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Satoru KOYANO[1], Yoshiro SAITO[1,2], Kimie SAI[1,3], Kouichi KUROSE[1,4], Shogo OZAWA[1,5], Toshiharu NAKAJIMA[6], Kenji MATSUMOTO[6], Hirohisa SAITO[6], Kuniaki SHIRAO[7], Teruhiko YOSHIDA[8], Hironobu MINAMI[9], Atsushi OHTSU[10], Nagahiro SAIJO[11] and Jun-ichi SAWADA[1,2]

[1]*Project Team for Pharmacogenetics,* [2]*Division of Biochemistry and Immunochemistry,* [3]*Division of Xenobiotic Metabolism and Disposition,* [4]*Division of Medicinal Safety Science,* [5]*Division of Pharmacology, National Institute of Health Sciences, Tokyo, Japan;* [6]*Department of Allergy and Immunology, National Research Institute for Child Health and Development, National Center for Child Health and Development, Tokyo, Japan;* [7]*Division of Internal Medicine, National Cancer Center Hospital,* [8]*Genetics Division, National Cancer Center Research Institute, National Cancer Center, Tokyo, Japan;* [9]*Division of Oncology/Hematology,* [10]*Division of GI Oncology/Digestive Endoscopy,* [11]*Deputy Director, National Cancer Center Hospital East, Chiba, Japan*

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## SNP Communication

# *Novel Genetic Polymorphisms in the NR3C1 (Glucocorticoid receptor) Gene in a Japanese Population*

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**Summary:** Glucocorticoid receptor, encoded by *NR3C1*, is a transcriptional regulator of many drug metabolizing enzymes and anti-inflammatory molecules. In order to identify genetic variations of the *NR3C1* gene, genomic DNA from 265 Japanese individuals was sequenced. Fifty genetic polymorphisms were identified, including 32 novel ones [3 were in coding exons, 17 in the introns, 4 in the 5′-untranslated region (UTR), and 8 in the 5′-flanking region]. The novel nonsynonymous variation was 420G>T (Lys140Asn), and the allele frequency was 0.004. We did not detect any nonsynonymous polymorphism reported previously in other races, including a relatively frequent SNP Asn363Ser found in Caucasians and African-Americans. Thus, ethnic differences between Japanese and other races are suggested to exist in *NR3C1*.

**Key words: *NR3C1*; glucocorticoid receptor; genetic polymorphisms; non-synonymous alteration**

## Introduction

Glucocorticoid receptor (GR), encoded by *NR3C1*, is a member of the nuclear hormone receptor family of transcription factors. In the cytosol, GRs are associated with heat-shock and other proteins, and the binding of glucocorticoid leads to their nuclear translocation and positive or negative regulation of various genes.[1,2] It is well known that GR causes anti-inflammatory effects through transcriptional activation of glucocorticoid-induced leucine zipper genes or transcriptional suppression of genes of inflammatory cytokines induced by NF-$\kappa$B or AP-1.[3-5] In addition, GR regulates expression of many drug metabolizing enzymes. For instance, it is reported that GR activates the transcription of drug metabolizing enzymes CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP3A4, and CYP3A5 through GREs in the promoter/enhancer regions of these genes, or induction of both pregnane X receptor and constitutive androstane receptor.[6-11]

The human *GR* gene is located on chromosome 5p31

On October, 18, 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/), PharmGKB (http://www.pharmgkb.org/do/) or GeneSNPs (http://www.genome.utah.edu/genesnps/) databases.

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*To whom correspondence should be addressed*: Satoru KOYANO, Ph.D., *Project Team for Pharmacogenetics, National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan. Tel. +81-3-5717-3831, Fax. +81-3-5717-3832, E-mail: skoyano@nihs.go.jp*

and contains 9 exons, including exon 9$\alpha$.[12] GR$\beta$ is an alternatively spliced form with exon 9$\beta$ replacing exon 9$\alpha$, which was initially identified in glucocorticoid-resistant human multiple myeloma cells and functions as a dominant negative type.[13] Recently, three promoters and three 5′-noncoding exon 1′s (exons 1A, 1B, and 1C) were identified in *NR3C1*.[14] Interindividual differences of cytochrome P450 enzymes are thought to result from differences in their expression levels and/or activities.[11] Therefore, it is possible that the altered transcriptional activity of GR associated with polymorphisms of *NR3C1* might affect the expression levels of target genes including P450 enzymes.

Several genetic polymorphisms have been reported in *NR3C1*.[15] A common single nucleotide polymorphism (SNP), IVS2-646G>C (*Bcl*I polymorphism; allele frequencies, 0.15–0.45.[15]) and a relatively frequent SNP, 1088A>G (Asn363Ser; allele frequencies, 0.03–0.07.[15]), were found in Caucasians and African-Americans and reported to increase cortisol sensitivity and insulin response, and to cause other metabolic disturbances.[16,17] Moreover, it has been reported that the Ile559Asn (1676T>A) GR$\alpha$ variant has a trans-dominant effect on the wild-type GR by inhibiting its nuclear translocation.[18] Furthermore, the Ile747Met (2241T>G) GR$\alpha$ variant causes autosomal dominant glucocorticoid resistance through abnormal interactions with the p160 steroid receptor coactivator.[19] We previously reported that the Ser651Phe (1952C>T) and 2314insA variants showed reduced and almost abrogated transcriptional activities, respectively, correlating with their protein expression levels.[20] Thus, it is suggested that the genetic polymorphisms in *NR3C1* at least partly contribute to the interindividual differences in GR transcriptional activity. However, little information on promoter and coding SNPs in the entire *NR3C1* gene has been available in any race.

In this study, the promoters, exons, and surrounding introns of *NR3C1* were sequenced from 265 Japanese subjects. Sequence analysis identified 50 genetic polymorphisms, including 32 novel polymorphisms.

## Materials and Methods

**Human DNA samples:** DNA was extracted from the blood leukocytes of 114 Japanese cancer patients (with lung, stomach, and colon cancers) administered irinotecan. Additional 151 Japanese subjects were patients with allergy. Their peripheral lymphocytes were immortalized using the Epstein-Barr virus, and genomic DNA was extracted from the immortalized cells. Written informed consent was obtained from all participating patients. The ethical review boards of the National Cancer Center, the National Center for Child Health and Development, and the National Institute of Health Sciences approved this study.

**Polymerase chain reaction (PCR) conditions and DNA sequencing:** First, the entire *NR3C1* gene was amplified by mixed primer sets (Mix 1 or Mix 2 in the "1st PCR" section) shown in **Table 1**. Amplification was performed from 200 ng of genomic DNA using 1.25 units of Z-Taq (Takara Bio Inc., Shiga, Japan) with 0.2 $\mu$M of the primer sets. Since exon 1B and 1C are highly GC-rich, this exon was amplified by utilizing the GC-buffer Kit (LA-Taq, Takara Bio, Inc.). The first round PCR conditions were 94°C for 10 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 1 min, and 72°C for 3 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 $\mu$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, 55°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**. 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 3 through 7 and 9$\beta$, the primer sets for the 2nd PCR were also used for sequencing. As for exons 1A, 1B, 1C (with the 5′-flanking region), 2, and 8–9$\alpha$, different primer sets were used for sequencing. All the detected variations were confirmed by repeating the PCR from the genomic DNA and sequencing the newly generated PCR products. All nucleotide changes were clearly discernable in the electropherograms. Hardy-Weinberg equilibrium for all the detected genetic polymorphisms was statistically analyzed by SNPAlyze Ver. 3.2 (DYNACOM Co., Kanagawa, Japan).

## Results and Discussion

NT_029289.10 (Genbank Accession number) was utilized as a reference sequence of *NR3C1*. Fifty genetic variations were identified, including 32 novel ones, from 265 Japanese individuals (see **Table 2**). Of the 32 single nucleotide variations, 3 were in the coding exons, 17 in the introns, 4 in the 5′-untranslated region (UTR), and 8 in the 5′-flanking region. All 50 genetic polymorphisms were in Hardy-Weinberg equilibrium.[21,22]

In the coding region, a novel nonsynonymous variation, 420G>T (Lys140Asn), was detected in exon 2. 420G>T (Lys140Asn) was detected from 2 cancer patients (stomach and small cell lung cancers) as heterozygotes. The electropherograms of the variation is shown in **Fig. 1**. The allele frequency was 0.004.

**Table 1.** Primer sequences for the analysis of *NR3C1*

| | | 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 | Exons 1B to 1C | ACCCTAAACCCACACAGCAC | 3947714 | TGGAGGGAAAGGGGACACTAG | 3945364 | 2351 |
| | Mix 2 | Exon 1A | TGAGCTTCCTACCAACAAAAGCA | 3979066 | TATCTGTGGCACACAAACTGCAC | 3977212 | 1855 |
| | | Exon 2 | CAAGCGATGTCTACCTGTGGGGG | 3947756 | AGAACCCTGTGAGCAAGACCTGT | 3941566 | 6191 |
| | | Exons 3 to 6 | CAGGCTTTCGACGTGTAGTCAGT | 3858584 | AACTGCAATGAGGTAAGGGGAAA | 3840562 | 18023 |
| | | Exons 7 to 9 | TGAAGAGCCTCTGATCACTTTGG | 3838930 | GAGGCATGGATGGGATAATCTTC | 3820214 | 18717 |
| 2nd PCR | | Exon 1A | CCTTTGCAACTTCTGCCATT | 3978607 | TTCAGCAGATATTCCCCCAG | 3977535 | 1073 |
| | | Exons 1B to 1C | CAGCACAACCTTTCCCAGAG | 3947701 | CCGTCCGAGGTCAGGTTCCT | 3945461 | 2241 |
| | | Exon 2 | TCAGCTGTGCAAATGGATTG | 3943587 | TGAGACATTATACTCTCCTG | 3941832 | 1756 |
| | | Exon 3 | TGCTAGCACTTGAAGCCAGA | 3856791 | CCTTATGCTCCCTGACCAAA | 3856260 | 532 |
| | | Exon 4 | TGTTTTTGACCTCTCGACCTC | 3852883 | TGTGTGTAAGAAGAACTGGTGGA | 3852384 | 500 |
| | | Exon 5 | TTCTCCTTTTCCATGTCACTTT | 3843418 | TGATTTGAAAAGCAGGGAGT | 3842784 | 635 |
| | | Exon 6 | CACAAGAGGGTTTGTGAGTCTT | 3841506 | TTGTTGCTTGCGACAGACTT | 3840896 | 611 |
| | | Exon 7 | GAGCAACTAGTAATAAGTTCTA | 3838405 | CAACAGAGATCCCTATGCAGC | 3837609 | 797 |
| | | Exons 8 to 9$\alpha$ | TGACTTCATCTTAACCTTTTAGTTCC | 3825248 | GAAGCACCAACCCATTTTCAC | 3823704 | 1545 |
| | | Exon 9$\beta$ | GGAAGGTGGTGCTTACATCC | 3822388 | CCACGTATCCTAAAAGGGCA | 3821703 | 686 |
| Sequencing | | Exon 1A | TGCAGATCAGATGTCTTGGG | 3978434 | AGTGCTTCCGTTGGACACAT | 3977742 | |
| | | Exon 1B | CAGCACAACCTTTCCCAGAG | 3947701 | CCGTCCGAGGTCAGGTTCCT | 3947110 | |
| | | | TTCGGAAGGGGGGCTATATT | 3947280 | ACAGCGGGCGGGCCACAAGA | 3946644 | |
| | | Exon 1C | TTCTGTGGGTGGAAGGAGAC | 3946028 | CCGTCCGAGGTCAGGTTCCT | 3945461 | |
| | | Exon 2 | TCAGCTGTGCAAATGGATTG | 3943587 | GCCCTTCAAATGTTGCTGTT | 3942825 | |
| | | | AGTGATGGGAAATGACCTGG | 3943053 | CAACGGGAATTGGTGGAATG | 3942263 | |
| | | | ACACTGCCCCAAGTGAAAAC | 3942825 | TGAGACATTATACTCTCCTG | 3941832 | |
| | | Exon 8 | TGACTTCATCTTAACCTTTTAGTTCC | 3825248 | ATTTCATGAATCACTGTAGTACCATAA | 3824741 | |
| | | Exon 9$\alpha$ | AGACATTTTTCCTAAGGCCAAT | 3824912 | CACAGACTTTGGGCACTGGT | 3824061 | |

* The position of the 5′ end of each primer on NT_029289.10

**Table 2.** Summary of *NR3C1* polymorphisms detected in a Japanese population

| SNP ID | | | | | Position | | | | Number of subjects | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| This Study | NCBI (dbSNP) | JSNP | EGP_SNPS (GeneSNPs) | Reference | Location | NT_029289.10 | From the translational initiation site or the nearest exon | Nucleotide change and flanking sequences (5' to 3') | Amino acid change | Wild-type | Hetero-zygote | Homo-zygote | Frequency |
| MPJ6_3C1001[a] | | | | | 5'-Flanking of Exon 1A | 3978027 | Exon1A-114[b] | TTTTCATTTCCT C/TCTATTGTGTGGT | | 239 | 26 | 0 | 0.049 |
| MPJ6_3C1002[a] | | | | | 5'-Flanking of Exon 1A | 3977977 | Exon1A-64[b] | CCCCATTCAAAA G/TGTACCTCTTCCT | | 239 | 26 | 0 | 0.049 |
| MPJ6_3C1003[a] | | | | | 5'-Flanking of Exon 1A | 3977966 | Exon1A-53[b] | AGGTACCTCTTC C/ATCTTCTCTTGCT | | 263 | 2 | 0 | 0.004 |
| MPJ6_3C1004[a] | | | | | Exon 1A (5'-UTR) | 3977783 | Exon1A+131[b] | AATCTTTCAAGC C/TCTGCAGGACCGT | | 264 | 1 | 0 | 0.002 |
| MPJ6_3C1005[a] | | | | | Exon 1A (5'-UTR) | 3977760 | Exon1A+154[b] | GTAAAATGCGCA T/CGTGTCCAACGGA | | 264 | 1 | 0 | 0.002 |
| MPJ6_3C1006[a] | | | | | 5'-Flanking of Exon 1B | 3947594 | Exon1B-643[c] | CGAGCTGGGGAA G/TGGAGCTGGGGCG | | 264 | 1 | 0 | 0.002 |
| MPJ6_3C1007 | rs10482603 | | | NR3C1000258 | 5'-Flanking of Exon 1B | 3947549 | Exon1B-598[c] | CCCCTCGCCCCAC G/AGCCCTCTCCTTT | | 255 | 10 | 0 | 0.019 |
| MPJ6_3C1008[a] | | | | | 5'-Flanking of Exon 1B | 3947253 | Exon1B-302[c] | TATTTCGCCAGC A/GACTTACTATTTC | | 256 | 9 | 0 | 0.017 |
| MPJ6_3C1009[a] | | | | | 5'-Flanking of Exon 1B | 3947113 | Exon1B-162[c] | TCGAGTGTGAGC A/GCATTGGGCGGGA | | 256 | 9 | 0 | 0.017 |
| MPJ6_3C1010 | rs3806855 | IMS-JST166183 | | | 5'-Flanking of Exon 1B | 3947065 | Exon1B-114[c] | GCGGCGCCTCCT T/GCTGCCGCCGCCG | | 241 | 22 | 2 | 0.049 |
| MPJ6_3C1011 | rs3806854 | IMS-JST166182 | | | 5'-Flanking of Exon 1B | 3947063 | Exon1B-112[c] | GGCGCCTCCTTC T/CGCCGCCGCCGCC | | 241 | 22 | 2 | 0.049 |
| MPJ6_3C1012[a] | | | | | 5'-Flanking of Exon 1B | 3947050_3947048 | Exon1B-99_-97[c] | GCCGCCGCCGCC GCC/-TCGCAGACTCGG | | 256 | 9 | 0 | 0.017 |
| MPJ6_3C1013 | rs5871845 | | | | Exon 1B (5'-UTR) | 3946886_3946885 | Exon1B+66_67[c] | GCGACTCCCCCC -/CGGGCCCAAAGTA | | 130 | 103 | 32 | 0.315 |
| MPJ6_3C1014[a] | | | | | 5'-Flanking of Exon 1C | 3945867 | Exon1C-35[d] | AGGGGCTCTCCC C/TCCACCCATGGAG | | 263 | 2 | 0 | 0.004 |
| MPJ6_3C1015[a] | | | | | Exon 1C (5'-UTR) | 3945824_3945823 | Exon1C+9_10[d] | TCCTTTTTTTAG -/AAAAAAAAAAATA | | 241 | 24 | 0 | 0.045 |
| MPJ6_3C1016[a] | | | | | Exon 1C (5'-UTR) | 3945815_3945814 | Exon1C+18_19[d] | TAGAAAAAAAAA -/TATATATTTCCCT | | 264 | 1 | 0 | 0.002 |
| MPJ6_3C1017[a] | | | | | Exon 2 | 3942921 | 420[e] | AGAGAACCCCAA G/TAGTTCAGCATCC | Lys140Asn | 263 | 2 | 0 | 0.004 |
| MPJ6_3C1018 | rs13306588 | | | | Exon 2 | 3942444 | 897[e] | GAAACTGGGCAC A/GGTTTACTGTCAG | (Thr299Thr) | 256 | 9 | 0 | 0.017 |
| MPJ6_3C1019 | rs13306589 | | | | Exon 2 | 3942276 | 1065[e] | TAATGTCATTCC A/GCCAATTCCCGTT | (Pro355Pro) | 264 | 1 | 0 | 0.002 |
| MPJ6_3C1020 | rs13306584 | | | | Intron 2 | 3942088 | IVS2+69 | ATGTAAATCTTC A/GTTGATTTATATG | | 257 | 8 | 0 | 0.015 |
| MPJ6_3C1021[a] | | | | | Intron 2 | 3942010 | IVS2+147 | TGAAAGTAGGAA A/GGGTAGACAACCT | | 223 | 39 | 3 | 0.085 |
| MPJ6_3C1022[a] | | | | | Intron 2 | 3856765_3856764 | IVS2-96_-95 | GCCAGAGTTCAC TG/-TGAGCATTCTGA | | 257 | 8 | 0 | 0.015 |
| MPJ6_3C1023[a] | | | | | Exon 3 | 3856612 | 1242[e] | AACAGCAACAAC A/GGGACCACCTCCC | (Thr414Thr) | 257 | 8 | 0 | 0.015 |
| MPJ6_3C1024[a] | | | | | Intron 3 | 3856423 | IVS3+80 | ATTTCCATTATA T/CGCAAAGCCCATG | | 241 | 22 | 2 | 0.049 |
| MPJ6_3C1025 | rs852979 | | | | Intron 3 | 3856334 | IVS3+169 | AGTATTTAAAAC A/GACAATACAAAAA | | 239 | 24 | 2 | 0.053 |
| MPJ6_3C1026 | rs3822376 | IMS-JST183280 | | | Intron 3 | 3852771 | IVS3-57 | CAGAGGCTGTCC T/GTATAAAATATGT | | 257 | 8 | 0 | 0.015 |
| MPJ6_3C1027[a] | | | | | Intron 3 | 3852741 | IVS3-27 | GACCTGTGAAAC T/CTTAATAGTGCCT | | 263 | 2 | 0 | 0.004 |
| MPJ6_3C1028 | rs6188 | | | 16) | Intron 4 | 3843280 | IVS4-16 | TTCTATGTATTT G/TTTTAAATACCCA | | 232 | 31 | 2 | 0.066 |
| MPJ6_3C1029 | | | | 20) | Exon 5 | 3843223 | 1510[e] | ATTCAGCAGGCC A/TCTACAGGAGTCT | Thr504Ser | 264 | 1 | 0 | 0.002 |
| MPJ6_3C1030 | rs3776216 | IMS-JST131921 | | | Intron 5 | 3842865 | IVS5+121 | TAGTTCTGGGGA C/TTATGAAGGGGAG | | 261 | 4 | 0 | 0.008 |
| MPJ6_3C1031[a] | | | | | Intron 5 | 3841355 | IVS5-42 | CAGAGATGATCT A/TCTGAAGAGTGTT | | 264 | 1 | 0 | 0.002 |
| MPJ6_3C1032 | rs6194 | | | | Exon 6 | 3841297 | 1764[e] | CAGGAACTTACA C/TCTGGATGACCAA | (His588His) | 240 | 23 | 2 | 0.051 |
| MPJ6_3C1033[a] | | | | | Intron 6 | 3841040 | IVS6+129 | TAGGATCTAGGC A/CAACTGGAAATAG | | 254 | 11 | 0 | 0.021 |
| MPJ6_3C1034[a] | | | | | Intron 6 | 3838355_3838351 | IVS6-265_-261 | TTAATTAAATAA TAAAA/-TAATACATGGCA | | 264 | 1 | 0 | 0.002 |
| MPJ6_3C1035[a] | | | | | Intron 6 | 3838342 | IVS6-251 | AAAATAATACAT G/AGCATGCATGATA | | 264 | 1 | 0 | 0.002 |
| MPJ6_3C1036[a] | | | | | Intron 6 | 3838291 | IVS6-200 | AGATTATTCATT A/TGTCTATTTAAAC | | 264 | 1 | 0 | 0.002 |
| MPJ6_3C1038 | | | | 20) | Exon 7 | 3838032 | 1952[e] | TGTATGTTTCCT C/TTGAGTTACACAG | Ser651Phe | 264 | 1 | 0 | 0.002 |
| MPJ6_3C1039 | rs258751 | | | 16) | Exon 8 | 3825216 | 2033[e] | AGTTCCTAAGGA C/TGGTCTGAAGAGC | (Asp678Asp) | 240 | 23 | 2 | 0.051 |
| MPJ6_3C1040[a] | | | | | Intron 8 | 3825044 | IVS8+25 | CATAAAGCCAAA T/CATAAGAGTTTTC | | 264 | 1 | 0 | 0.002 |
| MPJ6_3C1041[a] | | | | | Intron 8 | 3824988 | IVS8+81 | AATTATATACCA G/ACAGCGCCCCCAC | | 257 | 8 | 0 | 0.015 |
| MPJ6_3C1042[a] | | | | | Intron 8 | 3824860 | IVS8+209 | CTAGTCATGTGT A/TTATAAAATCACC | | 263 | 2 | 0 | 0.004 |
| MPJ6_3C1043 | rs258750 | | | | Intron 8 | 3824825 | IVS8+244 | AGAATTTAGTAA T/CACTGTTTTTAAA | | 232 | 31 | 2 | 0.066 |
| MPJ6_3C1044[a] | | | | | Intron 8 | 3824723 | IVS8-181 | TATATAATTCTA C/TATACAATCAAAG | | 257 | 8 | 0 | 0.015 |
| MPJ6_3C1045[a] | | | | | Intron 8 | 3824700 | IVS8-158 | AGAAATATAAAA T/CGTGTTTTGTACG | | 257 | 8 | 0 | 0.015 |
| MPJ6_3C1046[a] | | | | | Intron 8 | 3824551 | IVS8-9 | TTACCATATCTT C/GTCTTGCAGGTGG | | 257 | 8 | 0 | 0.015 |
| MPJ6_3C1047[a] | | | | | Intron 8 | 3824548 | IVS8-6 | CCATATCTTCTC T/CTGCAGGTGGTTG | | 264 | 1 | 0 | 0.002 |
| MPJ6_3C1048[a] | | | | | Exon 9 | 3824474 | 2250[e] | TATTGAATTCCC C/TGAGATGTTAGCT | (Pro750Pro) | 257 | 8 | 0 | 0.015 |
| MPJ6_3C1049 | rs6196 | IMS-JST006606 | | 16) | Exon 9 | 3824426 | 2298[e] | AAAATATTCAAA T/CGGAAATATCAAA | (Asn766Asn) | 240 | 23 | 2 | 0.051 |
| MPJ6_3C1051 | rs13306586 | | | | Exon 9 (3'-UTR) | 3822348 | 4376[e] | ACCATCTAATAG C/TGGGTTACTTTCA | | 257 | 8 | 0 | 0.015 |
| MPJ6_3C1052[a] | | | | | Intron 9 | 3822023 | IVS9 +45 | GATGGTGTGTAA C/TCCGGCTGGATAA | | 264 | 1 | 0 | 0.002 |

[a] Novel variations detected in our study.
[b] The starting nucleotide of exon 1A is numbered +1.
[c] The starting nucleotide of exon 1B is numbered +1.
[d] The starting nucleotide of exon 1C is numbered +1.
[e] A of the translation initiation codon ATG is numbered +1.

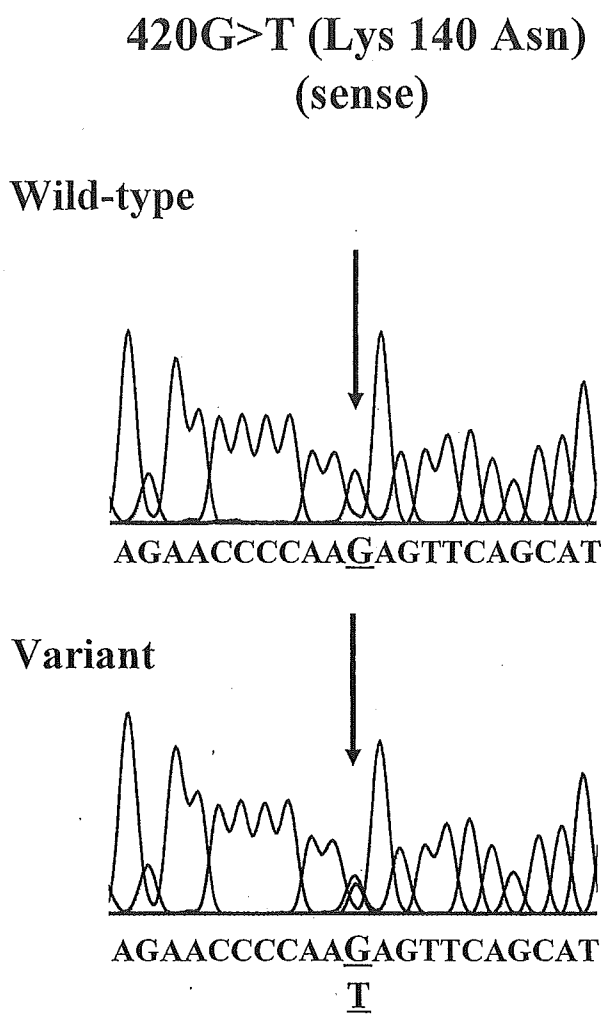


**Fig. 1.** Electropherograms for the novel nonsynonymous variation of *NR3C1*. (A) MPJ6_3C1017 (wild-type 420G/G; variant 420G/T). Arrows indicate the variant nucleotide position.

Lys-140 is located in the activation function-1 domain of the human GR protein. This domain interacts with the basal transcriptional machinery and is involved in both constitutive and inducible transactivation of target genes.[23] Thus, the amino acid substitution Lys140Asn might influence GR function. We are now elucidating its functional significance using a mammalian expression system.

Notably, among the 265 Japanese samples analyzed, we did not detect the relatively frequent polymorphism Asn363Ser found in Caucasians, including Dutch, and African-Americans.[15,16] Recently, Syed *et al.* reported that 295 South Asians living in the United Kingdom showed a low prevalence of the Asn363Ser polymorphism compared with an Europid population (0.003 *vs* 0.03 for allele frequency).[24] Their report and our present data clearly indicate that ethnic differences between Asian and other populations exist for the Asn363Ser polymorphism. Thus, the presence or absence of relatively frequent *NR3C1* polymorphisms of clinical importance should be further elucidated in the Asian populations.

Three sets of alternative promoter/exon 1 have been identified and several researchers have analyzed these promoter regions. Recently, Geng and Vedeckis reported that GRE in the 5′-regulatory region of exon 1A was involved in the autoregulation by GR.[25] As for the exon 1B and 1C promoters, proinflammatory cytokines were reported to regulate the GR transcription through the NF-$\kappa$B and AP-1 elements in the respective promoter regions.[26] We did not detect any polymorphisms in these elements themselves. In general, however, transcriptional regulation is very often conferred by many transcription factors and regulatory elements. In the present study, we found a number of polymorphisms near exons 1A, 1B, and 1C. It is important to elucidate whether these polymorphisms affect GR transcription and alter drug metabolism and/or glucocorticoid response.

GR is one of the key transcriptional regulators for many drug metabolizing enzymes and proinflammatory factors. Thus, our findings provide fundamental and useful information for genotyping *NR3C1* in the Japanese and could be utilized for determination of clinically important haplotypes.

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# *Genetic Variations and Haplotypes of UGT1A4 in a Japanese Population*

Mayumi SAEKI[1], Yoshiro SAITO[1,2], Hideto JINNO[1,3], Kimie SAI[1,4], Akiko HACHISUKA[2], Nahoko KANIWA[1,5], Shogo OZAWA[1,6], Manabu KAWAMOTO[7], Naoyuki KAMATANI[7], Kuniaki SHIRAO[8], Hironobu MINAMI[9], Atsushi OHTSU[10], Teruhiko YOSHIDA[11], Nagahiro SAIJO[12], Kazuo KOMAMURA[13,14], Takeshi KOTAKE[15], Hideki MORISHITA[15], Shiro KAMAKURA[13], Masafumi KITAKAZE[13], Hitonobu TOMOIKE[13] and Jun-ichi SAWADA[1,2]

[1]Project Team for Pharmacogenetics, [2]Division of Biochemistry and Immunochemistry, [3]Division of Environmental Chemistry, [4]Division of Xenobiotic Metabolism and Disposition, [5]Division of Medicinal Safety Science, [6]Division of Pharmacology, National Institute of Health Sciences, Tokyo, Japan [7]Division of Genomic Medicine, Department of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, Tokyo, Japan [8]Division of Internal Medicine, National Cancer Center Hospital, [11]Genetics Division, National Cancer Center Research Institute, National Cancer Center, Tokyo, Japan [9]Division of OncologyHematology, [10]Division of GI OncologyDigestive Endoscopy, [12]Deputy Director, National Cancer Center Hospital East, Chiba, Japan [13]Division of Cardiology, [14]Department of Cardiovascular Dynamics Research Institute, [15]Department of Pharmacy, National Cardiovascular Center, Osaka, Japan

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## SNP Communication

# *Genetic Variations and Haplotypes of UGT1A4 in a Japanese Population*

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[1]*Project Team for Pharmacogenetics,* [2]*Division of Biochemistry and Immunochemistry,* [3]*Division of Environmental Chemistry,* [4]*Division of Xenobiotic Metabolism and Disposition,* [5]*Division of Medicinal Safety Science,* [6]*Division of Pharmacology, National Institute of Health Sciences, Tokyo, Japan* [7]*Division of Genomic Medicine, Department of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, Tokyo, Japan* [8]*Division of Internal Medicine, National Cancer Center Hospital,* [11]*Genetics Division, National Cancer Center Research Institute, National Cancer Center, Tokyo, Japan* [9]*Division of Oncology/Hematology,* [10]*Division of GI Oncology/Digestive Endoscopy,* [12]*Deputy Director, National Cancer Center Hospital East, Chiba, Japan* [13]*Division of Cardiology,* [14]*Department of Cardiovascular Dynamics Research Institute,* [15]*Department of Pharmacy, National Cardiovascular Center, Osaka, Japan*

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

**Summary:** Nineteen genetic variations, including 11 novel ones, were found in exon 1 and its flanking region of the UDP-glucuronosyltransferase (UGT) 1A4 gene from 256 Japanese subjects, consisting of 60 healthy volunteers, 88 cancer patients and 108 arrhythmic patients. These variations include −217T>G and −36G>A in the 5′-flanking region, 30G>A (P10P), 127delA (43fsX22; frame-shift from codon 43 resulting in the termination at the 22nd codon, codon 65), 175delG (59fsX6), 271C>T (R91C), 325A>G (R109G), and 357T>C (N119N) in exon 1, and IVS1+1G>T, IVS1+98A>G and IVS1+101G>T in the following intron. Among them, 127delA and 175delG can confer early termination of translation, resulting in an immature protein that probably lacks enzymatic activity. Variation IVS1+1G>T is located at a splice donor site and thus may lead to aberrant splicing. Since we did not find any significant differences in the frequencies of all the variations among the three subject groups, the data were analyzed as one group. The allele frequencies of the novel variations were 0.006 for IVS1+101G>T, 0.004 for 30G>A (P10P) and 357T>C (N119N), and 0.002 for the 8 other variations. In addition, the two known nonsynonymous single nucleotide polymorphisms (SNPs), 31C>T (R11W) and 142T>G (L48V), were found at 0.012 and 0.129 frequencies, respectively. The SNP 70C>A (P24T), mostly linked with 142T>G (L48V) in German Caucasians, was not detected in this study. Sixteen haplotypes were identified or inferred, and some haplotypes were confirmed by cloning and sequencing. It was shown that most of 142T>G (L48V) was linked with −219C>T, −163G>A, 448T>C (L150L), 804G>A (P268P), and IVS1+43C>T, comprising haplotype **3a*; haplotype **4a* harbors 31C>T (R11W); 127delA (43fsX22) and 142T>G (L48V) were linked (haplotype **5a*); 175delG (59fsX6) was linked with 325A>G (R109G) (**6a* haplotype); and −219C>T, −163G>A, 142T>G (L48V), 271C>T (R91C), 448T>C (L150L), 804G>A (P268P), and IVS1+43C>T comprised haplotype **7a*. Our results provide fundamental and useful information for genotyping *UGT1A4* in the Japanese and probably Asian populations.

**Key words: UGT1A4; amino acid alteration; frameshift; splice donor site; drug metabolism**

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*To whom correspondence should be addressed*: Yoshiro SAITO, Ph.D., *Project Team for Pharmacogenetics, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan. Tel. +81-3-3700-9453, Fax. +81-3-3707-6950, E-mail: yoshiro@nihs.go.jp*

**Table 1.** Primers utilized for *UGT1A4* amplification and sequencing

| | Direction | Primer Name | Sequences | Location[a] |
|---|---|---|---|---|
| Amplification | forward | UGT1A4-1stF | TTAACAAAGTAGAAGGCAGTG | 135092 |
| | reverse | UGT1A4-1stR | TGAAAACTTGAAATACACTAGGC | 136460 |
| Sequencing | forward | UGT1A4-1stF | TTAACAAAGTAGAAGGCAGTG | 135092 |
| | forward | UGT1A4seqF2 | GGGCTGAGAGTGGAAAGGT | 135502 |
| | forward | UGT1A4seqF3 | TCCTTCCTCCTATATTCCTAAGTT | 135995 |
| | reverse | UGT1A4seqR1-2 | ATCAAATTCCTTCTGGGTCC | 135698 |
| | reverse | UGT1A4seqR2 | AAGGGGCAGAAAAAGTATGG | 136119 |
| | reverse | UGT1A4-1stR | TGAAAACTTGAAATACACTAGGC | 136460 |

[a]The 5′-end of each primer on AF297093.1.

On December 2, 2004, these variations were not found on the UDP Glucuronosyltransferase home page (http://som.flinders.edu.au/FUSA/ClinPharm/UGT/), the Japanese Single Nucleotide Polymorphisms (JSNP) (http://snp.ims.u-tokyo.ac.jp/), dbSNP in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP/), or PharmGKB (http://www.pharmgkb.org/do/) databases.

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

## Introduction

As phase II enzymes, the UDP-glucuronosyltransferase enzymes (UGTs) play crucial roles in the detoxification and elimination of a large number of endogenous and exogenous compounds.[1] Of the UGT1 and UGT2 subfamilies expressed in humans, the genes encoding UGT1As have a unique genetic structure consisting of at least 13 different exon 1′s, including four inactive ones, and the common exons 2 to 5 clustered on chromosome 2q37.[2] One of the exon 1′s can be spliced on to the common exons. The *N*-terminal domains (encoded by the exon 1′s) of the UGT1A proteins determine their substrate-binding specificity, and the common *C*-terminal domain (encoded by exons 2 to 5) is important for UDP-glucuronic acid binding.[3]

UGT1A4 is expressed in the liver, bile ducts, colon, small intestine, and pancreas.[1,4,5] UGT1A4 catalyzes the conjugation of exogenous amines and alcohols, including nicotine, sapogenins, imipramine, trifluoperazine, and tamoxifen.[1,6-9] In addition, many androgens and progestins are reported as endogenous substrates of UGT1A4.[6] Several genetic polymorphisms of *UGT1A4* were reported in the public databases. Among them, two nonsynonymous single nucleotide polymorphisms (SNPs), 70C>A (P24T) and 142T>G (L48V), were found in German Caucasians, and they were shown to be closely associated.[10] The variant enzymes (24T and 48V) had reduced *in vitro* activities for β-naphtylamine, benzidine, *trans*-androsterone, and dihydrotestosterone in a substrate-specific manner.[10]

In spite of the clinical importance of UGT1A4, there is no report on the comprehensive sequencing analysis for the genetic polymorphisms of *UGT1A4* in Asian populations, including the Japanese. In the present study, *UGT1A4* exon 1 was sequenced from 256 Japanese subjects. Eleven novel genetic variations were identified, including 4 nonsynonymous ones.

## Materials and Methods

**Human genomic DNA samples:** DNA was obtained from the blood leukocytes of 88 Japanese cancer patients and 108 Japanese arrhythmic patients. Written informed consent was obtained from all participating patients. DNA was also extracted from Epstein-Barr virus-transformed lymphoblastoid cells, for which blood samples were collected from 60 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 all the participating organizations approved this study.

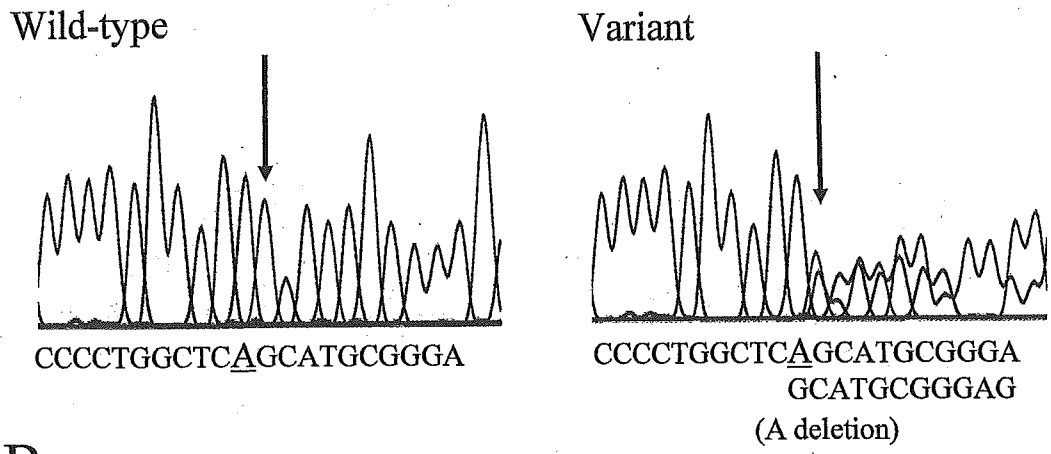
**PCR conditions for DNA sequencing:** First, exon 1 of *UGT1A4* was amplified from genomic DNA (100 ng) using 0.625 units of *Ex*-Taq (Takara Bio. Inc., Shiga, Japan) with 0.2 μM of amplification primers designed in the introns (**Table 1**). The PCR conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 1 min, and 72°C for 2 min, and then a final extension at 72°C for 7 min. These PCR products were then treated with a PCR Product Pre-Sequencing Kit (USB Co., Cleveland, OH, USA) and were directly sequenced on both strands using an ABI Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) (see **Table 1** for sequencing primers). The excess dye was removed by a DyeEx96 kit (Qiagen, Hilden, Germany). The eluates were analyzed on an ABI Prism 3700 DNA Analyzer (Applied Biosystems). All variations were confirmed by repeating

**Table 2.** Summary of *UGT1A4* polymorphisms detected in a Japanese population

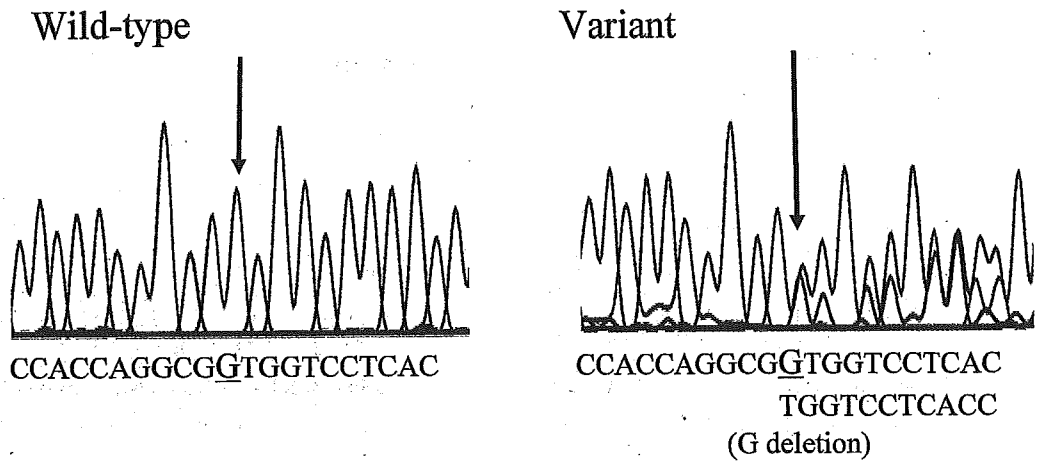
| SNP ID | | | | | Position | | | | Number of subjects | | | Frequency | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| This Study | dbSNP-NCBI database | JSNP database | Pharm GKB atabase[b] | Location | AF297093.1 | From the translational initiation site or from the end of exon 1 (IVS1+) | Nucleotide change and flanking sequences (5′ to 3′) | Amino acid change | Wild-type | Hetero-zygote | Homo-zygote | Total (n=256) | Healthy volunteers (n=60) | Cancer patients (n=88) | Arrhythmic patients (n=108) |
| MPJ6_U1A081 | rs3732219 | IMS-JST085729 | O | 5′-flanking | 135210 | −219 | GGGTCAGATGAG**C/T**TTTTCAAGATAG | | 195 | 54 | 7 | 0.133 | 0.133 | 0.142 | 0.125 |
| MPJ6_U1A082[a] | | | | 5′-flanking | 135212 | −217 | GTCAGATGAGC**T/G**TTCAAGATAGGC | | 255 | 1 | 0 | 0.002 | 0.000 | 0.000 | 0.005 |
| MPJ6_U1A083 | rs3732218 | IMS-JST085728 | O | 5′-flanking | 135266 | −163 | TAACGAAAGGCA**G/A**TTATAGATTAAT | | 195 | 54 | 7 | 0.133 | 0.133 | 0.142 | 0.125 |
| MPJ6_U1A084[a] | | | | 5′-flanking | 135393 | −36 | CAGGCACAGCGT**G/A**GGGTGGACAGTC | | 255 | 1 | 0 | 0.002 | 0.000 | 0.006 | 0.000 |
| MPJ6_U1A085[a] | | | | Exon 1 | 135458 | 30 | GGTTCCCCTGCC**G/A**CGGCTGGCCACA | P10P | 254 | 2 | 0 | 0.004 | 0.000 | 0.000 | 0.009 |
| MPJ6_U1A086 | rs3892221 | | O | Exon 1 | 135459 | 31 | GTTCCCCTGCCG**C/T**GGCTGGCCACAG | R11W | 250 | 6 | 0 | 0.012 | 0.025 | 0.011 | 0.005 |
| MPJ6_U1A087[a] | | | | Exon 1 | 135555 | 127 | AGCCCCTGGCTC**A/-**GCATGCGGGAGG | 43fsX22 | 255 | 1 | 0 | 0.002 | 0.000 | 0.000 | 0.005 |
| MPJ6_U1A088 | rs2011425 | | O | Exon 1 | 135570 | 142 | ATGCGGGAGGCC**T/G**TGCGGGAGCTCC | L48V | 197 | 52 | 7 | 0.129 | 0.133 | 0.148 | 0.111 |
| MPJ6_U1A089[a] | | | | Exon 1 | 135603 | 175 | GGCCACCAGGCG**G/-**TGGTCCTCACCC | 59fsX6 | 255 | 1 | 0 | 0.002 | 0.000 | 0.000 | 0.005 |
| MPJ6_U1A090[a] | | | | Exon 1 | 135699 | 271 | AAGGAATTTGATC**/T**GCGTTACGCTGG | R91C | 255 | 1 | 0 | 0.002 | 0.000 | 0.000 | 0.005 |
| MPJ6_U1A091[a] | | | | Exon 1 | 135753 | 325 | CATCTTCTGAAG**A/G**GATATTCTAGAA | R109G | 255 | 1 | 0 | 0.002 | 0.000 | 0.000 | 0.005 |
| MPJ6_U1A092[a] | | | | Exon 1 | 135785 | 357 | AATTATGAACAA**T/C**GTATCTTTGGCC | N119N | 254 | 2 | 0 | 0.004 | 0.008 | 0.006 | 0.000 |
| MPJ6_U1A093 | rs12468274 | | O | Exon 1 | 135876 | 448 | TTTGATGTGGTT**T/C**TAACAGACCCCG | L150L | 195 | 54 | 7 | 0.133 | 0.133 | 0.142 | 0.125 |
| MPJ6_U1A094 | rs2011404 | | O | Exon 1 | 135899 | 471 | CGTTAACCTCTGC**/T**[c]GGGGCGGTGCTG | C157C | 251 | 5 | 0 | 0.010 | 0.008 | 0.011 | 0.009 |
| MPJ6_U1A095 | rs3732217 | IMS-JST085727 | O | Exon 1 | 136232 | 804 | CTACCCCAGGCC**G/A**ATCATGCCCAAC | P268P | 195 | 54 | 7 | 0.133 | 0.133 | 0.142 | 0.125 |
| MPJ6_U1A096[a] | | | | Intron 1 | 136296 | IVS1+1 | CCACTATCTCAG**G/T**TCTGTATTGGTG | | 255 | 1 | 0 | 0.002 | 0.000 | 0.000 | 0.005 |
| MPJ6_U1A097 | rs2011219 | IMS-JST085726 | O | Intron 1 | 136338 | IVS1+43 | TTCCAGGCAAAA**C/T**ACTTTTTAAAAA | | 195 | 54 | 7 | 0.133 | 0.133 | 0.142 | 0.125 |
| MPJ6_U1A098[a] | | | | Intron 1 | 136393 | IVS1+98 | ACTTATCTTTCC**A/G**AAGATTTTATTT | | 255 | 1 | 0 | 0.002 | 0.000 | 0.006 | 0.000 |
| MPJ6_U1A099[a] | | | | Intron 1 | 136396 | IVS1+101 | TATCTTTCCAAA**G/T**ATTTTATTTTGG | | 253 | 3 | 0 | 0.006 | 0.008 | 0.006 | 0.005 |

[a]Novel variations detected in this study.
[b]The SNPs included in the PharmGKB database was shown as "O".
[c]T in the reference sequence.

A 127delA (43 fsX 22) (sense)




B 175delG (59 fsX 6) (sense)




C 271C>T (Arg 91 Cys) (antisense)

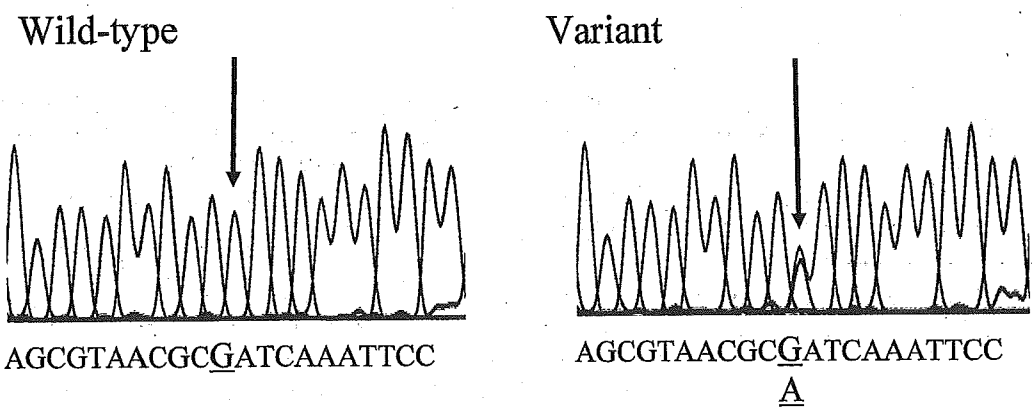



Fig. 1

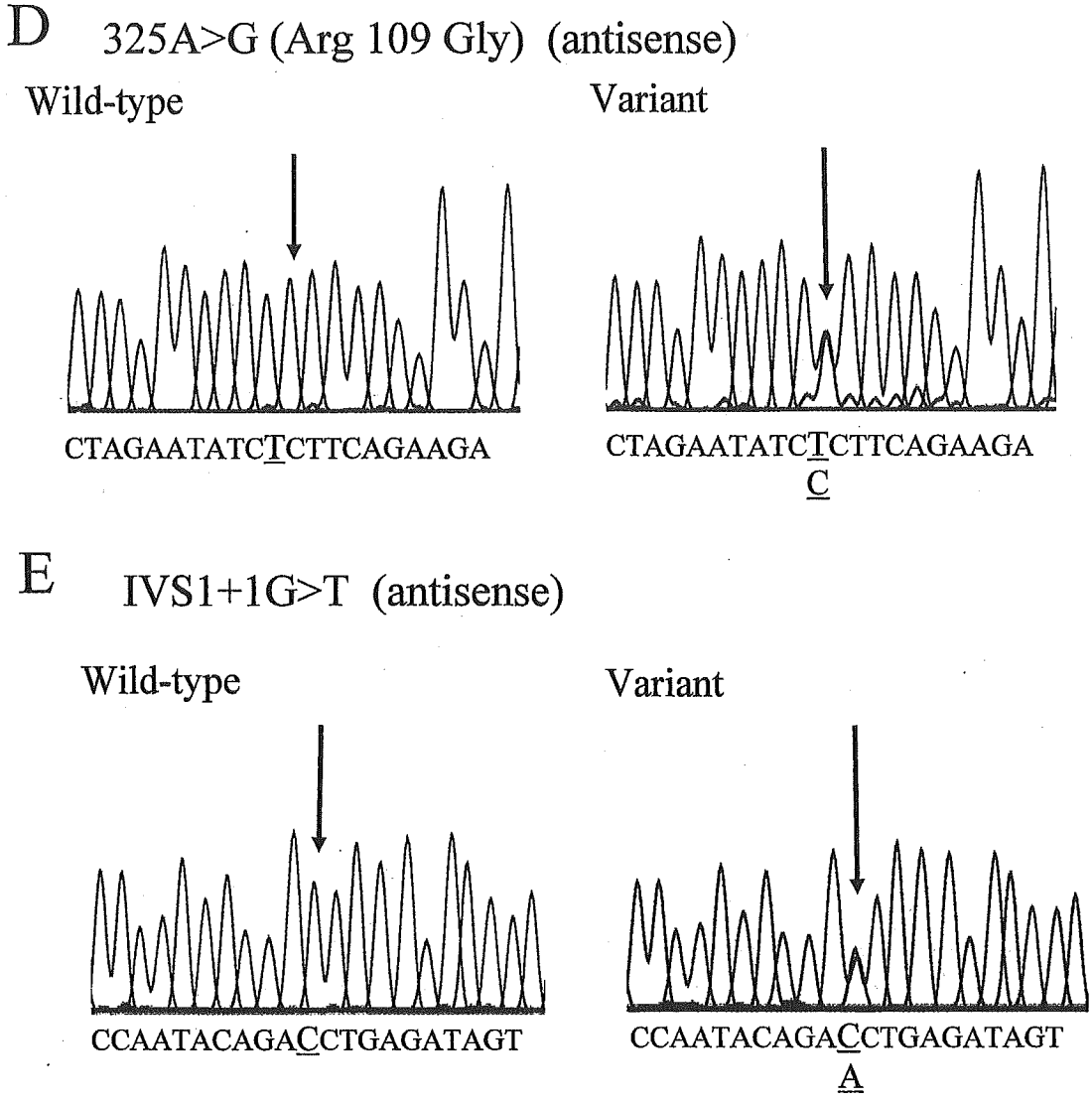



**Fig. 1.** The 4 novel genetic variations with amino acid substitutions and 1 splice donor site variation of human *UGT1A4*. (A) MPJ6_U1A087 (wild-type, 127A/A; variant, 127A/-). (B) MPJ6_U1A089 (wild-type, 175G/G; variant, 175G/-). (C) MPJ6_U1A090 (wild-type, 271C/C; variant, 271C/T). (D) MPJ6_U1A091 (wild-type, 325A/A; variant, 325A/G). (E) MPJ6_U1A096 (wild-type, IVS1+1G/G; variant, IVS1+1G/T). Arrows indicate the positions of the nucleotide changes.

the PCR on genomic DNA and sequencing 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).

**Linkage disequilibrium (LD) and haplotype analysis:** Hardy-Weinberg equilibrium analysis and LD analysis were performed by SNPAlyze software (Dynacom Co., Yokohama, Japan). Pairwise LDs were shown in rho square ($r^2$) values. Some of the haplotypes were unambiguous from the subjects with homozygous SNPs at all sites or a heterozygous SNP 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.[11] The haplotypes were described as a number plus a small alphabetical letter.

## Results and Discussion

*UGT1A4* exon 1 and its flanking regions (from −286 bases upstream of the translational start site to 112 bases downstream of the end of exon 1) were sequenced from 256 Japanese subjects. Genbank accession number AF297093.1 was utilized for the reference sequence. Nineteen polymorphisms were detected, including 11 novel ones (2 were in the 5′-flanking region, 6 in exon 1, and 3 in the following intron) (**Table 2**). All of the allelic frequencies were in Hardy-Weinberg equilibrium ($p=$

0.13 or over). Since we did not find any significant differences in the frequencies of all the variations among three subject groups ($p>0.25$ by $\chi^2$ test) and between two of the three groups ($p>0.13$ by $\chi^2$ test or Fisher's exact test), the data for all subjects were analyzed as one group.

We found two novel nonsynonymous variations, 271C>T (R91C) and 325A>G (R109G), and two novel deletions, 127delA (43fsX22) and 175delG (59fsX6), as individual heterozygotes at a 0.002 frequency. Among them, 127delA (43fsX22) and 175delG (59fsX6) are the frameshift variations starting from codon 43 and 59, respectively, resulting in early stop codons at the 22nd (*i.e.* codon 65) and the 6th (*i.e.* codon 65) codons, respectively. It is most likely that these variations generate an immature protein that probably has null activity. The functional significance of 271C>T (R91C) and 325A>G (R109G) is currently unknown. Additionally, IVS1+1G>T, which was found at a frequency of 0.002, was located at a splice donor site and thus may lead to aberrant splicing (**Fig. 1**).

We also detected two known nonsynonymous SNPs, 31C>T (R11W) and 142T>G (L48V), at 0.012 and 0.129 frequencies, respectively. The frequency of 142T>G (L48V) was almost comparable to that of German Caucasians (0.09).[10] L48V was reported to lead to a partial decrease in glucuronidation of $\beta$-naphthylamine and benzidine, a marked decrease in the activity to *trans*-androsterone, and no activity toward dihydrotestosterone *in vitro*.[10] The functional significance of SNP 31C>T (R11W) has not been reported yet.

High linkage disequilibrium ($r^2 \geqq 0.89$) was observed among −219C>T, −163G>A, 142T>G (L48V), 448T>C (L150L), 804G>A (P268P), and IVS1+43C>T. A perfect linkage ($r^2=1$) was found between 175delG and 325A>G (R109G), but found in only one subject. The $r^2$ values were below 0.014 between the other pairs of polymorphisms. The SNP 70C>A (P24T), mostly linked with 142T>G (L48V) in German Caucasians,[10] was not detected in this study. Thus, it must be clarified whether the differences in the linkage of those SNPs may lead to the ethnic differences in the enzymatic activities of UGT1A4. A similar kind of ethnic difference has been found in the **IB* haplotype, which harbors the three linked SNPs in the 3′-untranslated region of UGT1A common exon 5 found in a Japanese population.[12] In Caucasian and African-American populations, this linkage of the 3 SNPs was not complete, especially in African-Americans.[13]

Using the detected SNPs, haplotype analysis was then performed (**Table 3**). Since *UGT1A4*2* [70C>A (P24T)] and **3* [142T>G (L48V)] were defined in AF465196 and AF465197 (Genbank accession numbers), respectively, the novel haplotypes with amino acid changes, frameshift variations, or splice donor site

**Table 3.** *UGT1A4* haplotypes in a Japanese population

| Nucleotide change[a] | | -219 C>T | -217 T>G | -163 G>A | -36 G>A | 30 G>A | 31 C>T | 127 delA | 142 T>G | 175 delG | 271 C>T | 325 A>G | 357 T>C | 448 T>C | 471 C>T | 804 G>A | IVS1 +1 G>T | IVS1 +43 C>T | IVS1 +98 A>G | IVS1 +101 G>T | Frequency |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Amino acid change | | | | | | P10P | R11W | 43fsX22 | L48V | 59fsX6 | R91C | R109G | N119N | L150L | C157C | P268P | | | | | |
| Haplotypes[b] | *1a* | | | | | | | | | | | | | | | | | | | | 0.818 |
| | *1b* | | | | | | | | | | | | | | | | | | | | 0.010 |
| | *1c* | | | | | | | | | | | | | | | | | | | | 0.008 |
| | *1d* | | | | | | | | | | | | | | | | | | | | 0.006 |
| *1 | *1e* | | | | | | | | | | | | | | | | | | | | 0.856 |
| | *1f* | | | | | | | | | | | | | | | | | | | | 0.004 |
| | *1g* | | | | | | | | | | | | | | | | | | | | 0.002 |
| | *1h* | | | | | | | | | | | | | | | | | | | | 0.002 |
| | *1i* | | | | | | | | | | | | | | | | | | | | 0.002 |
| *3 | *3a* | | | | | | | | | | | | | | | | | | | | 0.123 |
| | *3b* | | | | | | | | | | | | | | | | | | | | 0.002 |
| *4 | *4a* | | | | | | | | | | | | | | | | | | | | 0.012 |
| *5 | *5a* | | | | | | | | | | | | | | | | | | | | 0.002 |
| *6 | *6a* | | | | | | | | | | | | | | | | | | | | 0.002 |
| *7 | *7a* | | | | | | | | | | | | | | | | | | | | 0.002 |
| *8 | *8a* | | | | | | | | | | | | | | | | | | | | 0.002 |

[a] A of the translational start codon of *UGT1A4* is numbered 1. AF297093.1 was used as the reference sequence.

[b] The haplotypes were described as a number plus a small alphabetical letter.

variation, were assigned as haplotypes **4* to **8*. Several haplotypes were first unambiguously assigned by homozygous SNPs at all sites (**1a* and **3a*) or a heterozygous SNP at only one site (**1b*, **1d*-**1i*, **3b*, **4a*, and **8a*). Separately, we estimated the diplotype configuration (a combination of haplotypes) for each subject by LDSUPPORT software. The diplotype configurations of 256 subjects were inferred with probabilities (certainty) of 0.9998 or over, except for one subject. The additionally inferred haplotypes were **1c*, **5a*, **6a*, and **7a*. As for one subject with a low probability (who had heterozygous SNPs of −219C>T, −163 G>A, 31C>T, 142T>G, 448T>C, 804G>A, and IVS1+43C>T), the diplotype was determined by the cloning and sequencing of DNA fragments. One chromosome had haplotype **3a* (consisting of −219C>T, −163 G>A, 142T>G, 448T>C, 804G>A, and IVS1+43C>T) and the other had haplotype **4a* (31C>T). Moreover, the data obtained by cloning and sequencing analysis confirmed the presence of haplotypes **5a* [127delA (43fsX22) and 142T>G (L48V)], **6a* [175delG (59fsX6) and 325A>G (R109G)], and **7a* [−219C>T, −163G>A, 142T>G (L48V), 271C>T (R91C), 448T>C (L150L), 804G>A (P268P), and IVS1+43C>T] (**Table 3**). The most frequent haplotype was **1a* (frequency: 0.818), followed by **3a* (0.123), **4a* (0.012) and **1b* (0.010). The frequencies of the other haplotypes were less than 0.01. Since 325A>G (R109G) was linked with 175delG (59fsX6), the enzymatic activity of this haplotype (**6a*) is probably null. The other SNP, 271C>T confers the R91C substitution. In human UGT1A4, eight cysteine residues were located in the lumenal domain.[3,14] Though the disulfide-bond formation and its significance are not clear in the UGT1A4, it has been reported that the reduction of disulfide-bonds of rat UGT1A6 with dithiothreitol increases its enzymatic activity in the liver microsomes.[15] On the other hand, the alterations of several lumenal cysteines into serine residues seem to reduce the UGT1A6 activity when the mutant enzymes were expressed in COS cells.[15] The effect of additional cysteine residue at codon 91 in the UGT1A4 should be determined in the future.

In conclusion, we detected 19 polymorphisms, including 11 novel ones, in *UGT1A4* from a Japanese population. Using the detected polymorphisms, 16 haplotypes were identified. Our results provide fundamental and useful information for genotyping *UGT1A4* in the Japanese, and probably Asian populations.

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# FUNCTIONAL CHARACTERIZATION OF THREE NATURALLY OCCURRING SINGLE NUCLEOTIDE POLYMORPHISMS IN THE *CES2* GENE ENCODING CARBOXYLESTERASE 2 (HCE-2)

Takashi Kubo, Su-Ryang Kim, Kimie Sai, Yoshiro Saito, Toshiharu Nakajima, Kenji Matsumoto, Hirohisa Saito, Kuniaki Shirao, Noboru Yamamoto, Hironobu Minami, Atsushi Ohtsu, Teruhiko Yoshida, Nagahiro Saijo, Yasuo Ohno, Shogo Ozawa, and Jun-ichi Sawada

*Project Team for Pharmacogenetics (T.K., S.-R.K., K.Sa., Y.S., S.O., J.I.S.), Division of Xenobiotic Metabolism and Disposition (K.Sa.), Division of Biochemistry and Immunochemistry (Y.S., J.I.S.), Division of Pharmacology (S.O.), and Deputy Director (Y.O.), National Institute of Health Sciences, Tokyo, Japan; Department of Allergy and Immunology, National Research Institute for Child Health and Development, Tokyo, Japan (T.N., K.M., H.S.); Division of Internal Medicine (K.Sh., N.Y.), National Cancer Center Hospital, Tokyo, Japan; Genetics Division (T.Y.), National Cancer Center Research Institute, Tokyo, Japan; and Division of Oncology Hematology (H.M.), Division of Gastrointestinal Oncology Digestive Endoscopy (A.O.), and Deputy Director (N.S.), National Cancer Center Hospital East, Chiba, Japan*

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**ABSTRACT:**

**Twelve single nucleotide polymorphisms (SNPs) in the human *CES2* gene, which encodes a carboxylesterase, hCE-2 [human carboxylesterase 2 (EC 3.1.1.1)], have been reported in the Japanese [S. R. Kim, T. Nakamura, Y. Saito, K. Sai, T. Nakajima, H. Saito, K. Shirao, H. Minami, A. Ohtsu, T. Yoshida, et al. (2003) *Drug Metab Pharmacokinet* 18:327–332). In this report, we have examined functional alterations of three SNPs, a nonsynonymous SNP (100C>T, R34W), an SNP at the splice acceptor site in intron 8 (IVS8-2A>G), and one newly discovered nonsynonymous SNP (424G>A, V142M). For the two nonsynonymous SNPs, the corresponding variant cDNAs were expressed in COS-1 cells. Both the R34W and V142M variants showed little esterase activities toward the anticancer agent irinotecan and two typical carboxylesterase substrates, *p*-nitrophenol acetate and 4-methylumbelliferyl acetate, although increased levels of cDNA-mediated protein expression were observed by Western blotting as compared with the wild type. To investigate a possible splicing aberration in IVS8-2A>G, an in vitro splicing assay was utilized and transcripts derived from *CES2* gene fragments of the wild type and IVS8-2A>G were compared. Sequence analysis of the cloned transcripts revealed that IVS8-2A>G yielded mostly aberrantly spliced transcripts, including a deleted exon or a 32-bp deletion proximal to the 5′ end of exon 9, which resulted in truncated hCE-2 proteins. These results suggested that 100C>T (R34W), 424G>A (V142M), and IVS8-2A>G are functionally deficient SNPs.**

Human carboxylesterases are members of serine esterases, metabolize ester, thioester, carbamate, and amide, and yield soluble acids and alcohols or amines (Satoh and Hosokawa, 1998; Satoh et al., 2002). Two major isoforms of human carboxylesterase, hCE-1 and CE-2, have been identified in the liver (Shibata et al., 1993; Schwer et al., 1997). The *CES2* gene encoding hCE-2 is located on chromosome 16q22.1 and consists of 12 exons (Fig. 1). hCE-2 has been shown to be expressed in relatively limited tissues, including the small intestine, colon, heart, kidney, and liver, whereas hCE-1 is ubiquitously distributed (Satoh et al., 2002; Xie et al., 2002). hCE-2 is relatively specific for heroin, cocaine (benzoyl ester), 6-acetylmorphine, procaine, and oxybutynin, although both isoforms show broad substrate specificities (Pindel et al., 1997; Takai et al., 1997; Satoh et al., 2002). hCE-2 has also been shown to catalyze the conversion of the antitumor drug, irinotecan, into its active metabolite SN-38 (7-ethyl-10-hydroxycamptothecin) (Takai et al., 1997; Humerickhouse et al., 2000). Data on the hepatic hydrolyzing activities of hCE-2 toward irinotecan revealed remarkable interindividual differences (Xu et al., 2002). With regard to genetic polymorphisms, including single nucleotide polymorphisms (SNPs) in the *CES2* gene, it has recently been shown that the allele and haplotype frequencies are significantly different between Europeans and Africans (Marsh et al., 2004). In our previous study, we found a number of SNPs, including a nonsynonymous SNP (100C>T causing R34W, MPJ6_CS2005) and an SNP at the splice acceptor site of intron 8 (IVS8-2A>G, MPJ6_CS2011), in the course of screening *CES2* SNPs from 153 Japanese individuals who were administered irinotecan or beclomethasone (Kim et

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**ABBREVIATIONS:** hCE-2, human carboxylesterase 2 (EC 3.1.1.1); SNP, single nucleotide polymorphism; RT, reverse transcriptase; PCR, polymerase chain reaction; AUC, area under the plasma concentration curve; *CES2*, the human carboxylesterase 2 gene; SN-38, 7-ethyl-10-hydroxycamptothecin; SN-38G, glucuronide conjugate of SN-38; HPLC, high-performance liquid chromatography; bp, base pair(s).

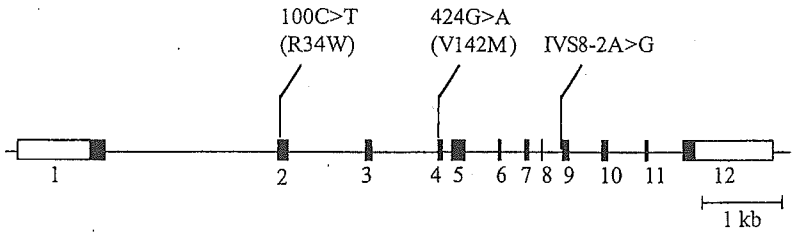


FIG. 1. Genomic structure of *CES2*. Three SNPs analyzed in this study are indicated with their position in the *CES2* gene. The 12 exons are shown by boxes; the regions corresponding to an open reading frame are shown as closed boxes; and the untranslated regions are shown as open boxes.

al., 2003). Additional nonsynonymous SNPs (424G>A, V142M, MPJ6_CS2015) have been discovered in further screening of *CES2* SNPs in Japanese allergic patients (Fig. 1).

As for the large ethnic differences of the *CES2* SNP frequencies pointed out by Marsh et al. (2004), the SNPs we found have not been elaborated in Marsh's studies, suggesting that the ones found by us were less frequent in Europeans and Africans. In the present study, we performed functional characterization of the two nonsynonymous SNPs using heterologous cell expression systems. To investigate the effect of SNP IVS8-2A>G on RNA splicing, a minigene assay was adopted. The results indicated that the 2 hCE-2 variants (R34W and V142M) almost completely lost the enzymatic activities toward irinotecan and two typical carboxylesterase substrates, *p*-nitrophenol acetate and 4-methylumbelliferyl acetate. The exon-intron junction SNP, IVS8-2A>G, was associated with aberrant splicing.

## Materials and Methods

**SNP Detection.** SNPs in the *CES2* gene (NT_010498.15 as a reference sequence) were surveyed by sequencing performed as described previously (Kim et al., 2003). In the present study, 81 Japanese cancer patients administered irinotecan, 72 Japanese asthmatic patients administered beclomethasone, and 12 Japanese allergic patients administered steroidal drugs, whose genomic DNAs were extracted from blood leukocytes, were analyzed for *CES2* SNPs. Each of the three SNPs elaborated in the present study was found separately as heterozygotes among the 165 subjects studied. The ethics committees of the National Cancer Center, National Center for Child Health and Development, and National Institute of Health Sciences approved this study. Written informed consent was obtained from all patients.

**Construction of Plasmids for a COS-1 Cell Expression System.** Wild-type *CES2* cDNA was obtained by the PCR amplification of a first-strand cDNA synthesized by a reverse transcriptase (RT) reaction from Human Liver PolyA+ RNA (BD Biosciences Clontech, Palo Alto, CA) using *CES2*-specific primers (5′-CTGGATCCGACCATGCGGCTGCACAG-3′ and 5′-ACAGGGAGCTACAGCTCTGTGT-3′, forward and reverse primers, respectively). The PCR was performed with 1.25 units of AmpliTaq Gold (Applied Biosystems, Foster City, CA) at 95°C for 10 min, followed by 30 cycles of 95°C for 30 s, 60°C for 1 min, and 72°C for 2 min. The resultant PCR products were cloned into a pCR3.1 vector by the TA cloning procedure (Invitrogen, Carlsbad, CA), and the sequence was confirmed in both directions. This expression plasmid was designated pCRhCE2/WT and was used as a template for the preparation of R34W and V142M plasmids. The variant plasmids were generated with a QuickChange PCR site-directed mutagenesis kit (Stratagene, La Jolla, CA). The primers for the respective variations were 5′-TCAGCCAGTCCCATCTGGACCACACACACGG-3′ for R34W and 5′-GATCCACACCATCATCGGCAGGTTAGAGCC-3′ for V142M (mutated sites are underlined). The sequence of each variant cDNA was confirmed.

**Construction of Plasmids for a His-Tagged hCE-2 Expression.** A histidine (His)-tagged hCE-2 expression plasmid was also constructed to obtain the wild-type hCE-2 protein. A *CES2* cDNA sequence without its signal peptide region was amplified from the pCRhCE2/WT vector by the PCR (Potter et al., 1998a,b) using a forward primer, 5′-CAAGATCTGCTTGTCCGGGGCCAGGGCCA-3′ (BglII site is underlined) and a reverse primer, 5′-CCGGTACCTACAGCTCTGTGTGTCTCTC-3′ (KpnI site is underlined). The amplified fragment was cloned into the pCR3.1 vector. After confirmation of the correct sequence, the cDNA fragment, digested with BglII and KpnI, was ligated into the pTrcHis B plasmid (Invitrogen) that was predigested with BglII and KpnI. This newly generated plasmid was designated pHisCES2.

**Construction of Plasmids for the Minigene Assay.** To construct plasmids for the minigene assay, a wild-type *CES2* gene fragment was amplified by PCR using genomic DNA as a template that was extracted from an irinotecan-administered cancer patient with the wild-type or variant (IVS8-2A>G) *CES2* genes. PCR primers used to amplify a *CES2* gene fragment containing exons 7 to 10 were 5′-GCACGCGTGGAGTGGTGGATGGGGTCTTC-3′ (forward primer, MluI site is underlined) and 5′-GCGTCGACGGCTGATGCTGGAACTCGTAGA-3′ (reverse primer, SalI site is underlined). The amplified fragments were cloned into a pCR3.1 vector. After confirmation of the correct sequence, the *CES2* fragment was digested with MluI and SalI and ligated into pCMV-TnT (Invitrogen) that had been digested with MluI and SalI. The plasmids containing the wild-type and IVS8-2G *CES2* fragments were designated pCMV-CES2WT and pCMV-CES2IVS8G, respectively. The sequence of the inserts was confirmed.

**Protein and mRNA Expressions of Wild-Type and Variant hCE-2s in COS-1 Cells.** COS-1 cells were seeded in 100-mm culture dishes. The cells were grown to reach approximately 70% confluency and rinsed with serum-free OPTI-MEM (Invitrogen) before transfection. The pCR3.1, pCR3.1/CES2 wild-type, pCR3.1/CES2 R34W, and pCR3.1/CES2 V142M plasmids (6 $\mu$g each) were transfected individually using the LipofectAMINE PLUS reagent (Invitrogen) as described previously (Murayama et al., 2004). The cells were harvested after 48 h and homogenized in 100 mM potassium phosphate buffer (pH 7.4). Cell homogenates were spun at 9000*g* for 10 min, and the resultant supernatants were then subjected to centrifugation at 105,000*g* for 1 h. The pellets were resuspended in a 250 mM sucrose solution and used as microsomes. mRNA expressions of the wild-type and variant (R34W and V142M) hCE-2 cDNA-transfected cells were determined by a reverse-transcription PCR method.

**Purification of His-Tagged hCE-2.** The expression plasmid pHisCES2 was introduced into *Escherichia coli* strain TOP10 (Invitrogen). Four hours after isopropyl $\beta$-D-thiogalactoside induction, the bacterial cells were harvested. The ProBond Purification System (Invitrogen) was used to purify His-tagged hCE-2 expressed in TOP10 cells. The purification was performed with a denaturing condition according to the manufacturer's protocol. The purified protein was kept at −80°C in 2% SDS and used as a standard in Western blotting. Protein concentration of the purified His-tagged hCE-2 was quantified colorimetrically using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA) and an hCE-2-specific band was confirmed by Western blotting.

**Western Blot Analysis.** Two or 4 $\mu$g of the microsomes from COS-1 cells were resolved by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Immunochemical detection of each hCE-2 protein was performed using rabbit anti-human hCE-2 raised against a peptide antigen (residues 539–555, KKALPQKIQELEEPEER) (diluted 1:2000). To verify that the samples were evenly loaded, the blot was subsequently treated with stripping buffer and reprobed with a polyclonal anti-calnexin antibody (diluted 1:4000; StressGen Biotechnologies, San Diego, CA). Visualization of these proteins was achieved with horseradish peroxidase-conjugated donkey anti-rabbit Ig (1:2000) and Enhanced Chemiluminescence-Plus reagents (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The densities of protein bands were quantified using His-tagged hCE-2 as a standard. Two, 4, and 8 ng of the His-tagged hCE-2 were applied

on the polyacrylamide gels for Western blotting. The amounts of wild type and the variants were within the range (2–8 ng) of the standard His-tagged hCE-2.

**Enzyme Assay.** A reaction mixture in a total volume of 200 μl contained 50 mM potassium phosphate buffer (pH 7.4) and several concentrations (1, 2, 5, 10, 20, and 50 μM) of irinotecan in the presence of 0.1 mg of microsomal proteins. Reactions were started by the addition of the substrate, incubated at 37°C for 10 min, and then terminated by the addition of 200 μl of methanol/5% perchloric acid (1:1) containing 0.29 μM camptothecin (internal standard). For the analysis of irinotecan and its metabolites, chromatographic separation was performed by an HP 1100 model HPLC system equipped with a fluorescence detector (G1321A; Hewlett Packard, Les Ulis, France). The HPLC analysis was performed as previously described (Sai et al., 2002). Carboxylesterase activity against *p*-nitrophenyl acetate was assayed colorimetrically. Briefly, a reaction mixture contained 0.5 M Tris-HCl (pH 8.0) and various concentrations (0.039, 0.078, 0.156, 0.313, 0.625, 1.25, 2.5, and 5 mM) of *p*-nitrophenyl acetate in the presence of microsomes of hCE-2-expressing cells. The initial rate of increase in the $OD_{405}$ was monitored as the production of *p*-nitrophenol. Catalytic activity was expressed as μmol/mg microsomal protein/min. Carboxylesterase activity against 4-methylumbelliferyl acetate was measured basically according to the method reported by Pindel et al. (1997). Briefly, a reaction mixture consisted of 90 mM $KH_2PO_4$ adjusted at pH 7.3, 40 mM KCl, and various concentrations (0.0625, 0.125, 0.25, 0.5, and 1 mM) of 4-methylumbelliferyl acetate in the presence of microsomes of hCE-2-expressing cells. The initial rate of increase in the $OD_{350}$ was monitored as the production of 4-methylumbelliferone. Catalytic activity was expressed as μmol/mg microsomal protein/min.

**Minigene Assay.** HepG2 cells were rinsed with serum-free OPTI-MEM (Invitrogen) before transfection. Either pCMV-CES2WT or pCMV-CES2IVS8G was added to the cells with LipofectAMINE PLUS reagent (Invitrogen). The cells were harvested after 48 h, and total RNA was extracted with an RNeasy Mini Kit (Qiagen, Hilden, Germany). RT-PCR was performed with a GeneAmp RNA PCR Kit (Applied Biosystems, Foster City, CA) using total RNA treated with DNaseI Amplification Grade (Invitrogen) as a template, an intron-skipping primer as a forward primer and a reverse primer having a portion of the exon 10 sequence (see Fig. 4A). The intron-skipping primer (5′-GAGGCACTGGGCAGGTGTCCACTC-3′) was designed to cover both the artificial introns of pCMVTnT. RT-PCR products were detected by 3% agarose gel electrophoresis. To analyze transcripts obtained by the minigene assay, the resultant RT-PCR products were cloned into pCR4-TOPO with the TOPO TA Cloning Kit for Sequencing (Invitrogen), and subsequent sequencing of the inserts derived from randomly isolated clones (greater than 100) was performed using an M13 reverse primer.

## Results

**SNPs in the *CES2* Gene.** As previously reported, we have found a nonsynonymous SNP (100C>T, leading to R34W) and an SNP at the splice acceptor site of intron 8 (IVS8-2A>G) by examining 165 Japanese individuals (Fig. 1). One novel nonsynonymous SNP was found in exon 4 in the present study. The cDNA position of the SNP was 424 (A of the translational start codon is position 1) and resulted in an amino acid alteration (V142M).

This heterozygous SNP was found in one subject. All three SNPs analyzed for 165 Japanese individuals in this paper were found separately as heterozygotes, and thus, they appeared at a frequency of 0.003.

**Protein Expression Levels and Enzymatic Activity of R34W and V142M.** As described under *Materials and Methods*, hCE-2 proteins in the cDNA-transfected cells were detected. Figure 2 illustrates Western blots of the hCE-2 and microsomal calnexin for the correction for the protein loading. The amounts of immunoreactive hCE-2 proteins levels were calculated based on the known amounts of His-tagged hCE-2 as a standard (Fig. 2, lane 2).

The variants, R34W and V142M, showed only a trace HPLC peak for SN-38, indicating that they almost completely lost the carboxylesterase activity toward irinotecan. The saturation curves of the

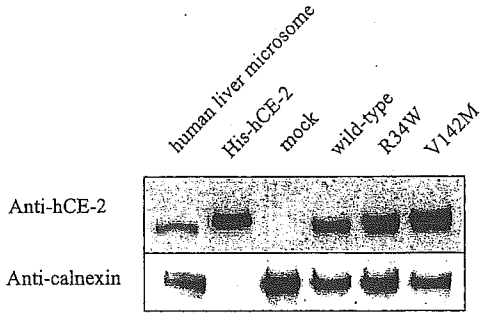


FIG. 2. Expression of wild-type and variant hCE-2 in COS-1 cells. Microsomes from the cDNA-transfected cells were subjected to SDS-polyacrylamide gel electrophoresis together with human liver microsomes (2 μg) and His-tagged hCE-2 (6 ng) as a standard. Detection was performed with a rabbit anti-human hCE-2 antiserum (upper) and anti-human calnexin antiserum (lower) as described under *Materials and Methods*. A representative result of three independent experiments is shown. Expression levels of R34W and V142M variants were higher than the wild type (252% and 360% of wild-type levels, respectively).

Michaelis-Menten kinetics are shown in Fig. 3, which also illustrates the inefficiency of the variants. The apparent kinetic parameters of wild-type hCE-2 were 1.228 ± 0.092 μM for $K_m$, 1.458 ± 0.0495 pmol/mg protein/min (91.57 ± 5.67 pmol/nmol hCE-2/min) for $V_{max}$, and 74.95 ± 3.79 μl/min/nmol hCE-2 for $V_{max}/K_m$. Their $V_{max}$ values were less than 5.0 in contrast to 91.57 pmol/nmol hCE-2/min for the wild type.

Table 1 summarizes the carboxylesterase function of the wild-type and its variant hCE-2s toward irinotecan, together with smaller-molecule and typical carboxylesterase substrates, *p*-nitrophenyl acetate and 4-methylumbelliferyl acetate. The catalytic activities toward

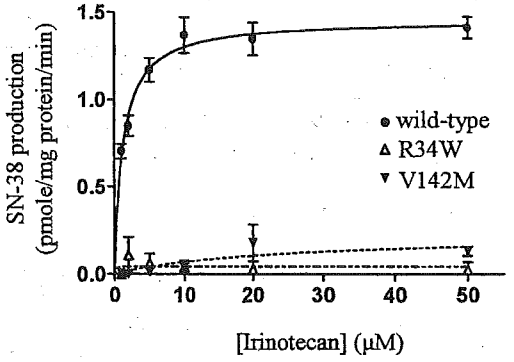


FIG. 3. Irinotecan-hydrolyzing activity of the R34W and V142M variants. Enzymatic formation of SN-38 from irinotecan by wild-type hCE-2 and its variants was measured by HPLC as described under *Materials and Methods*. Michaelis-Menten kinetics of SN-38 formation catalyzed by the wild-type hCE-2 and its variants are illustrated. No appreciable enzymatic activity was observed with the R34W and V142M variants.

TABLE 1

*$V_{max}$ values of wild-type hCE-2 and its variants toward irinotecan, p-nitrophenyl acetate, and 4-methylumberiferyl acetate*

Carboxylesterase activities toward the three substrates were determined as described under *Materials and Methods*. Concentrations used were 1 to 50 μM for irinotecan, 39 to 5000 μM for *p*-nitrophenyl acetate, and 62.5 to 1000 μM for 4-methylumbelliferyl acetate. $V_{max}$ values were determined by three independent experiments and were expressed as mean ± S.D. Data were expressed as pmol/mg microsomal protein/min for irinotecan, and as μmol/mg microsomal protein/min for *p*-nitrophenyl acetate and 4-methylumbelliferyl acetate.

| Substrates | Wild-Type | R34W | V142M |
|---|---|---|---|
| Irinotecan | 1.458 ± 0.0495 | <0.2 | <0.2 |
| *p*-Nitrophenyl acetate | 0.573 ± 0.0155 | 0.0513 ± 0.0073 | 0.0232 ± 0.0128 |
| 4-Methylumbelliferyl acetate | 0.193 ± 0.0068 | <0.03 | <0.03 |

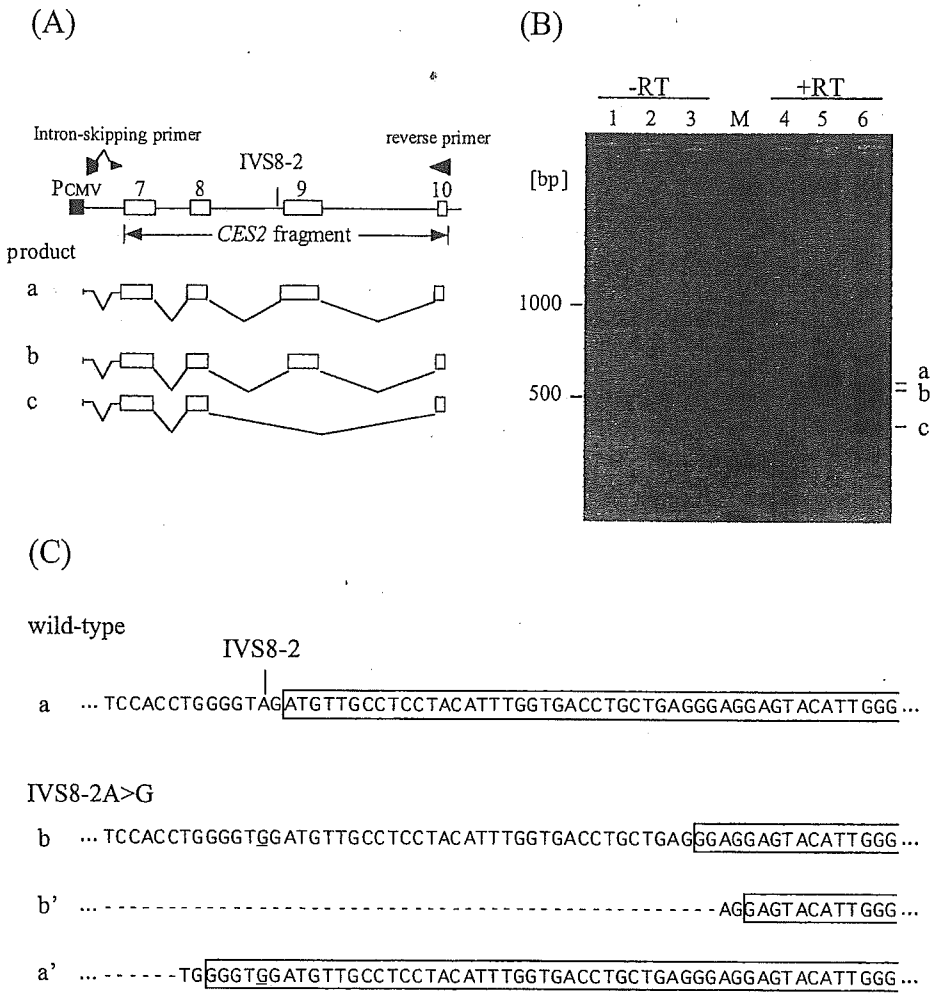


FIG. 4. In vitro splicing assay for IVS8-2A>G. The in vitro splicing assay for the wild-type and IVS8-2G variant mini *CES2* gene is described under *Materials and Methods*. A, schematic representation of the *CES2* gene fragments containing exons 7 to 10 with IVS8-2A (the wild type) and IVS8-2G and their transcripts. B, transcripts derived from the wild-type or IVS8-2G plasmids were amplified and analyzed on a high-resolution agarose gel. Lanes 1 and 4, mock; lanes 2 and 5, wild-type; lanes 3 and 6, IVS8-2G. Lanes 1 to 3, RT-PCR without reverse transcriptase; lanes 4–6, RT-PCR with reverse transcriptase; M, size marker. C, nucleotide sequences around the intron 8-exon 9 junction. The sequences for exon 9 in the normal (a) and aberrantly spliced transcripts are indicated as boxes. 32-bp (b) and 36-bp (b') deletions and the 6-bp insertion (a') from the IVS8-2G variant are shown.

all three substrates by R34W and V142M variants were catalytically much less efficient as compared with the wild type. $K_m$ values of the wild type for *p*-nitrophenyl acetate and 4-methylumbelliferyl acetate were 0.57 mM and 0.11 mM, respectively. The $K_m$ values, including that for irinotecan, were roughly similar to those reported by Sanghani et al. (2004) and Pindel et al. (1997).

**In Vitro Splicing Assay of IVS8-2A>G.** A minigene assay was performed with plasmids containing partial genomic sequences from exon 7 to exon 10, including the IVS8A>G SNP (Fig. 4A). The RT-PCR products were analyzed by electrophoresis in a high-resolving agarose gel (Fig. 4B). In the wild-type *CES2*-transfected HepG2 cells, a normally spliced mRNA was detected as a major product. In contrast, mRNA from the variant plasmid-transfected HepG2 cells revealed that a number of abnormally spliced mRNAs were generated. Sequences of the aberrant transcripts (Fig. 4, A and B, products a–c) were directly determined. The main transcripts found with the variant minigene were product c, with an exon 9 skipping, and product b, with a 32-bp deletion proximal to the 5′-end of exon 9 (Fig. 4C, product b). These splicing aberrations result in frameshifts and truncations of hCE-2. The sequence electropherogram for product b showed the presence of other minor transcripts.

To analyze the abnormal transcripts in detail, the RT-PCR products were cloned into a TA cloning vector. Subsequently, the cDNA sequences were determined for more than 100 clones. From the wild-type minigene, most of the mRNAs were spliced normally, as is schematically illustrated as product a in Fig. 4A. On the other hand, the major transcripts derived from the variant minigene were aberrant. In addition to exon skipping and a 32-bp deletion, another minor 36-bp deletion in the 5′-end of exon 9 was found (b′ in Fig. 4C). As a rare transcript, some clones of a 6-bp inserted transcript (a′ in Fig. 4C) were found. The major products b and c lack His-457 in the active site (Bencharit et al., 2002) and the C-terminal HXEL tetrapeptide (Robbi and Beaufay, 1991). Thus, these results suggest that this SNP causes a reduction in hCE-2 activity. Some aberrantly spliced mRNAs in IVS8-2A>G had a small deletion/insertion without a frameshift (e.g., the 36-bp deletion and the 6-bp insertion near the 5′-end of exon 9) but were, remarkably, rare as compared with the major 32-bp deletion.

## Discussion

The main point of this study is to functionally characterize three hCE-2 SNPs that we found among 165 Japanese subjects. Our functional characterization of the two nonsynonymous SNPs (R34W and V142M) revealed that the variants had an inefficient property as