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 9α	TGACTTCATCTTAACCTTTTAGTTCC	3825248	GAAGCACCAACCCATTTTCAC	3823704	1545
		Exon 9β	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 9α	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[a]	CCCCTCCCCCAC 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 -/C GGGCCCAAAGTA		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/GTTGATTATATG		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/ATTTAAATACCCA		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.

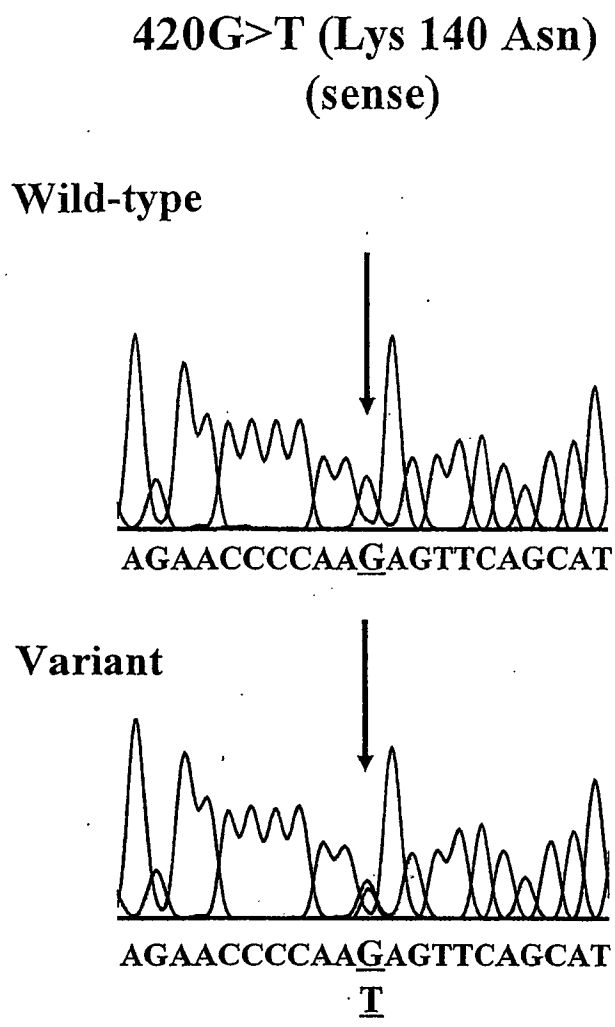

Fig. 1. Electropherograms for the novel nonsynonymous variation of *NR3C1*. (A) MPJ6_3C1017 (wild-type 420G/G; variant 420G/T). Arrows indicate the variant nucleotide position.

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-κB 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

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SNP Communication

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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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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.

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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			Nucleotide change and flanking sequences (5′ to 3′)	Amino acid change	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+)			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	GGGTCAGATGAGC/TTTTTCAAGATAG		195	54	7	0.133	0.133	0.142	0.125
MPJ6_U1A082[a]				5′-flanking	135212	−217	GTCAGATGAGCTT/GTTCAAGATAGGC		255	1	0	0.002	0.000	0.000	0.005
MPJ6_U1A083	rs3732218	IMS-JST085728	O	5′-flanking	135266	−163	TAACGAAAGGCAG/ATTATAGATTAAT		195	54	7	0.133	0.133	0.142	0.125
MPJ6_U1A084[a]				5′-flanking	135393	−36	CAGGCACAGCGTG/AGGGTGGACAGTC		255	1	0	0.002	0.000	0.006	0.000
MPJ6_U1A085[a]				Exon 1	135458	30	GGTTCCCCTGCCG/ACGGCTGGCCACA	P10P	254	2	0	0.004	0.000	0.000	0.009
MPJ6_U1A086	rs3892221		O	Exon 1	135459	31	GTTCCCCTGCCGC/TGGCTGGCCACAG	R11W	250	6	0	0.012	0.025	0.011	0.005
MPJ6_U1A087[a]				Exon 1	135555	127	AGCCCCTGGCTCA/-GCATGCGGGAGG	43fsX22	255	1	0	0.002	0.000	0.000	0.005
MPJ6_U1A088	rs2011425		O	Exon 1	135570	142	ATGCGGGAGGCCT/GTGCGGGAGCTCC	L48V	197	52	7	0.129	0.133	0.148	0.111
MPJ6_U1A089[a]				Exon 1	135603	175	GGCCACCAGGCGG/-TGGTCCTCACCC	59fsX6	255	1	0	0.002	0.000	0.000	0.005
MPJ6_U1A090[a]				Exon 1	135699	271	AAGGAATTTGATC/TGCGTTACGCTGG	R91C	255	1	0	0.002	0.000	0.000	0.005
MPJ6_U1A091[a]				Exon 1	135753	325	CATCTTCTGAAGA/GGATATTCTAGAA	R109G	255	1	0	0.002	0.000	0.000	0.005
MPJ6_U1A092[a]				Exon 1	135785	357	AATTATGAACAAT/CGTATCTTTGGCC	N119N	254	2	0	0.004	0.008	0.006	0.000
MPJ6_U1A093	rs12468274		O	Exon 1	135876	448	TTTGATGTGGTTT/CTAACAGACCCCG	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	CTACCCCAGGCCG/AATCATGCCCAAC	P268P	195	54	7	0.133	0.133	0.142	0.125
MPJ6_U1A096[a]				Intron 1	136296	IVS1+1	CCACTATCTCAGG/TTCTGTATTGGTG		255	1	0	0.002	0.000	0.000	0.005
MPJ6_U1A097	rs2011219	IMS-JST085726	O	Intron 1	136338	IVS1+43	TTCCAGGCAAAAC/TACTTTTTAAAAA		195	54	7	0.133	0.133	0.142	0.125
MPJ6_U1A098[a]				Intron 1	136393	IVS1+98	ACTTATCTTTCCA/GAAGATTTTATTT		255	1	0	0.002	0.000	0.006	0.000
MPJ6_U1A099[a]				Intron 1	136396	IVS1+101	TATCTTTCCAAAG/TATTTTATTTTGG		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.

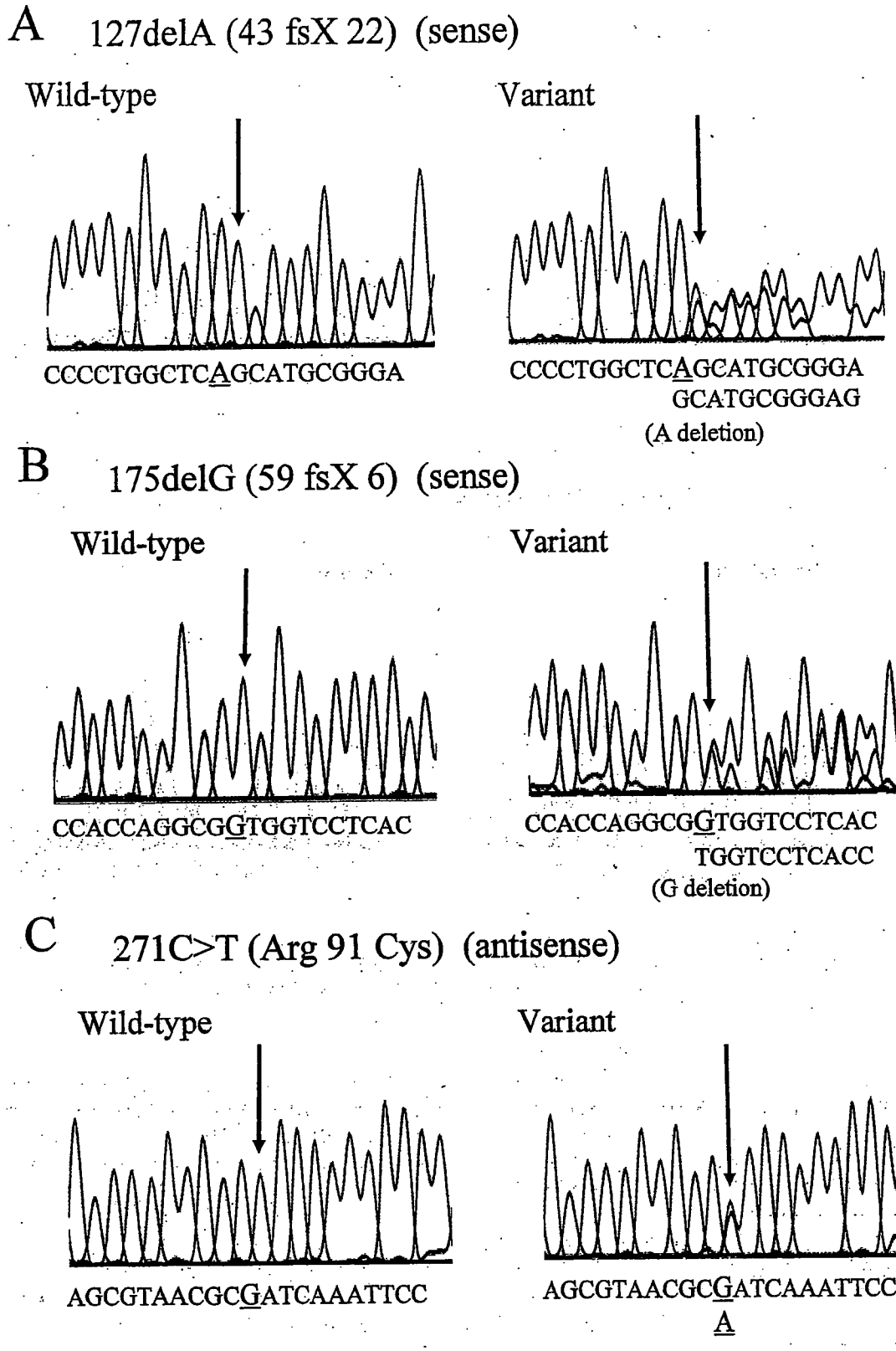
A
127delA (43 fsX 22) (sense)
Wild-type
Variant
CCCCTGGCTCAGCATGCGGGA
CCCCTGGCTCAGCATGCGGGA
GCATGCGGGAG
(A deletion)
B
175delG (59 fsX 6) (sense)
Wild-type
Variant
CCACCAGGCGGTGGTCCTCAC
CCACCAGGCGGTGGTCCTCAC
TGGTCCTCACC
(G deletion)
C
271C>T (Arg 91 Cys) (antisense)
Wild-type
Variant
AGCGTAACGCGATCAAATTCC
AGCGTAACGCGATCAAATTCC
A

Fig. 1

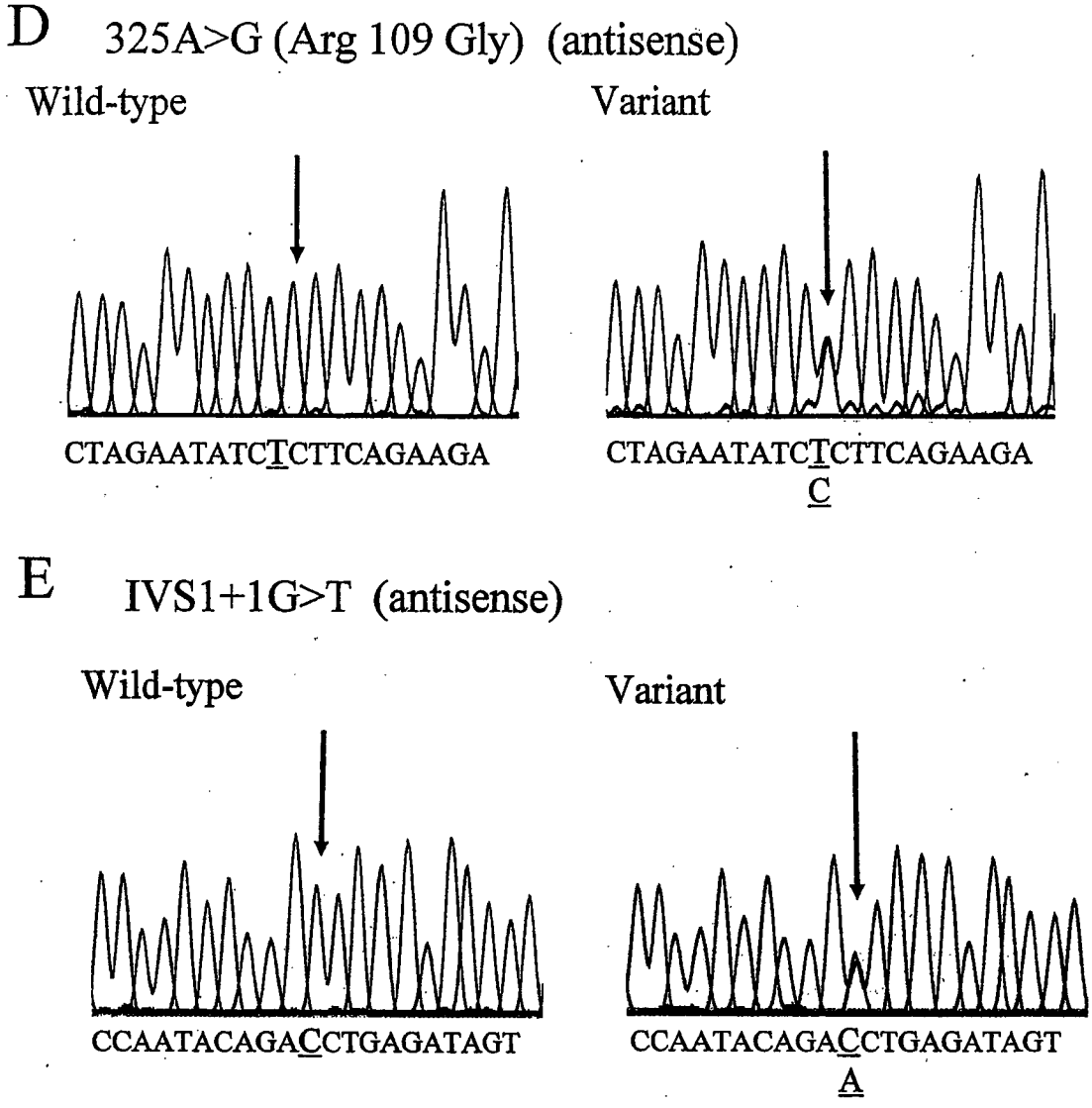

Fig. 1. The 4 novel genetic variations with amino acid substitutions and 1 splice donor site variation of human *UGT1A4*. (A) MPJ6_U1A087 (wild-type, 127A/A; variant, 127A/-). (B) MPJ6_U1A089 (wild-type, 175G/G; variant, 175G/-). (C) MPJ6_U1A090 (wild-type, 271C/C; variant, 271C/T). (D) MPJ6_U1A091 (wild-type, 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 χ^2 test) and between two of the three groups ($p>0.13$ by χ^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 β-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 **1B* 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	0.856
	*1b																				0.010	
	*1c																				0.008	
	*1d																				0.006	
*1	*1e																				0.004	
	*1f																				0.004	
	*1g																				0.002	
	*1h																				0.002	
	*1i																				0.002	
*3	*3a																				0.123	0.125
	*3b																				0.002	
*4	*4a																				0.012	0.012
*5	*5a																				0.002	0.002
*6	*6a																				0.002	0.002
*7	*7a																				0.002	0.002
*8	*8a																				0.002	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)

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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 hCE-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).

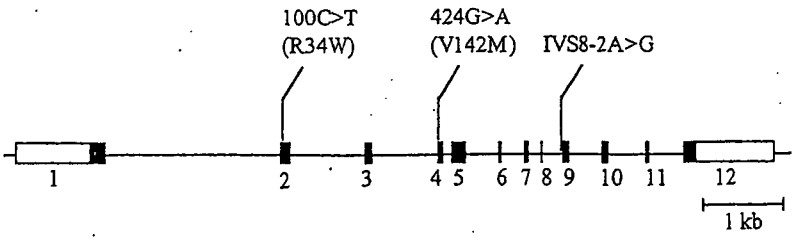

FIG. 1. Genomic structure of *CES2*. Three SNPs analyzed in this study are indicated with their position in the *CES2* gene. The 12 exons are shown by boxes; the regions corresponding to an open reading frame are shown as closed boxes; and the untranslated regions are shown as open boxes.

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 μg 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 β-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 μg 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

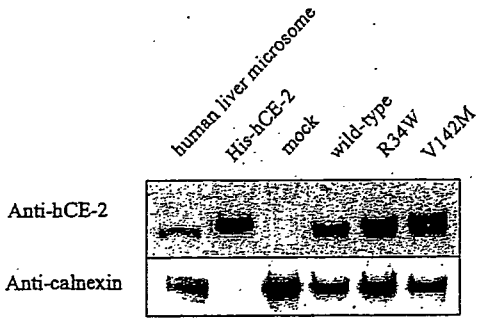

FIG. 2. Expression of wild-type and variant hCE-2 in COS-1 cells. Microsomes from the cDNA-transfected cells were subjected to SDS-polyacrylamide gel electrophoresis together with human liver microsomes (2 μg) and His-tagged hCE-2 (6 ng) as a standard. Detection was performed with a rabbit anti-human 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

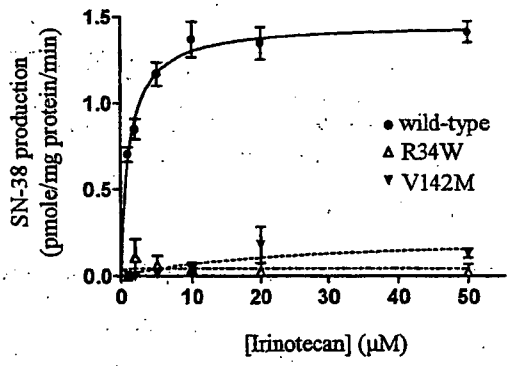

FIG. 3. Irinotecan-hydrolyzing activity of the R34W and V142M variants. Enzymatic formation of SN-38 from irinotecan by wild-type hCE-2 and its variants was measured by HPLC as described under *Materials and Methods*. Michaelis-Menten kinetics of SN-38 formation catalyzed by the wild-type hCE-2 and its 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

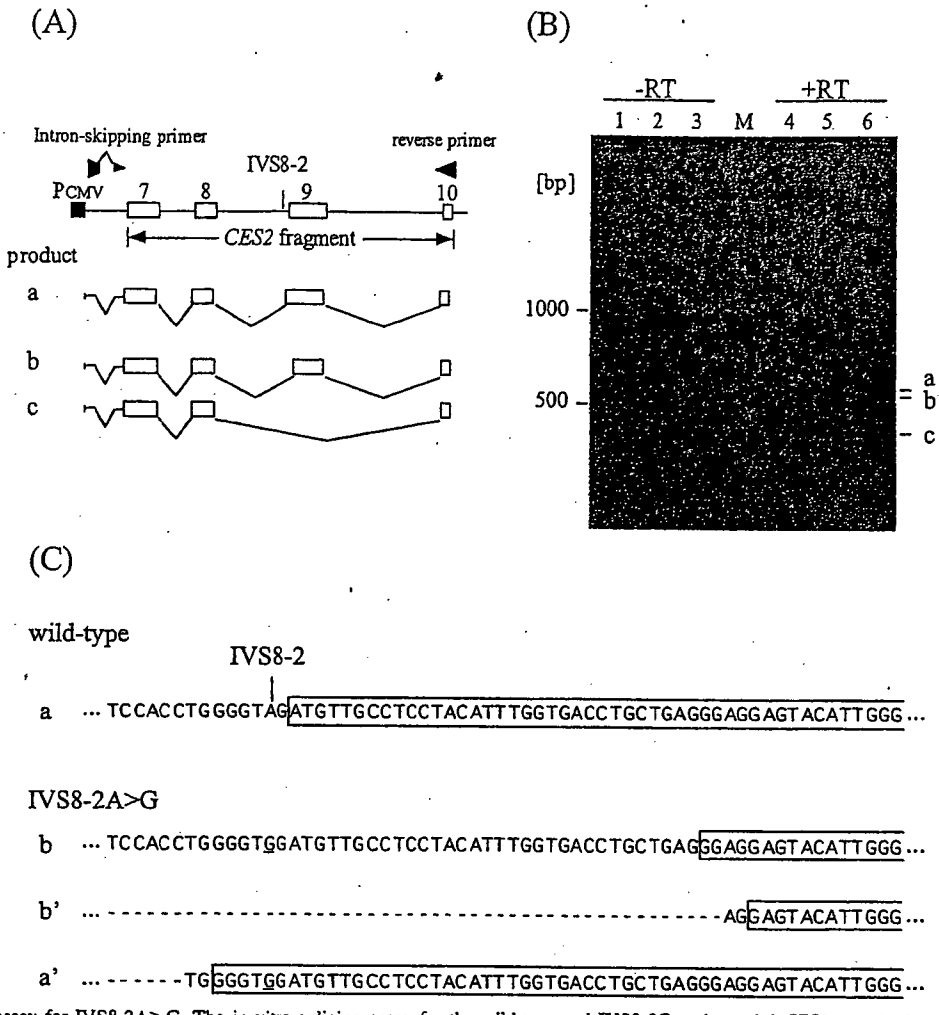

FIG. 4. In vitro splicing assay for IVS8-2A>G. The in vitro splicing assay for the wild-type and IVS8-2G variant mini *CES2* gene is described under *Materials and Methods*. A, schematic representation of the *CES2* gene fragments containing exons 7 to 10 with IVS8-2A (the wild type) and IVS8-2G and 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

carboxylesterases, at least toward irinotecan, *p*-nitrophenyl acetate, and 4-methylumbelliferyl acetate (Table 1). Expression levels of R34W and V142M variants were higher than those of the wild type (252% and 360% of wild-type levels, respectively). We measured mRNA levels for the wild-type and the variant hCE-2 cDNA-transfected cells. Levels of mRNA in the cells transfected with variant cDNAs (R34W and V142M) were comparable, whereas those in the wild-type cDNA-transfected cells were 68 to 75% of the cells transfected with the variant cDNAs. Therefore, the apparent lower expression of the wild-type hCE-2 may have occurred at the translational level. Another possibility is lower transfection efficiency of the hCE-2 wild-type plasmids.

With respect to amino acid residues Arg-34 and Val-142 of hCE-2, these are conserved in several animal species, including human (Schwer et al., 1997), rat (RefSeq accession: NP_598270), rabbit (Ozols, 1989), mouse, and monkey. Furthermore, both residues 34 and 142 have been shown to be located within the conserved domains of carboxylesterase type B. Particularly, residue 142 has been known to be located within a conserved domain common to esterase, lipase, and thioesterase families, as well as other human CES proteins, hCE-1 (Ketterman et al., 1989), hBr3 (Mori et al., 1999), and the recently reported CES3 (Sanghani et al., 2004). Based on the remarkable loss of catalytic function of R34W and V142M, we propose for the first time, to our knowledge, that the Arg-34 and Val-142 residues are critical for the catalytic function of hCE-2.

The in vitro splicing assay has been used to detect possible splicing errors due to base change(s) in the exon-intron junction of several genes, including steroid 17α-hydroxylase (CYP17) (Yamaguchi et al., 1997), CYP3A5 (Chou et al., 2001), and prostacyclin synthase (Nakayama et al., 2002). Generally, transcripts are obtained by the introduction of plasmids carrying the gene fragments containing the base change(s) in question, which is called a "minigene-assay". Our in vitro splicing assay revealed that the transcripts produced from the variant construct (IVS8-2G) were mostly aberrant. The most frequent transcripts from the variant had a 32-bp deletion at the 5′-end of exon 9. This deletion resulted in a frameshift; thus, the sequence downstream of residue Leu-441 was altered to produce a premature termination at codon 509.

The three amino acid residues, Ser-228, Glu-345, and His-457, in hCE-2 are highly conserved in carboxylesterase family proteins and are an active center of the enzyme. Moreover, most of the CES family proteins have a C-terminal HXEL tetrapeptide that is required for their endoplasmic reticulum retention. As compared with the wild-type hCE-2 (559 amino acids), the 32-bp deleted variant yields a protein of 508 amino acids that lacks His-457 in the active center and the C-terminal HXEL sequence. Thus, our findings, obtained by the in vitro splicing assay, suggest that the variant IVS8-2A>G might be a loss-of-function allele.

The SNPs, R34W and IVS8-2A>G, were found in irinotecan-administered Japanese cancer patients. The plasma concentrations of irinotecan, SN-38, and its glucuronide conjugate SN-38G were measured up to 24 h after a 90-min infusion of irinotecan. The metabolite/parent ratio of their areas under the plasma concentration curve (AUC) [i.e., the ratio of the areas under the plasma concentration curves of SN-38 plus SN-38G to irinotecan: $(AUC_{SN\text{-}38} + AUC_{SN\text{-}38G})/AUC_{irinotecan}$] could be considered as an estimate of the hepatic carboxylesterase activity. The heterozygous R34W patient, who suffered from colon cancer and was administered irinotecan, showed a low AUC ratio (K. Sai, unpublished data). On the other hand, the AUC ratio of the IVS8-2A>G patient who suffered from small cell lung cancer and was administered irinotecan was a little higher than the median value. Since the patient had a homozygous variant *ABCG2* (Glu141Lys), which was shown to be associated with the increase in AUC of diflomotecan, another topoisomerase I inhibitor (Sparreboom et al., 2004), bile excretion of SN-38 and SN-38G was presumably lower by the *ABCG2* variation, and as a result, plasma concentrations of SN-38 and SN-38G might have a tendency to increase (K. Sai, unpublished data). Thus, the clinical impact of IVS8-2A>G is unclear. All these *CES2* SNPs might be important for disease susceptibility as well, although it seems difficult to correlate certain diseases with SNPs with very low frequencies.

In conjunction with the large substrate-dependent interindividual variability, it could be very valuable knowledge if our analyzing three SNPs were associated with the large interindividual difference, since the difference is known to be as high as 5- to 45-fold (Hosokawa et al., 1995) or even 3- to 5-fold variability in irinotecan and *p*-nitrophenyl acetate, and butyrylthiocholine plasma hydrolytic activity (Guemei et al., 2001). However, considering the low SNP frequencies (0.003) and the low activities of the variants observed throughout irinotecan and the two typical carboxylesterase substrates, the contribution of our SNPs to the observed wide substrate-dependent interindividual difference is less likely.

In conclusion, two nonsynonymous SNPs (causing R34W and V142M) were identified as deficient alleles. An in vitro splicing assay also suggested that IVS8-2A>G might be a low-activity allele, although studies on more patients with the IVS8-2A>G are necessary to obtain conclusive data. The information on the remarkable functional changes in vitro caused by the three *CES2* SNPs would be useful for the modification of dosage regimens in irinotecan therapy.

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臨床試験におけるテーマ提起とプロトコール作成上の問題点
── 腹膜転移を有する胃がんを対象にした臨床試験 JCOG0106から ──

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はじめに

JCOG（Japan Clinical Oncology Group）消化器がん内科グループによる「腹膜転移を有する進行胃がんに対する 5-FU 持続静注療法 vs MTX＋5-FU 時間差療法による第Ⅲ相試験（JCOG0106）」は2002年10月に登録が開始され、現在進行中である。

当グループでは以前、「手術不能・再発進行胃がんを対象とした 5-FU 持続静注 vs 5-FU＋CDDP 併用療法の比較試験（JCOG9205）」を行ったが、その際適格規準を満たさず試験に登録できない主原因の検討を行った。これによると、第1位がＰＳ不良であり、第2位が腹膜転移の存在であった。腹膜転移例は手術不能・再発進行胃がんの約半数を占めていると推測されるが、腸管狭窄や腹水の存在が抗がん剤の毒性を遷延・重篤化させるという理由で胃がんに有効とされる 5-FU 系以外の薬剤（CDDP、CPT-11など）の使用が困難であった。以上のことから、我々は通常の進行胃がんとは別に腹膜転移を有する症例に対する標準治療を確立する必要があると考え、今回の試験を計画した。しかし、腹膜転移を対象とした試験は本邦のみならず、世界的にも初めての試みであったため、通常の試験にない工夫が必要であった。

本稿では、JOCG0106のプロトコール作成上問題となった点を挙げ、それにいかに対応したか述べたい。

Ⅰ．プロトコール作成上の問題点

「腹膜転移を有する進行胃がんに対する 5-FU 持続静注療法 vs MTX＋5-FU 時間差療法による第Ⅲ相試験（JCOG0106）」の主要評価項目は「生存期間」、副次的評価項目は「登録時経口摂取可能例が経口摂取不能となるまでの期間」、「登録時経口摂取不能例における経口摂取改善割合」、「有害事象発生割合」、「重篤な有害事象発生割合」である。腸管狭窄および腹水による食物摂取不良が腹膜転移例の特徴であるため、本試験では副次的評価項目に経口摂取に関する項目をおいた。

以上のコンセプトのもとプロトコールを作成したが、大きな問題点として、腹膜転移の定義、経口摂取に関する評価法、増悪の判定基準、説明文書における予後の表し方が問題となった。

Ⅱ．腹膜転移の定義

一般的に腹膜転移が進行すると腸閉塞や大量の腹水が出現し、安全性の点より通常の化学療法が行えなくなるが、このような腹膜転移例に対して安全で効果的な治療法の開発が本試験のコンセプトである。腹膜転移例に対する 5-FU の安全性はある程度確認されているため、5-FU 持続静注 vs MTX＋5-FU 併用療法といった 5-FU を含む治療法の比較が行われることとなった。

腹膜転移の進展形式として、開腹所見でしか確認できない時期から注腸（腸管の狭窄や壁不整を確認できる）やＣＴ（腹水や腹膜転移の腫瘤が確認できる）で所見がみられ始める時期を経て、最終的に腸閉塞、大量腹水が出現してくるというのが典型的パターンである。本試験の