Yoshimura M, Kimura T, Ishii M, Ishii K, Matsuura T et al (2008) Functional polymorphisms in carboxylesterase1A2 (*CES1A2*) gene involves specific protein 1 (Sp1) binding sites. Biochem Biophys Res Commun 369:939–942

Zhang L, Hu Z, Zhu C, Liu Q, Zhou Y et al (2009) Identification and characterization of an epididymis-specific gene, *Ces7*. Acta Biochim Biophys Sin 41:809–815

Zhen L, Rusiniak ME, Swank RT (1995) The beta-glucuronidase propeptide contains a serpin-related octamer necessary for complex formation with egasyn esterase and for retention within the endoplasmic reticulum. J Biol Chem 270:11912–11920

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Association of *carboxylesterase 1A* genotypes with irinotecan pharmacokinetics in Japanese cancer patients

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- Association of *UDP-glucuronosyltransferase 1A1 (UGT1A1)* genetic polymorphisms *6 and *28 with reduced clearance of SN-38 and severe neutropenia in irinotecan therapy was demonstrated in Japanese cancer patients.
- The detailed gene structure of *CES1* has been characterized.
- Possible functional SNPs in the promoter region have been reported.

WHAT THIS STUDY ADDS

- Association of functional *CES1* gene number with AUC ratio [(SN-38 + SN-38G)/irinotecan], an *in vivo* index of CES activity, was observed in patients with irinotecan monotherapy.
- No significant effects of major *CES1* SNPs on irinotecan PK were detected.

AIMS

Human carboxylesterase 1 (CES1) hydrolyzes irinotecan to produce an active metabolite SN-38 in the liver. The human *CES1* gene family consists of two functional genes, *CES1A1* (*1A1*) and *CES1A2* (*1A2*), which are located tail-to-tail on chromosome 16q13-q22.1 (*CES1A2-1A1*). The pseudogene *CES1A3* (*1A3*) and a chimeric *CES1A1* variant (*var1A1*) are also found as polymorphic isoforms of *1A2* and *1A1*, respectively. In this study, roles of *CES1* genotypes and major SNPs in irinotecan pharmacokinetics were investigated in Japanese cancer patients.

METHODS

CES1A diplotypes [combinations of haplotypes A (*1A3-1A1*), B (*1A2-1A1*), C (*1A3-var1A1*) and D (*1A2-var1A1*)] and the major SNPs (−75T>G and −30G>A in *1A1*, and −816A>C in *1A2* and *1A3*) were determined in 177 Japanese cancer patients. Associations of *CES1* genotypes, number of functional *CES1* genes (*1A1*, *1A2* and *var1A1*) and major SNPs, with the AUC ratio of (SN-38 + SN-38G)/irinotecan, a parameter of *in vivo* CES activity, were analyzed for 58 patients treated with irinotecan monotherapy.

RESULTS

The median AUC ratio of patients having three or four functional *CES1* genes (diplotypes A/B, A/D or B/C, C/D, B/B and B/D; $n = 35$) was 1.24-fold of that in patients with two functional *CES1* genes (diplotypes A/A, A/C and C/C; $n = 23$) [median (25th–75th percentiles): 0.31 (0.25–0.38) *vs.* 0.25 (0.20–0.32), $P = 0.0134$]. No significant effects of *var1A1* and the major SNPs examined were observed.

CONCLUSION

This study suggests a gene-dose effect of functional *CES1A* genes on SN-38 formation in irinotecan-treated Japanese cancer patients.

Introduction

Human carboxylesterases (CESs) are members of the α/β-hydrolase-fold family and are localized in the endoplasmic reticulum of many different cell types. These enzymes efficiently catalyze the hydrolysis of a variety of ester- and amide-containing chemicals as well as drugs (including prodrugs) to the respective free acids. They are involved in detoxification or metabolic activation of various drugs, environmental toxicants and carcinogens. CESs also catalyze the hydrolysis of endogenous compounds such as short- and long-chain acyl-glycerols, long-chain acyl-carnitine, and long-chain acyl-CoA esters. The two major CES families CES1 and CES2 have been identified in human tissues. CES1 is abundant in the liver and lung but not in the intestine, while CES2 is highly expressed in the intestine and kidney but has low expression in the liver and lung [1].

Human CES1 and CES2 are involved in producing a topoisomerase I inhibitor SN-38, an active metabolite of irinotecan which is clinically used for colorectal, lung and other cancers [2]. SN-38 is further inactivated by UDP-glucuronosyltransferase 1As (UGT1As) to produce SN-38 glucuronide (SN-38G). Irinotecan is also converted by cytochrome P450 3A4 (CYP3A4) to an inactive compound 7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino]carbonyloxycamptothecin (APC) (Figure 1).

Recent pharmacogenetic studies on irinotecan have revealed significant associations of *UGT1A1* polymorphisms **28* [–54_39A(TA)$_6$TAA>A(TA)$_7$TAA or –40_39insTA] and **6* [211G>A (G71R)], the latter being specifically detected in East Asians, with reduced clearance of SN-38 resulting in severe neutropenia [3–8]. These findings have led to the clinical application of genetic testing for *UGT1A1*28* in the United States (since August 2005) and for *UGT1A1*6* and **28* in Japan (since March 2009). In addition, possible additive effects of genotypes of the transporters for irinotecan and its metabolites, such as *ABCB1*, *ABCC2*, *ABCG2* and *SLCO1B1*, have been suggested [9–12]. We previously analyzed *CES2* polymorphisms in a Japanese

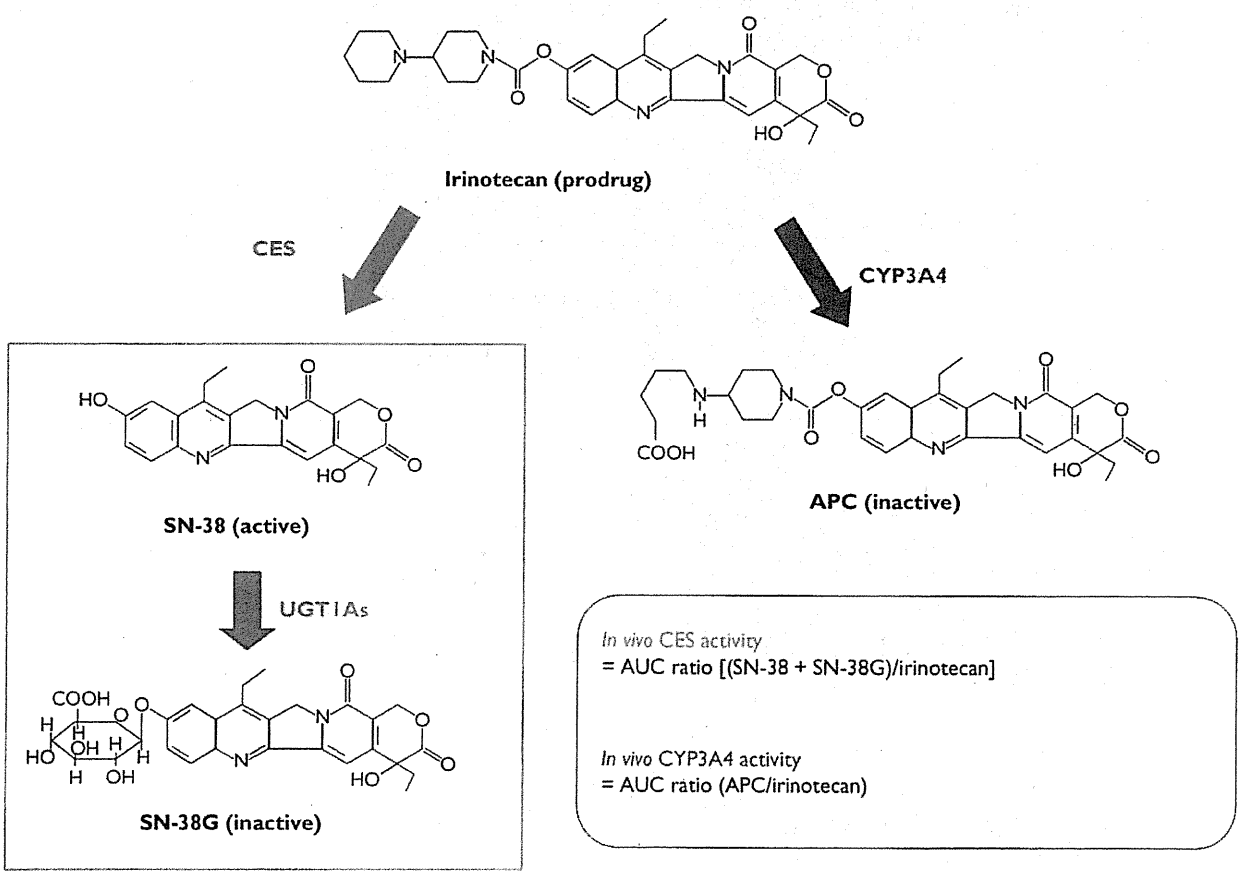

Figure 1

Metabolic pathway of irinotecan. The prodrug irinotecan is hydrolyzed by carboxylesterase (CES) to produce an active metabolite SN-38, and subsequently detoxified by UDP-glucuronosyltransferase 1As (UGT1As) to produce an inactive metabolite SN-38 glucuronide (SN-38G). Irinotecan is also metabolized by cytochrome P450 3A4 (CYP3A4) to produce another inactive metabolite APC

population and identified minor genetic variations which were associated with lower expression/function *in vitro* and *in vivo* [13, 14]. However, major *CES2* haplotypes (**1b* and **1c*) did not affect irinotecan pharmacokinetics (PK) [14]. Since CES1 is expressed at higher levels in the liver, a major organ for activating irinotecan, it is possible that *CES1* genotypes affect the plasma concentrations of irinotecan metabolites. However, their clinical relevance to irinotecan pharmacokinetics/pharmacodynamics has not yet been fully investigated.

Functional human *CES1* genes include *CES1A1* (*1A1*) and *CES1A2* (*1A2*), which are inversely located (tail-to-tail) on chromosome 16q13-q22.1 (*1A2-1A1*). Both *1A1* and *1A2* consist of 14 exons encoding 567 amino acids, and they have 98% homology with 5 nucleotide (4 amino acid) differences in exon 1, which encodes a signal peptide [1]. Recent studies also identified *CES1A1* variants (*var1A1*), in which exon 1 was replaced with exon 1 of *CES1A2*, and a pseudogene *CES1A3* (*1A3*; formerly referred to as *CES4*) replacing *CES1A2* [15, 16]. The *1A3* sequence from the promoter region to exon 1 is the same as that of *CES1A2*, but contains a stop codon in exon 3. The sequence downstream from exon 11 is highly homologous with that of *1A1* (NT_010498) [16]. Ethnic differences in these *CES1* genes (*1A1*, *var1A1*, *1A2* and *1A3*) have been reported [16].

Expression levels of CES1A2 mRNA were lower than those of CES1A1 mRNA in several tissues. This CES1A1 up-regulation could be mediated by additional Sp1 and C/EBP binding sites in the promoter region [17]. Transcript levels of CES1A2 derived from *var1A1* were reported to be higher than those from the original *1A2* [15, 16]. These findings suggest that polymorphisms in the upstream region of *CES1A1* or *var1A1* could affect their expression.

In addition to structural variations of the *CES1* gene family, several single nucleotide polymorphisms (SNPs) and small deletion/insertion variants were found. –816C in the *CES1A2* promoter region was reported to be associated with enhanced CES1A2 expression and imidapril efficacy [18]. Furthermore, –816A>C was found to be linked with several SNPs (–62T>C, –47G>C, –46G>T, –41C>G, –40A>G, –37G>C, –34del/G and –32G>T) in the proximal promoter region, leading to two additional Sp1 binding sites, and these additional sites were suggested to increase transcription of *1A2* [19].

In this context, this study investigated the clinical significance of *CES1* genotypes in irinotecan therapy. For this purpose, we analyzed the *CES1* genotypes (combinations of four *CES1A* isoforms) and major SNPs in the *CES1A1* exon 1 with its adjacent region and in the *CES1A2* and *1A3* promoter regions, which could be important for CES1 expression or function, in Japanese cancer patients treated with irinotecan, and then examined the associations of these *CES1* genotypes or SNPs with irinotecan PK.

Methods

Patients

Genetic analysis of 177 Japanese cancer patients who received irinotecan therapy at the National Cancer Center in Japan was performed. The patients were the same as those described in our previous study [7], where details on eligibility criteria for irinotecan therapy, patient profiles and irinotecan regimens were described. Since the AUC ratio [(SN-38 + SN-38G) : irinotecan], a parameter of *in vivo* CES activity, was influenced by irinotecan regimens [14], 58 patients receiving irinotecan monotherapy (100 mg m^{-2} weekly or 150 mg m^{-2} biweekly) from the 177 patients were primarily used for analysis of the association between *CES1* genotypes and irinotecan PK parameters. The patient set was the same as used in our previous study on *CES2* [14]. 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.

Determination of *CES1* genotypes and SNPs

For describing the *CES1* gene family, haplotypes A to D designated by Fukami *et al.* [16] were used (Figure 2): haplotype A, *CES1A3-CES1A1* (*1A3-1A1*); haplotype B, *CES1A2-CES1A1* (*1A2-1A1*); haplotype C, *CES1A3-CES1A1* variant (*1A3-var1A1*); and haplotype D, *CES1A2-CES1A1* variant (*1A2-var1A1*). To determine the diplotypes, combinations of haplotypes A to D, we sequenced *1A1/var1A1* exon 1 and its flanking region and the *1A2/1A3* promoter region of 177 patients. These regions are indicated in Figure 2, and a list of primers/probes is shown in Table 1.

For discrimination between *1A1* and *var1A1*, their exon 1s and flanking regions were sequenced (Figure 2a). Briefly, the first PCR was performed using 25 ng of genomic DNA with 0.625 units of Ex-Taq (Takara Bio. Inc., Shiga, Japan) and 0.2 μM of primers, Ces1-FP and Ces1-RP (Table 1a, first PCR). The PCR conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 2 min, and then a final extension at 72°C for 7 min. Then, the second PCR was performed with the primers, Ces1_seqF and Ces1_seqR (Table 1a, second PCR) under the same reaction conditions described above. The PCR products were treated with a PCR Product Pre-Sequencing Kit (USB Co., Cleveland, OH, USA) and directly sequenced on both strands using an ABI BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with the sequencing primers listed in Table 1a (sequencing). Excess dye was removed by a DyeEx96 kit (Qiagen, Hilden, Germany), and the eluates were analyzed on an ABI Prism 3730 DNA Analyzer (Applied Biosystems). The conditions of the PCR and sequencing procedures described in the following section were the same as described above unless otherwise noted.

1A2 and *1A3* were discriminated by the restriction fragment length polymorphism (RFLP) method for exon 5

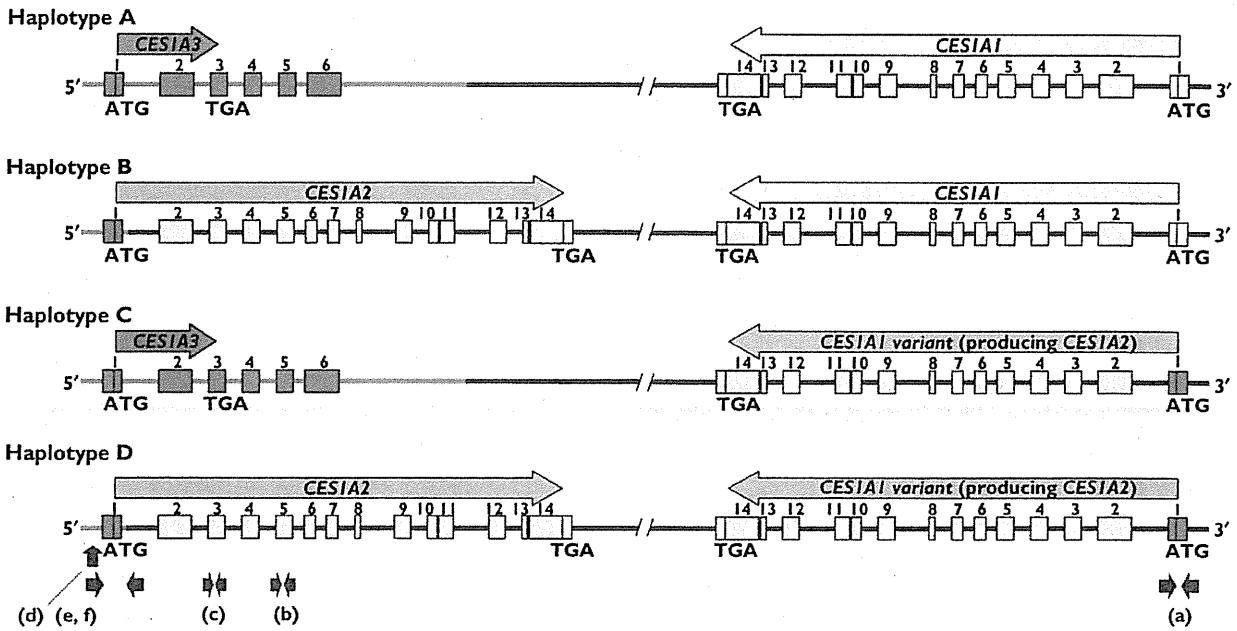

Figure 2

CES1 gene structure and haplotypes. The regions used for haplotype determination in this study are indicated with arrows (a–f)

reported by Fukami *et al.* [16] (Figure 2b). Briefly, the PCR was performed using a primer set (1A-int4F and 1A-int5AS) (Table 1b), and then the PCR products were digested with *Pvu*II to produce *CES1A3*-derived fragments (409 bp and 248 bp). UV intensity of the fragments stained with ethidium bromide was measured after electrophoresis (2% agarose gel). The number of *1A3* (0, 1 or 2) was also confirmed by direct sequencing of exon 5 using the same primer set. To verify that the *1A3* sequence is derived from the pseudogene, we confirmed the existence of a stop codon at codon 105 of *1A3* exon 3 (Figure 2c) in 11 randomly selected patients (heterozygous or homozygous) by amplification and sequencing using primers listed in Table 1c.

Genotyping for –816A>C in the *1A2* and *1A3* promoter region (Figure 2d) was conducted by the TaqMan method of Geshi *et al.* [18] (Table 1d) in all patients. We also examined attribution of –816C to *1A2* or *1A3* by specific amplifications from 5′-regions to intron 1 of the *1A2* and *1A3* (Figure 2e,f) in 23 randomly selected heterozygous patients. For specific amplifications, primers CES1A3-1A2_F1 and CES1A2 R1 for *CES1A2* (Table 1e) and primers CES1A3-1A2_F1 and CES1A3 R1 for *1A3* (Table 1f, first PCR) were used with 0.05 U μl^{-1} LA-Taq with GC buffer I (Takara Bio. Inc.); and for *1A3*, the second PCR using primers CES1A3-1A2_F2 and CES1A3 R2 (Table 1f, second PCR) was also conducted with 0.05 U μl^{-1} Ex-taq. Then, direct sequencing of the *1A2* and *1A3* PCR products was performed. Complete linkage among –816A>C and several SNPs in the proximal promoter region (between –62 to –32) [19] was confirmed for 11 randomly selected subjects.

All variations were confirmed by sequencing PCR products generated from new amplifications from genomic DNA. GenBank NT_010498.15 was used as the reference sequence for *CES1A1*, *CES1A3* and the promoter region of *CES1A2*, and AB119998.1 was used for exon 1 and its downstream region of *CES1A2*. The translational initiation site was designated as +1 to describe the polymorphism positions. Diplotype configuration was estimated with the LDSUPPORT software [20]. The diplotypes A/D and B/C could not be distinguished.

Pharmacokinetic data and association analysis

The area under the concentration–time curve (AUC) values for irinotecan and its metabolites, SN-38, SN-38G and APC, were previously obtained [4, 21]. The AUC ratio of SN-38 plus SN-38G to irinotecan [$AUC_{(SN\text{-}38\ +\ SN\text{-}38G)}/AUC_{irinotecan}$] was used as a parameter reflecting *in vivo* CES activity [14]. The AUC ratio of APC to irinotecan [$AUC_{APC}/AUC_{irinotecan}$] was used as a parameter for *in vivo* CYP3A4 activity [21].

Statistical significance (two-sided, $P < 0.05$) for associations between AUC ratios (or AUC/dose) and *CES1* genotypes or SNPs was determined by the Mann-Whitney test or the Jonckheere-Terpstra (JT) test using Prism version 4.0 (GraphPad Prism Software Inc. San Diego, CA, USA) and StatXact version 6.0 (Cytel Inc., Cambridge, MA). Correla-

Table 1

Primers and probes used in this study

Region (indicated in Figure 2)	Primer	Primer sequence	Reference
(a) *CES1A1* exon 1 and promoter region			This study
First PCR	Ces1-FP	5′-CCAGGCAAAACCTAGGAGTG-3′	
	Ces1-RP	5′-AGTACAGGGCGATCTCAGGA-3′	
Second PCR	Ces1_seqF	5′-GTATTTCCTTAGCCAGCGGTA-3′	
	Ces1_seqR	5′-CAGAGCCGGACCTGTTGT-3′	
Sequencing	Ces1_SF2	5′-AGAGCCTGGAAAGCTATGAAAA-3′	
	Ces1_SR	5′-TTTCTACGCATCTGCGCCCACC-3′	
(b) *CES1A1, 1A2* and *1A3* exon 5			[16]
PCR and sequencing	1A-int4F	5′-GCTCAGTAAATAGTTGCCAGTT-3′	
	1A-int5AS	5′-TCTCATCAGCATCACATCAAG-3′	
(c) *CES1A3* exon 3			This study
PCR and sequencing	CES1A3-15183F	5′-CAGGGAAGATCGTTGTATTGGTTT-3′	
	CES1A3-15974R	5′-TTCCTTCCACCACTAACATTATTG-3′	
Sequencing (additional primer)	CES1A3-15823R	5′-AAGATGTTCATTAAAGATGCACAG-3′	
(d) *CES1A2* and *1A3* -816A>C genotyping			[18]
PCR	F	5′-CCTTAATTTGGTGATTTCACATTGC-3′	
	R	5′-CAAGACATGGTTCAGCTTCTCAAG-3′	
TaqMan probe	FAM	5′-CATCACCCCTACTGC-3′	
	VIC	5′-CATCACACCTACTGCT-3′	
(e) *CES1A2* promoter region			This study
PCR	CES1A3-CES1A2_F1	5′-ATGATTTCCAGCTTCATCTACA-3′	
	CES1A2_R1	5′-GAGAGAACGTTCCCATGTCTTT-3′	
(f) *CES1A3* promoter region			This study
First PCR	CES1A3-CES1A2_F1	5′-ATGATTTCCAGCTTCATCTACA-3′	
	CES1A3_R1	5′-GCTTGAGTTTTCTTTACAGACA-3′	
Second PCR	CES1A3-CES1A2_F2	5′-AACAGTTTATAACCTGTTATTTTT-3′	
	CES1A3_R2	5′-TGCTTTGGATAAAGACAAGATGTT-3′	
Seqeuncing of *CES1A2/1A3* promoter region			
	CES1A3-CES1A2_F2	5′-AACAGTTTATAACCTGTTATTTTT-3′	
	CES1A3-CES1A2_R1	5′-CACACTTCCAATCTCAGGTAAA-3′	
	CES1A3-CES1A2_F3	5′-TTATGCCACAAGCAGTTGGGCG-3′	
	CES1A3-CES1A2_R2	5′-TCCAAGTCCAATTCCAAGTACGGA-3′	

NT_010498.15 was used as the reference sequence for *CES1A1, CES1A3* and the promoter region of *CES1A2*, and AB119998.1 was used for exon 1 and its downstream region of *CES1A2*.

tions between the AUC ratios [$AUC_{(SN\text{-}38\ +\ SN\text{-}38G)}/AUC_{irinotecan}$] and [$AUC_{APC}/AUC_{irinotecan}$] were analyzed by Spearman's rank correlation test. Multiplicity adjustment was not applied to bivariate analysis, and contributions of the candidate genetic markers to the AUC ratios [$AUC_{(SN\text{-}38\ +\ SN\text{-}38G)}/AUC_{irinotecan}$] were further determined by multiple regression analysis after logarithmic transformation of the AUC ratio. The variables examined were age, sex, body surface area, history of smoking or drinking, performance status, serum biochemistry (GOT, ALP, creatinine) at baseline, *CES1* genotypes and SNPs, *CES2*2* [100C>T(R34W)] or **5* [1A>T (M1L)] [13, 14], *UGT1A1*6* or **28* [7, 8], and the transporter haplotypes, *ABCB1*2* [2677G>T(A893A)], *ABCC2*1A* (−1774delG), *ABCG2*IIB* [421C>A (Q141K) and IVS12+49G>T] and *SLCO1A1*15·17* [521T>C (V174A)] [10]. The variables in the final models were selected by the forward and backward stepwise procedure at a significance level of 0.10 using JMP version 7.0.0 (SAS Institute, Inc., Cary, NC, USA). *UGT1A1*6 or *28* was grouped as '+' for stratifying patients: for example, homozygous *UGT1A1 *6 or *28* was depicted as *UGT+/+*.

Results

Genotypes and SNPs of CES1 gene family in Japanese

Frequencies of individual *CES1* genes and *CES1* diplotypes stratified according to the number of functional *CES1* genes are summarized in Table 2. The frequencies of the patients with two, three and four functional *CES1* genes were 44%, 47% and 9%, respectively, in all 177 patients.

By sequencing *1A1* and *var1A1* exon 1s and their flanking region, we detected four novel variations; three in the 5′-flanking region and one in the 5′-untranslated region (5′-UTR) (Table 3): −258C>T (allele frequency: 0.014), −233C>A (0.003), −161A>G (0.006) and −30G>A (0.042). Eleven nucleotide substitutions from the 5′-UTR to intron 1 at allele frequencies of 0.294–0.299 were closely linked with *var1A1* (Table 3). The SNP −816A>C found in the *1A2* and *1A3* promoter regions was genotyped by a TaqMan method [18], and the allele frequency of −816A>C in 177 subjects was 0.249 (Table 4). It was noted that −816C was detected only in patients with *1A3* (*1A3/1A2* and *1A3/1A3*),

Table 2

Frequency of *CES1* genes and diplotypes in Japanese cancer patients

CES1 diplotype	Number of *CES1* gene *1A1*	*var1A1*	*1A2*	*1A3*	Total*	Frequency (n = 177)†		Frequency (monotherapy: n = 58)†	
A/A	2	0	0	2	2	0.203	0.441	0.138	0.397
A/C	1	1	0	2		0.220		0.241	
C/C	0	2	0	2		0.017		0.017	
A/B	2	0	1	1	3	0.237	0.469	0.293	0.534
A/D or B/C	1	1	1	1		0.192		0.190	
C/D	0	2	1	1		0.040		0.052	
B/B	2	0	2	0	4	0.040	0.090	0.017	0.069
B/D	1	1	2	0		0.034		0.052	
D/D	0	2	2	0		0.017		0.000	
Frequency (n = 354)‡	0.703	0.297	0.325	0.675					
(monotherapy: n = 116)‡	0.690	0.310	0.336	0.664					

*Number of functional genes. †Number of subjects. ‡Number of chromosomes.

but not in the *1A2* homozygotes (*1A2/1A2*). In the *1A2/1A3* patients, 38 of the 39 patients having −816C were heterozygous for −816C (Table 4). These findings suggested a close association between −816C with *1A3*. Following specific amplifications of the regions from 5′-regions to intron 1 in *1A2* and *1A3* (Figure 2e,f) of 23 patients randomly selected from the 38 patients with −816A/C and *1A2/1A3*, we confirmed that −816C resided in the *1A3* gene (data not shown). Thus, −816A>C is the major SNP of *1A3* but very rare in *1A2*. In addition, the SNPs, −62T>C, −47G>C, −46G>T, −41C>G, −40A>G, −37G>C, −34del/G and −32G>T, in the proximal promoter region reported to be linked with −816A>C [19] were found to be completely linked with *1A3* (data not shown).

Association of CES1 genotypes with in vivo CES activity

CES1 diplotypes In patients treated with irinotecan monotherapy, we found the AUC ratios of patients with haplotypes A or C (having the *1A3* pseudogene) were lower than those without A or C, indicating functional *CES1* gene number dependency. The median AUC ratio of patients having three or four functional *CES1* genes was 1.24-fold of that in patients with two functional *CES1* genes [median (25th–75th percentiles): 0.31 (0.25–0.38) *vs.* 0.25 (0.20–0.32), P = 0.0134, Mann-Whitney test)] (Figure 3a). No significant differences were observed between *1A1* and *var1A1* (among *1A1/1A1*, *var1A1/1A1* and *var1A1/var1A1*). As we previously reported, the *CES2* variations, *CES2*5* [1A>T(M1L)] and *CES2*2* [100C>T(R34W)] [13, 14] showed low CES activity as indicated in Figure 3a.

Platinum-containing regimens themselves enhance renal excretion of irinotecan and its metabolites, especially SN-38G. No significant effect of *CES1* gene number on the AUC ratio was observed. However, it was noted that the median renal excretion ratio [(SN-38 + SN-38G)/irinotecan] in patients with four functional *CES1* genes was 1.37-fold higher than that in patients with two or three functional genes (P = 0.0217, Mann-Whitney test) (data not shown).

To exclude the possibility that the higher AUC ratio observed above (Figure 3a) was biased by CYP3A4, another metabolic enzyme for irinotecan, we analyzed the association between the (SN-38 + SN-38G)/irinotecan AUC ratio and the APC/irinotecan AUC ratio, an *in vivo* parameter of CYP3A4 activity [21], in patients treated with irinotecan monotherapy. The result showed no correlation between the two parameters (Spearman r = 0.126, P = 0.345).

CES1 SNPs Next, associations of the two *1A1* SNPs, −75G>T and −30G>A (Table 3) and *1A3*-816A>C with the AUC ratio [(SN-38 + SN-38G)/irinotecan] were analyzed. The effects of the SNPs were analyzed in patients stratified by the functional *CES1* gene number and also in all the patients receiving monotherapy. A −75G>T-dependent increase in the AUC ratio was observed in the whole group of patients (P = 0.027, JT test) (Figure 3b), and this trend was remarkable in patients with three or four functional *CES1* genes. No significant effect of −30G>A was observed (Figure 3c). As for −816C in *1A3*, no association between this SNP and the AUC ratio was evident in patients with two or three functional *CES1* genes (Figure 3d). In the platinum-containing regimens, no significant effects of these SNPs on the AUC ratio or the renal recovery ratio were observed (data not shown).

Multivariate analysis The contribution of *CES1* genotypes to the AUC ratio was further analyzed by multivariate analysis, using the patient background factors and polymorphisms including the haplotypes of *CES2*, *UGT1A1* and transporters as variables [7, 8, 10, 13, 14]. The final model revealed a significant association of the functional *CES1* gene number (n = 3 or 4) with the AUC ratio. Contributions of smoking history, irinotecan dose, hepatic and renal function were also detected while that of *ABCB1*2* (+/+) was

Table 3

Summary of genetic variations of *CES1A1* and *var 1A1* exon 1s and their flanking regions detected in this study

SNP identification				Position					
This study	NCBI (dbSNP)	JSNP	Location	NT_010498.15	From the translational initiation site or the nearest exon	Nucleotide change and flanking sequences (5′ to 3′)	Amino acid change	Allele frequency (n = 354)*	*CES1A1* variant (*CES1A2* type)
MPJ6_CS1001†			5′-flank	9481424	−258	ttgggcaagtttacagctct**C/T**gtaatctgacagtagagtc		0.014	
MPJ6_CS1002†			5′-flank	9481399	−233	atctgacagtagagtccaga**C/A**tggtttgatgaaagagggta		0.003	
MPJ6_CS1003†			5′-flank	9481327	−161	tagaagcccagggagatctg**A/G**ggaaagggagggcttttctg		0.006	
MPJ6_CS1004	rs3815583	IMS-JST175949	Exon1(5′-UTR)	9481241	−75	aactctgggcggggctgggc**G/T**ccagggctggacagcacagt		0.41	
MPJ6_CS1005	rs28429139		Exon1(5′-UTR)	9481212	−46	ggacagcacagtccctctga**A/G**ctgcacagagacctcgcagg		0.299	*var1A1*
MPJ6_CS1006	rs28494177		Exon1(5′-UTR)	9481205	−39	acagtccctctgaactgcac**A/G**gagacctcgcaggccccgag		0.299	*var1A1*
MPJ6_CS1007†			Exon1(5′-UTR)	9481196	−30	ctgaactgcacagagacctc**G/A**caggccccgagaactgtcgc		0.042	
MPJ6_CS1008	rs28520463		Exon1(5′-UTR)	9481187	−21	acagagacctcgcaggcccc**G/C**agaactgtcgcccttccacg		0.297	*var1A1*
MPJ6_CS1009	rs28499065		Exon1(5′-UTR)	9481186	−20	cagagacctcgcaggccccg**A/G**gaactgtcgcccttccacga		0.297	*var1A1*
MPJ6_CS1010	rs28515828		Exon1(5′-UTR)	9481168	−2	cgagaactgtcgcccttcca**C/G**gatgtggctccgtgccttta		0.299	*var1A1*
MPJ6_CS1011			Exon 1	9481156	11	cccttccacgatgtggctcc**G/C**tgcctttatcctggccactc	Arg4Pro	0.297	*var1A1*
MPJ6_CS1012			Exon 1	9481152	15	tccacgatgtggctccgtgc**C/T**tttatcctggccactctctc	Ala5Ala	0.297	*var1A1*
MPJ6_CS1013			Exon 1	9481151	16	ccacgatgtggctccgtgcc**T/C**ttatcctggccactctctct	Phe6Leu	0.297	*var1A1*
MPJ6_CS1014			Exon 1	9481148	19	cgatgtggctccgtgcctt**A/G**tcctggccactctctctgct	Ile7Val	0.297	*var1A1*
MPJ6_CS1015	rs28563878		Exon 1	9481133	34	tgcctttatcctggccactct**T/G**ctgcttccgcggcttggggt	Ser12Ala	0.297	*var1A1*
MPJ6_CS1016	rs12149359		Intron 1	9481099	IVS1+16	ttggggtgagtccttctgaa**A/G**tcaaaatgcggggcactttt		0.294	*var1A1*

*Number of chromosomes. †Novel variation detected in this study.

Table 4

Frequency of *CES1A2(/1A3)* promoter SNP −816A>C in Japanese cancer patients

CES1A2 and *1A3* Genotype	-816A>C Genotype	Number of subjects	Allele frequency
1A2/1A2	A/A	16	0/32 (0%)
	A/C	0	
	C/C	0	
1A2/1A3	A/A	44	40/166 (24.1%)
	A/C	38	
	C/C	1	
1A3/1A3	A/A	41	48/156 (30.8%)
	A/C	26	
	C/C	11	
Total		177	88/354 (24.9%)

not significant (Table 5). The *CES1* genotypes explained 22.6% of variability in the final model among all the variables and 11.3% of total variability in the AUC ratio.

Effects of CES1 genotypes on SN-38 AUC and toxicity

To clarify the clinical importance of *CES1* genotyping for irinotecan therapy, the effects of *CES1* genotypes or SNPs on AUC levels of the active metabolite SN-38 and neutropenia were examined in the non-*UGT*+/+ patients. In this non-*UGT*+/+ population, significantly higher AUC ratios of (SN-38 + SN-38G)/irinotecan were also observed in the patients with three or four functional *CES1* genes (P = 0.0234, Mann-Whitney test) as observed in all the patients treated with irinotecan monotherapy (Figure 3a). With increased number of functional *CES1* genes, an increasing trend of SN-38 AUC/dose was observed in patients receiving irinotecan monotherapy (1.4-fold for four genes *vs.* two genes; P = 0.080, JT test) (Figure 4). However, multiple regression analysis revealed no statistically significant contribution of *CES1* genotypes to SN-38 AUC/dose although *UGT1A1"*6 or *28"* and *ABCB1*2/*2* showed significant contributions [10]. Regarding neutropenia, a higher incidence (though statistically insignificant) for grade 3/4 neutropenia in patients with four functional *CES1* genes was observed (50% for four genes and 16% for two or three genes, P = 0.09, Fisher's exact test). The effects of the SNPs (−75G>T, −30G>A and −816A>C) on SN-38 AUC or incidence grade 3/4 neutropenia were not significant (data not shown). In platinum-containing regimens, no significant effects of the *CES1* genotypes on SN-38 AUC/dose or incidence of grade 3/4 neutropenia were detected in the non-*UGT*+/+ patients (data not shown).

Discussion

Recent pharmacogenetic studies on irinotecan have shown the clinical significance of *UGT1A1 *6* and **28* in Japanese patients [7, 8] and *UGT1A1*28* in Caucasians [5, 6] for severe neutropenia. Subsequent studies have revealed additional genetic factors including transporters [10–12]. However, the clinical importance of genotypes of the irinotecan-activating enzymes *CES1* and *CES2* is still uncertain.

Since the hydrolytic activity of CES2 for irinotecan was reported to be much higher than that of CES1 [2], most studies have focused on the clinical significance of *CES2* polymorphisms in irinotecan therapy [13, 14, 22]. We previously identified minor *CES2* genetic variations in Japanese, including *CES2*2* [100C>T (R34W)] and *CES2*5* [1A>T (M1L)] which caused low *in vitro* expression/function of CES2 [13, 14] and also exhibited reduced *in vivo* CES activity in irinotecan-treated patients [14] (also see Figure 3a). However, the major *CES2* haplotypes in Japanese, **1b* (IVS10-108G>A and 1749A>G, frequency = 0.233) and **1c* (−363C>G, IVS10-108G>A and IVS10-87G>A, frequency = 0.027), did not show any significant effects on irinotecan PK [14]. No clinical significance of *CES2* polymorphisms has been reported in Caucasians [22]. Neither *CES1* nor *CES2* SNPs affecting their mRNA expression in normal colonic mucosa were found in European and African populations [23]. Since precise structures of the *CES1* genes and their promoter regions had not been elucidated, evaluation of the roles of the *CES1* genotypes in irinotecan therapy has been rather difficult.

In the present study, the frequencies of individual *CES1* genes (*1A1, var1A1, 1A2* and *1A3*) (Table 2) were almost comparable with the previous report in the Japanese population (0.748, 0.252, 0.313 and 0.687, respectively) [16]. To our knowledge, the present study is the first report suggesting a possible effect of *CES1* genotypes on irinotecan PK. This study showed that the AUC ratio [(SN-38 + SN-38G)/irinotecan], and probably *in vivo* CES activity, was elevated depending on the number of functional *CES1* genes (*1A1, var1A1* and *1A2*) in patients treated by irinotecan monotherapy (100 or 150 mg m^{-2} irinotecan) (Figure 3a). This gene-dose effect was not clearly shown in the platinum-containing combination therapy (60–70 mg m^{-2} irinotecan), where renal excretion of irinotecan and its metabolites (especially SN-38G) is highly enhanced by a large volume of infusion fluid. However, the median renal excretion ratio [(SN-38 + SN-38G)/irinotecan] in patients with four functional genes was 1.37-fold higher than that in patients with two or three functional genes in the platinum-containing therapy (data not shown), supporting a partial but significant contribution of the CES1s to activate irinotecan. The present study showed no significant differences in the AUC ratios between *1A1* and *var1A1* (Figure 3a), indicating a common upstream region may be involved in regulation of gene expression of *1A1* and *var1A1*. The previous reports showed the expression levels of CES1A2 were lower than those of CES1A1 [17] and suggested that CES1A2 mRNA was derived mainly from transcription of *var1A1* rather than the original *1A2* [15, 16]. The present study, on the other hand, has suggested that the

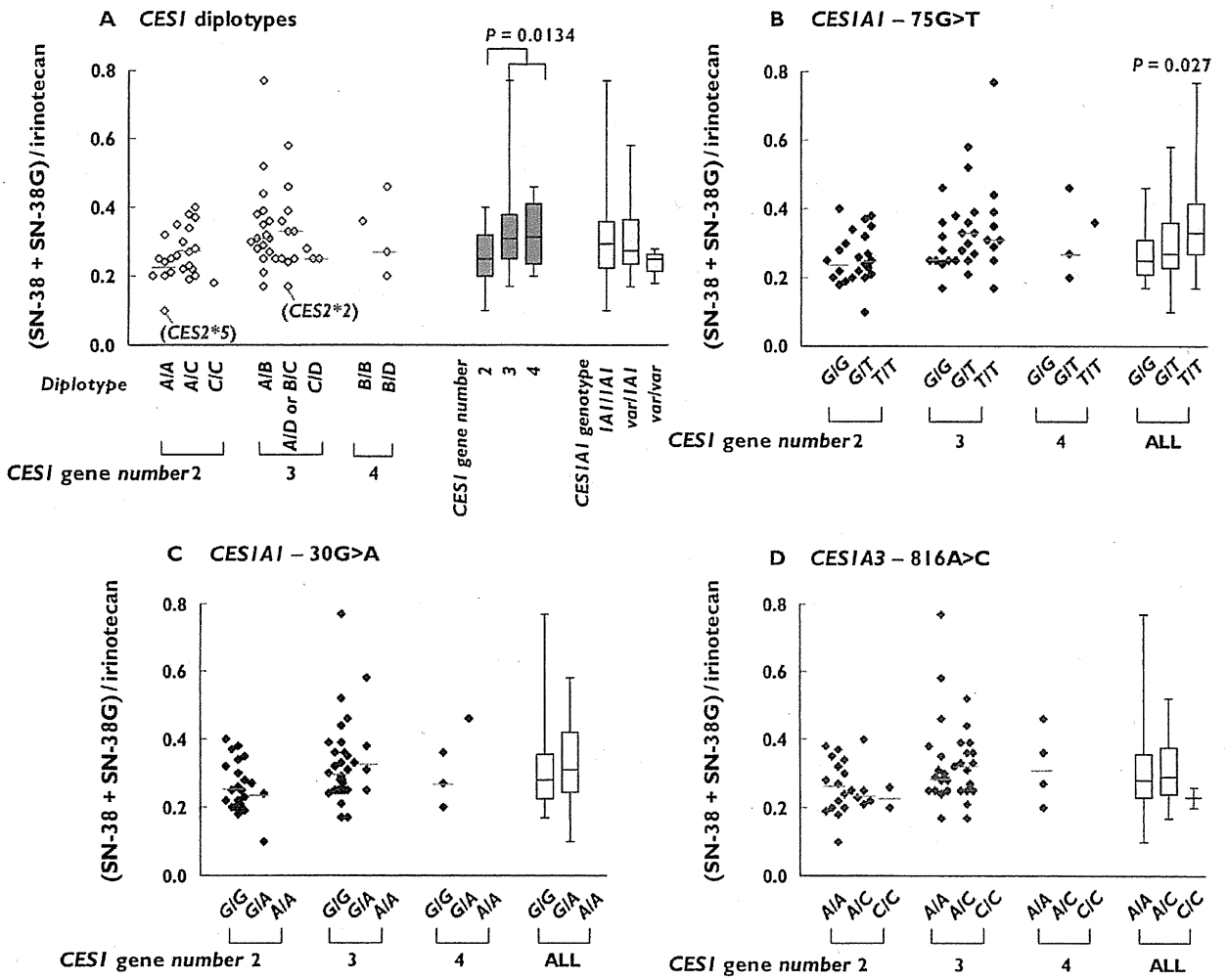

Figure 3

Association of *CES1* diplotypes (A) or SNPs (B–D) with AUC ratio [(SN-38 + SN-38G)/irinotecan], an *in vivo* index of CES activity, in Japanese cancer patients treated with irinotecan monotherapy (n = 58). '*CES1* gene number' means the number of functional genes (*1A1*, *var1A1* and *1A2*). Higher AUC ratios were observed in patients with three or four functional *CES1* genes than with two functional genes (P = 0.0134, Mann-Whitney test) in (A). Patients with *CES2*5* [*CES2* 1A>T (M1L)] (*CES2*5*) and *CES2*2* [*CES2* 100C>T (R34W)] (*CES2*2*) were found to have reduced CES activity in our previous study [13, 14]

Table 5

Multiple regression analysis of AUC ratio [(SN-38 + SN-38G)/irinotecan]* in Japanese cancer patients treated with irinotecan monotherapy

Variable	Coefficient	SE	P value
Smoking	0.073	0.034	0.0375
Initial dose of irinotecan (mg m^{-2})	−0.002	0.001	0.0005
Serum GOT and ALP†	0.082	0.027	0.0038
Serum creatinine (mg dl^{-1})	0.130	0.062	0.0399
*ABCB1*2*‡ (+/+)	0.042	0.024	0.0831
CES1 functional gene (n = 3 or 4)	0.038	0.016	0.0215

r^2 = 0.500, Intercept = −0.248, n = 58. * Values after logarithmic conversion were used. † Grade 1 or greater for both GOT and ALP. ‡ *ABCB1*2* [2677G>T (A893S)].

1A2 transcript could contribute to the total CES activity because the [(SN-38 + SN-38G)/irinotecan] AUC ratios of patients without *1A2* (with two functional *CES1* genes) were lower than those with *1A2* (with three or four functional genes) (Figure 3a). However, it must be noted that the increase in the AUC ratio by three or four functional *CES1* genes was only 20% compared with two functional genes (Figure 3a), and that such alterations might be masked by other non-genetic factors. In fact, hepatic and renal function, irinotecan dosage and smoking history were found to be potent contributors to this parameter (Table 5).

−816A>C SNP in *1A2* was reported to be associated with imidapril efficacy and a higher promoter activity for

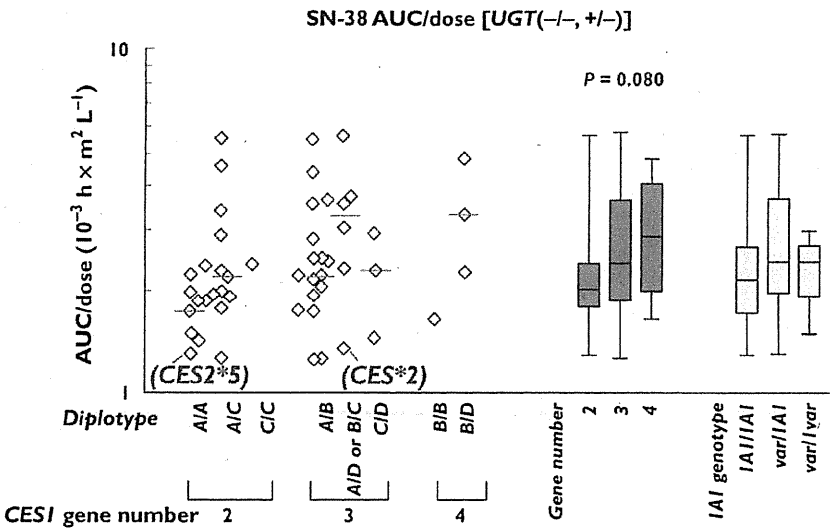

Figure 4

Association of *CES1* genotypes with SN-38 AUC/dose in *UGT*(–/– and +/–) patients treated with irinotecan monotherapy ($n = 51$).'*CES1* gene number' means the number of functional genes (*1A1*, *var1A1* and *1A2*). One patient with an outlying value who had *ABCB1*2* [2677G>T (A893S)] and **14* [2677G>T (A893S) and 1345G>A 230 (E448K)] was excluded from this analysis [10]. A slightly increasing trend in SN-38 AUC(/dose) was observed depending on functional *CES1* gene number. ($P = 0.080$, Jonckheere-Terpstra test). The patients with *CES2*5* [*CES2* 1A>T (M1L)] (*CES2*5*) and *CES2*2* [*CES2* 100C>T (R34W)] (*CES2*2*) [13, 14] are marked

CES1A2 [18] and had strong linkage with SNPs in the proximal promoter region (between –62 to –32) which resulted in additional Sp1 binding sites in the *1A2* promoter region [19]. However, our current study showed no significant effect of –816A>C on the AUC ratio. This can be explained by our finding that –816C and several linked SNPs were mostly located on the *CES1A3* psuedogene but not the functional *1A2* gene.

We newly detected three SNPs (–258C>T, –233C>A and –161A>G) in the 5'-flanking region and one SNP (–30 G>A) in the 5'-UTR of *CES1A1* (Table 3). The effect of –30 G>A on the AUC ratio was not significant (Figure 3c). The frequencies of three other SNPs in the 5'-flanking region were very low (0.003–0.014) which made statistical analysis difficult. These SNPs are not located in the putative transcriptional regulatory regions of *CES1A1*, the binding sites of transcription factors Sp1 and C/EBP [17]. The AUC ratios of the patients with these SNPs were within the 25th–75th percentiles except that slightly higher values were shown in the two -258T patients who received platinum-combination therapy (data not shown). Thus, clinical impact of these SNPs would be small.

With respect to the clinical importance of *CES1* genotyping for irinotecan therapy, the effects of *CES1* genotypes on the AUC level of the active metabolite SN-38 and incidence of grade 3/4 neutropenia should be considered. Since the patients homozygous for *UGT1A1*6 or *28* (*UGT+/+*: **6/*6*, **6/*28* and **28/*28*) showed higher SN-38 AUC/dose levels and severe neutropenia [7], we examined the effects of *CES1* genotypes and SNPs in the non-*UGT+/+* patients. Increasing trends of SN-38 AUC/dose (Figure 4) and incidence of grade 3/4 neutropenia were observed depending on the functional *CES1* gene number in patients with irinotecan monotherapy although statistical significance was not obtained. For the platinum-containing regimens, no significant effects of *CES1* genotypes were shown. Thus, although possible effects of the *CES1* genotypes on neutropenia could not be excluded in irinotecan monotherapy, this study was still insufficient to establish the clinical importance of *CES1* genotyping in irinotecan therapy. Since the sample size will be twice that of the present study to detect a statistically significant decrease of absolute neutrophil counts in the patients with four functional *CES1* genes, future clinical data obtained in a larger number of patients could clarify this point.

In conclusion, this study suggests that the total number of functional *CES1A* genes could influence the formation of the active metabolite of irinotecan in Japanese cancer patients.

Competing interests

HK has received lecture honorarium from Yakult Honsha, the manufacturer of irinotecan. HM has been paid by Yakult Honsha, the manufacturer of irinotecan, for speaking and research.

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REFERENCES

1 Hosokawa M. Structure and catalytic properties of carboxylesterase isozymes involved in metabolic activation of prodrugs. Molecules 2008; 13: 412–31.

2 Humerickhouse R, Lohrbach K, Li L, Bosron WF, Dolan ME. Characterization of CPT-11 hydrolysis by human liver carboxylesterase isoforms hCE-1 and hCE-2. Cancer Res 2000; 60: 1189–92.

3 Ando Y, Saka H, Ando M, Sawa T, Muro K, Ueoka H, Yokoyama A, Saitoh S, Shimokata K, Hasegawa Y. Polymorphisms of UDP-glucuronosyltransferase gene and irinotecan toxicity: a pharmacogenetic analysis. Cancer Res 2000; 60: 6921–6.

4 Iyer L, Das S, Janisch L, Wen M, Ramirez J, Karrison T, Fleming GF, Vokes EE, Schilsky RL, Ratain MJ. UGT1A1*28 polymorphism as a determinant of irinotecan disposition and toxicity. Pharmacogenomics J 2002; 2: 43–7.

5 Innocenti F, Undevia SD, Iyer L, Chen PX, Das S, Kocherginsky M, Karrison T, Janisch L, Ramírez J, Rudin CM, Vokes EE, Ratain MJ. Genetic variants in the UDP-glucuronosyltransferase 1A1 gene predict the risk of severe neutropenia of irinotecan. J Clin Oncol 2004; 22: 1382–8.

6 Han JY, Lim HS, Shin ES, Yoo YK, Park YH, Lee JE, Jang IJ, Lee DH, Lee JS. Comprehensive analysis of UGT1A polymorphisms predictive for pharmacokinetics and treatment outcome in patients with non-small-cell lung cancer treated with irinotecan and cisplatin. J Clin Oncol 2006; 24: 2237–44.

7 Minami H, Sai K, Saeki M, Saito Y, Ozawa S, Suzuki K, Kaniwa N, Sawada J, Hamaguchi T, Yamamoto N, Shirao K, Yamada Y, Ohmatsu H, Kubota K, Yoshida T, Ohtsu A, Saijo N. Irinotecan pharmacokinetics/pharmacodynamics and UGT1A genetic polymorphisms in Japanese: roles of UGT1A1*6 and *28. Pharmacogenet Genomics 2007; 17: 497–504.

8 Sai K, Saito Y, Sakamoto H, Shirao K, Kurose K, Saeki M, Ozawa S, Kaniwa N, Hirohashi S, Saijo N, Sawada J, Yoshida T. Importance of UDP-glucuronosyltransferase 1A1*6 for irinotecan toxicities in Japanese cancer patients. Cancer Lett 2008; 261: 165–71.

9 Sai K, Kaniwa N, Itoda M, Saito Y, Hasegawa R, Komamura K, Ueno K, Kamakura S, Kitakaze M, Shirao K, Minami H, Ohtsu A, Yoshida T, Saijo N, Kitamura Y, Kamatani N, Ozawa S, Sawada J. Haplotype analysis of ABCB1/MDR1 blocks in a Japanese population reveals genotype-dependent renal clearance of irinotecan. Pharmacogenetics 2003; 13: 741–57.

10 Sai K, Saito Y, Maekawa K, Kim SR, Kaniwa N, Nishimaki-Mogami T, Sawada J, Shirao K, Hamaguchi T, Yamamoto N, Kunitoh H, Ohe Y, Yamada Y, Tamura T, Yoshida T, Matsumura Y, Ohtsu A, Saijo N, Minami H. Additive effects of drug transporter genetic polymorphisms on irinotecan pharmacokinetics/pharmacodynamics in Japanese cancer patients. Cancer Chemother Pharmacol 2010; 66: 95–105.

11 Han JY, Lim HS, Park YH, Lee SY, Lee JS. Integrated pharmacogenetic prediction of irinotecan pharmacokinetics and toxicity in patients with advanced non-small cell lung cancer. Lung Cancer 2009; 63: 115–20.

12 Innocenti F, Kroetz DL, Schuetz E, Dolan ME, Ramírez J, Relling M, Chen P, Das S, Rosner GL, Ratain MJ. Comprehensive pharmacogenetic analysis of irinotecan neutropenia and pharmacokinetics. J Clin Oncol 2009; 27: 2604–14.

13 Kubo T, Kim SR, Sai K, Saito Y, Nakajima T, Matsumoto K, Saito H, Shirao K, Yamamoto N, Minami H, Ohtsu A, Yoshida T, Saijo N, Ohno Y, Ozawa S, Sawada J. Functional characterization of three naturally occurring single nucleotide polymorphisms in the CES2 gene encoding carboxylesterase 2 (HCE-2). Drug Metab Dispos 2005; 33: 1482–7.

14 Kim SR, Sai K, Tanaka-Kagawa T, Jinno H, Ozawa S, Kaniwa N, Saito Y, Akasawa A, Matsumoto K, Saito H, Kamatani N, Shirao K, Yamamoto N, Yoshida T, Minami H, Ohtsu A, Saijo N, Sawada J. Haplotypes and a novel defective allele of CES2 found in a Japanese population. Drug Metab Dispos 2007; 35: 1865–72.

15 Tanimoto K, Kaneyasu M, Shimokuni T, Hiyama K, Nishiyama M. Human carboxylesterase 1A2 expressed from carboxylesterase 1A1 and 1A2 genes is a potent predictor of CPT-11 cytotoxicity *in vitro*. Pharmacogenet Genomics 2007; 17: 1–10.

16 Fukami T, Nakajima M, Maruichi T, Takahashi S, Takamiya M, Aoki Y, McLeod HL, Yokoi T. Structure and characterization of human carboxylesterase 1A1, 1A2, and 1A3 genes. Pharmacogenet Genomics 2008; 18: 911–20.

17 Hosokawa M, Furihata T, Yaginuma Y, Yamamoto N, Watanabe N, Tsukada E, Ohhata Y, Kobayashi K, Satoh T, Chiba K. Structural organization and characterization of the regulatory element of the human carboxylesterase (CES1A1 and CES1A2) genes. Drug Metab Pharmacokinet 2008; 23: 73–84.

18 Geshi E, Kimura T, Yoshimura M, Suzuki H, Koba S, Sakai T, Saito T, Koga A, Muramatsu M, Katagiri T. A single nucleotide polymorphism in the carboxylesterase gene is associated with the responsiveness to imidapril medication and the promoter activity. Hypertens Res 2005; 28: 719–25.

19 Yoshimura M, Kimura T, Ishii M, Ishii K, Matsuura T, Geshi E, Hosokawa M, Muramatsu M. Functional polymorphisms in carboxylesterase1A2 (CES1A2) gene involves specific protein 1 (Sp1) binding sites. Biochem Biophys Res Commun 2008; 369: 939–42.

20 Kitamura Y, Moriguchi M, Kaneko H, Morisaki H, Morisaki T, Toyama K, Kamatani N. Determination of probability

distribution of diplotype configuration (diplotype distribution) for each subject from genotypic data using the EM algorithm. Ann Hum Genet 2002; 66: 183–93.

21 Sai K, Saito Y, Fukushima-Uesaka H, Kurose K, Kaniwa N, Kamatani N, Shirao K, Yamamoto N, Hamaguchi T, Kunitoh H, Ohe Y, Tamura T, Yamada Y, Minami H, Ohtsu A, Yoshida T, Saijo N, Sawada J. Impact of CYP3A4 haplotypes on irinotecan pharmacokinetics in Japanese cancer patients. Cancer Chemother Pharmacol 2008; 62: 529–37.

22 Charasson V, Bellott R, Meynard D, Longy M, Gorry P, Robert J. Pharmacogenetics of human carboxylesterase 2, an enzyme involved in the activation of irinotecan into SN-38. Clin Pharmacol Ther 2004; 76: 528–35.

23 Marsh S, Xiao M, Yu J, Ahluwalia R, Minton M, Freimuth RR, Kwok PY, McLeod HL. Pharmacogenomic assessment of carboxylesterases 1 and 2. Genomics 2004; 84: 661–8.

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RESEARCH ARTICLE

DNA methylation and its involvement in *carboxylesterase 1A1 (CES1A1)* gene expression

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Abstract

1. Carboxylesterase 1A1 (CES1A1) efficiently catalyses the hydrolysis of a substrate containing ester, amide, or thioester bonds. It is expressed at a high level in the human liver, but at a low level in the human kidney. In this study, we found the cause of this tissue-specific expression of the *CES1A1* gene using 5-aza-2'-deoxycytidine (5-aza-dC) and bisulfite sequencing.
2. Treatment of HEK293 cells, human embryonic kidney cells not expressing the *CES1A1* gene, with 5-aza-dC caused dramatic expression of the *CES1A1* gene. Bisulfite sequencing revealed that the region around the transcription start site (TSS) of the *CES1A1* gene was almost entirely methylated in HEK293 cells, whereas the region was almost entirely unmethylated in HepG2 cells, human hepatoma cells. The hypomethylated DNA molecules for the region were observed in HEK293 cells treated with 5-aza-dC. In the genome obtained from the kidney, the region downstream of the TSS was methylated compared with that obtained from the liver.
3. From these findings, it can be concluded that DNA methylation is involved in *CES1A1* gene expression and that the difference between *CES1A1* gene expression in the human kidney and that in the human liver may arise from the difference in DNA methylation levels in the region around the TSS.

Keywords: *Tissue-specific expression; CpG island; prodrug; oseltamivir*

Introduction

Mammalian carboxylesterases (CESs, EC 3.1.1.1) are encoded by a multigene family (Satoh et al. 2002) and are members of an α,β-hydrolase-fold family (Bencharit et al. 2003). CESs are ubiquitously expressed, particular in the liver, small intestine, kidney and lung, in various mammals, and the majority of CESs are localized to the endoplasmic reticulum (Satoh et al. 2002). CESs efficiently catalyse the hydrolysis of a substrate containing ester, amide, or thioester bonds. Therefore, they play an important role in the metabolic activation of prodrugs that are designed to improve bioavailability. According to the homology of amino acid sequence, we previously classified CESs into five major groups, CES1-5 (Satoh & Hosokawa 2006), and found that many of the identified CESs belong to the CES1 or CES2 family. The substrate specificities of the CES1 and CES2 families are very different. The CES1 isozyme mainly hydrolyses a substrate with a small alcohol group and large acyl group. In contrast, the CES2 isozyme mainly hydrolyses a substrate with a large alcohol group and small acyl group. The CES1 family can be divided into eight subfamilies (CES1A-H). CES1A1 is a human CES1A subfamily isozyme and is mainly expressed in the liver and lung. CES2A1 is a human CES2A subfamily isozyme and is mainly expressed in the small intestine and kidney (Hosokawa 2008).

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CESs are related to transporters and conjugation enzymes and they are involved in drug metabolism and disposition. For instance, CESs convert temocapril, an angiotensin-converting enzyme inhibitor, to the active metabolite temocaprilate, which is transported by a canalicular multispecific organic anion transporter (cMOAT, *ABCC2*) (Ishizuka et al. 1997). Furthermore CESs hydrolyse irinotecan hydrochloride (CPT-11), an anti-tumour drug, to the active metabolite SN-38, which is a good substrate for UDP-glucuronosyltransferase (UGT) (Sanghani et al. 2004).

It is important to understand the tissue-specific expression of CESs for design of ideal prodrugs that are efficiently hydrolysed in the target tissue and are associated with sufficient drug efficacy and few side-effects. The tissue-specific expression of CESs in the human kidney is involved in renal excretion, since CESs change the polarity of a prodrug. As mentioned above, CES2A1 is expressed in the human kidney. Therefore, it participates in renal excretion of a prodrug. Other drug-metabolizing enzymes that are expressed in the human kidney also play an important role in the metabolism of exogenous and endogenous compounds. For example, the glucuronidation of propofol is catalysed by UGT in the kidney (McGurk et al. 1998) and the secosteroid hormone 1,25-dihydroxyvitamin D3 (Calcitriol) is catalysed by 25-hydroxyvitamin D3 1-alpha-hydroxylase (CYP27B1) in the kidney (Zehnder et al. 1999). In this way, many drug-metabolizing enzymes expressed in the human kidney contribute to the metabolism of compounds. However, the *CES1A1* gene is poorly expressed in the human kidney. We previously reported that the transcription factor specificity protein (Sp) 1, which is ubiquitously expressed, can bind to the promoter region of the *CES1A1* gene, leading to transactivation of the promoter (Hosokawa et al. 2008). Hence, we have thought that there is a mechanism repressing *CES1A1* gene expression in the human kidney.

On the other hand, gene regulation by DNA methylation has been reported for some drug-metabolizing enzymes (Anttila et al. 2003; Gagnon et al. 2006). In mammals, DNA methylation occurs predominantly on cytosine in 5′-CpG-3′ dinucleotide and its methylation mark is propagated into both DNA strands after DNA replication. Approximately 40% of mammalian genes include CpG islands, regions with relatively high frequency of CpG nucleotides, in their promoters and exonic regions (Larsen et al. 1992). It is generally accepted that DNA methylation of a CpG island at a promoter region is closely associated with silencing of a gene expression. It is also known that DNA methylation in the region downstream of a transcription start site (TSS) causes dramatic reduction in gene expression (Graessmann et al. 1994; Hisano et al. 2003). Recently, it was proposed that a DNA methylation-free region extending several hundred bases downstream of the TSS may be a prerequisite for efficient transcription initiation (Appanah et al. 2007). The tissue-specific expression of a number of genes has been revealed by studies on DNA methylation (Kikuchi et al. 2007; Aoki et al. 2008).

The aim of the present study was to elucidate the cause of tissue-specific expression of the *CES1A1* gene, particularly the cause of the difference in gene expression in the human kidney and liver.

Materials and methods

Cell lines and human tissues

HEK293 cells, human embryonic kidney cells, and HuH-7 and HepG2 cells, human hepatoma cells, were used in this study. *CES1A1* gene is not expressed in HEK293 cells but is expressed in HuH-7 and HepG2 cells. The cell lines were cultured at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) foetal bovine serum and penicillin/streptomycin. Human kidney and liver were obtained from the National Disease Research Interchange (Philadelphia, PA, USA) through the Human and Animal Bridging Research Organization (HAB) (Chiba, Japan).

Treatment with DNA methylation inhibitor and histone deacetylase inhibitor

HEK293, HuH-7, and HepG2 cells were precultured for 24 h and then cultured for 3 days in medium containing 2 μM 5-aza-2′-deoxycytidine (5-aza-dC), a DNA methylation inhibitor (Sigma-Aldrich, St. Louis, MO, USA), diluted with PBS(−). Subsequently, total RNA was extracted. For histone deacetylase inhibition, 500 nM trichostatin A (TSA) (Wako, Osaka, Japan) diluted with ethanol was added to the cells 24 h before extraction of RNA.

Relative quantification of mRNA by real-time polymerase chain reaction (PCR)

Total RNA was extracted from the cell lines and tissues using an ISOGEN (Nippon Gene, Toyama, Japan) and then treated with DNase I (Invitrogen). Subsequently, cDNA was synthesized with the RNA using a ReverTra Ace® qPCR RT Kit (Toyobo, Osaka, Japan). Finally, the expression level of CES1A1 mRNA was analysed using a Realtime PCR Master mix (Toyobo) with an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster, CA). The specific primers were designed as follows: forward, 5′-GAGACCTCGCAGGCCCC-3′; reverse,

5′-GACGAACTTCCCCAGCACTT-3′. The fluorescent probe for CES1A1 was 5′-(FAM)-TCCGTGCCTTTATC-3′. Real-time PCR was performed under the following conditions: 50°C for 2 min, 95°C for 10 min, and 50 cycles of 95°C for 15 s and 56°C for 1 min. The CES1A1 mRNA expression was normalized with 18S rRNA expression:

Delta C_t (dC_t) = threshold cycle (C_t) for target amplification − C_t for reference amplification

In each cell line, the delta delta C_t (ddC_t) values were based on the average dC_t value of the cell line treated with 5-aza-dC alone, except that the ddC_t value of HuH-7 cells for Figure 1 was based on the average dC_t value of HEK293 cells treated with 5-aza-dC. The average mRNA expression ratio of three liver specimens was indicated as the result for the liver. The mRNA expression ratio of a specimen in which CES1A1 mRNA was always detected was selected as the representative value for the kidney. All assays were performed in triplicate. For improvement of the assay of CES1A1 mRNA used in our previous study (Hosokawa et al. 2008), *CES1A1* wild-type gene and a *CES1A1* gene variant were distinguished by sequencing and tissues that have only *CES1A1* wild-type gene were used in the present study. The *CES1A1* gene variant has the same exon 1 as that of the *CES1A3* gene instead of that of the *CES1A1* wild-type gene (Tanimoto et al. 2007; Fukami et al. 2008).

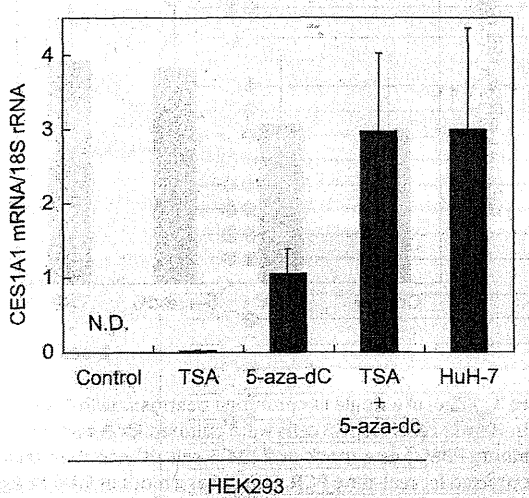

Figure 1. Effect of a single or combined treatment with 5-aza-dC and TSA in HEK293 cells. HEK293 cells were cultured for 3 days in medium containing PBS(-) as a mock or 2 μM 5-aza-dC and then total RNA was extracted for real-time PCR. Ethanol as a mock or 500 nM TSA was added to the cells 24 h before extraction of total RNA. CES1A1 mRNA expression was normalized with 18S rRNA. Each value is shown as the mean ± standard deviation (SD) of three independent experiments, which were performed in triplicate. Results for HuH-7 cells are shown for comparison with results for HEK293 cells treated with 5-aza-dC. N.D., The mRNA was not detected.

Determination of the CpG island

A search for the CpG island of the *CES1A1* gene was carried out using GENETYX-Mac Ver.12.2.0 software. The CpG island was determined using MethPrimer online software (Li & Dahiya 2002). The criteria used were island size > 200 bp, GC % > 50.0, and observed/expected CpG ratio > 0.6.

Bisulfite sequencing

Genomic DNA was extracted from the human kidney, human liver, HEK293 cells, and HepG2 cells using a FastPure™ DNA Kit (Takara Bio, Shiga, Japan). After purification, the genomic DNA was treated with sodium bisulfite using an Epitect™ Bisulfite Kit (Qiagen, Hilden, Germany). The target region (-512 to + 214) in the *CES1A1* gene was amplified from the genomic DNA by PCR using a GoTaq® Green Master Mix (Promega, Madison, WI, USA) and primers designed as follows: forward, 5′-TTGTGAAGTTAATTTAGGTTTTAGAAAGG-3′; reverse, 5′-AACCTATTATATCTTTACCTTTCTAC-3′. PCR was performed under the following conditions: 95°C for 2 min; 40 cycles of 95°C for 30 s, 53°C for 30 s, and 72°C for 1 min; and finally 72°C for 5 min. To perform hot start PCR, the reverse primer was added to the reaction mix when the reaction mix first reached 95°C. After being purified, the PCR products were cloned into a pGEM-T vector (Promega). Approximately ten clones for each tissue specimen were sequenced using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and a Montage™ SEQ_{96} Sequencing Reaction Cleanup Kit (Millipore, Billerica, MA, USA) on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). In addition, each of approximately 30 clones obtained from three independent experiments were sequenced for HEK293 cells and HEK293 cells treated with 5-aza-dC, and approximately 20 clones obtained from two independent experiments were sequenced for HepG2 cells. CpG methylation status was analysed by the web-based tool QUMA (Kumaki et al. 2008). Three human kidney specimens (age/sex, 47/male, 54/male, and 75/female) and three human liver specimens (age/sex, 62/female, 68/male, and 70/female) were used. Three TSSs of the *CES1A1* gene are known and the most upstream TSS was selected as TSS (+ 1) in the present study.

Enzyme assay

The activity of the hydrolysis for 0.1 mM *p*-nitrophenyl acetate (PNPA) was determined colorimetrically according to the method of Hosokawa et al. (1987). The cell culture and the treatment with 5-aza-dC and TSA were performed at the same condition of mRNA experiment

described above. Three independent experiments were performed.

Statistical analysis

All data of mRNA expression ratios were tested by the Smirnov–Grubbs test ($p<0.01$) and two extreme values were rejected. The results of bisulfite sequencing at each CpG site for the human kidney and liver were tested by Fisher's exact test, and that of the entire set of CpG sites for the human kidney and liver were tested by the Mann–Whitney *U*-test using the web-based tool QUMA (Kumaki et al. 2008). The results of enzyme assay were tested by Student's *t*-test.

Results

Comparison of CES1A1 mRNA expression levels in the human kidney, liver and the cell lines

We confirmed that the expression level of CES1A1 mRNA in the human liver is much higher than that in the kidney and that CES1A1 mRNA is detected in HuH-7 and HepG2 cells. When the ddC_t values of the kidney, liver and the cell lines were based on the average dC_t value of HuH-7 cells, the mRNA expression ratio (CES1A1 mRNA/18S rRNA) of the kidney, HuH-7, HepG2, and the liver was 0.106±0.0223, 1.11±0.508, 29.9±14.4 and 76.2±96.9 (mean ± standard deviation (SD)), respectively.

Activation of the CES1A1 gene in HEK293 cells by 5-aza-dC

CES1A1 mRNA was not detected in HEK293 cells by real-time PCR (Figure 1). To investigate whether the *CES1A1* gene in HEK293 cells is silenced by DNA methylation, we treated HEK293 cells with 2 μM 5-aza-dC for 3 days. As a result, we detected dramatic expression of CES1A1 mRNA in HEK293 cells (Figure 1). In addition, 500 nM TSA enhanced the expression in HEK293 cells treated with 5-aza-dC, but single treatment with TSA had little effect on mRNA expression in HEK293 cells. CES1A1 mRNA expression in HEK293 cells treated with 5-aza-dC and TSA was almost the same as the expression in HuH-7 cells alone. Furthermore, the dramatic enhancement of CES1A1 mRNA expression observed in HEK293 cells treated with 5-aza-dC was not observed in HuH-7 and HepG2 cells treated with 5-aza-dC (Figures 1–3).

Negative correlation between methylation and expression of the CES1A1 gene

To determine the methylation status of the *CES1A1* gene in the human kidney and liver and the cell lines, we performed bisulfite sequencing for the region of 726 bp (–512 to + 214) (Figure 4). The results revealed that the region around the TSS of the *CES1A1* gene is almost entirely methylated in HEK293 cells, whereas the region is almost entirely unmethylated in HepG2 cells (Figure 5). As a result of treatment with 2 μM

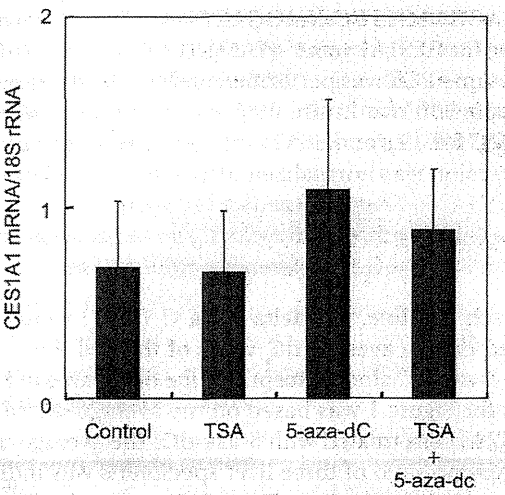

Figure 2. Effect of a single or combined treatment with 5-aza-dC and TSA in HuH-7 cells. HuH-7 cells were cultured for 3 days in medium containing PBS(–) as a mock or 2 μM 5-aza-dC and then total RNA was extracted for real-time PCR. Ethanol as a mock or 500 nM TSA was added to the cells 24 h before extraction of total RNA. CES1A1 mRNA expression was normalized with 18S rRNA. Each value is shown as the mean ± standard deviation (SD) of three independent experiments, which were performed in triplicate.

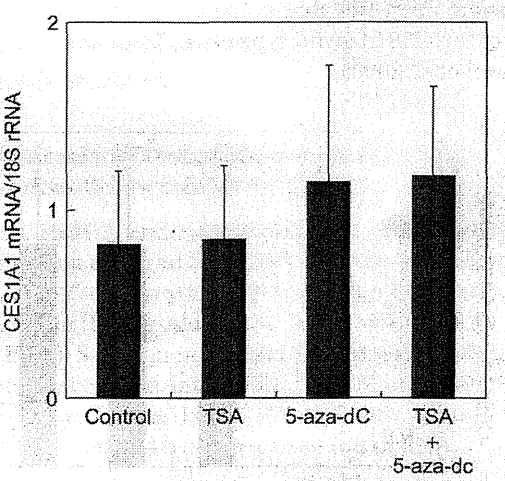

Figure 3. Effect of a single or combined treatment with 5-aza-dC and TSA in HepG2 cells. HepG2 cells were cultured for 3 days in medium containing PBS(–) as a mock or 2 μM 5-aza-dC and then total RNA was extracted for real-time PCR. Ethanol as a mock or 500 nM TSA was added to the cells 24 h before extraction of total RNA. CES1A1 mRNA expression was normalized with 18S rRNA. Each value is shown as the mean ± standard deviation (SD) of four independent experiments, which were performed in triplicate.

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-512 TTGTGAAGCTAATTCAGGCCTCAGAAAGGGGACTCGATGAAATTTAAGATCGCTTCCAAGCTTGAGAGCCTGGAAAGCTATGA
-429 AAACACAAGCCCTGGGAGCTGAGATATGTCCTAACTTACCCAGCTGAGCTGTGAGGTGTGAGTGGCTCTAACATTTTCCAGTTG
-345 TTTCTGAGGACCTCAGATCAAAGCTTCCCTTTGCCTAAAAGCATCTGCCTGCTGGTGTGGGCCCTTGGGGGCCGTCACAGTGCA
-261 CTGAGGTTAGAGTCCTGCAAGGGTGAACCCTTATGTAACAAGTAGTTGGGCAAGTTTACAGCTCTCTGTAATCTGACAGTAGAG
-177 TCCAGACTGGTTTGATGAAAGAGGGTAAACTGTGGGTGGGCGTGGCCTGAGGCCCCACTAGAAGCCCAGGGAGATCTGAGGAA
 -94 AGGGAGGGCTTTTCTGATCTCTCCCAATTAGAGGATTAGGCAATTGGCAGCGCAGGGCGGTAACTCTGGGCGGGGCTGGGCTC
 -11 CAGGGCTGGAC A GCACAGTCCCTCTGAACTGCACAGAGACCTCGCAGGCCCCGAGAACTGTCGCCCTTCCACGATGTGGCTC
                +1
+72 CGTGCCTTTATCCTGGCCACTCTCTCTGCTTCCGCGGCTTGGGGTGAGTCCTTCTGAAATCAAATATGCGGGGCACTTTTTGAAA
+157 TCCTTGTTCTGGGGCCGAGGTGGGCGCAGATGCGTAGAAAGGCAAAGACACAACAGGTC
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Figure 4. Positions of CpGs in the region of 726 bp (-512 to + 214) around the TSS of the *CES1A1* gene. Positions of CpGs are shown in boxes. A broken arrow with a box indicates TSS (+ 1). Exon 1 is shown in a shaded box. A part of the CpG island is underlined with a heavy line. ATG is underlined with a thin line.

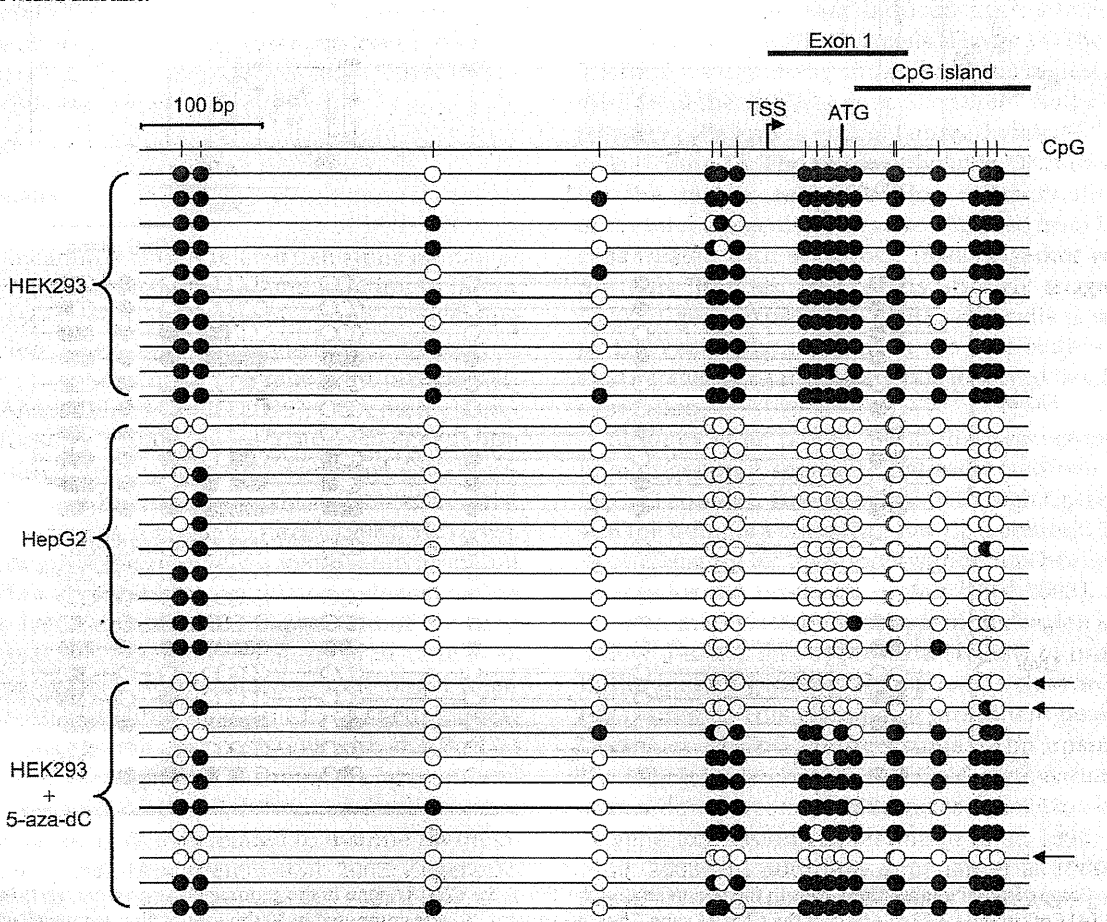

Figure 5. Appearance of hypomethylated molecules for the region around the TSS of the *CES1A1* gene in HEK293 cells treated with 5-aza-dC. Bisulfite sequencing was performed to determine the methylation pattern in the region from -512 to + 214. A broken arrow indicates TSS (+ 1). Each CpG position is depicted as a vertical bar. The methylated and unmethylated CpGs are depicted as closed circles and open circles, respectively. Ten representative molecules of each cell line are shown. Horizontal arrows indicate hypomethylated molecules.

5-aza-dC for 3 days, hypomethylated DNA molecules for the region accounted for approximately 30% of the total molecules that were obtained from HEK293 cells treated with 5-aza-dC. In the genome obtained from the kidney, the region downstream of the TSS was methylated compared with that obtained from the liver, although the difference in methylation levels between the kidney and liver was less clear than that

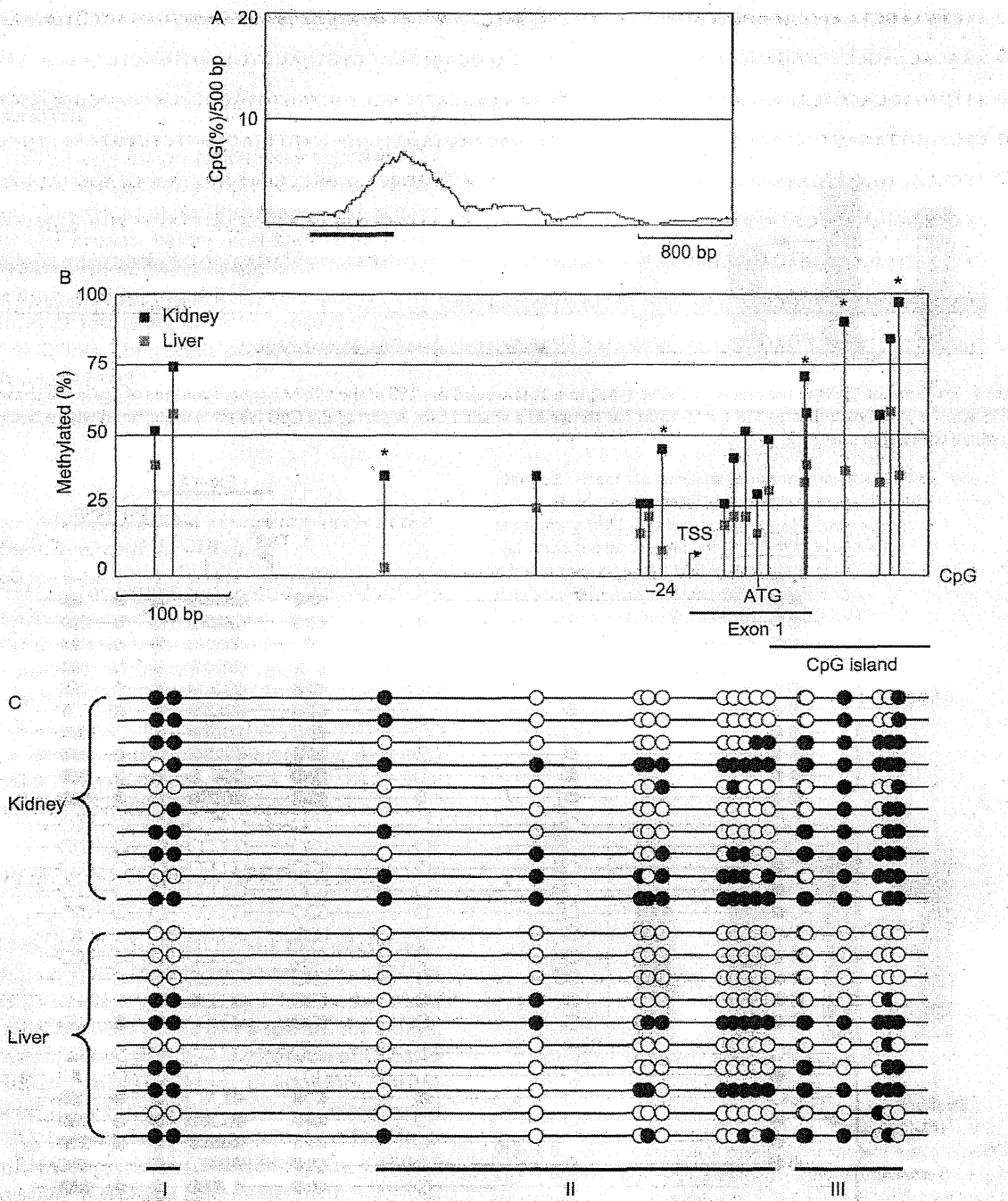

Figure 6. Comparison of methylation levels in the region around the TSS of the *CES1A1* gene in the genome obtained from the human kidney and liver. (A) CpG island (+ 72 to + 541) of the *CES1A1* gene. The CpG content (%) was computed in 500 bp overlapping segments across a 4-kb region. A heavy line indicates the amplified region of 726 bp. (B) Bisulfite sequencing was performed to determine the methylation pattern of the kidney and liver in the region of 726 bp (-512 to + 214). The methylated levels (%) of the kidney and liver are shown with the TSS (broken arrow), ATG, exon 1, and a part of the CpG island (+ 72 to + 214). The graph was made using approximately 30 molecules of each tissue. Each CpG position is depicted as a vertical bar. Asterisks indicate statistically significant differences in the methylation level between the human kidney and liver ($p<0.01$, Fisher's exact test). There was the statistically significant difference in the methylation level of the entire set of CpG sites between the kidney and liver ($p<0.01$, Mann–Whitney *U*-test). (C) The methylated and unmethylated CpGs are depicted as closed circles and open circles, respectively. Ten representative molecules of each tissue are shown.

between the cell lines (Figure 6). There was little difference in the methylation among individual liver or kidney specimens.

Activity of the hydrolysis of p-nitrophenyl acetate (PNPA)

As the results of the activity caused by single or combined treatment with 5-aza-dC and TSA to HEK293 cells, the values (nmol 10^{-6} cells min^{-1}) of control, TSA, 5-aza-dC, and TSA + 5-aza-dC were 1.67±0.425 (1.00-fold), 1.98±0.377 (1.18-fold), 2.10±0.0528 (1.26-fold), and 2.21±0.680 (1.32-fold) (mean ± SD), respectively. But statistically significant differences were not observed in the data of the activity of HEK293 cells. The result of the activity of HuH-7 cells was 13.3±1.95 nmol 10^{-6} cells min^{-1}. Correlation coefficient between PNPA hydrolase activity and mRNA expression level caused by single or combined treatment with 5-aza-dC and TSA to HEK293 cells was 0.789.

Discussion

DNA methylation is closely related to histone modifications such as deacetylation and methylation in mammals (Cameron et al. 1999). Histone proteins assemble into nucleosomes, and deacetylation of histone is important for silencing of the gene with the methylated promoter (Bird & Wolffe 1999). DNA methylation in the promoter region causes histone deacetylation by mediating some proteins such as methyl-CpG binding protein and histone deacetylase. As a result, the gene expression is repressed (Razin 1998). However, once silencing of the gene with a methylated promoter is established, inhibition of histone deacetylation generally cannot cause active expression of the gene (Cameron et al. 1999; Coffee et al. 1999; El-Osta et al. 2002). The present results showing that single treatment with TSA had little effect on *CES1A1* gene expression in HEK293 cells are consistent with the finding described above (Figure 1). The reason why treatment with a histone deacetylase inhibitor generally cannot cause active expression is related to histone methylation as described below (Peters et al. 2002; Zegerman et al. 2002). According to Kondo et al. (2003), methylation on lysine 9 (Lys-9) in histone H3 causes a repressive folded chromatin structure, affects the access of regulatory factors to chromatin, and leads to silencing of *P16*, *MLH1*, and *MGMT* genes in colorectal cancer. Treatment with 5-aza-dC decreased DNA methylation and Lys-9 methylation dramatically, increased Lys-9 acetylation slightly and Lys-4 methylation moderately, and reactivated gene expression (Kondo et al. 2003). Consistent with these findings obtained by treatment with 5-aza-dC, our results showed that treatment with 5-aza-dC allows HEK293 cells to activate *CES1A1* gene expression (Figure 1). Combined treatment with 5-aza-dC and TSA decreased DNA methylation and Lys-9 methylation and increased Lys-9 acetylation markedly and Lys-4 methylation, while single treatment with TSA increased Lys-9 acetylation and had no effect on Lys-9 or Lys-4 methylation (Kondo et al. 2003). The acetylation level of Lys-9 in the case of combined treatment was higher than that in the case of treatment with 5-aza-dC alone. Consequently, a high expression level of the gene was observed. In agreement with these findings about combined treatment, the results showed that the level of *CES1A1* gene expression induced by combined treatment with 5-aza-dC and TSA was approximately three times higher than that induced by treatment with 5-aza-dC alone in HEK293 cells (Figure 1). The results of bisulfite sequencing showed that the region around the TSS of the *CES1A1* gene is entirely methylated in HEK293 cells and is entirely unmethylated in HepG2 cells (Figure 5). The hypomethylated DNA molecules for the region accounted for approximately 30% of the total molecules that were obtained from HEK293 cells treated with 5-aza-dC. Taken together with the previous findings, our results strongly suggest that *CES1A1* gene expression in HEK293 cells is silenced by DNA methylation. This is the first study demonstrating that DNA methylation is involved in *CES* gene expression.

Saxonov et al. (2006) found that promoters in the human genome segregate naturally into two classes by CpG content. One is a class with high CpG content and the other is a class with low CpG content. To date, gene silencing by DNA methylation has been studied mainly for the promoter region (Bird 2002). Studies on DNA methylation for the promoter region seem to reflect the fact that approximately 70% of promoters in the human genome belong to the class with high CpG content. This class has a prominent peak in the frequency of CpG centred some 15 bp upstream of the TSS. It is also known that DNA methylation in the region downstream of TSS causes dramatic reduction in gene expression (Graessmann et al. 1994; Hisano et al. 2003). In addition, Appanah et al. (2007) proposed that methylation of the 3′ promoter-proximal region, approximately 300 bp downstream of the TSS, may dramatically reduce transcription initiation efficiency by relating alteration of the promoter chromatin structure. The findings about the region downstream of the TSS explain why the expression levels of the *CES1A1* gene in the human kidney and liver are different. In the genome obtained from the kidney, the region downstream of the TSS was methylated compared with that obtained from the liver (Figure 6).