning approximately 90 kb. Thus, the *CYP2C19* haplotypes were analyzed as one block.

Haplotype analysis was then performed (**Table 3**). The haplotypes registered to the CYP Allele Nomenclature Committee are shown with the numbers (**18* to **21*). The other haplotypes detected in this study are shown as the numbers plus small alphabetical letters. Several haplotypes were first unambiguously assigned by homozygous variations at all sites (**1d*, **2c*, and **3b*) or a heterozygous variation at only one site (**1g*, **1h*, **1k*, **1l*, **1m*, **1n*, and **18*). Separately, the diplotype configuration (a combination of haplotypes) for each subject was estimated by LDSUPPORT software. The additionally inferred haplotypes were six **1* subtype (**1e*, **1f*, **1j*, **1p* to **1r*), eight **2* subtypes (**2d* to **2l*), four **3* subtypes (**3c* to **3f*), **19*, **20*, and **21*. The determined/inferred haplotypes were summarized in **Table 3**. As for **20*, cloning of the amplified genomic DNA and subsequent sequencing confirmed that 1078G>A (D360N) was linked to the **3b* haplotype. The three subjects with heterozygous 481G>C (A161P) had heterozygous 681G>A (**2* allele) concurrently, and the haplotype harboring 481G>C (A161P) was inferred as a subtype of **2c* haplotype (designated as **21*). The most frequent haplotype was **1d* (frequency: 0.492), followed by **2c* (0.241), **3b* (0.115), **1e* (0.043), **1f* (0.022), and **1g* (0.014). The frequencies of the other haplotypes were less than 0.01.

Table 3. Haplotypes of *CYP2C19* in Japanese population



[a] A of the translational start codon of *CYP2C19* is numbered 1. NT_030059.12 was used as the reference sequence.
[b] Major allele, white; minor allele, gray
[c] The haplotypes are described as numbers (plus small alphabetical letters).
[d] The haplotypes inferred in only one subject are described with haplotype names and a question mark.

In conclusion, 48 genetic variations, including 27 novel ones, were detected in *CYP2C19* in a Japanese population. Using the detected variations, 31 haplotypes were determined and/or inferred. Our results would provide fundamental and useful information for genotyping *CYP2C19* in the Japanese and probably other Asian populations.

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Functional analysis of four naturally occurring variants of human constitutive androstane receptor

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Abstract

The human constitutive androstane receptor (CAR, NR1I3) is a member of the orphan nuclear receptor superfamily that plays an important role in the control of drug metabolism and disposition. In this study, we sequenced all the coding exons of the *NR1I3* gene for 334 Japanese subjects. We identified three novel single nucleotide polymorphisms (SNPs) that induce non-synonymous alterations of amino acids (His246Arg, Leu308Pro, and Asn323Ser) residing in the ligand-binding domain of CAR, in addition to the Val133Gly variant, which was another CAR variant identified in our previous study. We performed functional analysis of these four naturally occurring CAR variants in COS-7 cells using a *CYP3A4* promoter/enhancer reporter gene that includes the CAR responsive elements. The His246Arg variant caused marked reductions in both transactivation of the reporter gene and in the response to 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime (CITCO), which is a human CAR-specific agonist. The transactivation ability of the Leu308Pro variant was also significantly decreased, but its responsiveness to CITCO was not abrogated. The transactivation ability and CITCO response of the Val133Gly and Asn323Ser variants did not change as compared to the wild-type

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CAR. These data suggest that the His246Arg and Leu308Pro variants, especially His246Arg, may influence the expression of drug-metabolizing enzymes and transporters that are transactivated by CAR.

Keywords: Nuclear receptor; NR1I3; CAR; Constitutive androstane receptor; SNP; Amino acid alteration

Introduction

The constitutive androstane receptor (CAR) encoded by *NR1I3* is expressed predominantly in the liver [1] and it belongs to the nuclear receptor subfamily 1I. This subfamily also includes the pregnane X receptor (PXR) and the vitamin D receptor. CAR regulates transcription of the genes encoding drug/steroid-metabolizing enzymes and transporters, as well as other physiologically important enzymes [2–4]. CAR also regulates thyroid hormone and bilirubin metabolism [5,6]. In humans, CAR transactivates several major hepatic drug-metabolizing enzymes, such as the cytochrome P450s (CYPs) and transferase, including CYP2B6 [7], CYP3A4 [7,8], CYP2C9 [9,10], CYP2C19 [11], and UGT1A1 [12]. CAR forms a heterodimer with the retinoid X receptor (RXRα) and this binds to DNA motifs such as DR3, DR4, DR5, or ER6, of the target genes. It is noteworthy that, unlike most nuclear receptors, CAR is constitutively active in the absence of any added ligand [1,13]. However, ligand binding modulates the transcriptional activity of CAR. For example, 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3] thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime (CITCO) is an agonistic ligand of human CAR and it also triggers nuclear translocation [14].

NR1I3 is located on chromosome 1 and consists of nine exons. The DNA binding domain is encoded by exons 2, 3, and 4. A small hinge domain is encoded by a portion of exon 4, while the ligand-binding domain corresponds to exons 4–8 and a 5′ portion of exon 9 [15,16]. Recently, we sequenced all the coding exons of the *NR1I3* gene for 253 Japanese subjects and identified novel SNPs in the *NR1I3* gene [17]. These SNPs included one non-synonymous amino acid change that was localized to the ligand-binding domain of CAR. It is thought that SNPs may induce changes in the function or expression of the *NR1I3* gene, and this may explain variations in drug metabolism among humans. In this study, we analyzed the *NR1I3* sequence in an additional set of 334 Japanese subjects and found three novel, non-synonymous SNPs. We performed functional analysis of these four CAR variants using a reporter gene assay carried out with COS-7 cells.

Materials and methods

Human genomic DNA samples

The 334 subjects used in this study were Japanese cancer patients who were administered irinotecan or paclitaxel. Genomic DNA was extracted from blood leukocytes and was used as a template for the polymerase chain reaction (PCR). This study was approved by all the Ethnic Committees of the National Cancer Center, the National Cardiovascular Center, and the National Institute of Health Sciences. Written informed consent was obtained from all participants.

Conditions for PCR and DNA sequencing

The conditions and primers for PCR and DNA sequencing in these experiments were essentially the same as described previously [17]. Briefly, sequencing templates from the *NR1I3* gene were prepared by two rounds of PCR as described previously [17]. First, the entire region of the *NR1I3* gene (exon 1 to exon 9) was amplified by Z-Taq (Takara, Bio., Shiga, Japan) using each individual genomic DNA as a template (1st round PCR). Next, each exon was amplified by Ex-Taq (Takara, Bio) using the appropriate set of *NR1I3*-specific primers in the introns, and using the 1st round PCR product as a template. The PCR products were purified, and both strands were directly sequenced using the ABI BigDye Terminator Cycle Sequencing Kit Version 3.1 (Applied Biosystems, Foster City, CA, USA) and an ABI Prism 3730 DNA Analyzer (Applied Biosystems). The sequences were confirmed independently at least twice. Electropherograms were analyzed by two researchers.

Plasmids

Variant CAR (V133G, H246R, L308P, and N323S) expression plasmids were constructed by site-directed mutagenesis of the wild-type CAR (WT) expression plasmid [18] using the PCR-based QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). All the CAR constructs were introduced to the pcDNA6.2/nLumio-DEST mammalian Gateway vector to generate a six amino acids-Tag (Lumio tag) at the N-terminus, according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA) to facilitate in-gel detection of Lumio-tagged CAR proteins. All the coding regions of wild-type and variant CARs were confirmed by DNA sequencing. The luciferase reporter plasmid, pCYP3A4XREM 362/53Luc [19] that contains the XREM (xenobiotic-responsive enhancer module) and proximal promoter region of the *CYP3A4*

5′-flanking region (−7835 to −7208 and −362 to +53) including CAR responsive elements [8], was used for reporter gene assays.

Luciferase assay

COS-7 cells (African green monkey kidney cell line) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Hyclone Laboratories) under 5% CO_2 atmosphere at 37 °C. One day before transfection, the cells were plated onto 24-well plates at a density of 6×10^4 cells/well. The cells were transiently transfected with 0.175 μg each of the CAR expression plasmids, including the wild-type and the variants, together with 0.175 μg of the pCYP3 A4XREM 362/53Luc and 0.05 μg phRL-TK plasmid (encoding *Renilla* luciferase under control of the TK promoter; Promega, Madison, WI, USA) as the internal control. Mock transfections were carried out in parallel, using the pDNA6.2/nLumio-DEST without any inserts. All transfections were performed in 24-well plates using LipofectAmine2000 reagent (Invitrogen) according to the manufacturer's instructions. The total DNA was mixed in 50 μl of serum-free Opti-MEM I (Invitrogen). LipofectAmine2000 in 50 μl Opti-MEM I was added to the mixture and incubated for an additional 20 min. Cells were rinsed with growth medium without antibiotics, and incubated with 0.5 ml of the DNA/liposome mixture at 37 °C. Six hours after transfection, the medium was replaced with DMEM containing 10% charcoal-dextrane-treated FBS, and the cells were incubated at 37 °C overnight. Twenty-four hours after transfection, the cells were treated with the vehicle (dimethyl sulfoxide) or with various concentrations of the CITCO (0.1–10 μM) and then cultured for an additional 24 h. Cell lysates were prepared using the Dual-Glo Luciferase Assay System (Promega). Luciferase activity was then measured using an ARVO SX Multilabel Counter (Perkin-Elmer Wallac, Turku, Finland). All transfection efficiencies were normalized to the *Renilla* luciferase activity.

Detection and quantification of CAR variant proteins

Forty-eight hours after transfection with CAR expression plasmids, the cells were harvested and Lumio-tagged CAR proteins were detected using the Lumio Green Detection Kit (Invitrogen) as recommended by the manufacturer. Samples with 40 μg of total cellular protein were subjected to electrophoresis on NuPAGE Novex 4–20% Bis-Tris gel with MES buffer (Invitrogen). The fluorescent bands were then visualized and quantified using a fluorescent image analyzer FLA-3000 (Fuji film, Tokyo, Japan).

Results

Four non-synonymous SNPs in CAR

Previously, we identified the variant 398T > G in the *CAR* gene after screening 253 Japanese patients. This mutation leads to the amino acid alteration Val133Gly [17]. In this study, we surveyed an additional set of 334 Japanese subjects and identified novel non-synonymous SNPs 737A > G (His246Arg), 923T > C (Leu308 Pro), and 968A > G (Asn323Ser), found in two, one, and one subjects, respectively. The electropherograms of the three novel SNPs are shown in Fig. 1. All four SNPs were found as heterozygotes and all mapped to the ligand-binding domain (LBD), which is known to be the most important structural domain of nuclear receptors. We next looked for functional alterations resulting from these four amino acid substitutions using a luciferase reporter gene.

Expression of variant CAR proteins in COS-7 cells

COS-7 cells were transiently transfected with the wild-type and four variant CAR expression plasmids. The expression levels of the CAR proteins were determined by detection of the Lumio tag using the Lumio Green detection In Gel System. As shown in Fig. 2, the relative expression levels of all four variants were

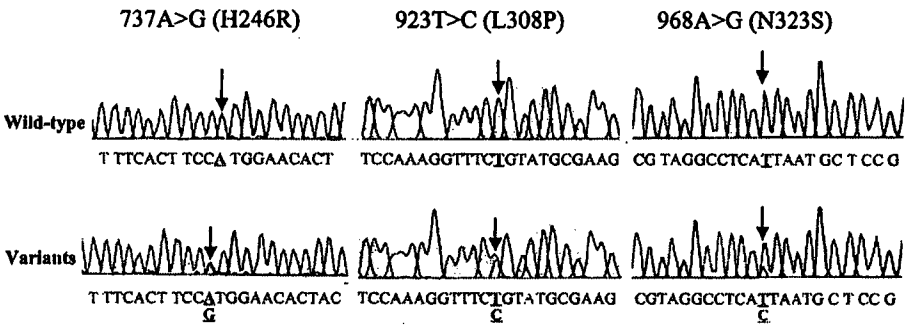

Fig. 1. Electropherograms (sense strands for 737A > G and 923T > C; antisense strand for 968A > G) of three novel non-synonymous variants of NR1I3. The first A of the translational initiation codon ATG in exon 2 is defined as position 1. Arrows indicate the nucleotide positions of variations.

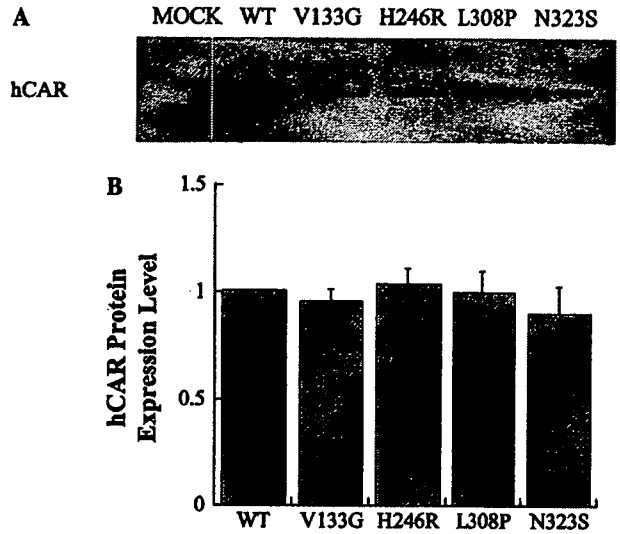

Fig. 2. Expression of the wild-type and variant CARs in COS-7 cells. (A) Aliquots (40 μg) of the pooled whole cell lysates from three independent preparations were loaded in each lane and Lumio Green detection was performed as described in Materials and methods. Whole cell lysates were prepared from COS-7 cells transfected with the empty expression vector (MOCK), the CAR wild-type plasmid (WT), or the variant expression plasmids as indicated. (B) Expression levels of CAR proteins are shown relative to that of the wild-type protein. Results are indicated as the means ± SD from three independent preparations.

equivalent to that of the wild-type protein, indicating that these substitutions do not affect protein stability and translational efficiency.

Reduced transactivation of the CYP3A4 promoter/enhancer by CAR variants

To evaluate the functional characteristics of the CAR variants with respect to transactivation, reporter gene assays were carried out. COS-7 cells were transiently transfected with a *CYP3A4* promoter/enhancer luciferase reporter plasmid that includes the CAR responsive elements [8], together with an expression plasmid for each CAR variant. As shown in Fig. 3, the His246Arg variant showed substantially less transactivation activity than that of the wild-type protein. The Leu308Pro variant also showed reduced activity. However, statistically significant differences in the transactivation levels of the Val133Gly and Asn323Ser variants versus the wild-type CAR were not observed. As the expression levels of the CAR proteins were similar (Fig. 2), this suggests that the reduced transactivation by these two CAR variants was due to a functional retardation associated with the amino acid alterations. Next, we investigated the effect of CITCO, which is a human CAR-specific agonist, on the activity of all the CAR variants. As shown in Fig. 4, all the variants except for His246Arg showed enhanced transactivations of the *CYP3A4* promoter/enhancer activity in the

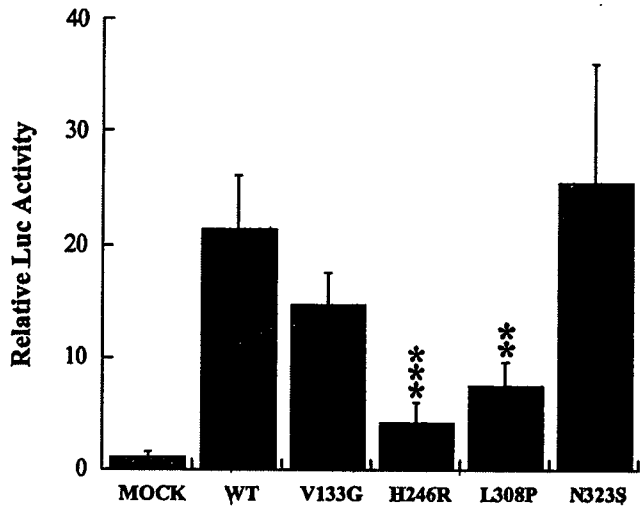

Fig. 3. Effect of CAR variants on the *CYP3A4* promoter/enhancer reporter activity. COS-7 cells were transfected with the *CYP3A4* luciferase reporter plasmid together with the empty expression vector (MOCK), the CAR wild-type plasmid (WT), or the variant expression plasmids as indicated. Data are shown as the means ± SD from four independent transfections. Statistically significant differences compared with the wild-type CAR are indicated by asterisks (**$p < 0.005$; ***$p < 0.0005$; by the one-way ANOVA and Dunnet's test).

presence of 0.1–10 μM of CITCO in COS-7 cells. In contrast, the CITCO-induced enhancement was completely abrogated in His246Arg.

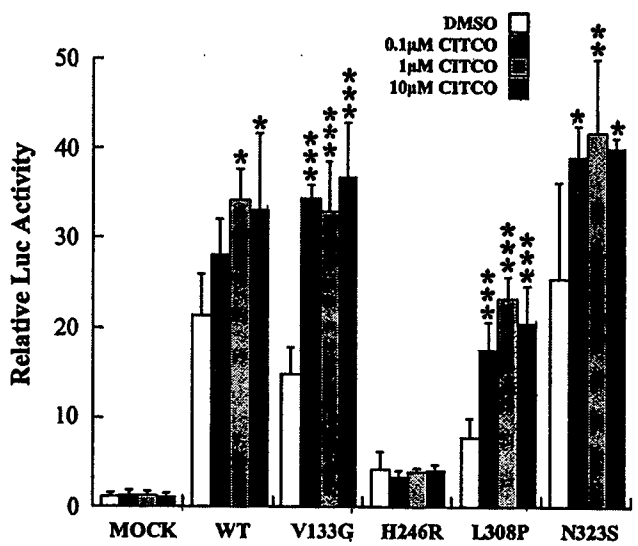

Fig. 4. Effect of CAR variants on the enhanced *CYP3A4* promoter/enhancer reporter activity in response to CITCO. COS-7 cells were transfected with the *CYP3A4* promoter/enhancer luciferase reporter plasmid together with the empty expression vector (MOCK), the CAR wild-type plasmid (WT), or the variant expression plasmids as indicated. After 24 h, the cells were further treated with CITCO (0.1–10 μM) for another 24 h. As a control, the cells were treated with vehicle (dimethyl sulfoxide, 0.4% v/v) alone (DMSO). Data are shown as the means ± SD from four independent transfections. Statistically significant differences compared with the activity of the DMSO-treated cells in each CAR are indicated by asterisks (*$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$; by the one-way ANOVA and Dunnet's test).