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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.

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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

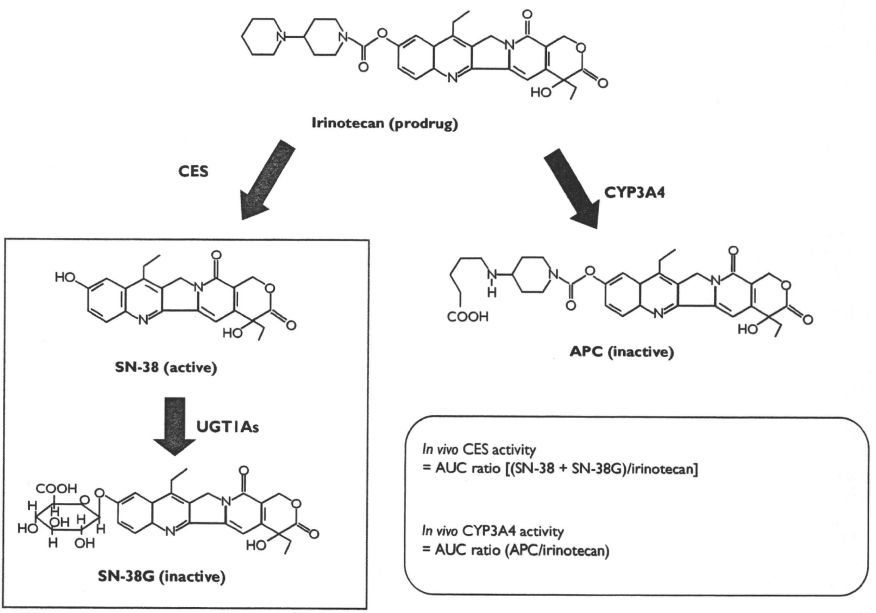

Figure 1

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

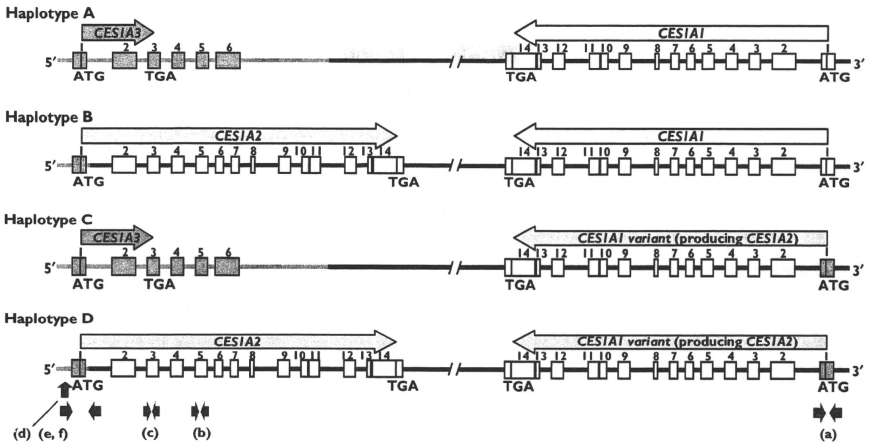

Figure 2

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**tgtaatctgacagtagagtc		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	acagagacctcgcaggccccc**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**atgtggctccgtgccttta		0.299	*var1A1*
MPJ6_CS1011			Exon 1	9481156	11	ccttccacgatgtggctcc**G/C**tgcctttatcctggccactc	Arg4Pro	0.297	*var1A1*
MPJ6_CS1012			Exon 1	9481152	15	tccacgatgtggctccgtgc**C/T**ttatcctggccactctctc	Ala5Ala	0.297	*var1A1*
MPJ6_CS1013			Exon 1	9481151	16	ccacgatgtggctccgtgcc**T/C**ttatcctggccactctctct	Phe6Leu	0.297	*var1A1*
MPJ6_CS1014			Exon 1	9481148	19	cgatgtggctccgtgccttt**A/G**tcctggccactctctctgct	Ile7Val	0.297	*var1A1*
MPJ6_CS1015	rs28563878		Exon 1	9481133	34	tgcctttatcctggccactctc**T/G**ctgcttccgcggcttggggt	Ser12Ala	0.297	*var1A1*
MPJ6_CS1016	rs12149359		Intron 1	9481099	IVS1+16	ttggggtgagtcctctctgaa**A/G**tcaaaatgcggggcacttttt		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

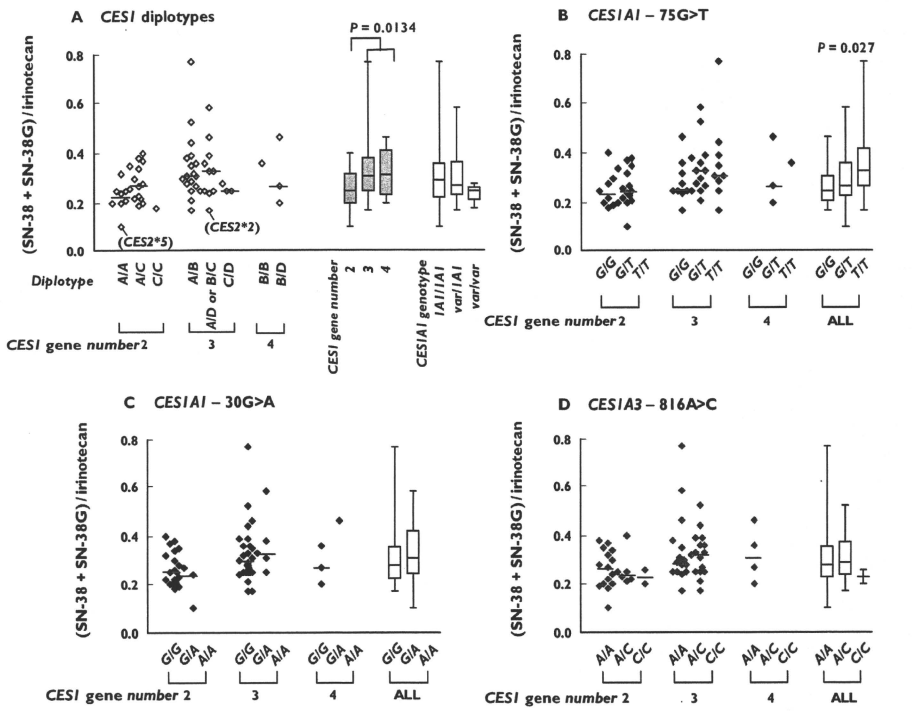

Figure 3

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

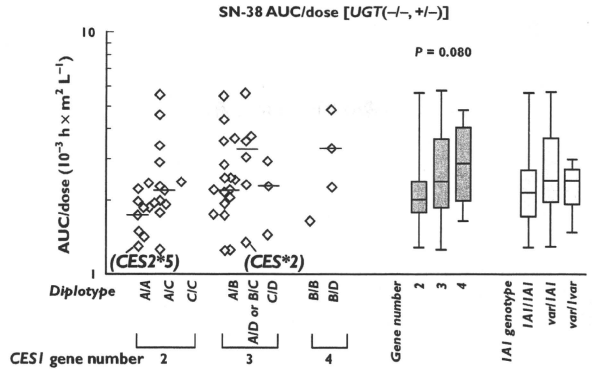

Figure 4

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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Original article

Second-line chemotherapy with irinotecan plus cisplatin after the failure of S-1 monotherapy for advanced gastric cancer

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Abstract

Background. **For advanced gastric cancer (AGC), second-line chemotherapy after the failure of S-1 has not yet been established. The present study aimed to retrospectively evaluate the efficacy and safety of irinotecan plus cisplatin (IP) therapy after the failure of S-1 in patients with AGC.**

Methods. **The subjects included 87 patients with AGC who received IP therapy as second-line chemotherapy. Irinotecan (70 mg/m^2) was administered by intravenous infusion followed by an intravenous infusion of cisplatin (80 mg/m^2) on day 1. On day 15, irinotecan (70 mg/m^2) alone was administered. The treatment was repeated every 4 weeks until disease progression, patient refusal, or severe adverse events.**

Results. **The median patient age was 62 years (range, 39–75 years), and the median number of treatment cycles was 3 (range, 1–9). Out of the 87 patients, 70 were assessable for clinical response. There were 2 complete responses and 18 partial responses. The overall response rate was 28.6% (95% confidence interval [CI], 18.4%-40.6%) and the disease control ratio was 70.0%. The median time to progression and median survival time from the first day of IP therapy were 4.3 months and 9.4 months, respectively. The 1-year survival rate was 34.6%. Severe (grade 3/4) leukopenia, neutropenia, anemia, and thrombocytopenia were observed in 34%, 40%, 28%, and 8% of patients, respectively. Grade 3/4 nonhematologic toxicities included anorexia (17%), febrile neutropenia (10%), diarrhea (6%), fatigue (5%), nausea (2%), and elevated creatinine (1%).**

Conclusions. **The combination of irinotecan plus cisplatin as second-line chemotherapy for AGC appears to be an effective and feasible treatment option after S-1 failure.**

Key words Irinotecan · Cisplatin · Gastric cancer · Second-line chemotherapy · S-1 failure

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Introduction

For the first-line treatment of advanced gastric cancer (AGC), the Japan Clinical Oncology Group (JCOG) reported the results of a three-arm phase III study comparing 5-fluorouracil (5-FU), irinotecan hydrochloride (CPT-11) plus cisplatin (CDDP) combination chemotherapy (IP), and S-1 [1]. The results showed that IP therapy did not demonstrate statistically significant superiority to 5-FU (median survival time [MST], 12.3 months vs 10.8 months; $P = 0.055$), although it was potentially promising.

In contrast, S-1 showed significant noninferiority to 5-FU (MST, 11.4 months vs 10.8 months; $P < 0.001$). Furthermore, Koizumi et al. [2] reported that in the S-1 plus CDDP versus S-1 in RCT in the treatment for stomach cancer (SPIRITS) trial, S1 plus CDDP established superiority over S-1 monotherapy (MST, 13.0 months vs 11.0 months, respectively; $P = 0.037$). However, another phase III study, comparing S-1 and S-1 plus CPT-11 (GC0301/TOP-002 trial), could not demonstrate a significant survival benefit for S1 plus CPT-11 [3].

According to these results, S-1 plus CDDP is suitable for first-line chemotherapy for AGC, and CPT-11-based regimens failed as first-line chemotherapy. Additionally, Sakuramoto et al. [4] have reported that S-1 is effective as adjuvant chemotherapy in patients who have undergone curative gastrectomy for locally advanced gastric cancer (adjuvent chemotherapy trial of TS-1 for gastric cancer; ACTS-GC-trial). Thus, S-1 is currently used for gastric cancer in both first-line and adjuvant settings. As such, it is expected that the number of S-1-refractory cases will increase in the near future, and therefore, establishing second-line chemotherapy for S-1-refractory AGC is very important.

However, there are few data for second-line IP therapy for AGC refractory to S-1. Therefore, we decided to retrospectively evaluate the efficacy and

safety of IP therapy in 87 patients who received IP therapy only after failure of S-1 monotherapy.

Patients, materials, and methods

Patient information

The subjects in this retrospective study included 87 patients with primary AGC who received IP therapy as second-line chemotherapy for unresectable or recurrent tumors at the National Cancer Center Hospital (Tokyo, Japan) between March 2001 and January 2007. The following inclusion criteria were used: (1) histologically proven adenocarcinoma of the stomach; (2) age 75 years or younger; (3) performance status (Eastern Cooperative Oncology Group) 0 to 2; (4) refractory to or unable to tolerate prior chemotherapy with S-1 monotherapy (given in 6-week cycles; 4 weeks of S-1 administration and 2 weeks' rest); (5) adequate organ function; (6) lack of massive ascites; and (7) written informed consent.

Treatment schedule

On day 1, CPT-11 (70 mg/m^2) was administered by intravenous infusion for 90 min, followed by intravenous infusion of CDDP (80 mg/m^2) for 120 min with adequate hydration. On day 15, CPT-11 (70 mg/m^2) alone was administered. The treatment was repeated every 4 weeks until disease progression, patient refusal to receive further treatment, or the occurrence of severe adverse event(s). Administration of CPT-11 on day 15 was delayed in the case of leukopenia or thrombocytopenia of grade 2 or more, diarrhea of grade 1 or more, or infection, until recovery from these adverse reactions. If the adverse reaction continued beyond day 22, CPT-11 was not given. If grade 4 leukopenia or thrombocytopenia or any grade 3/4 nonhematologic adverse reaction occurred, the doses of CPT-11 and CDDP were reduced to 60 mg/m^2 and 70 mg/m^2, respectively. If one of these severe adverse reactions occurred a second time, treatment was stopped. And if severe renal dysfunction (serum creatinine >2.0 mg/dl) developed, CDDP administration was halted, and CPT-11 monotherapy was continued until progression.

Clinical evaluation

Clinical response in measurable lesions was evaluated every 8 weeks by computed tomography (CT) using the Response Evaluation Criteria in Solid Tumors. Toxicity was evaluated according to the Common Terminology Criteria for Adverse Events (CTCAE), version 3.0. We defined overall survival (OS) as the number of days between the date of initial chemotherapy and the date of death or last follow-up visit. Time to progression was also measured from the beginning of treatment to the date of disease progression, which was evaluated by each physician. Survival analysis was performed using the Kaplan-Meier method, and differences between curves were analyzed using the log-rank test. The time to an event was calculated beginning with the start of treatment. All analyses were performed using the statistical software package StatView, version 5.0 (SAS Institute, Cary, NC, USA).

Results

Clinicopathological features

Patient clinicopathological characteristics are listed in Table 1. Between May 2000 and October 2006, 427 patients with AGC received first-line S-1 monotherapy. After failure, 298 patients subsequently received second-line chemotherapy. Of these, 96 patients received IP therapy, and we evaluated 87 patients who fulfilled the inclusion criteria (the excluded patients consisted of 7 patients aged >75 years and 2 who did not have adenocarcinoma). The primary reasons for discontinuation of S-1 therapy were progressive disease (n = 80 [92%]); followed by adverse events (n = 6 [7%]), including acneiform eruption (n = 3), anorexia (n = 2), edema (n = 1), and diarrhea (n = 1); and patient refusal (n = 1 [1%]). The median number of prior S-1 courses administered was 3 (range, 1–16). The median follow-up was 5.0 years (range, 2.4–8.2 years). The median number of IP cycles administered after S-1 failure was 3 (range, 1–9 cycles; total, 300 cycles).

Table 1. Patient characteristics (n = 87)

Factor	No. of patients	Percentage
Age		
Median (years)	62 (39–75)	
Sex		
Male	65	75
Female	22	25
ECOG performance status		
0/1/2	29/53/5	
Histological type		
Intestinal	46	53
Diffuse	41	47
Metastatic site		
Lymph node	53	61
Liver	44	51
Peritoneum	20	23
Lung	7	8
Bone	1	1
Discontinuation of S-1 therapy		
PD	80	92
Adverse event	6	7
Patient refusal	1	1

ECOG, Eastern Cooperative Oncology Group; PD, progressive disease

Table 2. Overall response (n = 70)

CR	PR	SD	PD	RR	DCR
2	18	29	21	28.6%[a]	70.0%[b]

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; RR, response rate; DCR, disease control ratio (CR+PR+SD/all)
[a]95% confidence interval (CI): 18.4–40.6
[b]95% CI: 57.9–80.4

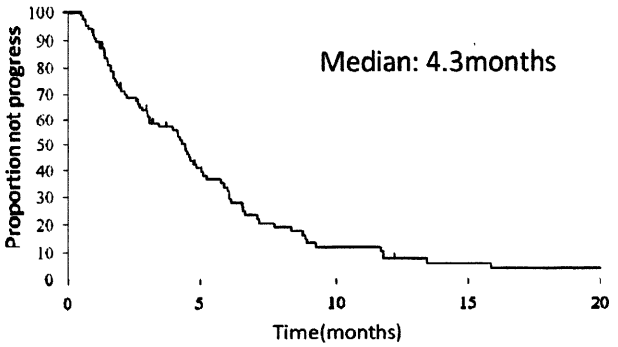

Fig. 1. Time to progression

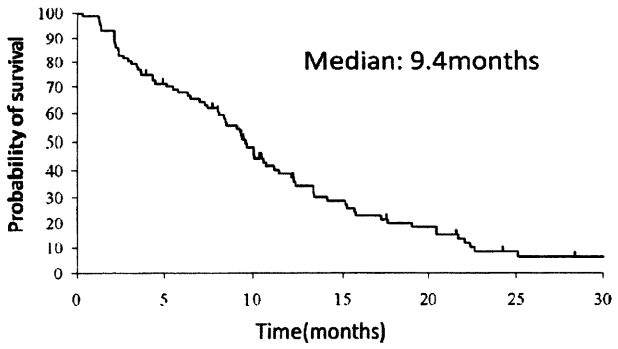

Fig. 2. Overall survival

Response and survival

A total of 70 patients had measurable lesions and were assessable for clinical response. There were 2 complete responses (CRs) and 18 partial responses (PRs). The overall response rate (ORR) was 28.6% (95% CI, 18.4–40.6%), and the disease control ratio was 70.0% (Table 2). The median time to progression (TTP) and MST from the first day of IP therapy were 4.3 months and 9.4 months, respectively (Figs. 1, 2). In addition, the MST from the first day of first-line S-1 was 14.3 months. The 1-year survival rate was 34.6%.

Toxicities

Toxicities experienced by patients treated with IP therapy are summarized in Table 3. Severe (grade 3/4) leukopenia, neutropenia, anemia, and thrombocytopenia were observed in 34%, 40%, 28%, and 8% of patients, respectively. Grade 3/4 nonhematologic toxicities included anorexia (17%), febrile neutropenia (10%), diarrhea (6%), fatigue (5%), nausea (2%), and elevated creatinine (1%).

Table 3. Adverse events (n = 87)

Grade	0/1	2	3	4	Grade ≥3 (%)
Leukopenia	38	19	24	6	34
Neutropenia	44	8	11	24	40
Anemia	42	21	18	6	28
Thrombocytopenia	73	7	5	2	8
Anorexia	52	20	15	0	17
Nausea	75	10	2	0	2
Diarrhea	76	6	5	0	6
Neutropenic fever	—	—	8	1	10
Fatigue	60	23	4	0	5
Creatinine	76	10	1	0	1

There were four patients (5%) who died less than 30 days from the initiation of therapy; one death was due to febrile neutropenia and infection, while the three other deaths were assumed to have been due to rapidly progressive disease.

Reasons for discontinuation and additional chemotherapy administered

The primary reasons for discontinuation of IP therapy were progressive disease (n = 73 [84%]), followed by adverse events (n = 9 [10%]), including renal dysfunction (n = 4), neutropenia (n = 1), anorexia (n = 1), liver dysfunction (n = 1), acneiform eruption (n = 1), anaphylactic shock (n = 1), patient refusal (n = 4 [5%]), and discontinuation because of CR (n = 1 [1%]).

A total of 46 (53%) patients received additional chemotherapy. The most commonly used regimens were paclitaxel monotherapy (n = 37 [80%]), docetaxel monotherapy (n = 6 [13%]), and mitomycin C (MMC)-based therapy (n = 3 [7%]).

Discussion

The clinical value of second-line chemotherapy for AGC remains controversial. However, in Japan, CPT-11 is widely used both as a single agent and as combined therapy with CDDP or MMC [5, 6]. Futatsuki et al. [7] reported that CPT-11 monotherapy (100 mg/m^2, weekly or 150 mg/m^2, biweekly) achieved ORRs of 20% (9/45) in previously treated gastric cancer patients, and 18.9% (7/37) in patients who were only pretreated with 5-FU. CPT-11 monotherapy therefore appears to be somewhat effective for 5-FU-refractory gastric cancer. In a more recent randomized phase III study, albeit of small

sample size (n = 40), Thuss-Patience et al. [8] reported that second-line CPT-11 monotherapy (250 to 350 mg/m^2, triweekly) significantly prolonged overall survival (OS) compared to best supportive care (BSC); the median survival in the CPT-11 arm was 123 days compared to 72.5 days for BSC; OS, hazard ratio [HR] = 2.85 (95% CI, 1.41–5.79); P = 0.0027. These results indicate that second-line chemotherapy using CPT-11 can now be considered as a treatment option in GC.

There have been two phase II studies evaluating IP therapy. Boku et al. [9] reported an ORR of 26.7% (4/15), and Ajani et al. [10] reported an ORR of 31% (9/29) and an MST of 5 months for AGC refractory to 5-FU therapy. In addition, in a retrospective study, Ueda et al. [11] reported a 28% (8/28) ORR, a progression-free survival (PFS) of 3.4 months, and an MST of 9.4 months.

Our present study had a selection bias, with comparatively few patients (23%) having peritoneal metastases, because such cases tend to be treated with taxanes; however, our results (28.6% ORR, TTP of 4.3 months, MST of 9.4 months, and 34.6% 1-year survival rate) indicate that second-line IP therapy for AGC appears to provide almost the same efficacy as that seen in other second-line trials, even for patients who have experienced S-1 failure.

We also demonstrated greater feasibility for IP therapy by using it in a second-line rather than first-line setting. In the first-line setting, IP therapy did not show statistically significant superiority to 5-FU because of its toxicity; more than 30% of patients receiving IP therapy discontinued for toxicity-related reasons, as opposed to fewer than 10% stopping for toxicity due to 5-FU and S-1 [1]. In the present study, grade 3/4 leukopenia or neutropenia were relatively mild, and only 10% of patients stopped treatment because of toxicity-related reasons. The reasons for these results may be that first, the duration of IP therapy is shorter in the second-line setting than in the first-line setting, and, second, in this study, dose reduction and discontinued treatment were carried out exactly according to protocol.

Another recent well-known IP regimen is biweekly CPT-11+CDDP. Koizumi et al. [12] reported on their phase I/II study using it as first-line therapy, where CPT-11 (60 mg/m^2) and CDDP (30 mg/m^2) were administered on days 1 and 15. In 2008, Nakae et al. [13] reported a phase II study of biweekly IP after S-1 failure. The ORR was 28.6%, and the median OS was 389 days. The most common grade 3/4 toxicities were: neutropenia (22.9%), anemia (11.4%), anorexia (14.3%), fatigue (8.6%), and diarrhea (2.9%). The efficacy and toxicity of this biweekly regimen were almost the same as those seen in our study. The biweekly regimen is available for outpatients; however, there are no phase III data on this regimen.

Currently, either CPT-11 (monotherapy or combined with CDDP) or taxanes [14–16] would be selected as second-line chemotherapy for AGC. However, it is not yet clear which of these regimens is most effective. Therefore, there are some currently ongoing clinical trials on the second-line treatment of AGC (after S-1 or S-1+CDDP failure), including phase III studies comparing CPT-11 and paclitaxel, CPT-11 alone and CPT-11+CDDP, CPT-11 alone and CPT-11+S-1, and so on. The results are anxiously awaited.

In conclusion, the combination of CPT-11 and CDDP as second-line chemotherapy for AGC appears to be effective and feasible, and should therefore be considered as a promising treatment option for patients who have experienced S-1 failure. Because CDDP has been widely used as first-line treatment for AGC patients, this regimen is suitable for patients who failed S-1 monotherapy used as adjuvant chemotherapy.

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Original Article

A Multicenter Phase-II Study of 5-FU, Leucovorin and Oxaliplatin (FOLFOX6) in Patients with Pretreated Metastatic Colorectal Cancer

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Objective: Infusional 5-fluorouracil and leucovorin with oxaliplatin is one of the standard regimens for patients with pretreated metastatic colorectal cancer, as well as for first-line chemotherapy. FOLFOX4 has shown its efficacy in pivotal trials, but patients must make twice-weekly hospital visits. FOLFOX6 is a more convenient regimen, requiring a visit once every two weeks. The objective of this study was to evaluate the efficacy and safety profile of FOLFOX6 in Japanese patients with pretreated colorectal cancer.

Method: Fifty-one metastatic colorectal cancer patients who failed to respond to first-line chemotherapy were enrolled in the study from April to July 2005. Oxaliplatin, 5-fluorouracil and *l*-leucovorin were administered every two weeks. Oxaliplatin (100 mg/m^2) and *l*-leucovorin (200 mg/m^2) were given intravenously over 2 h followed by 5-fluorouracil bolus 400 mg/m^2 i.v. and 46-h infusion of 2400 mg/m^2. The primary endpoint was the response rate.

Results: Two patients had no measurable lesions and were excluded from the efficacy analysis. Of the 49 eligible patients, one complete response and 6 partial responses were observed, resulting in a response rate (RR) of 14.3% (95% confidence interval: 5.9–27.2%). Median time to treatment failure and progression-free survival was 4.4 and 5.3 months, respectively. Overall survival was 11.4 months. The incidence of grade 2/3 (Debiopharm neurotoxicity criteria) peripheral neuropathy was 41.2%, whereas the overall incidence of grade 3/4 neutropenia was 43.2%.

Conclusion: The results of our study suggest that FOLFOX6 had an acceptable profile in terms of both efficacy and safety in previously treated colorectal cancer patients.

Key words: FOLFOX6 – second-line – colorectal cancer

INTRODUCTION

Colorectal cancer is the fourth most common cancer worldwide, and the number of patients affected by this disease continues to grow steadily (1–3). It is estimated that approximately 92 000 new cases of colorectal cancer are diagnosed each year in Japan (4).

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Infusional fluorouracil and leucovorin with oxaliplatin is one of the standard regimens for first- and second-line chemotherapy for metastatic colorectal cancer patients. The FOLFOX4 regimen has proved its superiority over infusional 5-FU with leucovorin (LV5FU2) in terms of RR (FOLFOX4: 9.9% vs LV5FU2: 0%), time to progression (TTP) (FOLFOX4: 4.6 vs LV5FU2: 2.7 months) and alleviation of tumour-related symptoms in patients with progressive colorectal cancer following irinotecan, fluorouracil and leucovorin (IFL) (5). For previously untreated patients, FOLFOX4 has also proved to be superior to IFL regimen in terms of RR (FOLFOX4: 45% vs IFL: 31%; $P = 0.002$), TTP (FOLFOX4: 8.7 vs IFL: 6.9 months; $P = 0.0014$) and median survival time (MST) (FOLFOX4: 19.4 vs IFL: 15.0 months; $P = 0.0001$) (6). FOLFOX4 showed a RR of 21.2% and overall survival (OS) of 11.5 months in 5-FU refractory patients (7). An RR of 9.9% and time to treatment failure (TTF) of 4.6 months was observed in patients who failed to respond to irinotecan-based therapy (5). On the other hand, FOLFOX6 showed an RR of 27% in 5-FU pretreated colorectal cancer patients and 15% in patients who failed to respond to FOLFIRI therapy (8,9).

Through the use of a central venous access device and infusional pump, patients are able to receive FOLFOX4 therapy on an outpatient basis, but the FOLFOX4 regimen requires two times hospital visit every 2 weeks and it is necessary to visit the hospital two times twice a week for the bolus 5-FU injection on Day 1 and Day 2. The FOLFOX6 regimen (8), in which oxaliplatin (100 mg/m^2) and *l*-leucovorin (200 mg/m^2) are given intravenously over 2 h followed by 5-FU bolus 400 mg/m^2 and 46-h infusion 2400 mg/m^2, is simpler and more convenient than the FOLFOX4 regimen. In this Phase-II study, we evaluated the FOLFOX6 regimen as second-line therapy for Japanese patients with metastatic colorectal cancer.

PATIENTS AND METHODS

Eligibility

The eligibility criteria were as follows: pathologically confirmed adenocarcinoma of the colon or rectum that was considered to be inoperable with at least one measurable metastasis (RECIST guidelines) (10); Eastern Cooperative Oncology Group (ECOG) performance status (PS) score of 0, 1 or 2; and age 20–75 years. Failure (disease progression/discontinuation due to toxicity) within 6 months of the last dose of first-line fluoropyrimidine and irinotecan treatment for metastatic disease or adjuvant therapy was required. Patients with previous oxaliplatin treatment were excluded. All patients had to meet the following laboratory criteria within 14 days before registration: white blood cells (WBC) ≥ 3000 /mm^3; platelet count $\geq 10 \times 10^9$/l; haemoglobin level ≥ 8.0 g/dl; aspartate aminotransferase (AST)/alanine aminotransferase (ALT) ≤ 100; total bilirubin ≤ 1.5 mg/dl; serum creatinine ≤ 1.1 mg/dl; and no major electrocardiogram abnormalities. Written informed consent was obtained from all patients. The study protocol was approved by the institutional review boards of the participating institutions.

Treatment Plan

FOLFOX6 consisted of oxaliplatin 100 mg/m^2 on Day 1, given as a 2-h infusion concurrent with *l*-LV 200 mg/m^2 followed by 5-FU 400 mg/m^2 injection and 2400 mg/m^2 given as a 46-h continuous infusion. Antiemetic prophylaxis with a $5HT_3$-receptor antagonist and steroid was also administered. The use of implantable ports and disposable pumps allowed chemotherapy administration on an outpatient basis.

Chemotherapy was delayed until recovery in cases of WBC $\geq 3.0 \times 10^9$/l, platelets $\geq 100 \times 10^9$/l, watery diarrhoea or for $\leq$ Grade 2 non-haematologic toxicity. If related Grade 3 non-haematologic toxicity, Grade 4 neutropenia or Grade 3/4 thrombocytopenia occurred at 2400 and 400 mg/m^2, 5-FU infusion and bolus dose was reduced to 2000 and 300 mg/m^2, respectively. Oxaliplatin dose was reduced to 75 mg/m^2 in the case of Grade 4 neutropenia, Grade 3/4 thrombocytopenia or Grade 3 diarrhoea. In the case of Grade 2 paresthaesia, oxaliplatin was reduced to 75 mg/m^2. In cases of persistent painful paresthaesia or Grade 3 neurotoxicity, oxaliplatin was omitted from the regimen. Treatment was terminated when disease progression was observed, Grade 4 non-hematological toxicity was observed, the patient refused to continue, or recovery from toxicity delayed the initiation of the second course by >3 weeks from the planned schedule.

Efficacy and Safety Evaluation

Computed tomography (CT) scans of measurable lesions were assessed within four weeks before registration in this study as the baseline and were repeated every four cycles. RECIST criteria were used to assess tumour response (10). Complete response (CR) was defined as complete disappearance of all clinically assessable disease for at least 4 weeks, and partial response as a decrease of at least 30% of the sum of the longest diameters of measurable lesions for at least 4 weeks. CT scans were taken 4 weeks later to confirm the response. Progression-free survival (PFS) was defined as the time from the date of registration to the first confirmation of disease progression, or death from any cause, and was censored at the last tumour assessment if a patient withdrew before progression. OS was defined as the time from registration to any death. TTF was defined as the time from registration to discontinuation of the protocol treatment.

Toxicity was assessed before starting each 2-week cycle using the Common Terminology Criteria for Adverse Events v.3.0 (11). The Debiopharm neurotoxicity criteria were used for sensory neurotoxicity: Grade 1 is short-lasting paresthaesia with complete regression within seven days, Grade 2 is persistent (>7 days) paresthaesia or dysesthesia without

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functional impairment and Grade 3 is persistent functional impairment.

Statistical Methods

The primary endpoint was the RR. The secondary endpoints were OS, time to TTF, PFS and toxicity. Thirty-nine patients were required to test the null hypothesis [overall response rate (ORR) = 5%] using a power of 80%, expected ORR of 15% and one-sided α-level of 5%. The target sample size of 45 patients was chosen with the expectation that a proportion of patients would prove to be ineligible for the efficacy analysis. ORRs were presented with a 95% confidence interval (CI). The probability of time-to-event parameters was estimated using the Kaplan–Meier method with 95% CI.

RESULTS

Patient Characteristics

Between April and July 2005, 51 patients were accrued from nine centres in Japan. All patients ($n = 51$) were included in the safety analysis. The patients' baseline characteristics are shown in Table 1. The median age was 60 years (range, 32–74 years). Twenty-six (51%), 24 (47%) and 1 (2%) patients showed ECOG PS of 0, 1 and 2, respectively. The median number of regimens received previously was two (range, 1–7). Forty-one (80%) of 51 patients had received irinotecan-containing therapy before registering in this study.

Table 1. Patient characteristics ($n = 51$)

Sex		
Male/female, *n* (%)	32 (19)	63 (37)
Age		
Median (range)	60 (32–74)	
ECOG performance status		
0/1/2	26/24/1	51/47/2
Primary tumour site		
Colon/rectum/other	25/24/2	49/47/4
Prior chemotherapy		
Median number (range)	2 (1–7)	
bolus 5-FU/LV	17	
IFL	16	
CPT-11 monotherapy	13	
FOLFIRI	10	
TS-1	12	
Prior CPT-11		
Yes/no	41/10	
Adjuvant therapy		
Yes/no	13/38	

ECOG, Eastern Cooperative Oncology Group.
IFL: CPT-11 + bolus 5-FU/LV.
FOLFIRI: CPT-11 + bolus 5-FU/LV + infusional 5-FU.

Treatment

FOLFOX6 was administered for a median duration of six cycles (range, 1–22 cycles). Median dose intensity (ratio of dose received to dose planned) was bolus 5-FU 0.848, infusional 5-FU 0.852 and oxaliplatin 0.845. Oxaliplatin dose reduction was required in 20 patients (39.2%), the major reasons for which were neutropenia ($n = 9$), sensory neuropathy ($n = 7$), vomiting ($n = 2$), febrile neutropenia ($n = 1$) and hand–foot syndrome ($n = 1$).

Efficacy

Two patients were excluded from the efficacy evaluation because of the absence of measurable lesions. At the data cut-off date (30 June 2008), the median duration of follow-up was 39.1 months. At this time, 40 patients had died from disease progression and all patients had discontinued the study medication. Of the 49 eligible patients, the ORR was 14.2% (95% CI: 5.9–27.2%). Of the ten patients with no prior irinotecan, the RR was 20%, whereas it was 12.8% in the 39 patients who had received irinotecan previously (Table 2). The median TTF and PFS was 4.3 months (95% CI: 3.9–4.8) and 5.3 months (95% CI: 4.3–6.2), respectively (Fig. 1). The median survival time was 11.4 months (95% CI: 9.4–13.3; Fig. 2). None of the patients had undergone surgery with curative intent.

Toxicity

Eleven (21.6%) patients withdrew from FOLFOX therapy due to adverse events. The reasons for discontinuation were insufficient recovery from hematological toxicity (five patients), peripheral sensory neuropathy (three patients), elevation of transaminase and grade 3 allergy and fatigue.

Table 2. Response

	Efficacy analysis population ($n = 49$)	Prior irinotecan	
		Yes ($n = 39$)	No ($n = 10$)
CR	1	0	1
PR	6	5	1
SD	17	13	4
PD	21	18	3
NE	4	3	1
ORR	7 (14.2%)	5 (12.8%)	2 (20.0%)

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluated; ORR, overall response rate.

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