camptothecin (SN-38), a topoisomerase inhibitor, by carboxylesterases (Humerickhouse et al., 2000) and further conjugated by hepatic uridine diphosphate glucuronosyltransferase to form the inactive metabolite SN-38 glucuronide (SN-38G) (Iyer et al., 1998). To a lesser extent, irinotecan is also converted to 7-ethyl-10-[4-*N*-(5-aminopentanoic acid)-1-piperidino]carbonyloxycamptothecin and 7-ethyl-10-(4-amino-1-piperidino)carbonyloxycamptothecin by cytochrome P450 3A4 (Dodds et al., 1998; Santos et al., 2000). Irinotecan and its metabolites are excreted by the efflux transporters, ABCB1 (P-glycoprotein), ABCC2 (canalicular multispecific organic anion transporter), and ABCG2 (breast cancer resistance protein), via a hepatobiliary pathway (Mathijssen et al., 2001). Although irinotecan metabolism is rather complex, hCE-2 is a key enzyme that determines the plasma levels of the active metabolite SN-38.

Hepatic hCE-2 activity toward irinotecan varies 3-fold in microsomes obtained from a panel of human livers (Xu et al., 2002). The activity loosely correlates with hCE-2 protein levels, but some microsomal samples showed unanticipated deviating activities. This result might be caused by genetic polymorphisms, such as single nucleotide polymorphisms (SNPs) in the *CES2* gene. Several SNPs and haplotypes have been reported for the *CES2* gene (Charasson et al., 2004; Marsh et al., 2004; Wu et al., 2004), and large ethnic differences in *CES2* SNP frequencies are found among Europeans, Africans, and Asian-Americans (Marsh et al., 2004).

Previously, 12 exons and their flanking regions of *CES2* were sequenced from 153 Japanese subjects, who received irinotecan or steroidal drugs, and 12 novel SNPs, including the nonsynonymous SNP, 100C>T ($Arg^{34}Trp$), and the SNP at the splice acceptor site of intron 8 (IVS8-2A>G) were found (Kim et al., 2003). In vitro functional characterization of these SNPs and an additional nonsynonymous SNP, 424G>A ($Val^{142}Met$), suggested that the ^{34}Trp and ^{142}Met variants were defective, and that IVS8-2G might be a low-activity allele (Kubo et al., 2005). In the present study, the same regions were sequenced from an additional 109 subjects (a total of 262 patients), and their haplotypes/diplotypes were determined/inferred. Then, associations between the haplotypes and pharmacokinetic parameters of irinotecan and its metabolites were analyzed for 177 cancer patients who were given irinotecan. Functional characterization of novel SNPs 1A>T and 617G>A, which were found in this study, was also performed by using a transient expression system with COS-1 cells.

Materials and Methods

Chemicals. Irinotecan, SN-38, and SN-38G were kindly supplied by Yakult Honsha Co. Ltd. (Tokyo, Japan).

Patients. A total of 262 Japanese subjects analyzed in this study consisted of 85 patients with allergies who received steroidal drugs and 177 patients with cancer who received irinotecan. The ethical review boards 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 participants.

DNA Sequencing. Total genomic DNA was extracted from blood leukocytes or Epstein-Barr virus-transformed lymphocytes and used as a template in the polymerase chain reaction (PCR). Sequence data of the *CES2* gene from 72 patients and 81 cancer patients were described previously (Kim et al., 2003). In addition, the *CES2* gene was sequenced from 13 allergic patients and 96 cancer patients. Amplification and sequencing of the *CES2* gene were performed as described previously (Kim et al., 2003). Rare SNPs found in only one heterozygous subject were confirmed by sequencing PCR fragments produced by amplification with a high-fidelity DNA polymerase KOD-Plus (Toyobo, Tokyo, Japan). GenBank accession number NT_010498.15 was used as the reference sequence.

Linkage Disequilibrium and Haplotype Analyses. LD analysis was performed by the SNPAlyze software (version 5.1; Dynacom Co., Yokohama, Japan), and a pairwise two-dimensional map between SNPs was obtained for the D' and rho square (r^2) values. All allele frequencies were in Hardy-Weinberg equilibrium. Some haplotypes were unambiguously assigned in the subjects with homozygous variations at all sites or a heterozygous variation at only one site. Separately, the diplotype configurations (combinations of haplotypes) were inferred by LDSUPPORT software, which determines the posterior probability distribution of the diplotype configuration for each subject on the basis of estimated haplotype frequencies (Kitamura et al., 2002). The haplotype groups were numbered according to the allele nomenclature systems suggested by Nebert (2000). The haplotypes harboring nonsynonymous or defective alleles were assigned as haplotype groups **2* to **6*. The subgroups were described as the numbers plus small alphabetical letters.

Administration of Irinotecan and Pharmacokinetic Analysis. The demographic data and eligibility criteria for 177 cancer patients who received irinotecan in the National Cancer Center Hospitals (Tokyo and Chiba, Japan) were described elsewhere (Minami et al., 2007).

Each patient received a 90-min i.v. infusion at doses of 60 to 150 mg/m^2, which varied depending on regimens/coadministered drugs: i.e., irinotecan dosages were 100 or 150 mg/m^2 for monotherapy and combination with 5-FU, 150 mg/m^2 for combination with mitomycin C (MMC), and 60 (or 70) mg/m^2 for combination with platinum anticancer drugs. Heparinized blood was collected before administration of irinotecan and at 0 min (end of infusion), 20 min, 1 h, 2 h, 4 h, 8 h, and 24 h after infusion. Plasma concentrations of irinotecan, SN-38, and SN-38G were determined as described previously (Sai et al., 2002). The AUCs from time 0 to infinity of irinotecan and its metabolites were calculated as described (Sai et al., 2004). Associations between genotypes and pharmacokinetic parameters including the AUC ratio (SN-38 + SN-38G)/CPT-11 were evaluated in 176 patients in whom pharmacokinetic parameters were obtained.

Construction of Expression Plasmids. The coding region of CES2L (long form) cDNA starts at an additional ATG translation initiation codon located 192 nucleotides upstream of the conventional ATG codon (Wu et al., 2003) and encodes a 623-amino acid protein found in the National Center for Biotechnology Information database (NP_003860.2). The wild-type CES2L cDNA was amplified by PCR from Human Liver QUICK-Clone cDNA (Clontech, Mountain View, CA) using *CES2*-specific primers, 5′-CACCCACCTATGACTGCTCA-3′ and 5′-AGGGAGCTACAGCTCTGTGT-3′. The PCR was performed with 1 unit of the high-fidelity DNA polymerase KOD-Plus and a 0.5 μM concentration of the *CES2* specific primers. The PCR conditions were 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 68°C for 3 min and then a final extension at 68°C for 5 min. The PCR products were cloned into the pcDNA3.1 vector by a directional TOPO cloning procedure (Invitrogen, Carlsbad, CA), and the sequences were confirmed in both directions. The resultant plasmid was designated pcDNA3.1/CES2L-WT. The 1A>T variation was introduced into pcDNA3.1/CES2L-WT by using a QuikChange Multi site-directed mutagenesis kit (Stratagene, La Jolla, CA) with the 5′-phosphorylated oligonucleotide, 5′-phospho-GAGACCAGCGAGCCGACCTTGCGGCTGCACAGACTTCG-3′ (the substituted nucleotide is underlined). The sequence of the variant cDNA was confirmed in both strands, and the resultant plasmid was designated pcDNA3.1/CES2L-A1T. Expression plasmids for the short-form wild-type (CES2S) and $Arg^{206}His$ variant CES2 were prepared and introduced into COS-1 cells according to the method described previously (Kubo et al., 2005).

Expression of Wild-Type and Variant CES2 Proteins in COS-1 Cells. Expression of wild-type and variant CES2 proteins in COS-1 cells was examined as described previously (Kubo et al., 2005). In brief, microsomal fractions (30 μg of protein/lane) or postmitochondrial fractions (0.4 μg of protein/lane) were separated by 8% SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Immunochemical detection of each type of CES2 protein was performed using rabbit anti-human CES2 antibody raised against a peptide antigen (residues 539–555, KKALPQKIQELEEPEER) (diluted 1:1500). To verify that the samples were evenly loaded, the blot was subsequently treated with a stripping buffer and reprobed with polyclonal anti-calnexin antibody (diluted 1:2000; Stressgen Biotechnologies Corp., San Diego, CA). Visualization of these proteins was achieved with horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:4000) and the Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life and Analytical Sciences, Boston, MA). Protein band densities were quantified with Diana III

and ZERO-Dscan software (Raytest, Straubenhardt, Germany). The relative expression levels are shown as the means ± S.D. of three separate transfection experiments.

Determination of CES2 mRNA by Real-Time RT-PCR. Total RNA was isolated from transfected COS-1 cells using the RNeasy Mini Kit (QIAGEN, Tokyo, Japan). After RNase-free DNase treatment of samples to minimize plasmid DNA contamination, first-strand cDNA was prepared from 1 μg of total RNA using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) with random primers. Real-time PCR assays were performed with the ABI7500 Real Time PCR System (Applied Biosystems) using the TaqMan Gene Expression Assay for *CES2* (Hs01077945_m1; Applied Biosystems) according to the manufacturer's instructions. The relative mRNA levels were determined using calibration curves obtained from serial dilutions of the pooled wild-type CES2 cDNA. Samples without reverse transcriptase were routinely included in the RT-PCR reactions to measure possible contributions of contaminating DNA, which was usually less than 1% of the mRNA-derived amplification. Transcripts of β-actin were quantified as internal controls using TaqMan β-Actin Control Reagent (Applied Biosystems), and normalization of CES2 mRNA levels were based on β-actin concentrations.

Enzyme Assay. CPT-11 hydrolyzing activity of the postmitochondrial supernatants (microsomal fraction plus cytosol) was assayed over the substrate concentration range of 0.25 to 50 μM as described previously (Kubo et al., 2005), except that the hydrolysis product, SN-38, was determined by the high-performance liquid chromatography method of Hanioka et al. (2001).

Statistical Analysis. Statistical analysis of the differences in the AUC ratios among *CES2* diplotypes, coadministered drugs. or irinotecan dosages was performed using the Kruskal-Wallis test, Mann-Whitney test, or Spearman rank correlation test (Prism 4.0, GraphPad Software, Inc., San Diego, CA). The *t* test (Prism 4.0) was applied to the comparison of the average values of protein expression and mRNA levels between wild-type and variant CES2.

Results

***CES2* Variations Detected in a Japanese Population.** Previously, the promoter region, all 12 exons, and their flanking introns of the *CES2* gene were sequenced from 72 allergic patients and 81 cancer patients and resulted in the identification of 12 novel SNPs (Kim et al., 2003). Additionally, the same region of *CES2* was sequenced from 13 allergic patients and 96 cancer patients. A total of 21 SNPs were found in 262 Japanese subjects (Table 1). Novel SNPs found in this study were −1233T>C, 1A>T, IVS2-71C>G, IVS7 + 27G>A, and IVS9 + 78C>T, but their frequency was low (0.002, identified in a single heterozygous subject for each SNP). The SNP 1A>T is nonsynonymous (M1L) and results in a substitution of the translation initiation codon ATG to TTG in the *CES2* gene. The other novel SNPs were located in the introns or the 5′-flanking region.

The nonsynonymous SNP 424G>A (V142M) reported by our group (Kubo et al., 2005) and another nonsynonymous SNP 617G>A (R206H) published in the dbSNP (rs8192924) and JSNP (ssj0005417) databases were found at a frequency of 0.002. Recently, several noncoding SNPs in *CES2* were also reported (Kim et al., 2003; Charasson et al., 2004; Marsh et al., 2004; Wu et al., 2004). Among them, the three SNPs, −363C>G in the 5′-UTR, IVS10-108(IVS10 + 406)G>A in intron 10, and 1749(*69)A>G in the 3′-UTR of exon 12, were found at frequencies of 0.031, 0.269, and 0.239, respectively, in this study.

LD and Haplotype Analysis. Using the detected SNPs, LD analysis was performed, and the pairwise values of r^2 and D' were obtained. A perfect linkage (r^2 = 1.00) was observed between SNPs −363C>G and IVS10-87G>A. A close association (r^2 = 0.85) was found between SNPs IVS10-108G>A and 1749A>G. Other associations were much lower (r^2 < 0.1). Therefore, the entire *CES2* gene was analyzed as one LD block. The determined/inferred haplotypes are summarized in Fig. 1 and are shown as numbers plus small

TABLE 1

Summary of SNPs in the CES2 gene in a Japanese population

SNP Identification			Location	Position		Nucleotide Change and Flanking Sequences (5′ to 3′)	Amino Acid Change	Allele Frequency
This Study	NCBI (dbSNP)	JSNP		NT_010498.15	From the Translational Initiation Site or from the Nearest Exon			
MPI6_CS2001			5′-Flanking	20582067	−1671[a]	CTGGAACAACTCG/CCTCCCCTCGGAA		0.010
MPI6_CS2002			5′-Flanking	20582484	−1254[a]	AACCACCACCGCT/CGATCCTAGCAGG		0.002
MPI6_CS2016[b]			5′-Flanking	20582505	−1233[a]	CAGGCGTGCGCTT/CCCGCTCCAACCC		0.002
MPI6_CS2003			Exon 1 (5′-UTR)	20582979	−759[a]	AAATGTTTGTCAA/GGTGGATAAATGA		0.006
MPI6_CS2004	rs11075646		Exon 1 (5′-UTR)	20583375	−363[a]	CCTCCTATCGATC/GCCCCAGCGCGCT		0.031
MPI6_CS2017[b]			Exon 1	20583738	1[a]	AGCGAGCCGACCA/TTGCGGCTGCACA	Met[1]Leu	0.002
MPI6_CS2005			Exon 2	20586162	100[a]	GCCAGTCCCATCC/TGGACCACACACA	Arg[34]Trp	0.002
MPI6_CS2021[b]			Intron 2	20587248	IVS2–71	GGTGGCTGGGAGC/GACCTCTGAACCC		0.002
MPI6_CS2015			Exon 4	20588325	424[a]	TGATTTCCCCAGG/ATGATGGTGTGGA	Val[142]Met	0.002
MPI6_CS2006		-	Intron 4	20588486	IVS4 + 29	GCTGGGCAACCCG/AGGCTGAGCGGGG		0.002
MPI6_CS2007			Exon 5	20588560	579[a]	CAAGCACGCAACC/TGGCAACTGGGGC	Thr[193]Thr (silent)	0.002
MPI6_CS2018	rs8192924	ssj0005417	Exon 5	20588598	617[a]	TGGCTGCACTACG/ACTGGGTCCAGCA	Arg[206]His	0.002
MPI6_CS2008			Exon 5	20588746	765[a]	CATGGAGAGTGGC/TGTGGCCCTCCTG	Gly[255]Gly (silent)	0.002
MPI6_CS2009			Intron 5	20589157	IVS5–69	CCTGTTCTTGCCC/TAGGGCCTTGGGC		0.017
MPI6_CS2019[b]			Intron 7	20589775	IVS7 + 27	AAGCCCACAAGTG/ACCTGGGGAGCCC		0.002
MPI6_CS2010			Intron 7	20589845	IVS7–25	CCCATCCCCAGCT/AACAGACTCTCTC		0.002
MPI6_CS2011			Intron 8	20590205	IVS8–2	TCCACCTGGGGTA/GGATGTTGCCTCC	Splicing defect	0.002
MPI6_CS2020[b]			Intron 9	20590429	IVS9 + 78	ACCTGCTGCTGTC/TCGGGTCAGCACT		0.002
MPI6_CS2012	rs2241409	IMS-JST1013275	Intron 10	20591293	IVS10–108	GGAAGAAAAAGCG/AGAGAAGCAGGAC		0.269
MPI6_CS2013	rs28382825		Intron 10	20591314	IVS10–87	GGACTGGGACCCG/AAGGTCTCGGGGG		0.031
MPI6_CS2014	rs8192925	ssj0005418	Exon 12 (3′-UTR)	20592196	1749 (*69)[a]	GTGCCCACACACA/GCCCACTAAGGAG		0.239

[a] A of the conventional translation initiation codon ATG in *CES2* (GenBank Y09616) is numbered 1, and the number in the parentheses indicates the position from the termination codon TGA.
[b] Novel variations detected in this study.

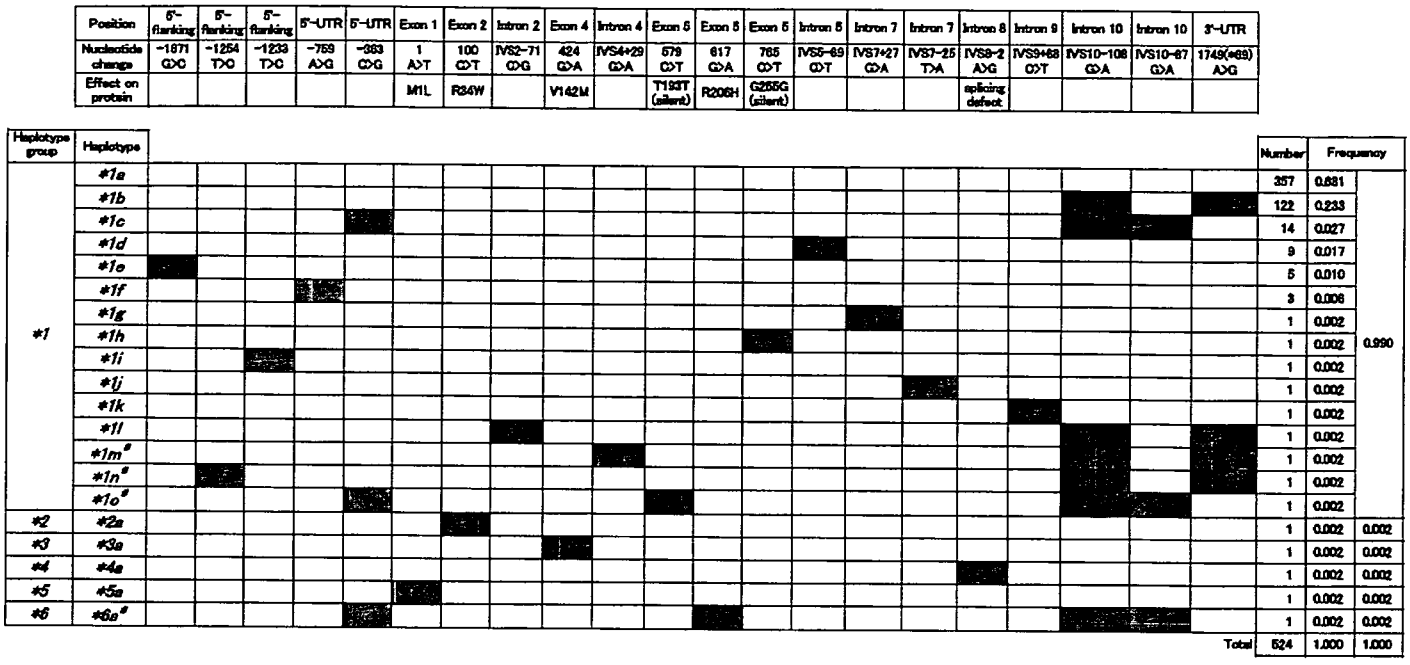

FIG. 1. Haplotypes of the *CES2* gene assigned for 262 Japanese subjects. The haplotypes assigned are described with lower case numbers and alphabetical letters. #, this haplotype was inferred in only one patient and is thus ambiguous.

alphabetical letters. Our nomenclature of haplotypes is distinct from those of previous studies (Charasson et al., 2004; Marsh et al., 2004; Wu et al., 2004). In this study, the haplotypes without amino acid changes and splicing defects were defined as the **1* group. The haplotypes harboring the nonsynonymous SNPs, 100C>T ($Arg^{34}Trp$), 424G>A ($Val^{142}Met$), 1A>T ($Met^{1}Leu$), and 617G>A ($Arg^{206}His$), were assigned as haplotypes **2, *3, *5*, and **6*, respectively. In addition, the haplotype harboring a SNP at the splice acceptor site of intron 8 (IVS8-2A>G) was assigned as haplotype **4*. Several haplotypes were first unambiguously assigned by homozygous variations at all sites (**1a* and **1b*) or heterozygous variation at only one site (**1d* to **1l, *2a, *3a, *4a*, and **5a*). Separately, the diplotype configurations (combinations of haplotypes) were inferred by LDSUPPORT software. The additionally inferred haplotypes were **1c* and **1m* to **1o*. The most frequent haplotype was **1a* (frequency, 0.681), followed by **1b* (0.233), **1c* (0.027), and **1d* (0.017). The frequencies of the other haplotypes were less than 0.01.

Association between *CES2* Genotypes and Irinotecan Pharmacokinetics. Next, the relationships between the *CES2* genotype and AUC ratio [(SN-38 + SN-38G)/CPT-11], a parameter of in vivo CES activity (Cecchin et al., 2005), in irinotecan-administered patients were investigated. The diplotype distribution of 176 patients, who received irinotecan and were analyzed for the AUC ratio, was similar to that of the 262 subjects. We examined preliminarily the effects of irinotecan dosage and comedication on the AUC ratio and obtained significant correlations of irinotecan dosage (Spearman $r = -0.559$, $p < 0.0001$) and comedication ($p < 0.0001$, Kruskal-Wallis test) with the AUC ratios. Because irinotecan dosages also depended on the drugs coadministered (see *Materials and Methods*), we finally stratified the patients with the coadministered drugs. As shown in Fig. 2, no significant differences in the median AUC ratios were observed among the **1* diplotypes in each group (p values in the Kruskal-Wallis test among **1a/*1a, *1a/*1b*, and **1b/*1b* were 0.260, 0.470, 0.129, and 0.072 for irinotecan alone, with 5-FU, with MMC and with platinum, respectively.). The relatively rare haplotype **1c*, which harbors −363C>G, did not show any associations with altered AUC ratio ($p = 0.756$ for irinotecan alone and $p = 0.230$ for irinotecan with platinum, Mann-Whitney test).

To estimate the effects of nonsynonymous SNPs on the metabolism of irinotecan, the AUC ratios in the patients carrying nonsynonymous SNPs were compared with the median AUC ratio of the **1/*1* patients. Three nonsynonymous SNPs, 100C>T ($Arg^{34}Trp$, **2*), 1A>T ($Met^{1}Leu$, **5*), and 617G>A ($Arg^{206}His$, **6*), and a SNP at the splice acceptor site of intron 8 (IVS8-2A>G, **4*) were found in 177 patients who received irinotecan. These SNPs were single heterozygotes. The AUC ratios of the patients with **2a/*1a* (0.17) and **5a/*1a* (0.10) in the monotherapy group were 60 and 36%, respectively, of the median value for the **1/*1* group (0.28) and substantially lower than the 25th percentile of the **1/*1* group (0.23) (Fig. 2). It must be noted that the **5a/*1a* patient had an extremely low AUC ratio. The AUC ratio of the **6* heterozygote who received cisplatin (0.25) was lower than the median value (0.37) but within the range for the **1/*1* group treated with platinum-containing drugs (Fig. 2). Regarding the effect of the heterozygous **4*, the AUC ratio (0.40) was not different from the median AUC ratio of the **1/*1* treated with platinum-containing drugs. To elucidate the effects of two novel amino acid substitutions, $Met^{1}Leu$ (**5*) and $Arg^{206}His$ (**6*), the functional analysis was conducted in vitro.

In Vitro Functional Analysis of the $Met^{1}Leu$ Variant. To clarify the functional significance of the novel variant $Met^{1}Leu$ (**5*), the protein expression level of CES2 carrying the nonsynonymous SNP 1A>T was examined. Wu et al. (2003) reported that transcription of CES2 mRNA was initiated from several transcriptional start sites, resulting in the expression of three *CES2* transcripts. Two longer transcripts carry a potential inframe translational initiation codon ATG at −192 that can encode an open reading frame (ORF) extending 64 residues at the amino terminus, as shown in the reference sequence in the National Center for Biotechnology Information database (NP_003860.2). Therefore, the expression of the CES2 protein from the long *CES2* ORF (CES2L), which encodes a potential 623 residue protein, was analyzed. Western analysis of membrane fraction proteins obtained from COS-1 cells

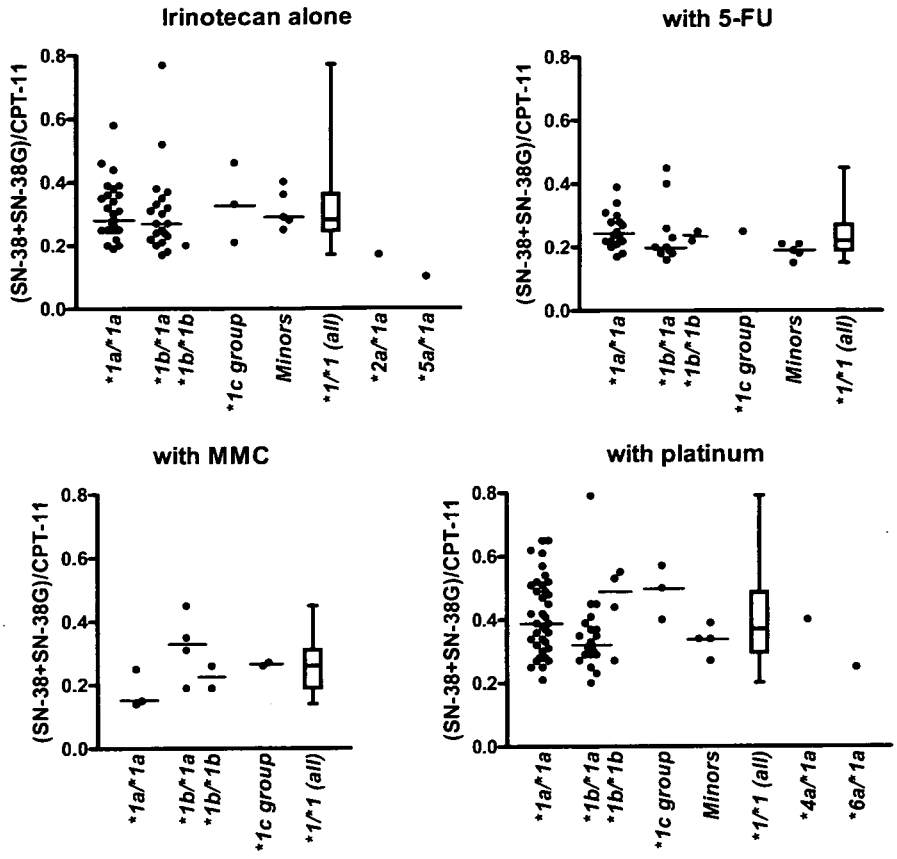

FIG. 2. Relationship between the *CES2* diplotypes and (SN-38 + SN-38G)/CPT-11 AUC ratios in Japanese cancer patients who received irinotecan. Each point represents an individual patient, and the median value in each genotype is shown with a horizontal bar. Distribution of the **1* group is shown by a box representing the 25th to 75th percentiles with a line at the median and bars representing the highest and lowest values. The **1c* group consists of **1c/*1a* and **1c/*1b*. "Minors" represents the heterozygous patients bearing minor **1* haplotypes (**1d, *1e, *1f, *1g, *1k*, and **1m*). Irinotecan alone, irinotecan monotherapy (n = 58); with 5-FU, combination therapy with 5-FU including tegafur (n = 35); with MMC, combination therapy with mitomycin C (n = 11); with platinum, combination therapy with either cisplatin (n = 62), cisplatin plus etoposide (n = 2), or carboplatin (n = 8).

transfected with the expression plasmid pcDNA3.1/CES2L-WT showed that the mobility (approximately 60 kDa) of the protein product from the CES2L cDNA was the same as that from the CES2S cDNA, which encodes a 559 residue protein (Kubo et al., 2005), and the CES2 protein in the human liver microsome (Fig. 3A). Western blot analysis of whole cell extracts also showed that CES2L yielded a single 60-kDa protein product (data not shown), indicating that translation of *CES2* was initiated from the second ATG codon of the *CES2L* ORF but not from the inframe translation initiation codon located at −192.

When the effect of the 1A>T SNP on the expression of the CES2 protein was examined by Western blotting (Fig. 3A), the relative expression levels of CES2 protein from cells transfected with plasmid pcDNA3.1/CES2L-A1T were 11.7 ± 2.4% (p = 0.0003) of the wild type. The mRNA expression levels determined by the TaqMan real-time RT-PCR assay were similar between the wild-type and variant CES2L cDNAs in COS-1 cells (Fig. 4A), indicating that the 1A>T SNP affects translational but not transcriptional efficiency. Thus, the $Met^{1}Leu$ variant was functionally deficient.

In Vitro Functional Analysis of the Arg^{206}His Variant. The known nonsynonymous SNP 617G>A changes an arginine to a histidine at residue 206. Western blot analysis of the postmitochondrial supernatant (including microsomes and cytosol) fractions obtained from COS-1 cells transfected with wild-type (CES2S) and Arg^{206}His variant CES2-expressing plasmids showed that the protein expression level of the Arg^{206}His variant was approximately 82 ± 7% (p = 0.017) of the wild-type (Fig. 3B). No significant differences in the mRNA expression levels determined by the TaqMan real-time RT-PCR assay were observed between the wild-type and 617G>A variant CES2s (82 ± 7%, p = 0.06) (Fig. 4B). Table 2 summarizes the apparent kinetic parameters for CPT-11 hydrolysis of wild-type and Arg^{206}His variant CES2.

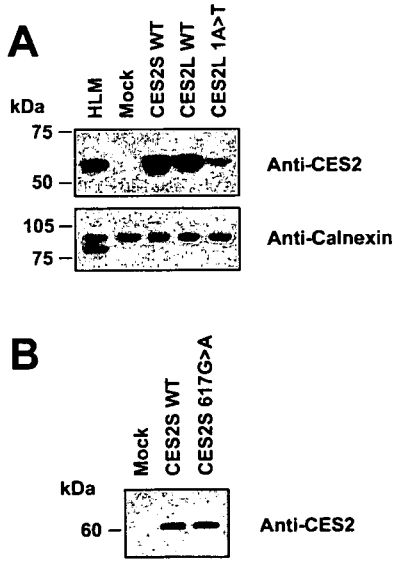

FIG. 3. Expression of CES2 protein from the wild-type and 1A>T (A) and 617G>A (B) variant *CES2* genes in COS-1 cells. Membrane fraction (A) or the postmitochondrial supernatant (B) from the cDNA-transfected cells was subjected to SDS-polyacrylamide gel electrophoresis, followed by transfer to the nitrocellulose membrane. Detection of CES2 and calnexin was performed with rabbit anti-human CES2 antiserum (A and B) and a rabbit anti-human calnexin antiserum (A) and horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody as described under *Materials and Methods*. A representative result from one of three independent experiments is shown. HLM, human liver microsomes.

Although a slight difference in the K_m values was obtained with statistical significance (p < 0.01), the kinetic parameters (V_{max} and V_{max}/K_m) were not significantly different when normalized by protein expression levels.

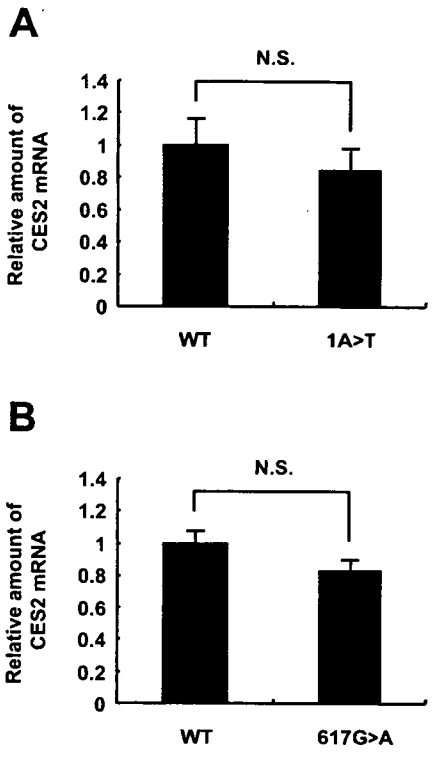

FIG. 4. Quantification of CES2 mRNA by TaqMan real-time RT-PCR in COS-1 cells transfected with wild-type (WT) and 1A>T (A) and 617G>A (B) variants. CES2 mRNA expression levels after 48 h were normalized with β-actin mRNA levels, and the mean level of the wild-type was set as 1.0. The results indicate the mean ± S.D. from three independent preparations. No significant difference in mRNA level was observed between the wild-type and variants ($p = 0.21$ an 0.06 in A and B, respectively).

Discussion

The present study provides comprehensive data on the haplotype analysis of the *CES2* gene, which encodes human carboxylesterase 2. From additional sequence analysis, a total of 21 SNPs including 4 nonsynonymous SNPs, 100C>T ($Arg^{34}Trp$), 424G>A ($Val^{142}Met$), 1A>T ($Met^{1}Leu$), and 617G>A ($Arg^{206}His$), and a SNP at the splice acceptor site of intron 8 (IVS8-2A>G) were found in 262 Japanese subjects. Among the nonsynonymous SNPs, in vitro functional analysis of the two nonsynonymous SNPs, 100C>T ($Arg^{34}Trp$) and 424G>A ($Val^{142}Met$), has already been performed to identify effects of these SNPs on expression levels and carboxylesterase activity. Kubo et al. (2005) showed that $Arg^{34}Trp$ and $Val^{142}Met$ variants had little carboxylesterase activity toward irinotecan, *p*-nitrophenyl acetate, and 4-methylumbelliferyl acetate, whereas expression levels of these variants were higher than those of the wild-type. An in vitro splicing assay using the *CES2* minigene carrying SNP IVS8-2A>G showed that IVS8-2A>G yielded mostly aberrantly spliced transcripts, resulting in the production of truncated CES2 proteins. These results have suggested that 100C>T ($Arg^{34}Trp$), 424G>A ($Val^{142}Met$), and IVS8-2A>G are functionally defective SNPs.

A novel SNP 1A>T found in this study changes the translation start codon ATG to TTG. Wu et al. (2003) identified three transcription start sites of *CES2*, resulting in the synthesis of three transcripts with either 78, 629, or 1187 nucleotides in the 5′-UTR. Another inframe ATG codon is present 192 nucleotides upstream of the conventional translational initiation codon, and two longer transcripts with 629 and 1187 nucleotides in the 5′-UTR can encode an ORF with 64 additional residues at the amino terminus (NP_003860.2). However, as shown in Fig. 3A, our in vitro experiment for the expression of CES2 showed that translation of CES2 mRNA started from the previously reported ATG codon but not from the inframe ATG codon at −192, when transiently expressed from the wild-type *CES2L* cDNA encoding a potential 623-amino acid CES2 protein in COS-1 cells. In vertebrate mRNAs, a purine residue in position −3 (A of the translational start codon is +1) is highly conserved and required for efficient translation (Kozak, 1991). The surrounding sequences of both ATG codons were accATGc for the functional ATG codon and cctATGa for the potential inframe ATG codon at −192. Thus, it is likely that their efficiencies of translation initiation depend on the flanking sequences of the translational start codon ATG.

When the expression levels between the wild-type and 1A>T variant were compared, the protein level of 1A>T was drastically reduced without changes in the mRNA levels, suggesting that the reduced protein level of the 1A>T variant might have been caused by its reduced translation initiation. It has been reported that alterations of the translational start codon ATG to TTG diminish or reduce the translation of growth hormone receptor (Quinteiro et al., 2002), protoporphyrinogen oxidase (Frank et al., 1999), low-density lipoprotein receptor (Langenhoven et al., 1996), and mitochondrial acetoacetyl-CoA thiorase (Fukao et al., 2003). Thus, it is likely that the 1A>T variation is a low-activity variation.

The functional effect of the known nonsynonymous SNP 617G>A ($Arg^{206}His$) was also investigated. The Arg^{206} residue is located in the α-helix within the catalytic domain and conserved among human carboxylesterases (Bencharit et al., 2002). However, no significant differences were found between the intrinsic enzyme activities of the wild-type and $Arg^{206}His$ variant for irinotecan hydrolysis.

In this study, 20 haplotypes of the *CES2* gene were identified. The most frequent haplotype was **1a* (frequency, 0.681), followed by **1b* (0.233), **1c* (0.027), and **1d* (0.017). Haplotype **1b* includes the polymorphisms IVS10-108G>A and 1749A>G, and haplotype **1c* harbors −363C>G, IVS10-108G>A, and IVS10-87G>A. The haplotype corresponding to **1b* in this study was found in Caucasians with a frequency of 0.086 (haplotypes 3 and 7 in Wu et al., 2004). Our **1c* corresponds to haplotypes 2 and 12 in Wu et al. (2004) and genotypes **1* and **6* in Charasson et al. (2004). Among the SNPs consisting of haplotype **1b* and **1c*, the three SNPs, −363C>G in the 5′-UTR, IVS10-108(IVS10 + 406)G>A in intron 10, and

TABLE 2

Kinetic parameters of CPT-11 hydrolysis by wild-type and $Arg^{206}His$ variant CES2 expressed in COS-1 cells

Results are expressed as the mean ± S.D. from four independent transfection experiments.

CES2	Apparent K_m	V_{max}	V_{max}/K_m	Normalized V_{max}[a]	Normalized V_{max}/K_m[a]
	μM	*pmol/min/mg protein*	*nl/min/mg protein*	*pmol/min/mg protein*	*nl/min/mg protein*
Wild-type	0.46 ± 0.01	3.45 ± 0.29	7.43 ± 0.54	3.46 ± 0.23	7.45 ± 0.50
$Arg^{206}His$	0.51 ± 0.02‡	2.81 ± 0.22†	5.53 ± 0.52‡	3.44 ± 0.16	6.77 ± 0.46

[a] V_{max} values were normalized by the relative protein expression level of the $Arg^{206}His$ variant (0.82 ± 0.07).
† Significantly different from that of the wild-type at $P < 0.05$.
‡ $P < 0.01$.

1749(*69)A>G in the 3′-UTR of exon 12, were previously reported, and their frequencies varied among several ethnic groups (Marsh et al., 2004; Wu et al., 2004). The frequency (0.269) of the **1b/*1c*-tagging SNP in Japanese, IVS10-108G>A, was comparable to that in African-Americans (0.263), but much higher than that in Asian-Americans (0.06) and European-Americans (0.063) (Wu et al., 2004). However, the **1b*-tagging SNP 1749A>G (0.239 in this study) was detected only in Asian-Americans with a low frequency (0.03) (Wu et al., 2004). The frequency of the **1c*-tagging SNP, −363C>G, also showed marked ethnic differences between Japanese (0.031) and Europeans (0.12) or Africans (0.33) (our data; Marsh et al., 2004). These findings indicate the existence of large ethnic difference in haplotype structures among African, European, and Japanese populations.

In this study, the relationship between the *CES2* genotypes and the (SN-38 + SN-38G)/CPT-11 AUC ratios of irinotecan-administered patients was analyzed. First, the relationship between the genotypes and the AUC ratios among the **1/*1* diplotypes in the patient group with or without coadministered drugs was assessed, and no significant differences in the AUC ratios were observed among the **1/*1* diplotypes in each group (Fig. 2). Wu et al. (2004) reported that the haplotype harboring SNP −363C>G that was homozygous appeared to have lower mRNA levels than the other haplotype groups. In this study, the haplotype having the SNP −363C>G was assigned haplotype **1c*. However, no functional differences were found between haplotype **1c* and the other **1* group haplotypes. Marsh et al. (2004) reported that IVS10-88C>T was associated with reduced RNA expression in colon tumor tissues. However, this SNP was not found in the present study with Japanese subjects.

The major **1* group haplotypes, **1a*, **1b*, and **1c*, account for 94% of Japanese *CES2* haplotypes. The current study revealed no association between the major *CES2* genotypes and changes in the AUC ratio, indicating that the variability in AUC ratio could not be interpreted by these haplotypes alone.

In irinotecan-administered patients, three nonsynonymous SNPs, 100C>T ($Arg^{34}Trp$, *2), 1A>T ($Met^{1}Leu$, *5), and 617G>A ($Arg^{206}His$, *6), and a SNP at the splice acceptor site of intron 8 (IVS8-2A>G, *4) were found as single heterozygotes. The patients heterozygous for $Arg^{34}Trp$ or $Met^{1}Leu$ showed substantially reduced AUC ratios. These results were consistent with in vitro functional analysis for the nonsynonymous SNPs by Kubo et al. (2005).

In the case of haplotype *6 harboring the nonsynonymous SNP, 617G>A ($Arg^{206}His$), the AUC ratio of the patient who received cisplatin was lower than the median value but within the range for the **1/*1* group treated with platinum-containing drugs. The protein expression level of the 206His variant was 82 ± 4%, and the $Arg^{206}His$ substitution itself showed no functional differences in intrinsic enzyme activity by in vitro functional analysis. Thus, the impact of the 617G>A ($Arg^{206}His$) SNP on irinotecan pharmacokinetics might be small.

On the other hand, the AUC ratio of the patient carrying the haplotype *4 was not different from the median value of the **1/*1* group treated with platinum-containing drugs. It is possible that other genetic factors might have increased the AUC ratio in this patient.

The patients with *4, *5, or *6 were found as single heterozygotes. Thus, further studies are needed to elucidate in vivo importance of the three haplotypes.

In conclusion, we have identified a panel of haplotypes of the *CES2* gene in a Japanese population using 21 genetic polymorphisms detected in this study and found that some rare haplotypes with nonsynonymous SNPs show a decreasing tendency toward enzymatic levels or activity. In vitro functional analysis for nonsynonymous SNPs showed that the 1A>T ($Met^{1}Leu$) SNP was a defective allele. These findings will be useful for further pharmacogenetic studies on efficacy and adverse reactions to CES2-activated prodrugs.

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SNP Communication

*Genetic Variations and Frequencies of Major Haplotypes in SLCO1B1 Encoding the Transporter OATP1B1 in Japanese Subjects: SLCO1B1*17 is More Prevalent Than *15*

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Summary: A liver-specific transporter organic anion transporting polypeptide 1B1 (OATP1B1, also known as OATP-C) is encoded by *SLCO1B1* and mediates uptake of various endogenous and exogenous compounds from blood into hepatocytes. In this study, 15 *SLCO1B1* exons (including non-coding exon 1) and their flanking introns were comprehensively screened for genetic variations in 177 Japanese subjects. Sixty-two genetic variations, including 28 novel ones, were found: 7 in the 5′-flanking region, 1 in the 5′-untranslated region (UTR), 13 in the coding exons (9 nonsynonymous and 4 synonymous variations), 5 in the 3′-UTR, and 36 in the introns. Five novel nonsynonymous variations, 311T>A (Met104Lys), 509T>C (Met170Thr), 601A>G (Lys201Glu), 1553C>T (Ser518Leu), and 1738C>T (Arg580Stop), were found as heterozygotes. The allele frequencies were 0.008 for 1738C>T (Arg580Stop) and 0.003 for the four other variations. Arg580Stop having a stop codon at codon 580 results in loss of half of transmembrane domain (TMD) 11, TMD12, and a cytoplasmic tail, which might affect transport activity. In addition, novel variations, IVS12-1G>T at the splice acceptor site and -3A>C in the Kozak motif, were detected at 0.003 and 0.014 frequencies, respectively. Haplotype analysis using -11187G>A, -3A>C, IVS12-1G>T and 9 nonsynonymous variations revealed that the haplotype frequencies for **1b*, **5*, **15*, and **17* were 0.469, 0.000 (not detected), 0.037, and 0.133, respectively. These data would provide fundamental and useful information for pharmacogenetic studies on OATP1B1-transported drugs in Japanese.

Key words: *SLCO1B1*; direct sequencing; novel genetic variation; amino acid change

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Introduction

Organic anion transporting polypeptide 1B1 (OATP1B1, also known as OATP-C, OATP2 and LST-1) is a liver-specific transporter expressed on the sinusoidal membrane and mediates uptake of various endogenous and exogenous compounds from blood into hepatocytes.[1,2] Exogenous compounds include several 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (such as pravastatin), an active metabolite of irinotecan SN-38, methotrexate, and rifampicin; endogenous substrates include bilirubin and bilirubin glucuronide, cholate, leukotriene C_4, and estradiol-17β-glucuronide.[1,2]

OATP1B1 protein (691 amino acid residues) is encoded by *SLCO1B1*, which consists of 15 exons (including non-coding exon 1) and spans approximately 109 kb on chromosome 12p12.2-p12.1. Similar to other OATP family members, this transporter is predicted to have 12 transmembrane domains (TMDs).[1,3]

Several genetic polymorphisms and haplotypes with functional significance are already known in *SLCO1B1*. In Japanese, two haplotypes with nonsynonymous variations **1b* and **15* have been frequently reported. The *SLCO1B1*1b* haplotype with 388A>G (Asn130Asp) has been shown to have no altered transport activity from *in vitro* expression systems.[2,4-7] Recently, however, an *in vivo* study has suggested that the area under the concentration-time curve (AUC) of pravastatin is significantly lower in **1b/*1b* subjects than in **1a/*1a* subjects, suggesting increased transport activity possibly through increased protein expression.[8] Another major haplotype, *SLCO1B1*15* harboring both 388A>G (Asn130Asp) and 521T>C (Val174Ala), has been reported to show impaired plasma membrane expression[6] and reduced transport activity *in vitro*,[6,7] probably due to the Val174Ala substitution.[2,4,6] The association of the **15* haplotype with significant increases in AUC was reported for pravastatin,[2,9] and irinotecan and SN-38.[10] The haplotype frequencies of **1b*, **5* (with 521T>C, Val174Ala), and **15* were reported to be 0.46–0.54, 0.00–0.01, and 0.10–0.15, respectively, in Japanese.[5,11]

Recently, the *SLCO1B1*17* haplotype having 388A>G (Asn130Asp), 521T>C (Val174Ala), and -11187G>A was also shown to increase the AUC of pravastatin[9] and likely reduces the pravastatin efficacy on cholesterol synthesis,[12] although the effect of -11187G>A on transcriptional activity has not been clarified *in vitro*. The frequency of **17* has not, however, been reported in Japanese.

In this study, all 15 exons and their surrounding introns were resequenced for comprehensive screening of genetic variations in *SLCO1B1*. Sequence analysis detected 62 variations including 5 novel nonsynonymous ones from 177 Japanese subjects. Haplotype frequencies of **1b*, **5*, **15*, and **17* were also estimated.

Materials and Methods

Human genomic DNA samples: One hundred seventy-seven Japanese cancer patients administered irinotecan participated in this study and provided written informed consent. The ethical review boards of the National Cancer Center and the National Institute of Health Sciences approved this study. Whole blood was collected from the patients prior to the administration of irinotecan, and genomic DNA was extracted from blood leukocytes by standard methods.

PCR conditions for DNA sequencing and haplotype analysis: First, two sets of multiplex PCR were performed to amplify all 15 exons of *SLCO1B1* from 100 ng of genomic DNA using 1.25 units of *Z-Taq* (Takara Bio. Inc., Shiga, Japan) with 0.2 μM each of the mixed primers (Mix 1 and Mix 2) designed in the intronic regions as listed in **Table 1** (1st PCR). Mix 1 contained primers for amplifying exons 1 and 2, and 12 to 14, and Mix 2 contained primers for exons 3 to 7, 8 to 11, and 15. The first PCR conditions consisted of 30 cycles of 98 °C for 5 sec, 55°C for 10 sec, and 72°C for 190 sec. Next, each exon was amplified separately by *Ex-Taq* (0.625 units, Takara Bio. Inc.) with appropriate primers (0.5 μM) designed in the introns (**Table 1**, 2nd PCR). The conditions for the second round PCR 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. For amplification of exons 10 and 13, PCR was carried out under the following conditions: 94°C for 5 min followed by 33 cycles of 94°C for 30 sec, 55°C for 1 min, and 72°C for 30 sec, and then a final extension at 72°C for 7 min. Following PCR, the 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 sequencing primers listed in **Table 1** (Sequencing). Excess dye was removed by a DyeEx96 kit (Qiagen, Hilden, Germany), and the eluates were analyzed on an ABI Prism 3700 DNA Analyzer (Applied Biosystems). All variations were confirmed by sequence analysis of PCR products generated by new amplification of the original genomic DNA templates.

As of July 18, 2007, the novel variations reported here are not found in the database of Japanese Single Nucleotide Polymorphisms (http://snp.ims.u-tokyo.ac.jp/), dbSNP in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP/), or PharmGKB Database (http://www.pharmgkb.org/).

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Table 1. Primer sequences used in this study

		Amplified or sequenced region	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Amplified region[a]	Length (bp)
1st PCR	Mix 1	Exons 1 and 2	ACTCTGGGGCTAAAACCTATTGGAC	CTGCTTGCCATAACATCTTGAGGGT	14041049_14055679	14,631
		Exons 12 to 14	CTAGGGCTTTTATTGATAGGCAGGT	AAACTTCCAGACTGTCTTCTACCAT	14127745_14137752	10,008
	Mix 2	Exons 3 to 7	TTTGTTGAGAAGAGACTGTTAGGCA	GGAAAATGGATGAAGAAGCACTGGA	14083429_14092020	8,592
		Exons 8 to 11	AAGGACAGCACCAAGCAATGAAGGA	CATTCAACTCAGCAATCCCACTACC	14106917_14119592	12,676
		Exon 15	CTGAGGAGAACTGTAATTTGATGTC	TTTCCAGAGGCAAGCATTTACAACT	14148708_14152868	4,161
2nd PCR		Exon 1	TAACAGGCATAATCTTTGGTCT	AAGGGCTCAGAATGTAAGCG	14041915_14043281	1,367
		Exon 2	TCCTTAGGCTAGAATTTGTGT	CAAAGTGAGTCTCAAGACATT	14053227_14053950	724
		Exon 3	TGGCTGAGTAGTAGTACCTG	ATCCTCACTATCAACATTTTCA	14084394_14084961	568
		Exon 4	TGAGTGGTCTAATGTAGGTGA	AGGTGTAAGTGTTGAGGTCTT	14086324_14086946	623
		Exon 5	ATCTTTCTTGCTGGACACTTC	TATTAAGGAATTTGTTACAGGG	14088485_14089158	674
		Exons 6 and 7	CATAAGAATGGACTAATACACC	GGGAGACATTTTACATTTGGTT	14090310_14091202	893
		Exon 8	TTCCTAGACAGTATCTGTTGC	CTTCCACTTGTTATGTGCTCA	14108658_14109289	632
		Exon 9	AGTTACAAAACAGCACTTACG	TCAGGAACTCATCTAAAATAAG	14112156_14112798	643
		Exon 10	CAGGGGTTAAAACCTAGATGA	ATCCATGTATTTCTCTAAGCC	14114169_14114904	736
		Exon 11	TGGCAAAGATGGAGAGCGTA	AGTCAAATGAGGTGCTTCTTA	14117563_14118256	694
		Exon 12	TTGTCCAAAAGAGTATGTGCT	CAGCCTTGAGAGTTCATAGT	14128668_14129375	708
		Exon 13	TTGACCCAGCAATCCAACTAT	CCTTTTTTTTTTCATCATACCTAGT[b]	14133788_14134290	503
		Exon 14	ATATTAACCAACATAACTTCCA	CCTTGAATCACAGTTTCTTCG	14136297_14136980	684
		Exon 15	GATGGCTTAACAGGGCTTGA	TGCGGCAAATGATCTAGGAA	14150619_14151844	1,226
Sequencing		Exon 1	TAACAGGCATAATCTTTGGTCT	AAGGGCTCAGAATGTAAGCG		
			TATGTGAGAGAAGGGTCTGTA	CTACAGGTTACATTGGCATTT		
			AAATGCCAATGTAACCTGTAG	CTGAAATAAAGTACAGACCCT		
		Exon 2	TCCTTAGGCTAGAATTTGTGT	CAAAGTGAGTCTCAAGACATT		
		Exon 3	TGGCTGAGTAGTAGTACCTG	ATCCTCACTATCAACATTTTCA		
		Exon 4	TGAGTGGTCTAATGTAGGTGA	AGGTGTAAGTGTTGAGGTCTT		
		Exon 5	ATCTTTCTTGCTGGACACTTC	TATTAAGGAATTTGTTACAGGG		
		Exon 6	TTAAGAGTTTACAAGTAGTTAAA	AAGCAATTTTACTAGATGCCAA		
		Exon 7	CTTCTTTGTATTTAGGTAATGTA	ATAGTATAAATAGGAGCTGGAT		
		Exon 8	TTCCTAGACAGTATCTGTTGC	CTTCCACTTGTTATGTGCTCA		
		Exon 9	AGTTACAAAACAGCACTTACG	TCAGGAACTCATCTAAAATAAG		
		Exon 10	TTGATAGGTGCAGCAAACCAC	GGAATAAAAGAATGTGTTTGAG		
		Exon 11	TCTTTTTGATATATGTCTATCAT	AGTCAAATGAGGTGCTTCTTA		
		Exon 12	TTGTCCAAAAGAGTATGTGCT	CAGCCTTGAGAGTTCATAGT		
		Exon 13	GTTCTAACCACTTCCTCATAG	CCTTTTTTTTTTCATCATACCTAGT		
		Exon 14	TCCTTTTTACCATTCAGGCTTA	ACTAAAATGAGATACGAGATTG		
		Exon 15	GATGGCTTAACAGGGCTTGA	TGCGGCAAATGATCTAGGAA		
			CACATCTTTTATGGTGGAAGT	AGGCTTATTTATACTTCCACC		

[a]The reference sequence is NT_009714.16.
[b]Mismatched nucleotides at the 5′ end are underlined.

Furthermore, rare SNPs found in single patients as heterozygotes were confirmed by sequencing the PCR fragments produced by the amplification with a high fidelity DNA polymerase KOD-Plus- (TOYOBO, Tokyo, Japan).

Hardy-Weinberg equilibrium was analyzed by SNPAlyze version 3.1 (Dynacom Co., Yokohama, Japan). Estimation of *SLCO1B1* haplotypes was performed by an expectation-maximization based program, LDSUPPORT software.[13]

Results and Discussion

Sequence analysis from 177 Japanese subjects resulted in the identification of 62 genetic variations, including 28 novel ones (**Table 2**). Of these variations, 7 were located in the 5′-flanking region, 1 in the 5′-untranslated region (UTR), 13 in the coding exons (9 nonsynonymous and 4 synonymous variations), 5 in the 3′-UTR, and 36 in the introns. All detected variations were in Hardy-Weinberg equilibrium ($p > 0.05$).

Of the 9 nonsynonymous variations, 5 variations were novel: 311T>A (Met104Lys), 509T>C (Met170Thr), 601A>G (Lys201Glu), 1553C>T (Ser518Leu), and 1738C>T (Arg580Stop). All of these variations were found as heterozygotes with frequencies of 0.008 for 1738C>T (Arg580Stop) and 0.003 for the four other variations. Arg580, residing in TMD11, is conserved among human, rat and mouse OATP families.[1] The change from arginine residue to the immature termination codon leads to loss of this conserved amino acid

Table 2. Summary of *SLCO1B1* variations detected in this study

SNP ID			Reference	Location	Position		Nucleotide change	Amino acid change	Allele frequency (n = 354)
This Study	dbSNP (NCBI)	JSNP			NT_009714.16	From the translational initiation site or from the end of the nearest exon			
MPJ6_SB1_001[a]				5′-flanking	14042128	-11355 (-1078)[b]	ccaatactctcaA/Gtaattaaccaag		0.003
MPJ6_SB1_002	rs4149015	ssj0003132	9, 15	5′-flanking	14042296	-11187 (-910)[b]	tatgtgtatacaG/Agtaaaagtgtgt		0.153
MPJ6_SB1_003[a]				5′-flanking	14042494	-10989 (-712)[b]	atctctactcaaG/Aaaaactttaac		0.076
MPJ6_SB1_004[a]				5′-flanking	14042530	-10953 (-676)[b]	cttcctttctccA/Tcaagtcaagtca		0.003
MPJ6_SB1_005	rs11835045			5′-flanking	14042793	-10690 (-413)[b]	atttgtcttaaaT/Ctatttcctattt		0.076
MPJ6_SB1_006[a]				5′-flanking	14042860	-10623 (-346)[b]	ttaaagaaaaaaA/-tcttatgccacc		0.003
MPJ6_SB1_007[a]				5′-flanking	14043018	-10465 (-188)[b]	aactaggttttaT/Catgtttgactag		0.003
MPJ6_SB1_008[a]				Intron 1	14043209	IVS1 + 65	ttcacggaagaaG/Catttttgatggtc		0.014
MPJ6_SB1_009	rs2010668	ssj0003141		Intron 1	14053267	IVS1-155	tcctactttttgtG/Ttccagcattgac		0.113
MPJ6_SB1_010[a]				5′-UTR	14053480	-3	atctatatttcaA/Ctcatggaccaaa		0.014
MPJ6_SB1_011[a]				Intron 2	14053635	IVS2 + 69	tagaaaagcaagT/Ctgttaaaaagaa		0.003
MPJ6_SB1_012[a]				Intron 2	14053648	IVS2 + 82	tgttaaaaagaaC/Tattatgtttcaa		0.003
MPJ6_SB1_013[a]				Intron 2	14053734	IVS2 + 168	aaaccagtctttT/Caatctgattaag		0.008
MPJ6_SB1_014	rs4149021	ssj0003142	9	Intron 2	14053759	IVS2 + 193	tatttctttggcG/Aaaatttttgatg		0.153
MPJ6_SB1_015	rs12812795		9	Intron 2	14053769	IVS2 + 203	gcgaaatttttgA/Ttgcttaatagtt		0.003
MPJ6_SB1_016[a]				Intron 2	14053807	IVS2 + 241	aatttagaaataT/Cttgatagcttc		0.006
MPJ6_SB1_017	rs12303784			Intron 2	14053814	IVS2 + 248	aaatattttgatA/Ggcttctctttgg		0.003
MPJ6_SB1_018[a]				Intron 2	14084429	IVS2-129	aaagggaaaactA/Gagtatggttttt		0.003
MPJ6_SB1_019[a]				Intron 2	14084478	IVS2-80	aaagaagaaagcT/Cattataattcca		0.008
MPJ6_SB1_020	rs2291073	JST-043317		Intron 3	14084788	IVS3 + 89	actggggtaaatT/Gtatctctcacag		0.271
MPJ6_SB1_021	rs2291074	JST-043318		Intron 3	14084923	IVS3 + 224	attctataatgcA/Gcaaagaatgatg		0.243
MPJ6_SB1_022[a]				Exon 4	14086569	311	gttgtttcattaT/Agggaattggagg	Met104Lys	0.003
MPJ6_SB1_023	rs4149036	ssj0003160		Intron 4	14086714	IVS4 + 97	ataggcagttacC/Attttgagaagat		0.427
MPJ6_SB1_024[a]				Intron 4	14088523	IVS4-161	cacttttacccaT/Ccacatctcttaa		0.017
MPJ6_SB1_025	rs2306283	JST-063865	4, 5, 9, 11, 15	Exon 5	14088712	388	gaaactaatatcA/Gattcatcagaaa	Asn130Asp	0.667
MPJ6_SB1_026	rs2306282	JST-063864	11	Exon 5	14088776	452	ttttatcactcaA/Gtagagcatcacc	Asn151Ser	0.034
MPJ6_SB1_027	rs4149044	ssj0003170	9	Intron 5	14088970	IVS5 + 165	cacagttcgcccA/Tttaacaacacag		0.427
MPJ6_SB1_028	rs4149045	ssj0003171	9	Intron 5	14088994	IVS5 + 189	ggtttaaactacG/Acgttttcactte		0.429
MPJ6_SB1_029	rs4149046	ssj0003172	9	Intron 5	14088996	IVS5 + 191	tttaaactacgcG/Attttcacttcta		0.331
MPJ6_SB1_030	rs4149096	ssj0003230	9	Intron 5	14090372_14090377	IVS5-107_112	aaattacttgtaCTTGTA/-aattaaaaaaaa		0.427
MPJ6_SB1_031[a]				Intron 5	14090469	IVS5-15	aaatgaaacactC/Gtcttatctacat		0.003
MPJ6_SB1_032[a]				Exon 6	14090511	509	ctgggtcatacaT/Cgtggatatatgt	Met170Thr	0.003
MPJ6_SB1_033	rs4149056	ssj0003182	4, 5, 9, 11, 15	Exon 6	14090523	521	tgtggatatatgT/Cgttcatgggtaa	Val174Ala	0.175
MPJ6_SB1_034	rs4149057	ssj0003183	9, 11, 15	Exon 6	14090573	571	cccatagtaccaT/Ctggggctttctt	Leu191Leu	0.333
MPJ6_SB1_035[a]				Exon 6	14090578	576	agtaccattgggG/Actttcttacatt	Gly192Gly	0.003
MPJ6_SB1_036	rs2291075	JST-043319	9, 11, 15	Exon 6	14090599	597	cattgatgatttC/Tgctaaagaagga	Phe199Phe	0.427
MPJ6_SB1_037[a]				Exon 6	14090603	601	gatgatttcgctA/Gaagaaggacatt	Lys201Glu	0.003
MPJ6_SB1_038	rs2291076	JST-043320	9	Intron 7	14090961	IVS7 + 33	gtaccatgataaC/Tgtctttctaagc		0.336
MPJ6_SB1_039			11	Exon 9	14112452	1007	tccttactaatcC/Gcctgtatgttat	Pro336Arg	0.006
MPJ6_SB1_040[a]				Intron 9	14114331	IVS9-68	ttgacatacattG/Ctgtttcatctat		0.003
MPJ6_SB1_041	rs4149099	JST-080069	9	Intron 10	14117669_14117670	IVS10-106_-107	tttatctacttt-/CTTttttccctctt		0.647
MPJ6_SB1_042			11	Intron 10	14117728_14117730	IVS10-46_-48	cttctttgtcttTTT/-cttctctctctc		0.003
MPJ6_SB1_043	rs4149070	ssj0003204	9	Intron 11	14128857	IVS11-170	gaaagaaatccaC/Gaaaactatttta		0.280
MPJ6_SB1_044	rs4149071	ssj0003205	9	Intron 11	14128938	IVS11-89	agtttgaacaagT/Cgagacttcacta		0.280
MPJ6_SB1_045	rs4149100	ssj0003234	9	Intron 11	14128952	IVS11-75	agacttcactaaA/-tataatgcaatg		0.395
MPJ6_SB1_046	rs4149072	ssj0003206	9	Intron 11	14128959	IVS11-68	actaaatataatG/Acaatgtatttgc		0.280
MPJ6_SB1_047			11	Intron 11	14129015	IVS11-12	catattttatacA/Gcaacgcttaagg		0.014
MPJ6_SB1_048[a]				Exon 12	14129082	1553	acagaaattactC/Tagcccatttggg	Ser518Leu	0.003
MPJ6_SB1_049	rs987839			Intron 12	14133812	IVS12-396	tccaactattggG/Atatctaccaaaa		0.316
MPJ6_SB1_050[a]				Intron 12	14134097	IVS12-111	ggggccattcaaC/Ttgtgagcttaat		0.020
MPJ6_SB1_051[a]				Intron 12	14134207	IVS12-1	tgtcttgtttcaG/Taattgttcaacc		0.003
MPJ6_SB1_052[a]				Exon 13	14134263	1738	tcaatggttataC/Tgagcactaggta	Arg580Stop	0.008
MPJ6_SB1_053	rs4149080	ssj0003214	9	Intron 13	14136533	IVS13-97	ctccaaatttttG/Caacttttattta		0.395
MPJ6_SB1_054	rs11045875		11	Intron 14	14136797	IVS14 + 50	gactatattaatT/Gcctaaaaaatat		0.011
MPJ6_SB1_055[a]				Intron 14	14150655	IVS14-232	tatatttttctcG/Attttatgaagaa		0.006
MPJ6_SB1_056[a]				Intron 14	14150656	IVS14-231	atatttttctcgT/Ctttatgaagaag		0.251
MPJ6_SB1_057[a]				Exon 15	14151004	1983	tgcatcagaaaaT/Cggaagtgtcatg	Asn661Asn	0.006
MPJ6_SB1_058[a]				3′-UTR	14151137	2116 (*40)[c]	tgtgtttccaaaC/Gagcattgcattg		0.011
MPJ6_SB1_059	rs4149085	ssj0003219		3′-UTR	14151264	2243 (*167)[c]	acaaactgtaggT/Cagaaaaaatgag		0.251
MPJ6_SB1_060	rs4149086	ssj0003220		3′-UTR	14151425	2404 (*328)[c]	aaacaaatgagtA/Gtcatacaggtag		0.025
MPJ6_SB1_061	rs4149087	ssj0003221	15	3′-UTR	14151536	2515 (*439)[c]	gaactataatacG/Taaggcctgaagt		0.333
MPJ6_SB1_062	rs4149088	ssj0003222		3′-UTR	14151560	2539 (*463)[c]	tctagcttggatG/Atatgctacaata		0.333

[a]Novel variations detected in this study.
[b]Intron 1 is skipped for counting.
[c]Positions are shown as * and bases from the translational termination codon TAA.

along with the subsequent half of TMD11, TMD12 and the cytoplasmic tail,[1] which very likely affects transport activity. Other variations 311T>A (Met104Lys), 509T>C (Met170Thr), 601A>G (Lys201Glu), and 1553C>T (Ser518Leu) are located in TMD3, TMD4, the short cytoplasmic loop between TMD4 and TMD5, and the large extracellular loop between TMD9 and TMD10, respectively.[1] Using the PolyPhen program (http://genetics.bwh.harvard.edu/pph/) to predict the functional effects of the four amino acid substitutions, three substitutions, Met104Lys, Met170Thr and Ser518Leu, were expected to alter the protein function based on the PSIC (position-specific independent count) score differences derived from multiple alignments. The functional significance of these 5 novel nonsynonymous variations should be clarified in the future. In addition, a novel variation at the splice acceptor site, IVS12-1 G>T, was detected at a 0.003 frequency. This variation might cause aberrant splicing of *SLCO1B1* pre-mRNA and thus influence the expression level of active protein. Furthermore, -3A>C might reduce translational efficiency since this purine-to-pyrimidine alteration results in a deviation from the Kozak motif, where the purine nucleotide at position -3 from the translational initiation codon is important.[14]

Four known variations, 388A>G (Asn130Asp), 452A>G (Asn151Ser), 521T>C (Val174Ala), and 1007C>G (Pro336Arg), were detected at 0.667, 0.034, 0.175, and 0.006 frequencies, respectively, which are similar to the Japanese data reported previously.[5,11] The allele frequencies of 521T>C (Val174Ala) in Japanese (0.11–0.18) are comparable to those in other Asian populations (0.04–0.25) and Caucasians (0.14–0.22), but higher than that in African-Americans (0.02).[10,15,16] The frequencies of 388A>G (Asn130Asp) in Japanese (0.63–0.67) are also similar to those in other Asians (0.57–0.88) and African-Americans (0.75), but higher than those in Caucasians (0.30–0.51).[10,15,16] Variations 452A>G (Asn151Ser) and 1007C>G (Pro336Arg) have not been reported in other ethnic populations. Analysis of these four known variations with PolyPhen program showed that only Val174Ala was expected to alter protein function, which is consistent with the previous functional analysis.[2,4,6] Variations 1454G>T (Cys485Phe) and 1628T>G (Leu543Trp) previously reported in Japanese were not detected in this study.[11,17] Hepatocyte nuclear factor 1α is known to transactivate *SLCO1B1* through binding to the promoter region (from -10432 to -10420 from the translational start codon);[18] however, no variation was found in this region.

Using -11187G>A, -3A>C, IVS12-1G>T and 9 nonsynonymous variations, diplotype configuration was estimated for each subject. The configuration was estimated with >0.99 probabilities for all but four subjects. The predicted haplotype frequencies for **1b* [harboring 388A>G (Asn130Asp)], **5* [harboring 521T>C (Val174Ala)], **15* [harboring 388A>G (Asn130Asp) and 521T>C (Val174Ala)] and **17* [harboring -11187G>A, 388A>G (Asn130Asp), and 521T>C (Val174Ala)] were 0.469, 0.000 (not detected), 0.037 and 0.133, respectively. The haplotype frequencies for **1b* and **5* are similar to those in the previous studies in Japanese.[5,11] The **17* frequency is higher than those in Chinese (0.085), Finnish Caucasians (0.069), Malay (0.029) and Indians (0.009).[15,16] It should be noted that 76% (n=47 alleles) of 521T>C (Val174Ala)-bearing haplotypes were assigned as **17*, and 21% (n=13) of them as **15*. The remaining two (3%) was estimated to exist with 1007C>G (Pro336Arg) and **17* variations [-11187G>A, 388A>G (Asn130Asp), and 521T>C (Val174Ala)] on the same chromosomes. The **17* ratio in 521T>C (Val174Ala)-bearing haplotypes is similar to that in Chinese (65%), but higher than those in Finnish Caucasians (34%), Malay (26%) and Indians (14%).[15,16] Variation 452A>G (Asn151Ser, n=12 alleles) or 1738C>T (Arg580Stop, n=3) were predicted to be on the **1a* background (no other variation).

In conclusion, 62 genetic variations were identified, including 28 novel ones, in *SLCO1B1*. One novel nonsynonymous variation results in a truncated protein and four novel nonsynonymous variations result in amino acids substitutions. In addition, novel variations IVS12-1 G>T at the splice acceptor site and -3A>C in the Kozak motif were detected. Approximately 76% of 521T>C (Val174Ala)-bearing haplotypes were assigned as **17* and the majority of the remaining haplotypes were **15*. This information would be useful for pharmacogenetic studies to investigate the associations of *SLCO1B1* variations with interindividual differences in drug disposition.

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ORIGINAL ARTICLE

Impact of *CYP3A4* haplotypes on irinotecan pharmacokinetics in Japanese cancer patients

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Abstract
Background and purpose Cytochrome P450 3A4 (CYP3A4) converts an anticancer prodrug, irinotecan, to inactive metabolites such as APC. However, the contribution of *CYP3A4* genetic polymorphisms to irinotecan pharmacokinetics (PK) and pharmacodynamics (PD) is not fully elucidated. In paclitaxel-administered cancer patients, an association of *CYP3A4*16B* harboring the low activity allele **16* [554C > G (Thr185Ser)] has been shown with altered metabolite/paclitaxel area under the plasma concentration–time curve (AUC) ratios, suggesting a possible impact of **16B* on the PK of other drugs. In this study, the effects of *CYP3A4* haplotypes including **16B* on irinotecan PK/PD were investigated in irinotecan-administered patients.
Methods The *CYP3A4* genotypes for 177 Japanese cancer patients who received irinotecan were defined in terms of

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4 major haplotypes, i.e., *1A* (wild type), **1G* (IVS10 + 12G > A), **16B* [554C > G (Thr185Ser) and IVS10 + 12G > A], and **18B* [878T > C (Leu293Pro) and IVS10 + 12G > A]. Associations of *CYP3A4* genotypes with irinotecan PK and severe toxicities (grade 3 diarrhea and grade 3 or 4 neutropenia) were investigated.

Results Area under the concentration–time curve ratios of APC/irinotecan, an in vivo parameter for CYP3A4 activity, were significantly higher in females than in males. The male patients with **16B* showed significantly decreased AUC ratios (APC/irinotecan) with 50% of the median value of the *non-*16B* male patients (no **16B*-bearing female patients in this study), whereas no significant alteration in the AUC ratios was observed in the patients with **18B*. A slight trend toward increasing AUC ratios (20%) was detected in both male and female patients bearing **1G*. Multivariate analysis confirmed contributions of *CYP3A4*16B* (coefficient ± SE = −0.18 ± 0.077, $P = 0.021$) and **1G* (0.047 ± 0.021, $P = 0.029$) to the AUC ratio. However, no significant association was observed between the *CYP3A4* genotypes and total clearance of irinotecan or toxicities (severe diarrhea and neutropenia).

Conclusion This study suggested that *CYP3A4*16B* was associated with decreased metabolism of irinotecan to APC. However, the clinical impact of *CYP3A4* genotypes on total clearance and irinotecan toxicities was not significant.

Keywords CYP3A4 · Haplotype · Irinotecan · Pharmacogenetics

Introduction

Human cytochrome P450 3A4 (CYP3A4) is a major CYP enzyme, abundant in the liver and intestine, and is involved in the metabolism of endogenous substances, including steroid hormones, and a variety of exogenous compounds such as environmental chemicals and pharmaceuticals. Large inter-individual differences in liver and intestinal CYP3A4 expression levels are known and thought to be caused by multiple factors including genetic variations, disease status, and modulation by exogenous stimuli, such as smoking, diet, and drugs [5, 18, 31]. The tissue-specific CYP3A4 expression is regulated by constitutive and inducible mechanisms via activation of the nuclear receptors, pregnane X receptor (PXR), constitutive androstane receptor (CAR), and vitamin D receptor (VDR) [5, 18]. Since approximately half of clinical drugs currently in use are metabolized by CYP3A4 [5, 33], it is important to find suitable biomarkers, including genetic polymorphisms, which can reflect in vivo CYP3A4 activity and predict individual responses to CYP3A4-metabolized drugs. Recent progress in pharmacogenetic research has led to the accumulation of knowledge about *CYP3A4* genetic variations responsible for altered expression or function. To date, more than 30 *CYP3A4* variations have been identified (http://www.cypalleles.ki.se/cyp3a4.htm), and large ethnic differences in their frequencies have been recognized. *CYP3A4*1B* (−392A > G), a single nucleotide polymorphism (SNP) in the 5′-flanking region, is found in Caucasians (2–9.6%) and African–Americans (35–67%), but not in Asians [16]. As relatively frequent coding SNPs, **2* [664T > C (Ser222Pro)] (2.7%) and **17* [566T > C (Phe189Ser)] (2%) were detected in Caucasians; **10* [520G > C (Asp174His)] in Caucasians (0.24–2%) and Mexicans (5%); **15* [485G > A (Arg162Gln)] (2–4%) in African–Americans; **16* [554C > G (Thr185Ser)] in East Asians (1.4–5%) and Mexicans (5%); **18* [878T > C (Leu293Pro)] (2.3–10%) in East Asians [2, 4, 17, 24]. We previously identified 25 *CYP3A4* haplotypes in a Japanese population [4]. The haplotypes **6* [including 830_831insA (Glu277fsX8)] (0.1%), **11* [including 1088C > T (Thr363Met)] (0.2%), **16B* [including 554C > G (Thr185Ser)] (1.4%), and **18B* [including 878T > C (Leu293Pro)] (2.8%) were identified, but **1B* (−392A > G) was not found. These findings indicate that ethnic-specific *CYP3A4* haplotypes must be taken into consideration in pharmacogenetic studies.

Irinotecan, an anticancer prodrug, is used for treatment of various cancers including lung and colon, and metabolized by CYP3A4 to produce inactive compounds such as APC (a major CYP3A4-mediated product) and NPC (a minor product) [6, 7]. An active metabolite SN-38 (a topoisomerase I inhibitor) is produced from the parent compound by carboxylesterases (CES) [28] and subsequently glucuronidated by UDP-glucuronosyltransferase 1As (UGT1As) to form inactive compound SN-38G [12] (Fig. 1). The parent compound and its metabolites are mainly excreted into the bile [29], where several ABC transporters, such as P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), and multidrug resistance-associated protein 2 (MRP2) are involved in excretion [30]. The dose-limiting toxicities of irinotecan are severe diarrhea and neutropenia, and high plasma concentrations of SN-38 and/or its accumulation in tissues are thought to cause these toxicities [3, 30]. Recent extensive pharmacogenetic studies on irinotecan, mostly focusing on the *UGT1A1* genotypes, have revealed important roles for *UGT1A1*28* and **6* in reduced in vivo UGT activity and enhanced toxicities [1, 8, 9, 11, 13, 22, 26]. On the other hand, CYP3A4 can modulate irinotecan pharmacokinetics (PK). Co-administration of ketoconazole, a CYP3A4 inhibitor and also a potent UGT1A1 inhibitor [34], with irinotecan resulted in a decreased value of the area under the concentration–time curve (AUC) for APC and also increased AUC for SN-38 [14]; and vice versa, co-administration of St. John's Wort,

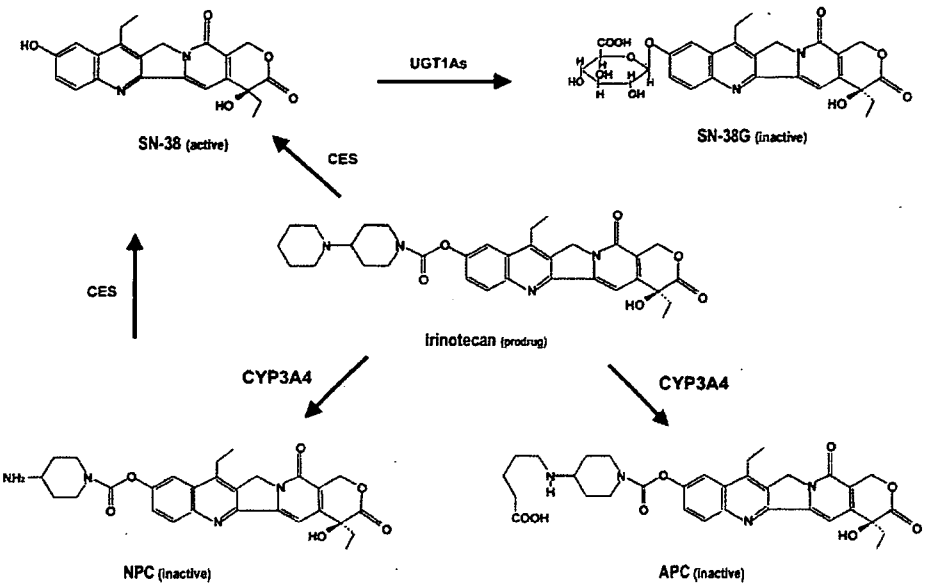


Fig. 1 Irintoecan metabolism in human liver. CYP3A4 mediates oxidation of irinotecan to produce inactive compounds, such as APC (a major CYP3A4-mediated product) and NPC (a minor product)

a CYP3A4 inducer, decreased the AUC of SN-38 [19]. A close association was also reported between in vivo CYP3A4 phenotypes and irinotecan clearance [21]. To date, however, no clinical impact by *CYP3A4* polymorphisms, such as **1B* (−392A > G) and **3* [1334T > C (Met445Thr)], has been demonstrated on irinotecan PK in Caucasians [20]. We previously found that **16* [554C > G (Thr185Ser)] caused decreased in vitro CYP3A4 activities [23]. Furthermore, a significant association of **16B* [harboring 554C > G (Thr185Ser)] was demonstrated with decreased AUC ratios of metabolite/paclitaxel, an in vivo parameter of CYP3A4 activity, in paclitaxel-administered Japanese patients [24].

In this study, to determine the clinical impact of the *CYP3A4* polymorphisms on irinotecan therapy, we identified the *CYP3A4* diplotypes of 177 Japanese cancer patients who received irinotecan and analyzed associations of the *CYP3A4* genotypes with irinotecan PK and toxicities.

Materials and methods

Patients and irinotecan treatment

One hundred seventy-seven patients with cancers who started irinotecan-containing therapy from 2002 to 2004 at two National Cancer Center Hospitals (Tokyo and Kashiwa, Japan) were enrolled for this pharmacogenetic study on irinotecan. This study was approved by the ethics committees of the National Cancer Center and the National Institute of Health Sciences, and written informed consent was obtained from all participants. No participant received irinotecan previously, and other eligibility criteria included: bilirubin < 2 mg/dl, aspartate aminotransferase (GOT) < 105 IU/l, alanine aminotransferase (GPT) < 120 IU/l, creatinine < 1.5 mg/dl, white blood cell count > 3000/μl, performance status of 0–2, and an interval of at least 4 weeks after the last session of chemotherapy (2 weeks after the last session of radiotherapy). Exclusion criteria were diarrhea, active infection, intestinal paralysis or obstruction, and interstitial pneumonitis. Irinotecan was administered as a single agent or in combination chemotherapy at the discretion of attending physicians. Doses and schedules were applied according to the approved treatment recommendations in Japan: intravenous 90-min infusion at a dose of 100 mg/m^2 weekly or 150 mg/m^2 biweekly for irinotecan-monotherapy, and 60 mg/m^2 weekly for combination therapy with cisplatin. Profiles of the patients and irinotecan regimens are summarized in Table 1.

Genotyping of *UGT1A1* and *CYP3A4*

DNA was extracted from pretreatment whole-blood samples taken from 177 patients who received irinotecan. Data on *UGT1A1* genetic polymorphisms obtained from the same set of DNA samples have been published elsewhere [22]. The *CYP3A4* genotypes for 88 patients were previously determined [4]. Additional *CYP3A4* genotyping for the remaining 89 patients was conducted using the pyrosequencing method described previously [24], and the *CYP3A4* diplotypes/haplotypes [4] were inferred using an expectation-maximization-based program, LDSUPPORT [15].

Pharmacokinetics and toxicities

Pharmacokinetic analysis for irinotecan in 176 patients (data on one patient was unavailable) was performed as

Table 1 Profiles of Japanese cancer patients in this study

			No. of patients
Patients for genotyping			177
(Male/female)			(135/42)
Age			
Mean/range	60.5/26–78		
Performance status	0/1/2		84/89/4
Combination therapy, tumor type and initial dose of irinotecan[a]			
Irinotecan monotherapy	Lung	100 (60–100)/w	21
	Colon	150 (120–150)/2w	28
	Others	100 (100–150)/w	7
With platinum-containing drug[b]	Lung	60 (50–90)/w	58
	Stomach	70/2w	9
	Others	60/w	5
With 5-fluorouracil (5-FU)/leucovorin (LV)[c] or tegafur/gimeracil/oteracil potassium[d]	Colon	100 (90–180)/w or 150/2w	34
	Others	90/w or 100/w	2
With mitomycin C (MMC)[e]	Stomach	150/2w	10
	Colon	150/2w	1
With amrubicin[f]	Lung	60/w	2

[a] The median value and range in the parentheses are shown. "/w" and "/2w" represent weekly and biweekly, respectively

[b] Mostly, cisplatin (60 or 80 mg/m^2) was administered after irinotecan treatment

[c] LV (10 mg/m^2) was administered right after irinotecan treatment and then followed by 5-FU treatment. (500 mg/m^2 injection); or LV (200 mg/m^2) was administered simultaneously with irinotecan and followed by 5-FU treatment (400 mg/m^2 bolus injection and 2.0–2.4 g/m^2 infusion)

[d] Tegafur (80 mg/m^2 per day)/gimeracil/oteracil potassium was administered twice (before irinotecan treatment and on the next day)

[e] MMC (5 mg/m^2) was administered just before irinotecan treatment

[f] Amrubicin (30 or 35 mg/m^2) was administered 24 h after irinotecan treatment

previously described [26]. Briefly, heparinized blood was collected before administration of irinotecan, and 0, 0.3, 1, 2, 4, 8, and 24 h after termination of the first infusion of irinotecan. Plasma concentrations of irinotecan and APC were determined by HPLC [25], and AUC_{inf} and other PK parameters were calculated using the trapezoidal method of the 202 non-compartmental model for a constant infusion in WinNonlin ver. 4.01 (Pharsight Corporation, Mountain View, CA, USA). As for the co-administered anti-cancer and other drugs which were administered within 1 week before irinotecan-treatment, no drugs significantly affected the PK parameters related to CYP3A4 activity. Information on foods and drinks taken by the patients which might induce or inhibit CYP3A4 activity was not available.

A complete medical history and data on physical examinations were recorded prior to irinotecan therapy. Complete blood cell counts with differentials and platelet counts, as well as blood chemistry, were measured once a week during the first 2 months of irinotecan treatment. Toxicities were graded according to the Common Toxicity Criteria of National Cancer Institute version 2. Association of genetic factors with irinotecan toxicities was analyzed primarily in patients who received irinotecan as a single agent.

Statistical analysis

Statistical analysis on the differences in PK parameters between sexes and among *CYP3A4* genotypes was performed using the Mann–Whitney test or Kruskal–Wallis test, and associations of *CYP3A4* genotypes with the irinotecan toxicities were assessed by the Chi-square test, using Prism version 4.0 (GraphPad Prism Software Inc. San Diego, CA, USA). $P = 0.05$ (two-tailed) was set as a significant level of difference. Multivariate analysis for the log-transformed AUC ratio (APC/irinotecan) was performed using age, sex, body surface area, dosage of irinotecan, history of smoking or drinking, performance status, co-administered drugs, serum biochemistry parameters at baseline, and genetic factors (including *CYP3A4* haplotypes and the *UGT1A1*6 or *28* haplotype obtained in our previous study [22]) as independent variables. Multivariate analysis on toxicities (grade 3 diarrhea or nadir of absolute neutrophil counts) was conducted for the patients who received irinotecan monotherapy, where the variables included dosing interval and the absolute neutrophil count at baseline, in addition to the other patient background and genetic factors described above. The variables in the final

models for both AUC ratio and toxicities were chosen by the forward and backward stepwise procedure at the significance level of 0.1 using JMP version 6.0.0 software (SAS Institute, Inc., Cary, NC, USA).

Results

Sex difference in PK parameters

Since hepatic CYP3A4 levels were reported to be significantly higher in females than in males [24, 32], we first analyzed the sex differences in the major PK parameters for irinotecan and APC, a major CYP3A4 metabolite (Table 2). As for irinotecan, lower total clearance and MRT, and higher AUC/dose were observed in females, but the differences (3, 5 and 3%, respectively) were not significant. A small but significant increase in C_{max}/dose for irinotecan was observed in females. This is attributable to the smaller distribution volume of females. On the other hand, the median values of AUC/dose and C_{max}/dose for APC of the females were significantly higher than those of the males (1.29- and 1.33-fold, respectively). The AUC ratio (APC/irinotecan), a parameter of in vivo CYP3A4 activity, was significantly higher (1.28-fold) in females than in males. These findings suggest that these differences may reflect the higher CYP3A4 activity in the females.

CYP3A4 genotypes

CYP3A4 diplotypes/haplotypes in 177 Japanese cancer patients were determined according to the previous definition [4]. The *CYP3A4* haplotypes found in this population were **1A* (wild type), **1G* (IVS10 + 12G > A alone), **16B* [554C > G (Thr185Ser) and IVS10 + 12G > A], and **18B* [878T > C (Leu293Pro) and IVS10 + 12G > A]. In the current study, neither **6* [830_831insA (Glu277fsX8)] nor **11* [1088C > T (Thr363Met)] were found. The frequencies of **1G*, **16B*, and **18B* were 0.215, 0.014, and 0.020 (Table 3), and they were comparable to those obtained in previous reports [4, 24]. Note that the haplotypes **16B* and **18B* were detected only in male patients.

Associations of *CYP3A4* genotypes with PK parameters

Considering the significant sex difference in APC levels, associations between the *CYP3A4* genotypes and PK parameters were analyzed for each sex separately. In male patients, no significant differences among the *CYP3A4* genotypes were observed for total clearance and MRT of irinotecan (Fig. 2a, b). In females, a slightly but significantly lower (10%) median value for MRT of irinotecan was observed in patients bearing **1G* compared with those carrying the wild type (**1A*/**1A*) ($P = 0.022$, Mann–Whitney test) (Fig. 2b), whereas no significant **1G*-dependency was observed for total clearance (Fig. 2a). No significant

Table 3 Frequencies of *CYP3A4* haplotypes (A) and diplotypes (B) for Japanese cancer patients in this study

(A) Haplotype group[a]	No. of chromosomes ($N = 354$)	Frequency
**1A*	266	0.751
**1G*	76	0.215
**16B*	5	0.014
**18B*	7	0.020
(B) Diplotype	**No. of patients ($N = 177$)**	**Frequency**
**1A/*1A*	100	0.565
**1G/*1A*	55	0.311
**1G/*1G*	10	0.056
**16B/*1A*	4	0.023
**16B/*1G*	1	0.006
**18B/*1A*	7	0.040

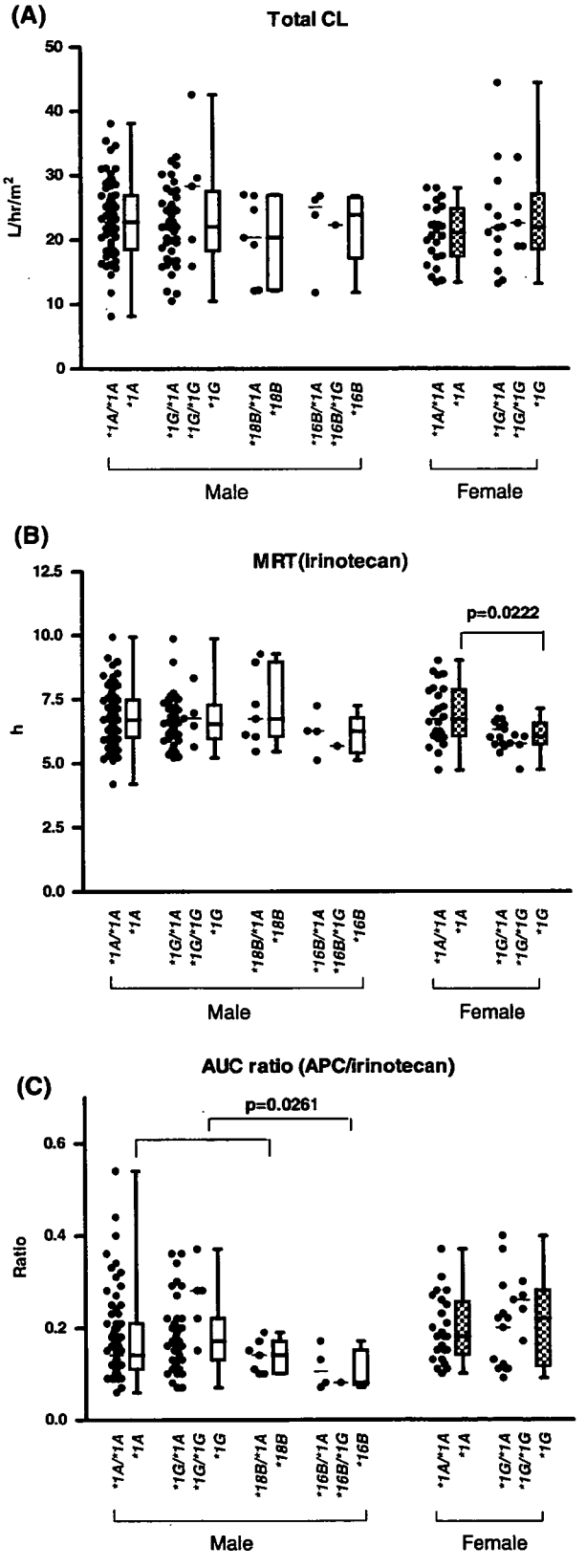
[a] Groups based on tagging SNPs of major haplotypes previously defined [4]; **1A* wild type, **1G* IVS10 + 12G > A; **16B* 554C > G (Thr185Ser) and IVS10 + 12G > A; **18B* 878T > C (Leu293Pro) and IVS10 + 12G > A

Table 2 Pharmacokinetic parameters for irinotecan-administered Japanese patients and sex differences

Parameters	Male ($N = 134$) Median (25–75‰)	Female ($N = 42$) Median (25–75‰)	P value[a]
Irinotecan			
Total CL (l/h per m^2)	22.6 (18.5–26.9)	21.8 (17.8–25.1)	0.242
AUC/dose (10^{-3} h m^2 per l)	44.4 (37.3–54.1)	45.8 (39.8–55.8)	0.242
C_{max}/dose (10^{-3} m^2 per l)	10.0 (8.96–11.3)	11.4 (10.4–12.4)	0.0003
MRT (h)	6.61 (6.01–7.40)	6.29 (5.78–7.12)	0.202
APC			
AUC/dose (10 h m^2 per l)	6.72 (5.23–9.49)	8.66 (6.57–13.1)	0.0071
C_{max}/dose (10^{-3} m^2 per l)	0.560 (0.430–0.805)	0.745 (0.610–1.14)	0.0007
AUC ratio (APC/irinotecan)	0.151 (0.114–0.210)	0.194 (0.132–0.266)	0.0179

CL clearance; *MRT* mean residence time

[a] Mann–Whitney test



◀ **Fig. 2** Association of *CYP3A4* genotypes with irinotecan pharmacokinetics in Japanese cancer patients. The values of mean residence time (MRT) of irinotecan in female patients were significantly lower in those with **1G* than those with the wild-type (**1A/*1A*) ($P = 0.0222$, Mann–Whitney test). The levels of the AUC ratio (APC/irinotecan), a parameter of CYP3A4 activity, in male patients were significantly lower in those with **16B* than those without **16B* ($P = 0.0261$, Mann–Whitney test)

differences in C_{max}/dose for irinotecan among the genotypes were observed in both males and females (data not shown). Regarding the AUC ratio (APC/irinotecan) in males, a significantly lower median value (50%) was observed in patients with **16B* than patients without **16B* (i.e., *non-*16B* patients) ($P = 0.0261$, Mann–Whitney test) (Fig. 2c). In contrast, no significant changes in the AUC ratio (APC/irinotecan) were detected in the male **18B* heterozygotes. In both males and females, a higher median AUC ratio (20%), without statistical significance, was observed in **1G*-bearing patients (**1G/*1A* and **1G/*1G*) than wild-type patients (**1A/*1A*). As for C_{max}/dose of APC, similar trends were observed (without statistical significance): 35% decrease in the median value for **16B* compared with *non-*16B*; 10 and 20% increases in males and females, respectively, for **1G* compared with the wild type (data not shown).

Multivariate analysis of PK parameters

To further clarify contributions of the *CYP3A4* polymorphisms to APC generation, multivariate analysis was conducted on the AUC ratio (APC/irinotecan) data, where variables included patient backgrounds, irinotecan regimens, and *CYP3A4* (**1G, *16B* and **18B*) and *UGT1A1 (*6 or *28)* haplotypes. Significant contributions of *CYP3A4*16B* (coefficient ± SE = -0.18 ± 0.077, $P = 0.021$) and **1G* (0.047 ± 0.021, $P = 0.029$) to the AUC ratio (APC/irinotecan) were confirmed, in addition to the contributions of two patient background factors, sex (female) and hepatic function (serum GOT and ALP) (Table 4). No significant associations were observed between the *CYP3A4* polymorphisms and total clearance or MRT of irinotecan (data not shown).

Associations of *CYP3A4* genotypes with toxicities

Severe irinotecan toxicities, grade 3 diarrhea and grade 3 or 4 neutropenia, were monitored in 176 patients during 2 months after starting irinotecan therapy. Since incidences of severe toxicities depended on the irinotecan regimens used and a higher incidence of severe neutropenia with comedication was evident [22], associations of the *CYP3A4*

Table 4 Multivariate analysis of AUC ratio (APC/irinotecan)

Variable	Coefficient	SE	*P* value
Female	0.040	0.016	0.0132
Serum GOT and ALP[a]	0.110	0.021	<0.0001
Serum creatinine[b]	0.132	0.071	0.0651
*CYP3A4*16B*	−0.180	0.077	0.0213
*CYP3A4*1G*	0.047	0.021	0.0291

The values after logarithmic conversion were used

R^2 0.225; Intercept −0.794; *N* 176

[a] Grade 1 or greater scores in both serum GOT and ALP before irinotecan treatment

[b] The absolute value (mg/dl) before irinotecan treatment

haplotypes with toxicities were evaluated in patients who received irinotecan monotherapy. Because there was no sex difference in the incidences of severe toxicities, the patients with irinotecan monotherapy were not stratified by sex. Furthermore, significant contributions of *UGT1A1*6* and **28* to neutropenia were previously demonstrated [22]. Therefore, the incidence of severe neutropenia was also evaluated among the wild-type patients without *UGT1A1*6 or *28* (*UGT −/−*). No significant differences in the incidences of severe diarrhea and neutropenia were observed among the *CYP3A4* diplotypes of all or *UGT −/−* patients with irinotecan monotherapy (Table 5). It must be noted that the **16B*-bearing patient ($N = 1$) treated with irinotecan monotherapy did not experience either toxicity. Similarly, for **1G* and **18B*, no statistically significant change in the neutropenia or diarrhea incidence was observed. Multivariate analysis also revealed no significant contribution of the *CYP3A4* polymorphisms to severe diarrhea (logistic model) or absolute neutrophil count nadir (data not shown).

Table 5 Association of *CYP3A4* genotypes with severe toxicities in irinotecan monotherapy

Diplotype	Diarrhea[a]/total (%)	Neutropenia[b]/total (%)	
	All	All	UGT-/-[c]
**1A/*1A*	3/27 (11.1)	5/27 (18.5)	2/11 (18.2)
**1G/*1A*	2/20 (10.0)	5/20 (25.0)	1/9 (11.1)
**1G/*1G*	0/3 (0.0)	2/3 (66.7)	0/0 (–)
**16B/*1A*	0/1 (0.0)	0/1 (0.0)	0/0 (–)
**18B/*1A*	1/4 (25.0)	2/4 (50.0)	0/1 (0.0)
P value[d]	0.8571	0.289	

[a] Grade 3

[b] Grade 3 or 4

[c] Wild type without *UGT1A1 *6 or *28*

[d] Chi-square test

Discussion

In the current study, the higher in vivo CYP3A4 activity in females than in males [24, 32] was suggested from the CYP3A4-mediated APC formation. Since correlations between in vivo CYP3A4 activity and irinotecan PK parameters have been reported [14, 19, 21], clinical impact of *CYP3A4* polymorphisms on irinotecan PK has been presumed. In this study, we demonstrated for the first time a role of *CYP3A4*16B* [554C > G (Thr185Ser) and IVS10 + 12G > A] in reduced APC generation (Fig. 2; Table 4). This finding is concordant with the findings of our previous studies showing a reduced in vitro activity of CYP3A4 by **16* [23] and altered AUC ratios of metabolite/paclitaxel in paclitaxel-administered Japanese patients bearing **16B* [24]. These findings indicate that *CYP3A4*16* could modulate pharmacokinetics of other drugs which are metabolized by CYP3A4. On the contrary, **18B* [878T > C (Leu293Pro) and IVS10 + 12G > A] did not alter the AUC ratios (APC/irinotecan) in irinotecan-administered patients. This also coincides with our previous finding that showed no clinical impact of **18B* on the metabolite/paclitaxel AUC ratio [24].

In the current study, an increasing trend in the AUC ratios (APC/irinotecan) by **1G* (IVS10 + 12G > A) was detected in both males and females, although their increases were small (20% in the median values). In accordance with this tendency, significant reduction in MRT of irinotecan by **1G* was observed in females, whereas this was not significant in males. At present, the reason of this sex-difference in MRT is not clear. Our previous haplotype analysis of the *CYP3A4* and *CYP3A5* regions revealed that *CYP3A4*1G* is mostly linked to *CYP3A5*1* but rarely to *CYP3A5*3* [3] which is a defective allele [10, 16, 17, 33]. Therefore, there is a possibility that *CYP3A5* polymorphisms rather than *CYP3A4*1G* contribute to irinotecan PK. However, this speculation is unlikely because CYP3A5 produces only a very minor metabolite of irinotecan, a de-ethylated product [27]. Since the effect of **1G* was relatively small and was not shown in case of paclitaxel [23], the clinical importance of **1G* should be further evaluated in pharmacogenetic studies on other drugs.

Contrary to the clear reduction in APC production, changes in the PK parameters for the parent compound, i.e., total clearance and Cmax of irinotecan, were not affected by the *CYP3A4* haplotypes. Furthermore, multivariate analysis revealed no associations of the *CYP3A4* haplotypes with the AUC ratio of (SN-38 + SN-38G)/irinotecan, an in vivo parameter for CES activity, and with the AUC ratio of SN-38 (SN-38/irinotecan) (data not shown). We previously observed that the total clearance of irinotecan was affected by other non-genetic factors, such as age, smoking, hepatic and renal functions, and co-administered drugs