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Title

Haplotypes and a novel defective allele of *CES2* found in a Japanese population

Su-Ryang Kim, Kimie Sai, Toshiko Tanaka-Kagawa, Hideto Jinno, Shogo Ozawa, Nahoko Kaniwa, Yoshiro Saito, Akira Akasawa, Kenji Matsumoto, Hirohisa Saito, Naoyuki Kamatani, Kuniaki Shirao, Noboru Yamamoto, Teruhiko Yoshida, Hironobu Minami, Atsushi Ohtsu, Nagahiro Saijo, and Jun-ichi Sawada

Project Team for Pharmacogenetics (S.-R.K., K.Sa., H.J., S.O., N.Kan., Y.S., J.S.), Division of Biosignaling (K.Sa.), Division of Environmental Chemistry and Exposure Assessment (T.T.-K., H.J.), Division of Pharmacology (S.O.), Division of Medicinal Safety Sciences (N.Kan.), Division of Biochemistry and Immunochemistry (Y.S., J.S.), National Institute of Health Sciences, Tokyo, Japan; Department of Allergy and Immunology, National Research Institute for Child Health and Development (K.M., H.S.), National Children's Medical Center (A.A.), National Center for Child Health and Development, Tokyo, Japan; Division of Genomic Medicine (N.Kam.), Department of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, Tokyo, Japan; Division of Internal Medicine (K.Sh., N.Y.), National Cancer Center Hospital, Genetics Division (T.Y.), National Cancer Center Research Institute, Tokyo, Japan; and Division of Oncology/Hematology (H.M.), Division of GI Oncology/Digestive Endoscopy (A.O.), Deputy Director (N.S.), National Cancer Center Hospital East, Chiba, Japan.

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Running Title

a) Running Title

Haplotypes and novel variants of human *CES2*

b) Corresponding author

Su-Ryang Kim, Ph.D.,

Project Team for Pharmacogenetics, National Institute of Health Sciences,

1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

Tel: +81-3-5717-3831, Fax: +81-3-5717-3832, E-mail: kim@nihs.go.jp

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d) Abbreviations

SNP, single nucleotide polymorphisms; RT, reverse transcriptase; AUC, area under plasma concentration curve; hCE-2, human carboxylesterase 2 (EC 3.1.1.1); *CES2*, human carboxylesterase 2 gene

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Abstract

Human carboxylesterase 2 (hCE-2) is a member of the serine esterase superfamily and responsible for hydrolysis of a wide variety of xenobiotic and endogenous esters. hCE-2 also activates an anticancer drug, irinotecan (CPT-11), into its active metabolite, SN-38. In this study, a comprehensive haplotype analysis of the *CES2* gene, which encodes hCE-2, in a Japanese population was conducted. Using 21 single nucleotide polymorphisms (SNPs), including 4 nonsynonymous SNPs, 100C>T (Arg34Trp, **2*), 424G>A (Val142Met, **3*), 1A>T (Met1Leu, **5*), and 617G>A (Arg206His, **6*), and a SNP at the splice acceptor site of intron 8 (IVS8-2A>G, **4*), 20 haplotypes were identified in 262 Japanese subjects. In 176 Japanese cancer patients that received irinotecan, associations of *CES2* haplotypes and changes in a pharmacokinetic parameter, (SN-38+SN-38G)/CPT-11 AUC ratio, were analyzed. No significant association was found among the major haplotypes of the **1* group lacking nonsynonymous or defective SNPs. However, patients with nonsynonymous SNPs, 100C>T (Arg34Trp) or 1A>T (Met1Leu), showed substantially reduced AUC ratios. *In vitro* functional characterization of the SNPs was conducted and showed that the 1A>T SNP affected translational, but not transcriptional, efficiency. These findings are useful for further pharmacogenetic studies on CES2-activated prodrugs.

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Introduction

Human carboxylesterases are members of the serine esterase superfamily and are responsible for hydrolysis of a wide variety of xenobiotic and endogenous esters. They metabolize esters, thioesters, carbamates, and amides to yield soluble acids and alcohols or amines (Satoh and Hosokawa, 1998; Satoh et al., 2002). In the human liver, two major isoforms of carboxylesterase, hCE-1and hCE-2, have been identified (Shibata et al., 1993; Schwer et al., 1997). hCE-2 is a 60-kD monomeric enzyme with a pI value of approximately 4.9, and shares 48% amino acid sequence identity with hCE-1 (Pindel et al., 1997, Schwer et al., 1997, Takai et al., 1997). The *CES2* gene, which encodes hCE-2, is located on chromosome 16q22.1 and consists of 12 exons. Distribution of hCE-2 is relatively limited to several tissues, such as the small intestine, colon, heart, kidney, and liver, while hCE-1 is ubiquitously expressed (Satoh et al., 2002; Xie et al., 2002).

Although both hCE-1 and hCE-2 show broad substrate specificities, hCE-2 is relatively specific for heroin, cocaine (benzoyl ester), 6-acetylmorphine, procaine, and oxybutynin (Satoh et al., 2002; Pindel et al., 1997; Takai et al., 1997). In addition, hCE-2 is reported to play a major role in the metabolic activation of the anti-tumor drug irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxycamptothecin: CPT-11). Irinotecan is a water-soluble derivative of the plant alkaloid camptothecin and widely used for treatment of several types of cancer. Irinotecan is converted to 7-ethyl-10-hydroxycamptothecin (SN-38), a topoisomerase inhibitor, by carboxylesterases (Humerickhouse et al., 2000) and further conjugated by hepatic uridine diphosphate glucuronosyltransferase (UGT) 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

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acid)-1-piperidino]carbonyloxycamptothecin (APC) and 7-ethyl-10-(4-amino-1-piperidino)carbonyloxycamptothecin (NPC) by cytochrome P450 3A4 (Santos et al., 2000, Dodds et al., 1998). Irinotecan and its metabolites are excreted by the efflux transporters, ABCB1 (P-gp), ABCC2 (cMOAT), and ABCG2 (BCRP), 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 were administered irinotecan or steroidal drugs, and 12 novel SNPs, including the nonsynonymous SNP, 100C>T (Arg34Trp), 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 (Val142Met), suggested that the 34Trp and 142Met 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

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its metabolites were analyzed for 177 irinotecan-administered cancer patients. 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.

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Materials and Methods

Chemicals

Irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxycamptothecin: CPT-11), 7-ethyl-10-hydroxycamptothecin (SN-38) and SN-38 glucuronide (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 allergic patients who were administered steroidal drugs and 177 cancer patients who were administered irinotecan. The ethical review board 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 allergic patients and 81 cancer patients were described previously (Kim et al., 2003). Additionally, the *CES2* gene was sequenced from 13 allergic patients and 96 cancer patients. Amplification and sequencing of the *CES2* gene were performed as previously described (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 (LD) and haplotype analyses

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LD analysis was performed by the SNPAlyze software (Ver 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 based on 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 intravenous infusion at doses of 60 to 150 mg/m^2, which varied depending on regimens/co-administered 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).

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Associations between genotypes and pharmacokinetic parameters including the AUC ratio (SN-38+SN-38G)/CPT-11 were evaluated in 176 patients in which PK 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 NCBI 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 a high fidelity DNA polymerase KOD -Plus- (TOYOBO, Tokyo, Japan) and 0.5 μM of the *CES2* specific primers. The PCR conditions were 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 60°C for 30 sec, 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-GAGACCAGCGAGCCGACC<u>T</u>TGCGGCTGCACAGACTTCG-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 Arg206His

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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). Briefly, microsomal fractions (30 μg protein/lane) or post-mitochondrial fractions (0.4 μg protein/lane) were separated by 8% sodium dodecyl sulfate-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 Inc., 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 the ZERO-Dscan software (Raytest, Straubenhardt, Germany). The relative expression levels were shown as the mean ± 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). Following 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

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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 ß-actin were quantified as internal controls using TaqMan ß-actin Control Reagent (Applied Biosystems), and normalization of CES2 mRNA levels were based on ß-actin concentrations.

Enzyme assay

CPT-11 hydrolyzing activity of the post-mitochondrial supernatants (microsomal fraction plus cytosol) was assayed over the substrate concentration range of 0.25 - 50 µM as described previously (Kubo et al., 2005), except that the hydrolysis product, SN-38, was determined by the HPLC method of Hanioka *et al*. (2001).

Statistical analysis

Statistical analysis of the differences in the AUC ratios among *CES2* diplotypes, co-administered 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, GraphPad Software, Inc.) was applied to the comparison of the average values of protein expression and mRNA levels between wild-type and variant CES2.

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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 non-synonymous (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 non-synonymous SNP 424G>A (V142M) reported by our group (Kubo et al., 2005), and another non-synonymous SNP 617G>A (R206H) published in the dbSNP (rs8192924) and JSNP (ssj0005417) databases were found at a frequency of 0.002. Recently, several non-coding 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.

Linkage disequilibrium (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).

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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 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 (Arg34Trp), 424G>A (Val142Met), 1A>T (Met1Leu), and 617G>A (Arg206His), 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* genotypes and AUC ratios [(SN-38+SN-38G)/CPT-11], a parameter of in vivo CES activity (Cecchin et al., 2005), in irinotecan (CPT-11)-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 preliminarily examined the effects of irinotecan dosage and co-medication on the AUC ratio and obtained significant correlations of irinotecan dosage (Spearman r = -0.559, $p < 0.0001$) and co-medication ($p < 0.0001$, Kruskal-Wallis test) with the AUC ratios. Since irinotecan dosages also

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depended on the drugs co-administered (see Materials and Methods), we finally stratified the patients with the co-administered 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 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 to the median AUC ratio of the **1/*1* patients. Three nonsynonymous SNPs, 100C>T (Arg34Trp, **2*), 1A>T (Met1Leu, **5*), and 617G>A (Arg206His, **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 25 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, Met1Leu (**5*) and Arg206His (**6*), the functional analysis was conducted *in vitro*.

In vitro functional analysis of the Met1Leu variant

To clarify the functional significance of the novel variant Met1Leu (*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 NCBI 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 transfected with the expression plasmid pcDNA3.1/CES2L-WT showed that the mobility (about 60 kD) 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-kD 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 Met1Leu variant was functionally deficient.

In vitro functional analysis of the Arg206His variant

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The known nonsynonymous SNP 617G>A changes an arginine to a histidine at residue 206. Western blot analysis of the post-mitochondrial supernatant (including microsomes and cytosol) fractions obtained from COS-1 cells transfected with wild-type (CES2S) and Arg206His variant CES2-expressing plasmids showed that the protein expression level of the Arg206His 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 Arg206His variant CES2. 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.

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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 (Arg34Trp), 424G>A (Val142Met), 1A>T (Met1Leu), and 617G>A (Arg206His), 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 (Arg34Trp) and 424G>A (Val142Met), has already been performed to identify effects of these SNPs on expression levels and carboxylesterase activity. Kubo *et al*. (2005) showed that Arg34Trp and Val142Met variants had little carboxylesterase activity toward irinotecan, *p*-nitrophenyl acetate, and 4-methylumbelliferyl acetate, whereas expression levels of these variants were higher than 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 (Arg34Trp), 424G>A (Val142Met), 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 open reading frame (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

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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 (Arg206His) was also investigated. The Arg206 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 Arg206His 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

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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 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 co-administered 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

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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 (Arg34Trp, **2*), 1A>T (Met1Leu, **5*), and 617G>A (Arg206His, **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 Arg34Trp or Met1Leu 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 (Arg206His), the AUC ratio of the patient that 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 76 ± 5%, and the Arg206His substitution itself showed no functional differences in intrinsic enzyme activity by *in vitro* functional analysis. Thus, the impact of the 617G>A (Arg206His) 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.

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Since the patients with **4*, **5* or **6* were found as single heterozygote, 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 towards enzymatic levels or activity. *In vitro* functional analysis for nonsynonymous SNPs showed that the 1A>T (Met1Leu) 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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Footnotes

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Figure legends

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 thus ambiguous.

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).

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 post-mitochondrial 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 in the Materials and Methods. A representative result from one of three independent experiments is shown. HLM,

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human liver microsomes.

Fig. 4. Quantification of CES2 mRNA by TaqMan real-time RT-PCR in COS-1 cells transfected with wild-type 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).

Table 1. Summary of SNPs in the *CES2* gene in a Japanese population

SNP ID				Position				
This study	NCBI (dbSNP)	JSNP	Location	NT_010498.15	From the translational initiation site or from the nearest exon	Nucleotide change and flanking sequences (5' to 3')	Amino acid change	Allele frequency
MPJ6_CS2001			5'-Flanking	20582067	-1671[b]	CTGGAACAACTC**G/C**CTCCCCTCGGAA		0.010
MPJ6_CS2002			5'-Flanking	20582484	-1254[b]	AACCACCACCGC**T/C**GATCCTAGCAGG		0.002
MPJ6_CS2016[a]			5'-Flanking	20582505	-1233[b]	CAGGCGTGCGCT**T/C**CCGCTCCAACCC		0.002
MPJ6_CS2003			Exon 1 (5'-UTR)	20582979	-759[b]	AAATGTTTGTCA**A/G**GTGGATAAATGA		0.006
MPJ6_CS2004	rs11075646		Exon 1 (5'-UTR)	20583375	-363[b]	CCTCCTATCGAT**C/G**CCCCAGCGCGCT		0.031
MPJ6_CS2017[a]			Exon 1	20583738	1[b]	AGCGAGCCGACC**A/T**TGCGGCTGCACA	Met1Leu	0.002
MPJ6_CS2005			Exon 2	20586162	100[b]	GCCAGTCCCATC**C/T**GGACCACACACA	Arg34Trp	0.002
MPJ6_CS2021[a]			Intron 2	20587248	IVS2-71	GGTGGCTGGGAG**C/G**ACCTCTGAACCC		0.002
MPJ6_CS2015			Exon 4	20588325	424[b]	TGATTTCCCCAG**G/A**TGATGGTGTGGA	Val142Met	0.002
MPJ6_CS2006			Intron 4	20588486	IVS4+29	GCTGGGCAACCC**G/A**GGCTGAGCGGGG		0.002
MPJ6_CS2007			Exon 5	20588560	579[b]	CAAGCACGCAAC**C/T**GGCAACTGGGGC	Thr193Thr (silent)	0.002
MPJ6_CS2018	rs8192924	ssj0005417	Exon 5	20588598	617[b]	TGGCTGCACTAC**G/A**CTGGGTCCAGCA	Arg206His	0.002
MPJ6_CS2008			Exon 5	20588746	765[b]	CATGGAGAGTGG**C/T**GTGGCCCTCCTG	Gly255Gly (silent)	0.002
MPJ6_CS2009			Intron 5	20589157	IVS5-69	CCTGTTCTTGGC**C/T**AGGGCCTTGGGC		0.017
MPJ6_CS2019[a]			Intron 7	20589775	IVS7+27	AAGCCCACAAGT**G/A**CCTGGGGAGCCC		0.002
MPJ6_CS2010			Intron 7	20589845	IVS7-25	CCCATCCCCAGC**T/A**ACAGACTCTCTC		0.002
MPJ6_CS2011			Intron 8	20590205	IVS8-2	TCCACCTGGGGT**A/G**GATGTTGCCTCC	splicing defect	0.002
MPJ6_CS2020[a]			Intron 9	20590429	IVS9+78	ACCTGCTGCTGT**C/T**CGGGTCAGCACT		0.002
MPJ6_CS2012	rs2241409	IMS-JST1013275	Intron 10	20591293	IVS10-108	GGAAGAAAAAGC**G/A**GAGAAGCAGGAC		0.269
MPJ6_CS2013	rs28382825		Intron 10	20591314	IVS10-87	GGACTGGGGACC**G/A**AGGTCTCGGGGG		0.031
MPJ6_CS2014	rs8192925	ssj0005418	Exon 12 (3'-UTR)	20592196	1749 (*69)[b]	GTGCCCACACAC**A/G**CCCACTAAGGAG		0.239

[a]Novel variations detected in this study.

[b]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.

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Table 2. Kinetic parameters of CPT-11 hydrolysis by wild-type and Arg206His variant CES2 expressed in COS-1 cells[a]

CES2	Apparent K_m (μM)	V_{max} (pmol/min/mg protein)	V_{max}/K_m (nl/min/mg protein)	Normalized V_{max} [b] (pmol/min/mg protein)	Normalized V_{max}/K_m [b] (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
Arg206His	0.51 ± 0.02[‡]	2.81 ± 0.22[†]	5.53 ± 0.52[‡]	3.44 ± 0.16	6.77 ± 0.46

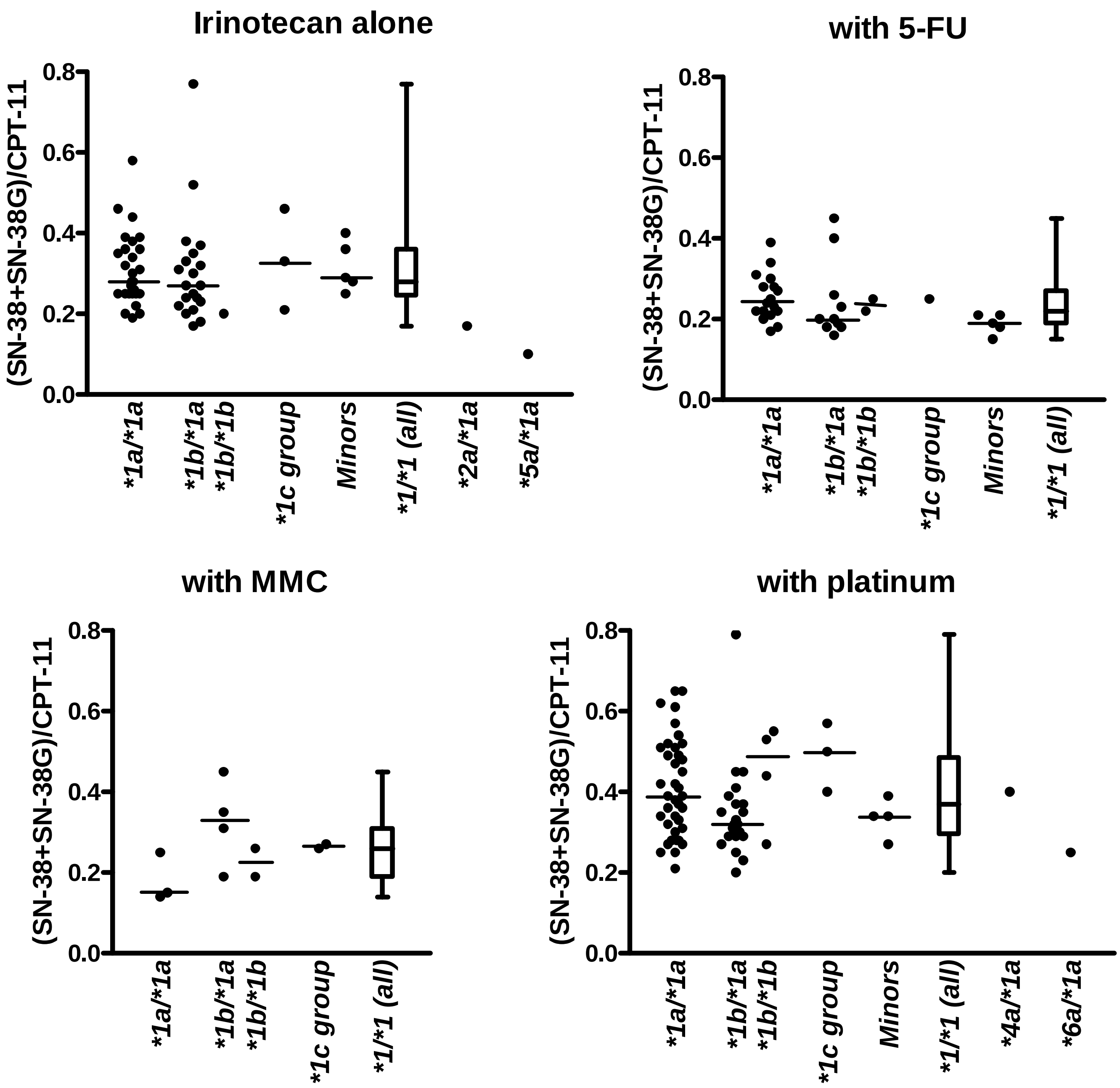
[a]Results are expressed as the mean ± SD from four independent transfection experiments. † and ‡, significantly different from that of the wild-type at $p < 0.05$ and $p < 0.01$, respectively.

[b]V_{max} values were normalized by the relative protein expression level of the Arg206His variant (0.82 ± 0.07).

Fig. 1

	Position	5'-flanking	5'-flanking	5'-flanking	5'-UTR	5'-UTR	Exon 1	Exon 2	Intron 2	Exon 4	Intron 4	Exon 5	Exon 5	Exon 5	Intron 5	Intron 7	Intron 7	Intron 8	Intron 9	Intron 10	Intron 10	3'-UTR			
	Nucleotide change	−1671 G>C	−1254 T>C	−1233 T>C	−759 A>G	−363 C>G	1 A>T	100 C>T	IVS2−71 C>G	424 G>A	IVS4+29 G>A	579 C>T	617 G>A	765 C>T	IVS5−69 C>T	IVS7+27 G>A	IVS7−25 T>A	IVS8−2 A>G	IVS9+88 C>T	IVS10−108 G>A	IVS10−87 G>A	1749(*69) A>G			
	Effect on protein						M1L	R34W		V142M		T193T (silent)	R206H	G255G (silent)				splicing defect							
Haplotype group	Haplotype																						Number	Frequency	
*1	*1a																						357	0.681	
*1	*1b																						122	0.233	
*1	*1c																						14	0.027	
*1	*1d																						9	0.017	
*1	*1e																						5	0.010	
*1	*1f																						3	0.006	
*1	*1g																						1	0.002	
*1	*1h																						1	0.002	0.990
*1	*1i																						1	0.002	
*1	*1j																						1	0.002	
*1	*1k																						1	0.002	
*1	*1l																						1	0.002	
*1	*1m#																						1	0.002	
*1	*1n#																						1	0.002	
*1	*1o#																						1	0.002	
*2	*2a																						1	0.002	0.002
*3	*3a																						1	0.002	0.002
*4	*4a																						1	0.002	0.002
*5	*5a																						1	0.002	0.002
*6	*6a#																						1	0.002	0.002
																						Total	524	1.000	1.000

Fig. 2



Irinotecan alone
(SN-38+SN-38G)/CPT-11
0.0
0.2
0.4
0.6
0.8
*1a/*1a
*1b/*1a
*1b/*1b
*1c group
Minors
*1/*1 (all)
*2a/*1a
*5a/*1a
with 5-FU
(SN-38+SN-38G)/CPT-11
0.0
0.2
0.4
0.6
0.8
*1a/*1a
*1b/*1a
*1b/*1b
*1c group
Minors
*1/*1 (all)
with MMC
(SN-38+SN-38G)/CPT-11
0.0
0.2
0.4
0.6
0.8
*1a/*1a
*1b/*1a
*1b/*1b
*1c group
*1/*1 (all)
with platinum
(SN-38+SN-38G)/CPT-11
0.0
0.2
0.4
0.6
0.8
*1a/*1a
*1b/*1a
*1b/*1b
*1c group
Minors
*1/*1 (all)
*4a/*1a
*6a/*1a

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Fig. 3

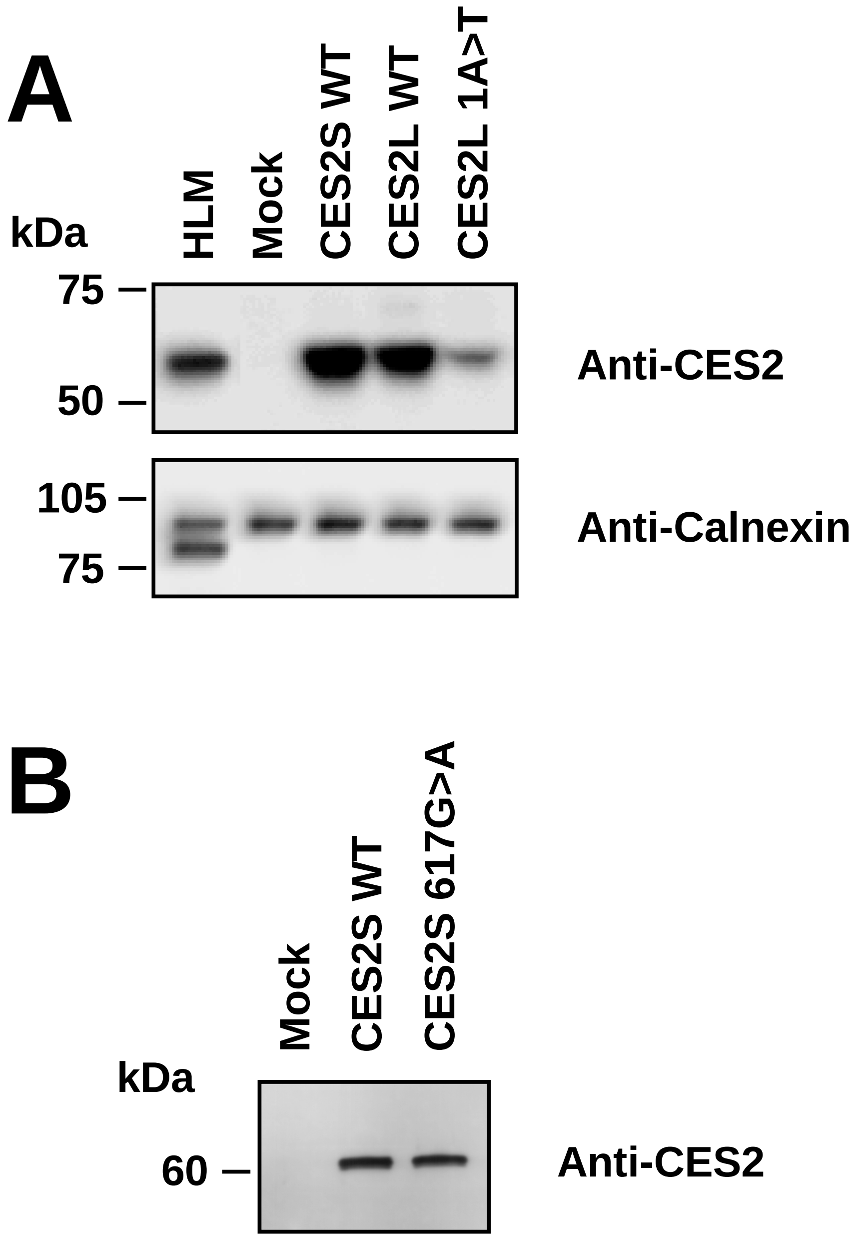

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Fig. 4