

Title

Functional Characterization of Three Naturally Occurring Single Nucleotide Polymorphisms in the *CES2* gene encoding carboxylesterase 2 (hCE-2).

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Running Title

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Three Naturally Occurring SNPs in the *CES2* encoding hCE-2.

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d) Abbreviations

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

Abstract

Twelve single nucleotide polymorphisms (SNPs) in the human *CES2* gene, which encodes a carboxylesterase, hCE-2, have been reported in the Japanese (Kim et al., *Drug Metab Pharmacokinet* 18:327-332, 2003). In this report, we have examined functional alterations of three SNPs, a nonsynonymous SNP (100C>T, R34W), an SNP at the splice acceptor site in intron 8 (IVS8-2A>G), and one newly discovered nonsynonymous SNP (424G>A, V142M). For the two nonsynonymous SNPs, the corresponding variant cDNAs were expressed in COS-1 cells. Both the R34W and V142M variants showed little esterase activities toward the anti-cancer agent irinotecan and 2 typical carboxylesterase substrates, *p*-nitrophenol acetate and 4-methylumbelliferyl acetate, although increased levels of cDNA-mediated protein expression were observed by Western blotting as compared with the wild-type. To investigate a possible splicing aberration in IVS8-2A>G, an *in vitro* splicing assay was utilized and transcripts derived from *CES2* gene fragments of the wild-type and IVS8-2A>G were compared. Sequence analysis of the cloned transcripts revealed that IVS8-2A>G yielded mostly aberrantly spliced transcripts, including a deleted exon or a 32-bp deletion proximal to the 5' end of exon 9, which resulted in truncated hCE-2 proteins. These results suggested that 100C>T (R34W), 424G>A (V142M), and IVS8-2A>G are functionally deficient SNPs.

Introduction

Human carboxylesterases are members of serine esterases, metabolize ester, thioester, carbamate, and amide and yield soluble acids and alcohols or amines (Saitoh and Hosokawa, 1998; Satoh et al., 2002). Two major isoforms of human carboxylesterase, hCE-1 and hCE-2, have been identified in the liver (Shibata et al., 1993; Schwer et al., 1997). The *CES2* gene encoding hCE-2 is located on chromosome 16q22.1 and consists of 12 exons (Fig. 1). hCE-2 has been shown to be expressed in relatively limited tissues, including the small intestine, colon, heart, kidney, and liver, while hCE-1 is ubiquitously distributed (Satoh et al., 2002; Xie et al., 2002). hCE-2 is relatively specific for heroin, cocaine (benzoyl ester), 6-acetylmorphine, procaine, and oxybutynin, although both isoforms show broad substrate specificities (Satoh et al., 2002; Pindel et al., 1997; Takai et al., 1997). hCE-2 has also been shown to catalyze the conversion of the anti-tumor drug, irinotecan, into its active metabolite SN-38 (7-ethyl-10-hydroxy-camptothecin) (Takai et al., 1997; Humerickhouse et al., 2000). Data on the hepatic hydrolyzing activities of hCE-2 toward irinotecan revealed remarkable inter-individual difference (Xu et al., 2002). With regard to genetic polymorphisms including single nucleotide polymorphisms (SNPs) in the *CES2* gene, it has recently been shown that the allele and haplotype frequencies are significantly different between Europeans and Africans (Marsh et al., 2004). In our previous study, we found a number of SNPs, including a nonsynonymous SNP (100C>T causing R34W, MPJ6_CS2005) and an SNP at the splice acceptor site of intron 8 (IVS8-2A>G, MPJ6_CS2011) in the course of screening *CES2* SNPs from 153 Japanese individuals, who were administered irinotecan or beclomethasone (Kim et al., 2003). Additional nonsynonymous SNPs (424G>A, V142M, MPJ6-CS2015) have been discovered in

further screening of *CES2* SNPs in Japanese allergic patients (Fig. 1).

As the large ethnic differences of the *CES2* SNP frequencies pointed out by Marsh et al. (Marsh et al., 2004), the SNPs we found have not been elaborated in the Marsh's literature, suggesting that the ones found by us were less frequent in Europeans and Africans.

In the present study, we performed functional characterization of the two nonsynonymous SNPs using heterologous cell expression systems. To investigate the effect of SNP IVS8-2A>G on RNA splicing, a minigene assay was adopted. The results indicated that the 2 hCE-2 variants (R34W and V142M) almost completely lost the enzymatic activities toward irinotecan and 2 typical carboxylesterase substrates, *p*-nitrophenol acetate and 4-methylumbelliferyl acetate. The exon-intron junction SNP, IVS8-2A>G, was associated with aberrant splicing.

Materials and Methods

SNP detection

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

Construction of plasmids for a COS-1 cell expression system

Wild-type *CES2* cDNA was obtained by the PCR amplification of a first strand cDNA synthesized by a reverse transcriptase (RT) reaction from Human Liver PolyA+ RNA (Clontech, Palo Alto, CA) using *CES2* specific primers (5'CTGGATCCGACCATGCGGCTGCACAG3' and 5'ACAGGGAGCTACAGCTCTGTGT3', forward and reverse primers, respectively). The PCR was performed with 1.25 units of AmpliTaq Gold (Applied Biosystems) for 95 ° C for 10 min, followed by 30 cycles of 95 ° C for 30 sec, 60 ° C for 1 min, and 72 ° C for 2 min. The resultant PCR products were cloned into a pCR3.1 vector by the TA cloning procedure (Invitrogen, Carlsbad, CA) and the sequence was confirmed in both directions. This expression plasmid was designated pCRhCE2/WT and was used as

a template for the preparation of R34W and V142M plasmids. The variant plasmids were generated with a QuickChange PCR site-directed mutagenesis kit (Stratagene, La Jolla, CA). The primers for the respective variations were 5'TCAGCCAGTCCCATCTGGACCACACACACGG3' for R34W and 5'GATCCACACCATCATCGGCAGGTTAGAGCC3' for V142M (mutated sites are underlined). The sequence of each variant cDNA was confirmed.

Construction of plasmids for a His-tagged hCE-2 expression

A histidine (His)-tagged hCE-2 expression plasmid was also constructed to obtain the wild-type hCE-2 protein. A *CES2* cDNA sequence without its signal peptide region was amplified from the pCRhCE2/WT vector by the PCR (Potter PM et al., 1998a, Potter PM et al., 1998b) using a forward primer, 5'CAAGATCTGCTTGTCCGGGGCCAGGGCCA3' (*Bgl*III site is underlined) and a reverse primer, 5'CCGGTACCTACAGCTCTGTGTGTCTCTC 3' (*Kpn*I site is underlined). The amplified fragment was cloned into the pCR3.1 vector. After confirmation of the correct sequence, the cDNA fragment, digested with *Bgl*III and *Kpn*I, was ligated into the pTrcHis B plasmid (Invitrogen) that was predigested with *Bgl*III and *Kpn*I. This newly generated plasmid was designated pHisCES2.

Construction of plasmids for the minigene assay

To construct plasmids for the minigene assay, a wild-type *CES2* gene fragment was amplified by PCR using genomic DNA as a template that was extracted from an irinotecan-administered cancer patient with the wild-type or variant (IVS8-2A>G) *CES2* genes. PCR primers used to amplify a *CES2* gene fragment containing exons 7 to 10

were 5'GCACGCGTGGAGTGGTGGATGGGGTCTTC3' (forward primer, *Mlu*I site is underlined) and 5'GCGTCGACGGCTGATGCTGGA⁸ACTCGTAGA3' (reverse primer, *Sal*I site is underlined). The amplified fragments were cloned into a pCR3.1 vector. After confirmation of the correct sequence, the *CES2* fragment was digested with *Mlu*I and *Sal*I and ligated into pCMV-TnT (Invitrogen) that had been digested with *Mlu*I and *Sal*I. The plasmids containing the wild-type and IVS8-2G *CES2* fragments were designated pCMV-CES2WT and pCMV-CES2IVS8G, respectively. The sequence of the inserts was confirmed.

Protein and mRNA expressions of wild-type and variant hCE-2s in COS-1 cells

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

Purification of His-tagged hCE-2

The expression plasmid pHisCES2 was introduced into *Escherichia coli* strain TOP10

(Invitrogen). Four hours after IPTG induction, the bacterial cells were harvested. The ProBond Purification System (Invitrogen) was used to purify His-tagged hCE-2.9 expressed in TOP10 cells. The purification was performed with a denaturing condition according to the manufacturer's protocol. The purified protein was kept at -80°C in 2% SDS and used as a standard in Western blotting. Protein concentration of the purified His-tagged hCE-2 was quantified colorimetrically using the Protein Assay (Bio-Rad, Hercules, CA) and an hCE-2-specific band was confirmed by Western blotting.

Western blot analysis

Two or four μg of the microsomes from COS-1 cells were resolved by 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Immunochemical detection of each hCE-2 protein was performed using rabbit anti-human hCE-2 raised against a peptide antigen (residues 539-555, KKALPQKIQELEEPEER) (diluted 1:2000). To verify that the samples were evenly loaded, the blot was subsequently treated with stripping buffer and reprobed with a polyclonal anti-calnexin antibody (diluted 1:4000; Stressgen Biotechnologies Inc., San Diego, CA). Visualization of these proteins was achieved with horseradish peroxidase-conjugated donkey anti-rabbit Ig (1:2000) and Enhanced Chemiluminescence-Plus reagents (Amersham Biosciences Inc., Piscataway, NJ). The densities of protein bands were quantified using His-tagged hCE-2 as a standard. Two, four, and eight ng of the His-tagged hCE-2 were applied on the polyacrylamide gels for Western blotting. The amounts of wild-type and the variants were within the range (2-8 ng) of the standard His-tagged hCE-2.

Enzyme assay.

A reaction mixture in a total volume of 200 μ L contained 50 mM potassium phosphate buffer (pH 7.4), and several concentrations (1, 2, 5, 10, 20 and 50 μ M) of irinotecan in the presence of 0.1 mg of microsomal proteins. Reactions were started by the addition of the substrate, incubated at 37°C for 10 min, and then terminated by the addition of 200 μ L of methanol/5% perchloric acid (1:1) containing 0.29 μ M camptothecin (internal standard). For the analysis of irinotecan and its metabolites, chromatographic separation was performed by an HP 1100 model HPLC system equipped with FLD (G1321A; Hewlett Packard, Les Ulis, France). The HPLC analysis was performed as previously described (Sai et al., 2002). Carboxylesterase activity against p-nitrophenyl acetate was assayed colorimetrically. Briefly, a reaction mixture contained 0.5 M Tris-HCl (pH 8.0), and various concentrations (0.039, 0.078, 0.156, 0.313, 0.625, 1.25, 2.5, and 5 mM) of p-nitrophenyl acetate in the presence of microsomes of hCE-2-expressing cells. Initial rate of increase in the OD₄₀₅ was monitored as the production of p-nitrophenol. Catalytic activity was expressed as μ mol/mg microsomal protein/min. Carboxylesterase activity against 4-methylumbelliferyl acetate was measured basically according to the method reported by Pindel et al (Pindel et al., 1997). Briefly, a reaction mixture consisted of 90 mM KH₂PO₄ adjusted at pH 7.3, 40 mM KCl, and various concentrations (0.0625, 0.125, 0.25, 0.5, and 1 mM) of 4-methylumbelliferyl acetate in the presence of microsomes of hCE-2-expressing cells. Initial rate of increase in the OD₃₅₀ was monitored as the production of 4-methylumbelliferone. Catalytic activity was expressed as μ mol/mg microsomal protein/min.

Minigene assay.

HepG2 cells were rinsed with serum-free OPTI-MEM (Invitrogen) before transfection. Either pCMV-CES2WT or pCMV-CES2IVS8G was added to the cells with LipofectAMINE PLUS reagent (Invitrogen). The cells were harvested after 48 h, and total RNA was extracted with an RNeasy Mini Kit (Qiagen, Hilden, Germany). RT-PCR was performed with a GeneAmp RNA PCR Kit (Applied Biosystems, Foster City, CA) using total RNA treated with DNaseI Amplification Grade (Invitrogen) as a template, an intron-skipping primer as a forward primer and a reverse primer having a portion of the exon 10 sequence (Fig. 4A). The intron-skipping primer (5'GAGGCACTGGGCAGGTGTCCACTC3') was designed to cover both the artificial introns of pCMVTnT. RT-PCR products were detected by 3% agarose gel electrophoresis. To analyze transcripts obtained by the minigene assay, the resultant RT-PCR products were cloned into pCR4-TOPO with the TOPO TA Cloning Kit for Sequencing (Invitrogen), and subsequent sequencing of the inserts derived from randomly isolated clones (greater than 100) was performed using an M13 reverse primer.

Results

SNPs in the *CES2* gene

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

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

Protein expression levels and enzymatic activity of R34W and V142M

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

The variants, R34W and V142M, showed only trace HPLC peak for SN-38, indicating they almost completely lost the carboxylesterase activity toward irinotecan. The saturation curves of the Michaelis-Menten kinetics are shown in Fig. 3 that also illustrates the inefficiency of the variants. The apparent kinetic parameters of wild-type hCE-2 were: $1.228 \pm 0.092 \mu\text{M}$ for K_m , $1.458 \pm 0.0495 \text{ pmole/mg protein/min}$ ($91.57 \pm 5.67 \text{ pmol/nmole hCE-2/min}$) for V_{max} , and $74.95 \pm 3.79 \mu\text{l/min/nmole hCE-2}$ for V_{max}/K_m . Their V_{max} values were less than 5.0 in contrast to $91.57 \text{ pmol/nmole hCE-2/min}$ for

the wild-type.

Table 1 summarizes the carboxylesterase function of the wild-type and its variant hCE-2s toward irinotecan together with smaller molecule and typical carboxylesterase substrates, *p*-nitrophenyl acetate and 4-methylumbelliferyl acetate. The catalytic activities toward all the three substrates by R34W and V142M variants were catalytically much less efficient as compared with the wild-type. K_m values of the wild-type for *p*-nitrophenyl acetate and 4-methylumbelliferyl acetate were 0.57 mM and 0.11 mM, respectively. The K_m values including that for irinotecan were roughly similar to those reported by Sanghani et al. (Sanghani et al., 2004) and Pindel et al. (Pindel et al., 1997).

***In vitro* splicing assay of IVS8-2A>G**

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

To analyze the abnormal transcripts in detail, the RT-PCR products were cloned into a TA cloning vector. Subsequently, the cDNA sequences were determined for more than

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

Discussion

The main point of this study is to functionally characterize three hCE-2 SNPs that we found among 165 Japanese subjects. Our functional characterization of the two nonsynonymous SNPs (R34W and V142M) revealed that the variants had an inefficient property as carboxylesterases at least toward irinotecan, *p*-nitrophenyl acetate and 4-methylumbelliferyl acetate (Table 1). Expression levels of R34W and V142M variants were higher than the wild-type (252% and 360% of wild-type levels, respectively). We measured mRNA levels for the wild-type and the variant hCE-2 cDNA-transfected cells. Levels of mRNA in the cells transfected with variant cDNAs (R34W and V142M) were comparable, whereas those in the wild-type cDNA-transfected cells were 68-75 % of the cells transfected with the variant cDNAs. Therefore, the apparent lower expression of the wild-type hCE-2 may have occurred at the translational level. Another possibility is lower transfection efficiency of the hCE-2 wild-type plasmids.

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

The *in vitro* splicing assay has been used to detect possible splicing errors due to base

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

The three amino acid residues, Ser-228, Glu-345, and His-457, in hCE-2 are highly conserved in carboxyesterase family proteins and are an active center of the enzyme. Moreover, most of the CES family proteins have a C-terminal HXEL tetrapeptide that is required for their ER retention. As compared to the wild-type hCE-2 (559 amino acids), the 32-bp deleted variant yields a protein of 508 amino acids that lacks His457 in the active center and the C-terminal HXEL sequence. Thus, our findings obtained by the *in vitro* splicing assay suggest that the variant IVS8-2A>G might be a loss-of-function allele.

The SNPs, R34W and IVS8-2A>G, were found in irinotecan-administered Japanese cancer patients. The plasma concentrations of irinotecan, SN-38, and its glucuronide conjugate SN-38G were measured up to 24 h after a 90 min-infusion of irinotecan. The metabolite/parent ratio of their area under plasma concentration curves (AUC) {i.e., the ratio of the areas under the plasma concentration curves of SN-38 plus SN-38G to irinotecan: $(AUC_{SN-38} + AUC_{SN-38G}) / AUC_{irinotecan}$ } could be considered as an estimate of the hepatic carboxylesterase activity. The heterozygous R34W patient, who suffered from colon cancer and was administered irinotecan, showed a low AUC ratio (unpublished data). On

the other hand, the AUC ratio of the IVS8-2A>G patient that suffered from small cell lung cancer and was administered irinotecan was a little higher than the median value. Since the patient had a homozygous variant *ABCG2* (Glu141Lys), which was shown to be associated with the increase in AUC of diflomotecan, another topoisomerase I inhibitor (Sparreboom et al., 2004), bile excretion of SN-38 and SN-38G was presumably lower by the *ABCG2* variation, and as a result, plasma concentrations of SN-38 and SN-38G might have a tendency to increase (unpublished data). Thus, the clinical impact of IVS8-2A>G is unclear. All these *CES2* SNPs might be important for disease susceptibility as well, although it seems difficult to correlate certain diseases with SNPs with very low frequencies.

In conjunction with the large substrate-dependent inter-individual variability, it could be very valuable knowledge if our analyzing 3 SNPs were associated with the large inter-individual difference, as the difference is known to be as high as 5- to 45-fold (Hosokawa et al., 1995) or even 3- to 5-fold variability in irinotecan and *p*-nitrophenyl acetate, and butyrylthiocholine plasma hydrolytic activity (Guemei et al., 2001). However, considering the low SNP frequencies (0.003) and the low activities of the variants observed throughout irinotecan and the 2 typical carboxylesterase substrates, contribution of our SNPs to the observed wide substrate-dependent inter-individual difference is less likely.

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

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Footnotes

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Legends for figures.

Fig. 1. Genomic structure of *CES2*.

Three SNPs analyzed in this study are indicated with their position in the *CES2* gene. The 12 exons are shown by shaded boxes and the corresponding region with an open reading frame is shown as closed boxes, and the untranslated regions are shown as open boxes.

Fig. 2. Expression of wild-type and variant hCE-2 in COS-1 cells.

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

Fig. 3. Irinotecan-hydrolyzing activity of the R34W and V142M variants.

Enzymatic formation of SN-38 from irinotecan by wild-type hCE-2 and its variants was measured by HPLC as described in the Materials and Methods. Michaelis-Menten kinetics of SN-38 formation catalyzed by the wild-type hCE-2 and its 27 variants are illustrated. No appreciable enzymatic activity was observed with the R34W and V142M variants.

Fig. 4. *In vitro* splicing assay for IVS8-2A>G.

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

Table 1 Vmax values of wild-type hCE-2 and its variants toward irinotecan, *p*-nitrophenyl acetate and 4-methylumbelliferyl acetate.

substrates	wild-type	R34W	V142M
Irinotecan	1.458 ± 0.0495	<0.2	<0.2
<i>p</i> -Nitrophenyl acetate	0.573 ± 0.0155	0.0513 ± 0.0073	0.0232 ± 0.0128
4-Methylumbelliferyl acetate	0.193 ± 0.0068	<0.03	<0.03

Carboxylesterase activities toward the three substrates were determined as described in Materials and Methods. Concentrations used were: 1 to 50 μ M for irinotecan, 39 to 5000 μ M for *p*-nitrophenyl acetate and 62.5 to 1000 μ M for 4-methylumbelliferyl acetate. Vmax values were determined by three independent experiments and were expressed as mean \pm SD. Data were expressed as pmol/mg microsomal protein/min for irinotecan, and as μ mol/mg microsomal protein/min for *p*-nitrophenyl acetate and 4-methylumbelliferyl acetate.

Fig. 1

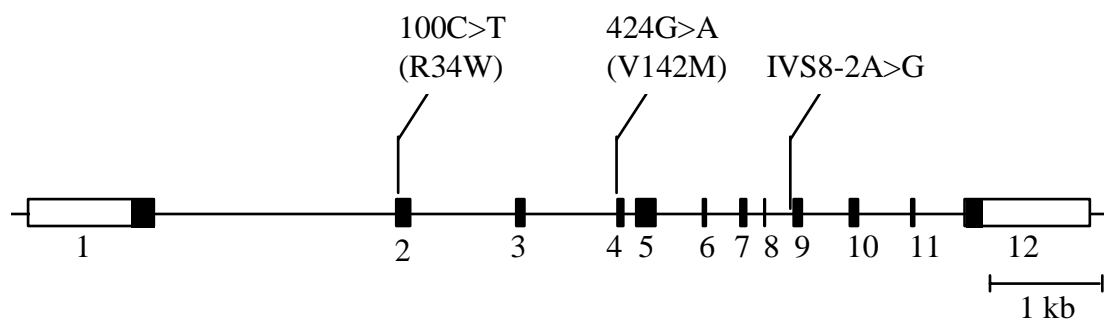


Fig. 2

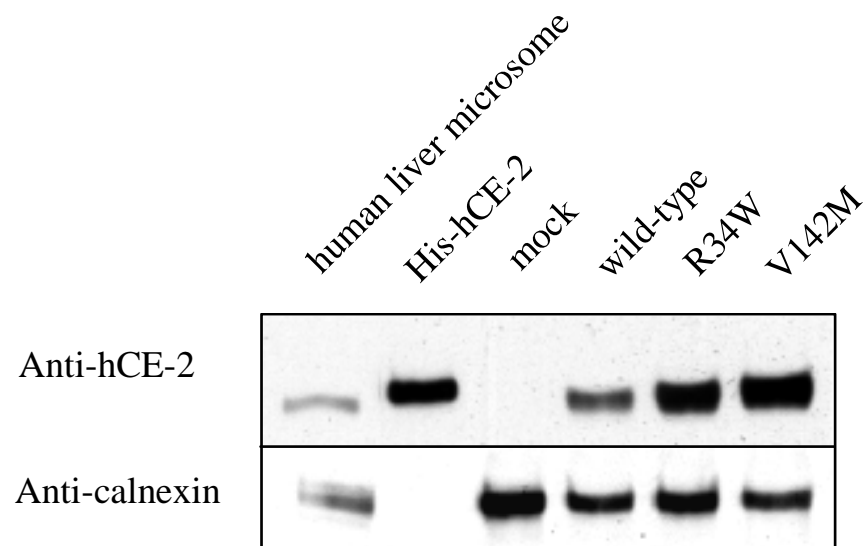


Fig. 3

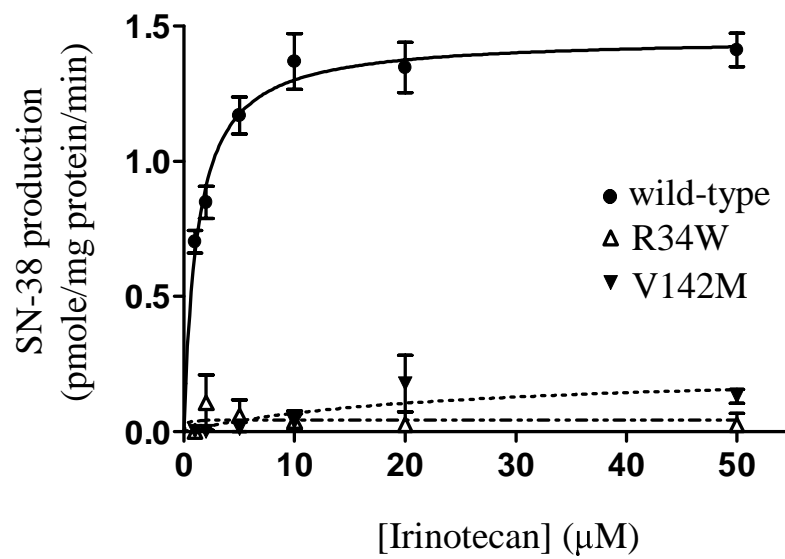


Fig. 4

