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.

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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)₆TAA>A(TA)₇TAA 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 *PvuII* 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 per-

formed. 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 [$\text{AUC}_{\text{SN-38} + \text{SN-38G}} / \text{AUC}_{\text{irinotecan}}$] was used as a parameter reflecting *in vivo* CES activity [14]. The AUC ratio of APC to irinotecan [$\text{AUC}_{\text{APC}} / \text{AUC}_{\text{irinotecan}}$] 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) <i>CES1A1</i> exon 1 and promoter region	First PCR	Ces1-FP Ces1-RP	This study
	Second PCR	Ces1_seqF Ces1_seqR	
	Sequencing	Ces1_SF2 Ces1_SR	
		5'-CCAGGCAAAACCTAGGAGTG-3' 5'-AGTACAGGGCGATCTCAGGA-3' 5'-GTATTTCTTAGCCAGCGGA-3' 5'-CAGAGCCGGACCTGTTGT-3' 5'-AGAGCCTGAAAAGCTATGAAAA-3' 5'-TTTCTACGCATCTGCGCCACC-3'	
(b) <i>CES1A1</i> , <i>1A2</i> and <i>1A3</i> exon 5	PCR and sequencing	1A-int4F 1A-int5AS	[16]
(c) <i>CES1A3</i> exon 3	PCR and sequencing	CES1A3-15183F CES1A3-15974R	This study
	Sequencing (additional primer)	CES1A3-15823R	
		5'-CAGGGAAGATCGTTGATTGGTTT-3' 5'-TTCCTTCCACCACTAATATTG-3' 5'-AAGATGTTCTTAAAGATGCACAG-3'	
(d) <i>CES1A2</i> and <i>1A3</i> -816A>C genotyping	PCR	F R	[18]
	TaqMan probe	FAM VIC	
		5'-CCTTAATTTGGTGATTTACATTGC-3' 5'-CAAGACATGGTTCAGCTTCTCAAG-3' 5'-CATCACCCCTACTGC-3' 5'-CATCACACCTACTGCT-3'	
(e) <i>CES1A2</i> promoter region	PCR	CES1A3-CES1A2_F1 CES1A2_R1	This study
(f) <i>CES1A3</i> promoter region	First PCR	CES1A3-CES1A2_F1 CES1A3_R1	This study
	Second PCR	CES1A3-CES1A2_F2 CES1A3_R2	
	Sequencing of <i>CES1A2/1A3</i> promoter region	CES1A3-CES1A2_F2 CES1A3-CES1A2_R1 CES1A3-CES1A2_F3 CES1A3-CES1A2_R2	
		5'-ATGATTTCAGCTTCATCTACA-3' 5'-GAGAGAACGTTCCCATGTCTTT-3' 5'-ATGATTTCAGCTTCATCTACA-3' 5'-GCTTGAGTTTCTTACAGACA-3' 5'-AACAGTTTATAACCTGTTATTTT-3' 5'-TGCTTTGGATAAAGACAAGATGTT-3' 5'-AACAGTTTATAACCTGTTATTTT-3' 5'-CACACTTCCAATCTCAGGTAAA-3' 5'-TTATGCCACAAGCAGTTGGGCG-3' 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 $[AUC_{(SN-38 + SN-38G)}/AUC_{irinotecan}]$ 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 $[AUC_{(SN-38 + SN-38G)}/AUC_{irinotecan}]$ 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 2Frequency of *CES1* genes and diplotypes in Japanese cancer patients

<i>CES1</i> diplotype	Number of <i>CES1</i> gene		1A2	1A3	Total*	Frequency (n = 177)†		Frequency (monotherapy: n = 58)‡	
	1A1	var1A1							
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		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)
This study	NCBI (dbSNP)	JSNP	Location	NT_010498.15						
MPJ6_CS1001†			5'-flank	9481424	-258	ttggcaagttaacagctctCTtgtaacttgacagtagatc			0.014	
MPJ6_CS1002†			5'-flank	9481399	-233	atctgacagtagagtcagaCAgtggtttgtagaagaggga			0.003	
MPJ6_CS1003†			5'-flank	9481327	-161	tagaagccagaggagatctgAGggaaaggagggttttctg			0.006	
MPJ6_CS1004	rs3815583	IMS-JST175949	Exon1(5'-UTR)	9481241	-75	aactctggcggggctgggcGTccagggtcgacacacagt			0.41	var1A1
MPJ6_CS1005	rs28429139		Exon1(5'-UTR)	9481212	-46	ggacagcacagctctctgaaAGctgacagagacacctgcagg			0.299	var1A1
MPJ6_CS1006	rs28494177		Exon1(5'-UTR)	9481205	-39	acagtcctctgaactgcacAGgagacctcgagggcccgag			0.299	var1A1
MPJ6_CS1007†			Exon1(5'-UTR)	9481196	-30	ctgaactgcacagagacctcGACagggcccgagaactgtgc			0.042	
MPJ6_CS1008	rs28520463		Exon1(5'-UTR)	9481187	-21	acagagacctcgagggcccG/Cagaactgctgacctccag			0.297	var1A1
MPJ6_CS1009	rs28499065		Exon1(5'-UTR)	9481186	-20	cagagacctcgagggcccGAGgaactgtgaccttccacga			0.297	var1A1
MPJ6_CS1010	rs28515828		Exon1(5'-UTR)	9481168	-2	cgagaactgtgaccttccacC/Ggagtggtctcgaccttta			0.299	var1A1
MPJ6_CS1011			Exon 1	9481156	11	cccttccacagatggtctccG/Ctgcctttatctggccactc		Arg4Pro	0.297	var1A1
MPJ6_CS1012			Exon 1	9481152	15	tccacagatgggtctccgC/Tttatctggccactcttc		Ala5Ala	0.297	var1A1
MPJ6_CS1013			Exon 1	9481151	16	ccacagatgggtctcggtccT/Cttatctggccactctctct		Phe6Leu	0.297	var1A1
MPJ6_CS1014			Exon 1	9481148	19	cgatgggtccggtctttA/Gcttcggccactctctctgct		Ile7Val	0.297	var1A1
MPJ6_CS1015	rs28563878		Exon 1	9481133	34	tgcctttatctcgccactctT/Gctgtctccggtctggggt		Ser12Ala	0.297	var1A1
MPJ6_CS1016	rs12149359		Intron 1	9481099	IVS1+16	ttgggtgagtcctcttgaaAGtcaaaatgcgggacctttt			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

<i>CES1A2</i> and <i>1A3</i>	–816A>C	Number of subjects	Allele frequency
Genotype	Genotype		
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 *CES1*s 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
<i>ABCB1*2</i> † (+/+)	0.042	0.024	0.0831
<i>CES1</i> 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* pseudogene 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 ($-30G>A$) in the 5'-UTR of *CES1A1* (Table 3). The effect of $-30G>A$ 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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