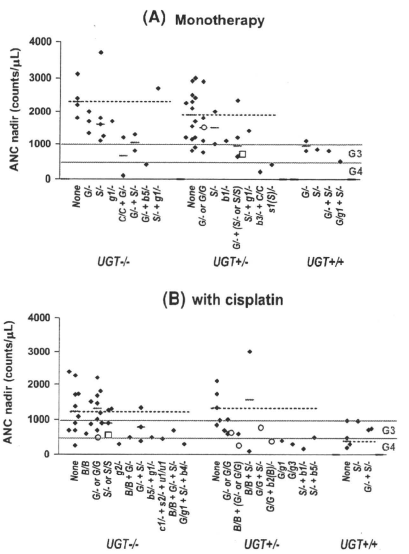

Fig. 2 Additive effects of transporter haplotypes/variations on ANC nadirs in irinotecan monotherapy (**a**) and combination therapy with cisplatin (**b**). *UGT+ = UGT1A1*6* or **28*; *B = ABCB1*2*; *C = ABCC2*1A*; *G = ABCG2#IIB* (*open circle*, *#IIB/#IIB*); *S = SLCO1B1*15 · 17* (*open square*, **15 · 17/*15 · 17*); *b1–u1* = minor variations listed in Table 3. **a** *None = non-*(*C*, *G*, *S* or *minors*), **b** *None = non-*(*B*, *G*, *S* or *minors*). The *bar* in each genotype represents the median. The *dotted lines* in each *UGT* genotype show the median values of patients without any selected transporter polymorphisms/variations (*None*). The *lines* (G3 and G4) represent the border of grade 3 and 4 neutropenia

In the irinotecan monotherapy, the increasing effect of *ABCB1*2/*2* (block 2) on SN-38 AUC/dose was evident while contributions of *ABCB1 BJL* (block 1), *ABCB1*1b* (block 3), *ABCG2#IIB* and *SLCO1B1*15 · 17* were not significant in the multivariate analysis. For neutropenia, additive effects were suggested for *ABCC2*1A/*1A*, *ABCG2#IIB*, *SLCO1B1*15 · 17*, and possibly some minor genetic variations in addition to *UGT1A1*6* or **28* (Fig. 2a). The association of *ABCB1*2* (block 2) with grade 3 diarrhea was also observed.

In the combination therapy with cisplatin, an increase in the SN-38 AUC/dose by *ABCB1*2* and for a decrease by *ABCB1*1b* were observed, but the multivariate analysis did not show their significant contributions. Regarding neutropenia, additive effects of *ABCB1*2/*2*, *ABCG2#IIB/#IIB*, and possibly, *SLCO1B1*15 · 17/*15 · 17* and some minor variations were suggested (Fig. 2b).

Thus, in both regimens, the associations of *ABCB1*2* (block 2) with higher SN-38 AUC/dose levels and toxicities (diarrhea or neutropenia), and additive effects of *ABCG2#IIB* and *SLCO1B1*15 · 17* with *UGT1A1*6* or **28* on neutropenia were observed. The current study also suggests that combination genotypes with two or more genes could have a greater effect on neutrophil count reduction than a single gene, indicating a quantitative property of multiple genetic factors affecting phenotype. These findings could partly explain a large interindividual variation in irinotecan toxicities within each *UGT* genotype.

In this study, influences of the transporter genotypes on SN-38 AUC/dose did not always correlate to an influence on neutropenia as observed in the combination therapy with cisplatin and in the case of *ABCB1*2* (block 2) in the monotherapy. Although weak negative correlations were observed between the SN-38 AUC level and ANC nadir, the SN-38 AUC values of patients who exhibited grade 3/4 neutropenia (ANC nadir < 1,000 counts/μL) were fairly diverse, especially in the combination therapy with cisplatin (Fig. 3). It is likely that the extent of toxicities depends not only on systemic exposure levels of the active metabolite for which hepatic UGT activity is a large contributor, but also on the elimination from the target cells (neutrophil progenitor cells or enterocytes) where transporter function might be more critical.

Our previous study showed the association of *ABCB1* block 2 **2* [1236C>T, 2677G>T (A893S) and 3435C>T] with lower renal clearance of irinotecan and its metabolites [16]. The current data obtained in the irinotecan monotherapy also suggest higher AUC/dose for irinotecan, SN-38G, and SN-38 with *ABCB1*2/*2*. Since a high affinity of P-gp for irinotecan is known, lower elimination rate of irinotecan could also result in higher plasma levels of its metabolites. Other studies have also suggested associations of the haplotype 1236T–2677T (corresponding to our **2* group in this study) with a reduced excretion rate of P-gp substrates [37] and SN-38 [25], and associations of the haplotype 2677T–3435T (corresponding to our **2* group in this study) with paclitaxel-induced neutropenia [38].

For *ABCC2*, *ABCC2* −1774delG, a tagging SNP of **1A*, was reported to be associated with low promoter activity and cholestatic or mixed-type hepatitis [32]. Patients with *ABCC2*1A/*1A* together with *ABCB1*2/*2* or *ABCG2#IIB* showed higher values of SN-38 AUC (Fig. 1) and neutropenia in the monotherapy (Fig. 2a), but these trends were not evident in the *UGT−/−* patients treated with cisplatin-combination therapy (data not shown). Thus, the effects of *ABCC2* might be dependent on combinations with other genetic and non-genetic factors. Conflicting clinical outcomes of *ABCC2* 3972C>T, a marker of **1C/G*, were reported to cause higher AUC of irinotecan and its

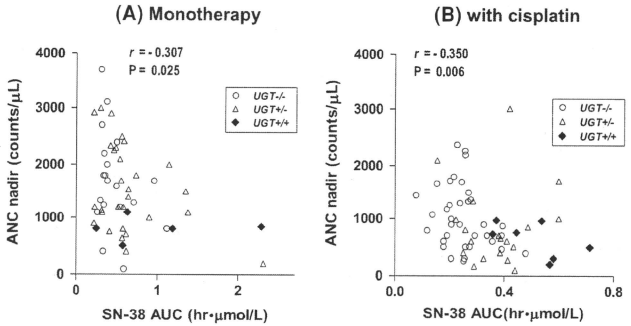

Fig. 3 Correlations between SN-38 AUC and ANC nadir in patients in irinotecan monotherapy (**a**) and combination therapy with cisplatin (**b**). *r* Spearman's rank correlation coefficient

metabolites in Caucasians treated with irinotecan monotherapy [18] and to lower the incidence of grade 3 diarrhea in Koreans treated with a combination therapy of irinotecan and cisplatin [24]. In the current study, no significant association of *ABCC2*1C/G* on PK/PD was observed in the monotherapy. Although a high incidence of grand 3/4 neutropenia was observed in patients with *ABCC2*1C/G* in the combination therapy with cisplatin, most patients also had *ABCG2#IIB* (data not shown); thus, the effect of *ABCC2*1C/G* remains obscure.

For *ABCG2*, the current study examined the association with the combinatorial haplotypes consisting of the three previously defined block haplotypes [28]. *ABCG2#IIB* contains the non-synonymous SNP 421C>A (Q141K), which was detected at higher frequencies in Asians and was reported to cause reduced expression of BCRP in vitro [36, 39–41]. In clinical studies, the association of 421C>A (Q141K) with higher plasma levels of diflomotecan was shown in Caucasians [42]. However, an association of this SNP with irinotecan PK/PD had not been shown [19, 24]. An association of 421C>A (Q141K) alone with irinotecan PK/PD was not significant in our hands (data not shown), but *#IIB* containing both 421C>A (Q141K) and IVS12 + 49G>T showed a moderate association with neutropenia. It is unclear whether the additional SNP IVS12 + 49G>T itself or another unknown linked SNP is causative for the reduced function. *ABCG2#IIIC* contains a non-synonymous SNP 34G>A (V12M) which has no influence on BCRP expression or activity in vitro [36, 39–41]. Our study showed no influence of *ABCG2#IIIC* on the SN-38 AUC/dose levels and neutropenia in the irinotecan monotherapy (data not shown), but did show a decreasing trend in grade 3/4 neutropenia in the combination therapy with cisplatin. In contrast, a report on Korean patients suggested the association of *ABCG2* 34G>A (V12M) with a higher incidence of grade 3 diarrhea in a combination therapy of irinotecan and cisplatin [24].

Among *SLCO1B1* polymorphisms, 521T>C (V174A), a tagging SNP of **15 · 17*, was demonstrated to reduce in vitro SN-38 influx [7], and clinical studies in Asians also showed its relevance to a higher SN-38 AUC and severe neutropenia in combination therapy of irinotecan with cisplatin [22–24]. Our results support these previous findings. Note that our **15 · 17* mainly consists of **17* [containing −11187G>A, 521T>C (V174A) and 388A>G (N130D)].

Taken together, the clinical data on transporter genotypes show variability among the studies. The reasons for these conflicting findings might be partly attributed to the ethnic differences in transporter genotypes and the regimens used. In addition, non-genetic factors, such as disease status and inflammation [43, 44], hepatic or renal function [45], and co-administered or pre-administered drugs, may also influence the clinical outcome.

The current study suggests combined effects of multiple haplotypes/variations on neutropenia. From clinical aspects of irinotecan therapy, the benefit of additional genotyping of transporters to predict severe toxicities should be clarified. Regarding grade 3 and 4 neutropenia, positive prediction values for two or more candidate genotypes including *UGT* (+) (Fig. 2) were 46 and 89% in the monotherapy and the cisplatin-combination therapy, respectively, which are low compared with *UGT+/+* (80 and 100%, respectively). Regarding grade 4 neutropenia, positive predictive values for these candidate genotypes were 15 and 41% in the monotherapy and the cisplatin-combination therapy, respectively, while for *UGT+/+*, they were 0 and 43%, respectively. Further studies using a

larger population size are needed to further elucidate the roles of these candidate markers.

In conclusion, the current study suggests there are additive effects for several transporter genotypes on the SN-38 AUC level and the reduction of neutrophil counts in irinotecan therapy. The clinical benefits of additional genotyping of these candidate markers should be further delineated.

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

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

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

WHAT THIS STUDY ADDS

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

AIMS

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

METHODS

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

RESULTS

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

CONCLUSION

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

Introduction

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

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

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

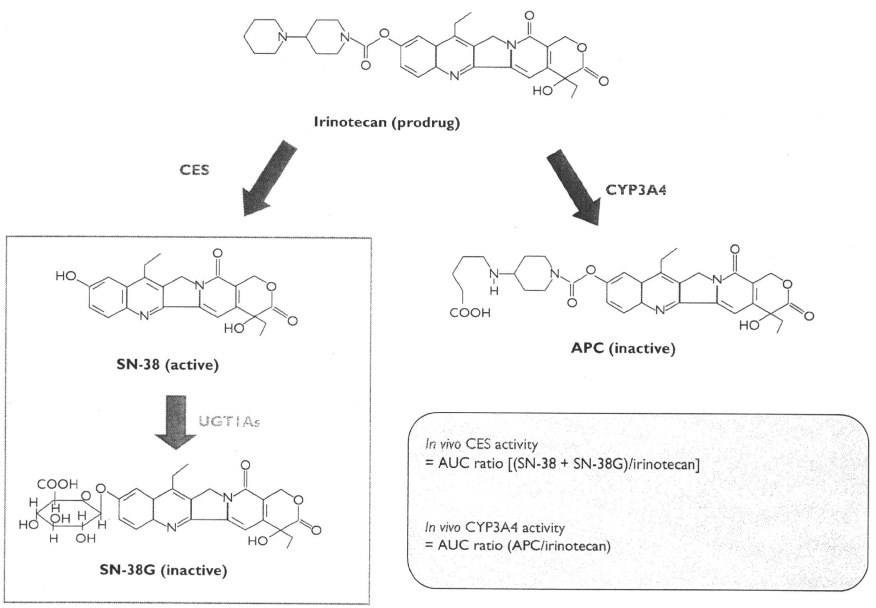

Figure 1

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

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

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

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

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

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

Methods

Patients

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

Determination of *CES1* genotypes and SNPs

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

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

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

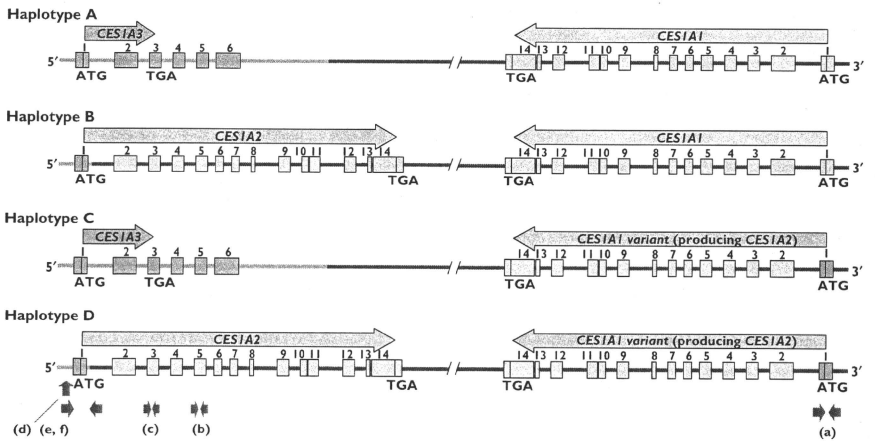

Figure 2

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

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

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

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

Pharmacokinetic data and association analysis

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

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

Table 1

Primers and probes used in this study

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

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

tions between the AUC ratios [$AUC_{(SN\text{-}38\ +\ SN\text{-}38G)}/AUC_{irinotecan}$] and [$AUC_{APC}/AUC_{irinotecan}$] were analyzed by Spearman's rank correlation test. Multiplicity adjustment was not applied to bivariate analysis, and contributions of the candidate genetic markers to the AUC ratios [$AUC_{(SN\text{-}38\ +\ SN\text{-}38G)}/AUC_{irinotecan}$] were further determined by multiple regression analysis after logarithmic transformation of the AUC ratio. The variables examined were age, sex, body surface area, history of smoking or drinking, performance status, serum biochemistry (GOT, ALP, creatinine) at baseline, *CES1* genotypes and SNPs, *CES2*2* [100C>T(R34W)] or **5* [1A>T (M1L)] [13, 14], *UGT1A1*6* or **28* [7, 8], and the transporter haplotypes, *ABCB1*2* [2677G>T(A893A)], *ABCC2*1A* (−1774delG), *ABCG2*IIB* [421C>A (Q141K) and IVS12+49G>T] and *SLCO1A1*1b-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	ttgggcaagtttacagctctC/Ttgtaatctgacagtagagtc		0.014	
MPJ6_CS1002†			5′-flank	9481399	−233	atctgacagtagagtccagaC/Atggtttgatgaaagagggta		0.003	
MPJ6_CS1003†			5′-flank	9481327	−161	tagaagcccagggagatctgA/Ggaaaagggagggcttttctg		0.006	
MPJ6_CS1004	rs3815583	IMS-JST175949	Exon1(5′-UTR)	9481241	−75	aactctgggcggggctgggcG/Tccagggctggacagcacagt		0.41	
MPJ6_CS1005	rs28429139		Exon1(5′-UTR)	9481212	−46	ggacagcacagtccctctgaA/Gctgcacagagacctcgcagg		0.299	*var1A1*
MPJ6_CS1006	rs28494177		Exon1(5′-UTR)	9481205	−39	acagtccctctgaactgcacA/Ggagacctcgcaggccccgag		0.299	*var1A1*
MPJ6_CS1007†			Exon1(5′-UTR)	9481196	−30	ctgaactgcacagagacctcG/Acaggccccgagaactgtcgc		0.042	
MPJ6_CS1008	rs28520463		Exon1(5′-UTR)	9481187	−21	acagagacctcgcaggccccG/Cagaactgtcgcccttccacg		0.297	*var1A1*
MPJ6_CS1009	rs28499065		Exon1(5′-UTR)	9481186	−20	cagagacctcgcaggccccgA/Ggaactgtcgcccttccacga		0.297	*var1A1*
MPJ6_CS1010	rs28515828		Exon1(5′-UTR)	9481168	−2	cgagaactgtcgcccttccaC/Ggatgtggctccgtgccttta		0.299	*var1A1*
MPJ6_CS1011			Exon 1	9481156	11	ccctccacgatgtggctccG/Ctgcctttatcctggccactc	Arg4Pro	0.297	*var1A1*
MPJ6_CS1012			Exon 1	9481152	15	tccacgatgtggctccgtgcC/Tttatcctggccactctctc	Ala5Ala	0.297	*var1A1*
MPJ6_CS1013			Exon 1	9481151	16	ccacgatgtggctccgtgccT/Cttatcctggccactctctct	Phe6Leu	0.297	*var1A1*
MPJ6_CS1014			Exon 1	9481148	19	cgatgtggctccgtgccttA/Gtcctggccactctctctgct	Ile7Val	0.297	*var1A1*
MPJ6_CS1015	rs28563878		Exon 1	9481133	34	tgcctttatcctggccactctcT/Gctgcttccgcggcttggggt	Ser12Ala	0.297	*var1A1*
MPJ6_CS1016	rs12149359		Intron 1	9481099	IVS1+16	ttggggtgagtcctctgaaA/Gtcaaaatgcggggcacttttt		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

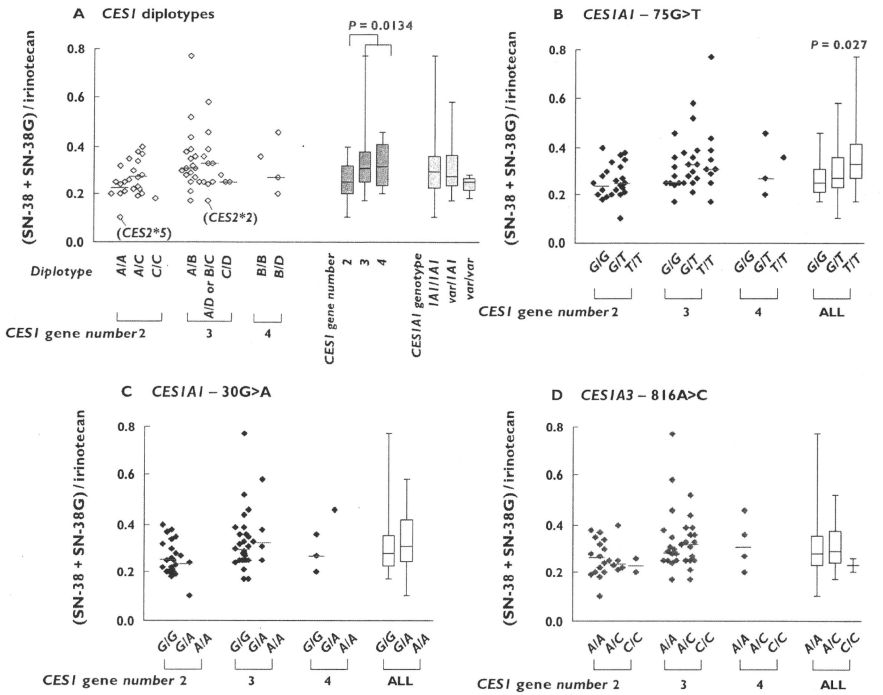

Figure 3

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

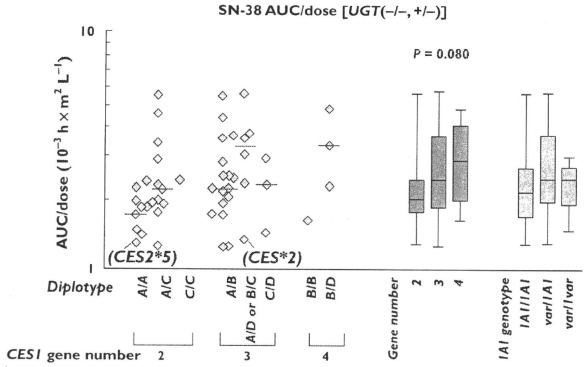

Figure 4

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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INTERNAL MEDICINE

□ REVIEW ARTICLE □

Recent Development of Molecular-Targeted Drugs in Lung Cancer

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Abstract

Numerous molecular target drugs have been introduced for the treatment of advanced malignancies. In the treatment of lung cancer, epidermoid growth factor receptor-tyrosine kinase inhibitors (EGFR-TKI) demonstrate striking antitumor activity in selected EGFR mutation positive patients. Patient selection by biomarker is extremely important to obtain successful results. The anti-vascular endothelial growth factor (VEGF) antibody, bevacizumab, shows a markedly increased response rate, progression free survival of advanced non-squamous cell lung cancer when combined with cytotoxic drugs. The classification of lung cancer is rapidly changing based on the advances in molecular biology. Here, the recent development of new molecular target drugs against lung cancer is thoroughly reviewed in addition to EGFR-TKIs and bevacizumab with special emphasis on the clinical application.

Key words: molecular target drugs, lung cancer, EGFR-TKI, VEGF, angiogenesis

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Introduction

In recent years, the understanding of cancer at the molecular level has progressed, and numerous genes and proteins which play important roles in the growth, invasion and metastasis of tumors have been identified. Furthermore, by setting these genes and proteins as the targets, small molecular weight drugs called signal transduction inhibitors (e.g., tyrosine kinase inhibitors), monoclonal antibodies, etc., have been developed for the treatment of cancer(s). Numerous molecular-targeted drugs have been developed, including epidermal growth factor receptor (EGFR) inhibitors, vascular endothelial growth factor (VEGF) antibodies, etc, have also been developed for the treatment of non-small cell lung cancer (NSCLC), and a number of clinical studies on these new drugs have been conducted towards the goal of their clinical application.

1. Treatment targeted at EGFR

EGFR is a transmembrane-type receptor protein composed of an extracellular ligand-binding domain, a transmembrane domain, and an intracellular tyrosine kinase domain. When a growth factor binds to this receptor, a dimer is formed and the downstream signal transduction system is activated, resulting in cancer cell proliferation, metastasis, vascularization and apotosis, etc (1-3).

Excessive EGFR expression has been reported to be detected in 32-81% of all cases of NSCLC (4-6). Two therapeutic strategies designed to inhibit the EGFR signal transduction system have been developed. One is to use EGFR tyrosine kinase inhibitors (EGFR-TKIs) which are low molecular weight compounds that bind to the ATP-binding site of intracellular tyrosine kinase, inhibiting the self-phosphorylation of EGFR. The other strategy is to use monoclonal antibodies that bind specifically to the extracellular domain of EGFR, thereby inhibiting ligand binding to EGFR.

1) EGFR tyrosine kinase inhibitors

a. Gefitinib

The results of randomized phase II clinical studies of gefitinib in previously treated cases of NSCLC were reported in 2002. In the Iressa Dose Evaluation in Advanced Lung Cancer (IDEAL)-1 study, carried out primarily in Europe and Japan, the response rate was 18.4% in the 250 mg/day

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group and 19.0% in the 500 mg/day group (7). Also in IDEAL-2, carried out in the USA, the response rates were almost the same between the 250 mg/day group (11.8%) and the 500 mg/day group (8.8%), and there was no difference in the survival period between the two dose groups (8). Toxicity was lower in the 250 mg/day group than in the 500 mg/day group, and the dose level of 250 mg/day was adopted as the recommended dose level. In a subgroup analysis, the response rate was significantly higher in females, patients with adenocarcinoma, and Japanese patients. On the basis of these results, the Japanese regulatory authority approved the use of gefitinib in 2002, earlier than in other countries around the world.

The Iressa Survival Evaluation in Advanced Lung Cancer (ISEL) was a large-scale phase III clinical study in which 1,692 previously treated patients with NSCLC were randomly allocated to the gefitinib and the placebo group. The results revealed that the response rate was significantly higher in the gefitinib group than in the placebo group (8% vs. 1%, p<0.0001). Of the primary endpoints, the median survival time (MST) and one-year survival rate were 5.1 months and 21%, respectively, in the placebo group and 5.6 months and 27% in the gefitinib group, respectively, with no significant difference between the two groups (p=0.087) (9). In the subgroup analysis, however, gefitinib was shown to extend the survival in non-smokers (MST: 8.9 months vs. 6.1 months, p=0.012) and Asian patients (MST: 9.5 months vs. 5.5 months, p=0.01). A randomized phase III clinical study (V-15-32) aimed at confirming the non-inferiority of gefitinib to docetaxel (DOC) was carried out in Japan, involving 490 previously treated patients with NSCLC. The response rate was significantly higher in the gefitinib group (22.5%) than in the DOC group (12.8%) (p=0.009). The median progression-free survival (mPFS) was 2.0 months in both groups. The MST (a primary endpoint) was 14.0 and 11.5 months in the two groups, respectively. The hazard ratio (HR) was 1.12 (95% confidence interval [CI]: 0.89-1.40). Thus, the study did not demonstrate non-inferiority of gefitinib to DOC (10). In addition, a report was published of a randomized phase III clinical study (INTEREST) carried out in 24 countries (Europe, USA and Asia), comparing gefitinib with DOC in 1,433 previously treated patients with NSCLC. In that study, the response rate did not differ significantly between the gefitinib group (9.1%) and the DOC group (7.6%) (p=0.33), and there was no significant difference in the mPFS either between the gefitinib group (2.2 months) and the DOC group (2.7 months) (p=0.47). In the analysis of the overall survival period, the primary endpoint, the HR was 1.020 (95%CI: 0.905-1.150) and did not exceed the preset upper limit (1.154), thus endorsing the non-inferiority of gefitinib to DOC (11). In the evaluation of toxicity, the gefitinib group most frequently developed skin eruptions and diarrhea, while the DOC group most frequently developed decreased blood neutrophil count, myasthenia, and alopecia. In 2008, interesting results were reported from a phase III clinical study (Iressa Pan Asia Study: IPASS) comparing gefitinib therapy with carboplatin (CBDCA) + paclitaxel (PTX) therapy, each administered as the initial therapy (to be described in detail later) (12). Furthermore, a randomized phase III clinical study (WJTOG 0203) was carried out in Japan, comparing platinum-based chemotherapy (3-6 cycles) with platinum-based chemotherapy (3 cycles) + sequential gefitinib therapy in 598 previously untreated patients with NSCLC. In that study, mPFS was significantly longer in the sequential therapy group (4.60 months) than in the platinum-based chemotherapy alone group (4.27 months) (p<0.001), while the overall survival period (a primary endpoint) did not differ significantly between the two groups (MST: 12.89 months vs. 13.68 months, p=0.10). In a subset analysis, the overall survival period of adenocarcinoma patients was extended by the sequential therapy (MST: 14.33 months vs. 15.42 months, p=0.03) (13). In a randomized phase II clinical study in which 97 previously untreated patients with NSCLC were divided into two groups, one group receiving oral gefitinib therapy after 4 cycles of CBDCA + PTX therapy until exacerbation of the condition and the other receiving oral gefitinib therapy (until exacerbation of the condition) followed by 4 subsequent cycles of CBDCA + PTX therapy, the overall survival period (a primary endpoint) differed little between the two groups (MST: 18.8 months vs. 17.2 months) (14).

b. Erlotinib

In a phase II clinical study of erlotinib monotherapy involving 57 previously treated patients of NSCLC showing positive immunostaining of the tumor cells for EGFR, the response rate was 12.3% and the MST was 8.4 months (5). In this study, the results suggested that the overall survival period was probably correlated with the incidence and severity of skin eruptions (15).

In sharp contrast to the findings of the above-mentioned studies on gefitinib were the results obtained in a phase III clinical study comparing erlotinib with BSC. In this phase III comparative study (BR.21) carried out by the National Cancer Institute of Canada Clinical Trial Group (NCIC), 731 previously treated patients with NSCLC were allocated randomly to the erlotinib group and the placebo group at a ratio of 2:1. In analysis of the primary endpoints, erlotinib was significantly superior in terms of both the overall survival (MST: 6.7 months in the erlotinib group vs. 4.7 months in the placebo group, p<0.001) and the progression-free survival (2.2 months in the erlotinib group vs. 1.8 months in the placebo group, p<0.001) (16). On the basis of the results of this study, erlotinib was adopted as one of the standard therapies for previously treated cases of NSCLC. Following publication of the results of this study, erlotinib was approved in 2004 in the USA and in 2007 in Japan. Regarding the discrepancy of the results between ISEL and BR.21, the influence of pharmacological differences has been pointed out, such as the difference in the dose level (erlotinib dose level equal to the MTD and gefitinib dose level equivalent to about 1/3 of the MTD) and the difference in the affinity for EGFR (17). In addition, a phase IV clini-

Table 1. Phase II Study of EGFR- TKI in EGFR Mutation (+) Patients

	n	EGFR-TKI	RR (%)	mPFS (M)	MST (M)
Morita S (I-CAMP)	148	gefitinib	76.4	9.7	24.3
Sirera R	193	erlotinib	70.8	12.0	22.0
Sequist LV	34	gefitinib	55	9.2	17.5

EGFR-TKI: Epidermal Growth Factor Receptor-Tyrosine Kinase Inhibitor

RR: Response Rate

mPFS: median Progression-Free Survival

MST : Median Survival Time

M: Month

cal study (TRUST) was carried out on erlotinib monotherapy for NSLCLC. In an analysis of the interim results of this study covering 6,809 patients, the response rate was 13% and mPFS was 3.5 months, close to the results obtained in the BR.21 study (18). At a meeting of ASCO in 2009, the results of a phase III clinical study (SATURN) comparing maintenance erlotinib therapy with placebo were reported, demonstrating the superiority of erlotinib in terms of the PFS as a primary endpoint (19). At present, clinical studies such as a phase III clinical study of erlotinib with chemotherapy of pemetrexed (PEM) or DOC as secondary chemotherapy and a phase III clinical study (RADIANT) comparing erlotinib with placebo as postoperative adjuvant therapy are ongoing. Interesting results from these studies are expected.

2) Predictors of responses to EGFR-TKI

EGFR gene mutations are reported as the most important factor predictive of the responses of NSCLC to EGFR-TKI (20-22). More than forty mutations of the EGFR gene in exon 18-21 of the tyrosine kinase domain have been reported. Among others, deletion of 5 amino acids in exon 19 and the L858R point mutation of exon 21 are reported to account for more than 80% of all mutations of the EGFR gene (3, 23). EGFR gene mutations have also been reported to be correlated with clinical factors associated with a high sensitivity to EGFR-TKI, such as adenocarcinoma, female gender, non-smoker and Asian race (20, 24, 25). In addition, the results of a phase II clinical study of EGFR-TKI in patients carrying EGFR gene mutations have been reported (Table 1) (26-28). In 2008, the results of an integrated analysis of the results of 7 Japanese phase II clinical studies of gefitinib (I-CAMP) were reported. In that analysis, EGFR-TKI therapy yielded excellent outcomes in 148 patients carrying gene mutations, with a response rate of 76.4%, mPFS of 9.7 months, and MST of 24.3 months (26). The responses of the gene mutation-positive cases to this therapy were also favorable in other studies, suggesting that the presence of EGFR gene mutation serves not only as a predictor of the response to treatment, but also as a prognostic factor (29).

There are also reports on the usefulness of the number of EGFR gene copies, evaluated by fluorescence *in situ* hybridization (FISH), as a predictor of the response to treatment (30-32). In an evaluation of patients registered with the BR.21 study, amplification of gene copies was significantly correlated with the response rate to erlotinib, whereas the presence of gene mutation was not correlated with the response rate (33). In a similar analysis of cases registered with the ISEL study, patients with gene copy amplification tended to have a longer survival period following gefitinib therapy, although the difference was not statistically significant (p=0.07). In that analysis, it was not possible to evaluate the correlation of the presence of gene mutations with survival, because the number of gene mutation-positive cases was not sufficiently large (34). In Western countries, the number of gene copies is often used as a predictor of response to treatment, because the frequency of gene mutations is low.

KRAS gene mutation is seen in 20-40% of cases of NSCLC and has been reported to serve as a predictor of a poor response to EGFR-TKI and chemotherapy (35, 36) but to date there is not sufficient evidence.

3) EGFR-TKI as a means of primary treatment

a. EGFR-TKI monotherapy

The results of a phase II clinical study on gefitinib conducted on previously untreated patients with NSCLC in National Cancer Center Hospital East have been reported. Of the 40 patients eligible for the study, 40% were female, 75% had adenocarcinoma and 20% were non-smokers. The response rate was 30%, MST was 13.9 months, and the one-year survival rate was 55%. However, death from acute lung disorders as an adverse event occurred in 10% of all patients (37). EGFR-TKI monotherapy also did not yield promising results in other phase II studies which did not incorporate careful patient selection (38). In addition, the results of phase II studies of the efficacy of initial treatment with EGFR-TKIs incorporating patient selection have also been reported. In a phase II study of gefitinib in 36 non-smokers with adenocarcinoma, the response rate was as high as 69%. The mPFS was 8.3 months and the estimated one-year survival rate was 73%, representing more favorable results as compared to the results of previously reported studies on standard chemotherapy (39). Furthermore, a phase II study on gefinitib as the initial chemotherapy was carried

Table 2. Randomized Controlled Trial of EGFR-TKI vs Platinum Doublet in EGFR Mutation(+) Patients

Trial	n	EGFR-TKI	Chemotherapy	Primary Endpoint	Results
NEJ002	320	gefitinib	CBDCA + PTX	PFS	Positive
WJOG3405	200	gefitinib	CDDP + DOC	PFS	Positive
EURTAC	146	erlotinib	platinum-doublet*	PFS	On going
ML20981	150	erlotinib	CBDCA + GEM	PFS	On going

*GEM + CDDP, DOC + CDDP, GEM + CBDCA, DOC + CBDCA

EGFR-TKI: Epidermal Growth Factor Receptor-Tyrosine Kinase Inhibitor

PFS: Progression-Free Survival

CBDCA: Carboplatin

PTX: Paclitaxel

CDDP: Cisplatin

DOC: Docetaxel

GEM: Gemcitabine

out in Japan, in 30 patients with NSCLC satisfying one of the following requirements: 1) EGFR gene mutation-positive elderly patients; and 2) patients with poor performance status (PS) who were not candidates for standard chemotherapy. In that study, the outcome was excellent, with a response rate of 66% and MST of 17.8 months, and number of treatment-associated deaths was zero (40).

A phase III study (Iressa Pan Asia Study: IPASS) was carried out in 10 East Asian countries including Japan to compare gefitinib therapy with carboplatin (CBDCA) + paclitaxel (PTX) therapy as the first-line treatment in patients with clinical factors (adenocarcinoma; non-smoker or light smoker) possibly associated with a high sensitivity to EGFR-TKIs. In this study, 1,217 patients were allocated randomly into two groups, and the response rate was significantly higher in the gefitinib group (43.0%) than in the CBDCA + PTX group (32.2%) (p=0.0001). In the analysis of the PFS (the primary endpoint), the HR was 0.741 (95% CI: 0.651-0.845, p<0.0001) and the outcome was significantly better in the gefitinib group. However, since the survival curves for the two groups crossed each other, the interpretation of the data was controversial. When the patients of this study were divided according to the presence/absence of EGFR gene mutation, the crossing of the survival curves disappeared, and the PFS was significantly longer in the gene mutation-positive group. Data on the patient overall survival in this study have not yet been reported because they are still premature (12). At present, four phase III studies comparing EGFR-TKIs with standard chemotherapy in EGFR gene mutation-positive patients are under way and preliminary results of two studies have been reported (Table 2) (41, 42). EGFR-TKIs are now viewed as useful alternatives for first-line chemotherapy in EGFR gene mutation-positive patients with NSCLC, although they have not been demonstrated to be superior to platinum-based therapy in overall survival.

b. Combined EGFR-TKI + chemotherapy

In regard to studies conducted to evaluate the significance of combining EGFR-TKI with chemotherapy, the Iressa NSCLC Trial Assessing Combination Therapy (INTACT)-1 (43) and INTACT-2 (44) have been carried out using gefitinib as the EGFR-TKI. These studies, however, failed to endorse the significance of administering gefitinib in combination with chemotherapy. Similarly, phase III studies of erlotinib administered in combination with chemotherapy have been carried out, however, no enhancement of the efficacy with the use of erlotinib in combination with chemotherapy was demonstrated in either the Tarceva Lung Cancer Investigation (TALENT) study (45) or the Tarceva Responses in Conjunction with Taxol and Carboplatin (TRIBUTE) study (46) (Table 3). However, subgroup analysis of the data from the TRIBUTE study revealed a significant extension of the survival period in non-smokers following the addition of erlotinib to the chemotherapeutic regimen (MST: 22.5 months vs. 10.1 months, p=0.01). Therefore, it would seem valuable to conduct similar studies on appropriately selected patients for further evaluation.

4) Toxicity of EGFR-TKIs

The major toxicities of EGFR-TKIs are skin disorders (eruption, dry skin, pruritus, etc.), diarrhea, and liver dysfunction. Interstitial lung disease (ILD) is a toxicity that needs the greatest attention. The incidence of this adverse reaction is reported to be about 3.5-5% and some of the risk factors for its onset are advanced age, male gender, poor PS, positive smoking history and the presence of underlying interstitial disease (47, 48). When EGFR-TKIs are used, it is essential to take into account the risk of onset of ILD.

5) Second-generation EGFR-TKIs

Recurrence of disease occasionally takes place within about 12 months after successful treatment with EGFR-TKIs (gefitinib, erlotinib, etc.) even in gene mutation-positive cases (49). This has been explained by the development of tumor resistance to EGFR-TKIs through the development of secondary EGFR gene mutations such as mutation of T790