Table 4. Non-hematologic toxicities (all courses)

Dose levels	Level 1 (200 mg/m^2)		Level 2 (260 mg/m^2)		Level 3 (300 mg/m^2)		All	
No. of patients (no. of courses)	$n = 3$ (9)		$n = 6$ (23)		$n = 3$ (14)		$n = 12$ (46)	
CTCAE grade	1–2	3	1–2	3	1–2	3	1–2	3
Sensory neuropathy	1	0	5	1	1	2	7	3
Alopecia	3	0	4	0	3	0	10	0
Myalgia	0	0	6	0	3	0	9	0
Rash	2	0	4	0	1	0	7	0
Arthralgia	1	0	4	0	2	0	7	0
Asthenia	2	0	2	0	2	0	6	0
Motor neuropathy	0	0	3	0	2	0	5	0
Nausea	2	0	1	0	1	0	4	0
Anorexia	3	0	1	0	0	0	4	0
Vomiting	1	0	2	0	0	0	3	0
Diarrhea	2	0	0	0	0	0	2	0
Stomatitis	0	0	0	0	2	0	2	0

Grade 4 toxicities were not observed.

Table 5. Anti-tumor response

	Tumor type	Prior taxane therapy	Response
Level 1 (200 mg/m^2)	NSCLC	+	PD
	NSCLC	+	PR
	Parotid gland	+	PD
Level 2 (260 mg/m^2)	NSCLC	+	PD
	NSCLC	−	PR
	Ovary	+	PD
	NSCLC	+	PR
	Colon	−	PD
	Thymoma	−	SD
Level 3 (300 mg/m^2)	Bladder	−	SD
	NSCLC	+	NE
	Pharyngeal and esophageal	+	SD

PD, progressive disease; PR, partial response; SD, stable disease; NE, not evaluable.

Response

Eleven of 12 patients were evaluable for anti-tumor response (Table 5). Partial responses were observed in three NSCLC patients. Of them, two of whom had received docetaxel-containing chemotherapy prior to the study. The first patient, entered at Level 1, had received 6 courses of ABI-007, and the second and third patients, entered at Level 2, 11 and 6 courses, respectively. The both responders in Level 2 attained disease control until the treatment discontinuation due to the sensory neuropathy.

Pharmacokinetics

Blood samples for PK analysis were available from all of 12 patients following the first course of treatment. A semi-log plot of the mean values of paclitaxel concentration for each dose level vs. time is shown in Fig. 1. After 30 min infusion of ABI-007, the concentration of paclitaxel began to decrease immediately upon cessation of the infusion with $t_{1/2}$ of 17.3–27.3 h in the whole blood, which is nearly comparable with that of standard dose of solvent-based paclitaxel (6), and the decline of paclitaxel concentration from maximum was multiphasic.

The mean PK parameters of paclitaxel are summarized in Table 6. C_{max}, AUC_{0-t} and AUC_{inf} of paclitaxel when administered as a 30 min infusion of ABI-007 increased with increasing dosage. CL and Vz of the blood sample showed the small inter-patient variability, and the mean $\pm$ SD values (CV%) for CL and Vz at the dose level of 260 mg/m^2 were 18.1 ± 2.33 (12.9 CV%) (l/h/m^2) and 510 ± 96.8 (19.0 CV%) (l/m^2), respectively. These values slightly decreased with increased dosage. It was considered that there was no remarkable difference in calculated values of PK parameters between whole blood and plasma. Regression analysis suggested the dose-proportionality of ABI-007 within the dose range in this study (R^2 of $C_{max} = 0.4470$, R^2 of

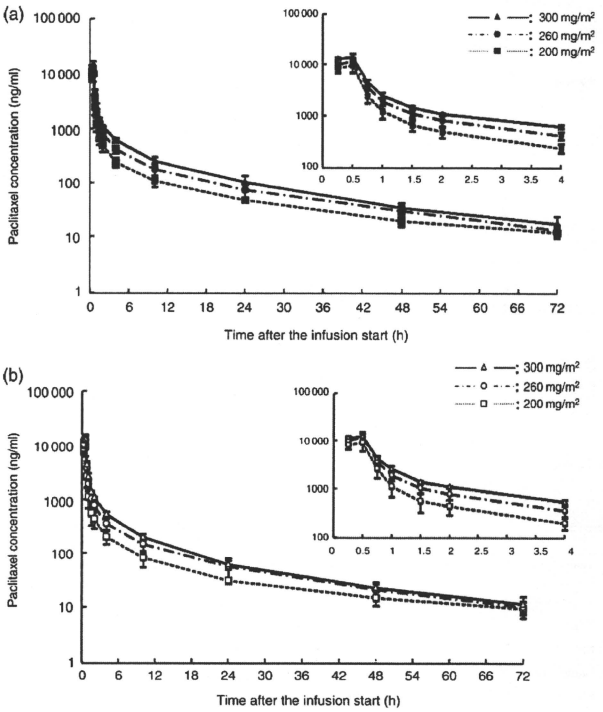

Figure 1. (a) Mean whole blood concentration–time profiles of paclitaxel. (b) Mean plasma concentration–time profiles of paclitaxel.

$AUC_{inf} = 0.7177$); however, it was difficult to establish the linearity due to those narrow dose range and small data size.

DISCUSSION

In the Phase I study where ABI-007 was administered in Q3W schedule in Japanese patients, no DLT occurred at any dose level of 200, 260 and 300 mg/m^2. Because MTD was not reached by the 3 + 3 rule, selection of RD was attributed to the consideration of reasonable tolerability, toxicities and PK profile. Since paclitaxel treatment was characterized for the cumulative neurotoxicity, dose selection also took into account the development of sensory neuropathy throughout the study. Consequently, 260 mg/m^2 was reassessed as potential RD and established as RD in the absence of applicable DLT. Outcome of sensory neuropathy in all treatment courses also provided the justification for the feasibility of 260 mg/m^2 (Table 7). Among 260 and 300 mg/m^2 cohorts, every patient experienced neuropathic events, in which Grade 3 or 4 event was more frequent in 300 mg/m^2 (two out of three patients) than in 260 mg/m^2 cohorts (one out of six patients). Moreover, all the three patients in 300 mg/m^2 cohort discontinued the treatment due to neuropathic events as opposed to two out of six patients in 260 mg/m^2 cohort.

In terms of treatment-related toxicities, Grade 3 or 4 neutropenia was experienced in 15 of 46 treatment courses (32%). Nonetheless, no febrile neutropenia was observed. Median duration of recovery from Grade 3 or 4 to <Grade 2 was 6.5 days (range, 3–14). No treatment delay was caused by neutropenia. In addition, platelet decrease ≥Grade 2 was not observed throughout the study. On the whole, hematological toxicities were mild. In regard to sensory neuropathy, the median time to the first indication or exacerbation from the baseline was 7 days, which

was relatively early to that of solvent-based paclitaxel. Especially for Grade 3 sensory neuropathy, the indication or exacerbation fell within the first week of the first course, ranging from 3 to 6 days; the time to improve from Grade 3 to Grade 2 or 1 was 21, 26 and 46 days in the respective cases. Although the improvement tended to delay when compared with median 22 days in a previous Phase III study (4), it still remains controversial because of the great difference in the sample sizes between the two studies. Meanwhile, other non-hematological toxicities including mucositis—the DLT of the US Phase I study—were generally mild to moderate up to 300 mg/m^2.

PK profiles of ABI-007 have revealed the small inter-patient variability, and the AUC and C_{max} of paclitaxel increased with increasing the dosage. In whole blood samples, there was a significant correlation between the doses and PK parameters. The linearity was uncertain in the face of wide confidence interval (CI) with small sample size, however, presumable from the other reported data showing the linearity over a wide dose range: 80–300 mg/m^2 (2) and PK equality between Japanese and western population (3).

Anti-tumor response was demonstrated in the patients with NSCLC including the patients who had received prior taxane-containing therapy.

Multiple previous studies of ABI-007 also reported the promising data in the patients with NSCLC. In a Phase II trial, 260 mg/m^2 of ABI-007 was administered alone in the same Q3W schedule as our study in the first-line setting, overall response rate was 16.3% (95% CI, 5.24–27.31%) and the disease control rate was 48.8% (95% CI, 33.90–63.78%) (8). More recently, weekly (QW) schedule of ABI-007 was also reported: 125 mg/m^2 of ABI-007 was administered in monotherapy on days 1, 8 and 15 every 4 weeks, the response rate was 30% ((95% CI, 16–44%) and the disease control rate was 50% (95% CI, 35–66%) (9). Despite the higher incidence of ≥Grade 3 neutropenia and sensory neuropathy relative to the Q3W schedule, QW schedule was well tolerated and active.

In conclusion, no DLT observed at any dose levels, and ABI-007 was well tolerated up to 300 mg/m^2 in Japanese

Table 6. PK parameters of paclitaxel

	200 mg/m^2 (n = 3)		260 mg/m^2 (n = 6)		300 mg/m^2 (n = 3)	
	Mean	CV (%)	Mean	CV (%)	Mean	CV (%)
Whole blood						
C_{max} (ng/ml)	9430	28.3	11 635	13.0	13 833	15.3
AUC_{inf} (ng h/ml)	10 360	22.0	14 593	13.7	19 138	12.2
$t_{1/2}$ (h)	24.3	10.9	19.5	7.9	18.3	1.9
CL (l/h/m^2)	19.9	21.6	18.1	12.9	15.8	11.2
Vz (l/m^2)	689	15.3	510	19.0	417	9.7
Plasma						
C_{max} (ng/ml)	9040	34.0	12 000	17.6	12 700	20.5
AUC_{inf} (ng h/ml)	9146	29.6	13 330	20.7	16 271	11.2
$t_{1/2}$ (h)	29.0	17.7	20.8	19.5	19.8	9.8
CL (l/h/m^2)	23.1	26.4	20.2	21.5	18.6	10.6
Vz (l/m^2)	935	11.7	620	36.9	527	7.0

PK, pharmacokinetic; CV, coefficient of variation; C_{max}, maximum concentration; AUC_{inf}, area under the concentration–time curve up to ∞ hours; $t_{1/2}$, terminal elimination half-life; CL, clearance; Vz, volume of distribution based on terminal phase.

Table 7. Grade change in sensory neuropathy (all courses)

Level	Case	Before administration	Course no.										
			1	2	3	4	5	6	7	8	9	10	11
Level 1	1-2	0	0	0	1	1	1	1[a]	—	—	—	—	—
Level 2	2-1	1	2	—	—	—	—	—	—	—	—	—	—
	2-2	0	1	1	1	1	1	1	1	1	2	2	3[a]
	2-3	0	0	1	—	—	—	—	—	—	—	—	—
Level 3	3-1	0	1	1	2	2	2	3[a]	—	—	—	—	—
	3-2	0	1	1	1	2	2[a]	—	—	—	—	—	—
	3-3	0	2	2	3[a]	—	—	—	—	—	—	—	—
Level 2	2-4	0	1	1	1	2	2	2[a]	—	—	—	—	—
	2-5	0	1	—	—	—	—	—	—	—	—	—	—
	2-6	0	1	1	—	—	—	—	—	—	—	—	—

—, end of study.
[a]Study-off due to sensory neuropathy.

patients. RD in this schedule was determined to be 260 mg/m^2 in consideration of efficacy, toxicities and similarity of PK profile in the western studies. Additional studies of single-agent ABI-007 and platinum-based combinations are warranted.

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Conflict of interest statement

Hironobu Minami and Tomohide Tamura receive remuneration for the lectures from Taiho Pharmaceutical (Tokyo, Japan).

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A Phase 1 Clinical Study of Temsirolimus (CCI-779) in Japanese Patients with Advanced Solid Tumors

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Objective: Temsirolimus (CCI-779) is a novel inhibitor of the mammalian target of rapamycin. This Phase 1 study was aimed at investigating the maximum-tolerated dose, toxicity, pharmacokinetics and antitumor activity in Japanese patients with advanced solid tumors.
Methods: Temsirolimus was given as a 30 min intravenous infusion once a week. Patients with solid tumors not amenable to standard forms of treatment were eligible. Dose escalation of temsirolimus was planned from 15, 45, 80 to 165 mg/m^2. The pharmacokinetics of temsirolimus and sirolimus in whole blood were examined for cycles 1, 2, 4 and 5 of treatment.
Results: Ten patients (median age 60.5 years; range 41–69 years) with advanced solid tumors were enrolled. Their primary cancers were renal cell carcinoma (five patients), lung cancer (three patients) and colorectal cancer (two patients). The major toxicities were hypophosphatemia diarrhea, hyperglycemia, stomatitis, pyrexia, elevated aspartate aminotransferase, rash, reduced neutrophil count, elevated alanine aminotransferase, anorexia, hypertriglyceridemia and somnolence. Two of three patients who received temsirolimus 45 mg/m^2 developed dose-limiting toxicities of Grade 3 stomatitis (one patient) and Grade 3 diarrhea (two patients). The maximum-tolerated dose was 15 mg/m^2. The peak blood concentrations of temsirolimus and sirolimus, a major active metabolite, increased in a dose-dependent manner. The area under the concentration-versus-time curve of sirolimus, but not temsirolimus, increased in a dose-dependent manner.
Conclusions: The recommended dose for Phase 2 clinical studies of temsirolimus in Japanese patients with advanced solid tumors is 15 mg/m^2 intravenously once a week.

Key words: Phase 1 study – CCI-779 – temsirolimus – advanced solid tumor

INTRODUCTION

Temsirolimus (CCI-779), a novel inhibitor of mammalian target of rapamycin (mTOR), is an ester analogue of the immunosuppressive agent sirolimus (rapamycin, trade name: Rapamune®, sale: Pfizer Inc.), which was approved for the prophylaxis of organ rejection in patients receiving renal transplants in the USA. Temsirolimus inhibits several key signal transduction pathways regulating G1 phase of the cell cycle by inhibiting the activity of mTOR, a cell cycle regulatory kinase, and ultimately blocks progression from G1 to S phase of the cell cycle (1). Temsirolimus is also reported to show an inhibitory effect on the growth of various tumor cells, especially on the tumor cells with mutation or deletion of *PTEN* (phosphatase and tensin homolog deleted on chromosome 10) tumor suppressor gene (2–10).

On the basis of the inhibitory effect of temsirolimus on the growth of tumor cells in preclinical studies, clinical studies of temsirolimus have been conducted in patients with a variety of malignant tumors, including advanced renal cell carcinoma (13–20,22,23). In the USA, temsirolimus has been approved for the treatment of advanced renal cell carcinoma. In Europe, temsirolimus has been approved for the

first-line treatment of patients with advanced renal cell carcinoma who have at least three of six poor prognostic factors (Trade name: Torisel®, INN: temsirolimus, sale: Pfizer Inc. (21,24)). The recommended dose of temsirolimus is 25 mg/body infused intravenously over a 30–60 min period once a week.

On the basis of the above results, a Phase 1 study was also conducted in Japan to investigate the safety, tolerability, pharmacokinetics and antitumor activity of temsirolimus in patients with advanced solid tumors. Two different temsirolimus schedules were used in US, European and international studies (daily for 5 days every second week and once a week) and showed that temsirolimus was well tolerated and had antitumor activity (14,15,22,23). The weekly administration was chosen for the conduct of this clinical study in Japan because this is the schedule that has been approved in the USA and Europe and it is a more convenient schedule for patients than daily for 5 days every second week.

PATIENTS AND METHODS

Study Design

This was an open-label, single-center, Phase 1 study. The primary objective was to investigate the safety and tolerability of temsirolimus administered by a 30 min intravenous infusion once a week. The secondary objective was to collect preliminary data of the pharmacokinetics and antitumor activity of temsirolimus.

The study protocol was approved by the institutional review board of the National Cancer Center, and the study was conducted from October 2002 to March 2005 in accordance with Good Clinical Practice.

Patients

Patients were enrolled in the study only if they met the following entry criteria:

Inclusion criteria were: (i) histologically and cytologically confirmed diagnosis of advanced solid tumors; (ii) refractory to standard therapy or no appropriate therapy; (iii) measurable lesion; (iv) at least 4 weeks since any prior chemotherapy, hormonal therapy, radiation therapy and/or surgery, and any other investigational agent use (at least 6 weeks since nitrosourea and mitomycin C); (v) available to be hospitalized from the day before the first administration through the fourth week; (vi) age $\geq$20 and $<$75 years at the time of consent; (vii) neutrophil count $\geq$1500/mm^3, platelet count $\geq$100 000/mm^3, hemoglobin $\geq$8.5 g/dl; (viii) adequate renal function: serum creatinine $<$1.5 mg/dl, creatinine clearance (24 h method) $\geq$50 ml/min; (ix) adequate hepatic function: total bilirubin $<$2 mg/dl, glutamic oxaloacetic transaminase (aspartate aminotransferase, AST) and glutamic pyruvic transaminase (alanine aminotransferase, ALT) $<$3 times the institutional upper limit of the normal range; (x) serum cholesterol $\leq$350 mg/dl, triglyceride $\leq$500 mg/dl; (xi) performance status 0–2; (xii) estimated life expectancy of at least 3 months and (xiii) written informed consent for the study.

Exclusion criteria were: (i) concomitant use of CYP3A4 inhibitors or inducers, such as anticonvulsants and rifampicin, where the treatment could not be discontinued or switched to a different medication; (ii) symptomatic brain metastasis; (iii) positive HIV antibody, HBs antigen or HCV antibody; (iv) infection requiring systemic medication; (v) complications that were difficult to control by medication or other therapies; (vi) use of immunosuppressive agents within 3 weeks prior to enrollment; (vii) regular use of steroids; (viii) concurrent angina, myocardial infarction within 6 months prior to starting study or heart disease that was difficult to control with medication or other therapies; (ix) history of hypersensitivity to diphenhydramine or its structurally related antihistamine drugs; (x) history of hypersensitivity to polysorbate 80 or polyethylene glycol contained in the diluent of the investigational drug, (xi) women who were pregnant or lactating, or not willing to use acceptable contraception during the study and for at least 3 months following the last study drug administration and (xii) inappropriate patients for the study for any other reason such as screening test results by the investigator.

Dosage and Administration

Temsirolimus was supplied by Wyeth K.K. (Tokyo, Japan) in 5 ml vials for intravenous injection; each contained 25 mg/ml of temsirolimus, co-packaged with a specific diluent.

Diphenhydramine, an antihistamine drug, was intravenously infused at a dose of 30 mg over 30 min $\sim$1 h before the temsirolimus administration to prevent hypersensitivity reactions. Approximately 30 min after the diphenhydramine infusion, temsirolimus was administered by a 30 min intravenous infusion.

The doses of temsirolimus were 15, 45, 80 or 165 mg/m^2. The 15, 45 and 165 mg/m^2 doses correspond to flat doses of 25, 75 and 250 mg. The latter were used once a week in a Phase 2 clinical study in kidney cancer patients and tolerability and efficacy were observed at all doses (14). Thus, these doses were used in this study of Japanese patients. In addition, 80 mg/m^2 was used as an intermediate dose between 45 and 165 mg/m^2 so that there would not be a 3.7-fold increase in dose in the escalation. Temsirolimus was administered once a week as used previously (14,15,22) and at least three administrations were planned. The fourth or subsequent administrations of temsirolimus were allowed to continue until tumor progression or unacceptable toxicity occurred.

Dose Escalation Method

On the basis of the Guidelines for Clinical Evaluation of Anticancer Drugs (11), three patients were administered temsirolimus in each dose group. Additional patients were

treated at that dose level or dose escalation was performed according to the number of patients with unacceptable toxicity.

Definition of Dose-limiting Toxicity

Tolerability of temsirolimus was assessed on the basis of the safety evaluation of the initial three-weekly administrations. When the following events related to temsirolimus occurred before the fourth administration, the event was defined as unacceptable toxicity: (i) Grade 3 or 4 non-hematologic toxicity (exclusion: nausea or vomiting without the use of appropriate antiemetic drugs, and serum triglycerides <1500 mg/dl recovering to Grade 2 by the next week), (ii) Grade 4 thrombocytopenia, (iii) Grade 4 neutropenia lasting 5 days or more, (iv) Grade 4 febrile neutropenia or (v) delay of administration for 2 weeks or more due to prolonged toxicity.

The severity of toxicities was assessed according to National Cancer Institute Common Toxicity Criteria (NCI-CTC Version 2.0, 30 April 1999).

Efficacy Evaluation

On the basis of the guidelines of Response Evaluation Criteria in Solid Tumors (RECIST) (12), best overall response was assessed.

Pharmacokinetics

Blood samples were collected at 0, 0.25, 0.5, 1, 2, 4, 6, 24, 72, 96 and 168 h (just before the time of second administration) after the first administration of temsirolimus and at the time of fifth administration (168 h after the fourth administration). Blood concentrations of temsirolimus and sirolimus, a major active metabolite, were measured by Taylor Technology Inc. (NJ, USA) using a validated liquid chromatography/tandem mass spectrometry method. The limit of detection was 0.25 ng/ml for each compound. The pharmacokinetic parameters of temsirolimus and sirolimus in whole blood, including maximum concentration (C_{max}), time to maximum concentration (t_{max}), terminal half-life ($t_{1/2}$), area under the concentration-versus-time curve (AUC), apparent clearance (corrected for unknown fraction of drug metabolized; CL/f_m) and apparent volume of distribution at steady state (Vd_{ss}/f_m), were calculated using a non-compartmental analysis technique.

RESULTS

Patients' Characteristics

Ten patients with solid tumors were enrolled in this study (Table 1). The median age was 60.5 years with a range of 41–69 years. All patients had at least one prior chemotherapy and seven patients also had surgery for primary tumors.

Table 1. Patients' characteristics

	Number of patients ($n = 10$)
Sex	
Male	6
Female	4
Age (years)	
≥ 40 to <50	2
≥ 50 to <60	2
≥ 60 to <70	6
Median (range)	
60.5 (41–69)	10
Performance status	
0	2
1	8
Number of prior	
1	3
Chemotherapy	
2–3	2
Regimens	
≥ 4	5
Prior treatment	
Chemotherapy	1
Chemotherapy + radiation	2
Chemotherapy + surgery	7
Primary cancer	
Renal cell carcinoma	5
Lung cancer	1
Small cell lung cancer	1
Non-small cell lung cancer	1
Sigmoid colon cancer	1
Colorectal cancer	1

The primary cancer of five patients was renal cell carcinoma. Three patients had lung cancer and two had colon/colorectal cancer. With regard to prior chemotherapy, five patients had received four or more regimens.

Dose Escalation and Unacceptable Toxicity

The starting dose of 15 mg/m^2 was administered to three patients. One subject discontinued temsirolimus treatment after the first administration because of Grade 4 gastrointestinal perforation. The Efficacy and Safety Evaluation Committee determined that this adverse event was not an unacceptable toxicity, and the patient was excluded from tolerability evaluation and another patient was enrolled. The other three patients in the 15 mg/m^2 dose group did not develop unacceptable toxicity and the escalated dose of

45 mg/m^2 was administered to three patients. Two of them developed unacceptable toxicity. Grade 3 stomatitis and diarrhea occurred in one patient and Grade 3 diarrhea occurred in the other patient. According to the prescribed procedure, further dose escalation was discontinued, and three additional patients were enrolled in the starting dose of 15 mg/m^2 to confirm tolerability. None of these three additional patients developed unacceptable toxicity. From these results, temsirolimus administered as a monotherapy once a week at an intravenous dose of 15 mg/m^2 was judged to be acceptable.

In the 15 mg/m^2 dose group, six of seven patients received three or more weekly administrations of temsirolimus. Three received 15-, 26- and 47-weekly administrations. The patient in the 45 mg/m^2 dose group who did not have unacceptable toxicity received four-weekly administrations of temsirolimus.

Toxicities

All patients (10/10) experienced at least one adverse event related to temsirolimus administration (Table 2). A greater percentage of patients who received temsirolimus 45 mg/m^2 experienced Grade 3 or 4 adverse events (100%) than those who received 15 mg/m^2 (29%).

Temsirolimus-related adverse events of any grade that occurred in any cycle in at least six patients were hypophosphatemia (90.0%), diarrhea (80.0%), hyperglycemia (80.0%), stomatitis (70.0%), pyrexia (70.0%), elevated AST (70.0%), rash (70.0%), reduced neutrophil count (60.0%), elevated ALT (60.0%), anorexia (60.0%), hypertriglyceridemia (60.0%) and somnolence (60.0%) (Table 2).

The adverse events of Grade 3 or higher that were related to temsirolimus treatment are as follows. In the period from the first to the third dose of temsirolimus, two patients had diarrhea and one patient each had a perforated digestive tract, stomatitis, malaise, hyperglycemia, hypokalemia or hypophosphatemia (Table 2). In the fourth and subsequent administrations, two patients had supraventricular arrhythmia and one each had pulmonary infection and hypercholesterolemia. The perforated digestive tract, malaise, hypercholesterolemia and supraventricular arrhythmia (one patient) occurred in the 15 mg/m^2 group and the diarrhea, stomatitis, hyperglycemia, hypophosphatemia, hypokalemia, pulmonary infection and supraventricular arrhythmia (one patient) occurred in the 45 mg/m^2 group. These adverse events resolved when temsirolimus treatment was stopped and the patients were treated appropriately.

For Grade 2 or lower adverse events of hemoglobin decreased, hematocrit decreased, alkaline phosphatase increased, epistaxis and onychia, the frequency of occurrence increased with the number of administrations of temsirolimus. However, for the adverse events of Grade 3 or higher, the frequency of occurrence did not increase with the number of administrations.

The reasons for discontinuing temsirolimus treatment in this trial were adverse events (four patients), progressive disease (PD; five patients) and other (one patient). Diarrhea, perforated digestive tract, stomatitis and supraventricular arrhythmia were the adverse events that led to discontinuation. No patients died in this trial.

Antitumor Activity

All 10 patients who received temsirolimus were eligible for this study and were included in efficacy evaluation. Eight patients had stable disease (SD) and two patients had PD as their best overall responses. Of those with SD, 71% (5/7) received temsirolimus 15 mg/m^2 and 100% (3/3) received temsirolimus 45 mg/m^2. The median length of SD was 35 days (range 20+ to 371 days, Table 3). Three patients with renal cell carcinoma who received temsirolimus 15 mg/m^2 had a long period of SD (113–371 days).

Pharmacokinetics

Whole blood samples were collected from seven patients in the 15 mg/m^2 dose group and three patients in the 45 mg/m^2 dose group for measurement of concentrations of temsirolimus and sirolimus. After the intravenous infusion of temsirolimus was completed, concentrations of temsirolimus in whole blood decreased more rapidly than those of sirolimus. After a single dose of temsirolimus, mean C_{max} for temsirolimus increased in a dose-dependent manner but mean AUC did not (Table 4). Mean Vd_{ss} for temsirolimus increased with increasing dose from 83.85 l after the 15 mg/m^2 dose to 162.9 l after the 45 mg/m^2 dose. Mean CL also increased with increasing dose (8.48 l/h after 15 mg/m^2 and 27.19 l/h after 45 mg/m^2). Mean terminal half-life for temsirolimus was 14.77 and 13.47 h for 15 and 45 mg/m^2, respectively.

After a single dose of temsirolimus, sirolimus mean C_{max} and AUC were increased with increasing dose; however, they did not increase in a dose-proportional manner (Table 4). A similar dose-proportional increase in sirolimus was observed in the previous study (22). Mean Vd_{ss}/f_m and CL/f_m also appeared to increase with increasing dose. Mean terminal half-life for sirolimus was 67.04 and 59.15 h for 15 and 45 mg/m^2, respectively, and, therefore, was longer than $t_{1/2}$ for temsirolimus. After a single dose of temsirolimus, mean AUC_{sum} also exhibited a dose-dependent increase. Mean AUC_{ratio} was 2.94 after the 15 mg/m^2 dose and 4.79 after the 45 mg/m^2 dose. Inter-subject variability was generally modest after single and multiple doses of temsirolimus.

DISCUSSION

This Phase 1 trial in Japanese patients with advanced solid tumors showed that 15 mg/m^2 temsirolimus infused intravenously once a week was well tolerated. The result of evaluation by dose groups demonstrated that none of the patients

Table 2. Temsirolimus-related adverse events, including laboratory abnormalities, reported in any cycle in at least 30% of patients who received temsirolimus

Adverse event	Temsirolimus 15 mg/m^2 ($n = 7$)		Temsirolimus 45 mg/m^2 ($n = 3$)		Total ($n = 10$)	
	All grades[a] (n)	Grades 3 and 4[a] (n)	All grades[a] (n)	Grades 3 and 4[a] (n)	All grades[a] (n)	Grades 3 and 4[a] (n)
Any	7	2	3	3	10	5
Diarrhea	6		2	2	8	2
Nausea	2		1		3	
Stomatitis	5		2	1	7	1
Vomiting	3				3	
Malaise	2	1	3		5	1
Pyrexia	4		3		7	
Pharyngitis	3				3	
Weight loss	3		2		5	
Anorexia	3		3		6	
Dysgeusia	2		1		3	
Somnolence	4		2		6	
Headache	3		2		5	
Nasal bleeding	4		1		5	
Rash	5		2		7	
Flushing	3		2		5	
Hypertension	3		1		4	
Phlebitis	3				3	
Leukocytes decreased	2		3		5	
Lymphocytes decreased	2		1		3	
Monocytes increased	2		1		3	
Reduced neutrophil count	3		3		6	
Platelets decreased	2		2		4	
Hemoglobin decreased	3		2		5	
Hematocrit decreased	3		1		4	
Elevated ALT	4		2		6	
Elevated AST	5		2		7	
Creatinine increased	2		2		4	
LDH increased	3		2		5	
Alkaline phosphatase increased	3		1		4	
Hypercholesterolemia	4	1	1		5	1
Hyperglycemia	5		3	1	8	1
Hypertriglyceridemia	5		1		6	
Hypophosphatemia	6		3	1	9	1

In the 15 mg/m^2 group, one patient each had perforated digestive tract or supraventricular arrhythmia. In the 45 mg/m^2 group, one patient each had hypokalemia, pulmonary infection or supraventricular arrhythmia. ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase.
[a]NCI-CTC Ver 2.0.

in 15 mg/m^2 dose group experienced unacceptable toxicity, whereas two of three patients experienced Grade 3 stomatitis and diarrhea in 45 mg/m^2 dose group. For single doses of 15 or 45 mg/m^2, the exposure of temsirolimus and its major active metabolite sirolimus in whole blood increased in a dose-dependent but subproportional manner. Inter-subject variability between single (the first) and multiple (the fourth) dosing periods was low. The pharmacokinetic parameters for

temsirolimus and sirolimus that were measured in this study were very similar to those measured in the Phase 1 study of patients with solid tumors treated with intravenous temsirolimus on a weekly schedule (22) and in the Phase 2 study of patients with renal cell carcinoma treated with temsirolimus on a weekly schedule (14).

Table 3. Individual efficacy results of patients treated with temsirolimus

Temsirolimus dose	Primary cancer	Best overall response	Time to progression (days)
15 mg/m^2	Lung cancer	PD	20
	Colorectal cancer	PD	20
	Small cell lung cancer	SD	20+
	Sigmoid colon cancer	SD	23+
	Renal cell carcinoma	SD	113
	Renal cell carcinoma	SD	244
	Renal cell carcinoma	SD	371
45 mg/m^2	Renal cell carcinoma	SD	20+
	Renal cell carcinoma	SD	27+
	Non-small cell carcinoma	SD	42+

SD, stable disease; PD, progressive disease.

The common temsirolimus-related adverse events were hypophosphatemia, diarrhea, hyperglycemia, stomatitis, pyrexia, elevated AST, rash, reduced neutrophil count, elevated ALT, anorexia, hypertriglyceridemia and somnolence. These adverse events also have been reported in other studies of patients treated with temsirolimus (14,15,22,23). Because only 10 Japanese patients were treated in this study, we cannot accurately determine whether the incidence of these adverse events in this population is similar to the incidence in populations in other studies.

In the Phase 3, international, interferon-α-controlled study of temsirolimus conducted in patients with advanced renal cell carcinoma, the patients administered temsirolimus alone at the dose of 25 mg/body (15 mg/m^2) demonstrated a significant improvement in overall survival and progression-free survival when compared with the patients administered interferon-α alone (15). There was no significant difference in the objective response rate (percentage of patients with complete and partial responses) for patients treated with temsirolimus or interferon-α but patients treated with

Table 4. Pharmacokinetic parameters following intravenous administration of temsirolimus

	Single dose		Multiple doses[a]	
	15 mg/m^2	45 mg/m^2	15 mg/m^2	45 mg/m^2[b]
Temsirolimus (mean ± SD)				
C_{max} (ng/ml)	1013.9 ± 316.3	1793.3 ± 421.6	912.0 ± 50.0	1580.0
t_{max} (h)	0.51 ± 0	0.34 ± 0.2	0.43 ± 0.2	0.25
$t_{1/2}$ (h)	14.77 ± 0.68	13.47 ± 1.09	13.76 ± 0.15	11.65
AUC (ng h/ml)[c]	2873 ± 358	2750 ± 250	2203 ± 203	2403
CL (l/h)	8.48 ± 1.73	27.19 ± 6.37	11.54 ± 1.30	24.66
Vd_{ss} (l)	83.85 ± 10.91	162.9 ± 26.67	88.29 ± 9.61	153.4
Sirolimus (mean ± SD)				
C_{max} (ng/ml)	89.1 ± 40.5	157.3 ± 37.1	118.7 ± 28.5	190.0
t_{max} (h)	7.53 ± 11.3	1.87 ± 1.9	2.03 ± 1.7	0.52
$t_{1/2}$ (h)	67.04 ± 17.37	59.15 ± 28.94	71.36 ± 12.24	35.33
AUC (ng h/ml)[c]	8168 ± 2089	13 524 ± 9763	9061 ± 1788	7772
CL/f_m (l/h)	3.05 ± 0.61	7.11 ± 3.41	2.41 ± 0.90	7.37
Vd_{ss}/f_m (l)	189.6 ± 23.46	325.2 ± 103.4	139.0 ± 15.55	273.6
Composite				
AUC_{ratio} (sirolimus:temsirolimus)	2.94 ± 1.08	4.79 ± 3.03	5.20 ± 1.69	3.35
AUC_{sum} (ng h/ml)	11 041 ± 1935	16 274 ± 9970	13 583 ± 2715	10 469

SD, standard deviation; AUC, area under the curve.
[a]Following the fourth administration.
[b]A blood sample from only one patient was available.
[c]AUC for single dose means $AUC_{0-\infty}$ and for multiple doses means $AUC_{0-\tau}$.

temsirolimus had a significantly higher clinical benefit rate (percentage of patients with complete and partial responses and SD for at least 24 weeks) than did patients treated with interferon-α. Thus, long-term SD was an important component of the temsirolimus response in patients with advanced renal cell carcinoma. For patients treated with temsirolimus, the objective response rate was 9% and the clinical benefit rate was 32%. In this Phase 1 study, three (3) patients with renal cell carcinoma had long-term SD lasting 113–371 days. The fact that 3 of 10 Japanese patients had long-term SD and none had partial or complete responses agrees with the results obtained in the Phase 3 study and suggests that the antitumor activity of temsirolimus in Japanese cancer patients is similar to that in renal cell carcinoma patients of the international population.

From the above results, tolerability at 15 mg/m^2 was confirmed, and the recommended dose for Japanese patients with advanced solid tumors was determined to be temsirolimus 15 mg/m^2 as an intravenous infusion once a week. Further clinical studies will be required to investigate the safety and efficacy of temsirolimus in detail.

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Conflict of interest statement

None declared.

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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

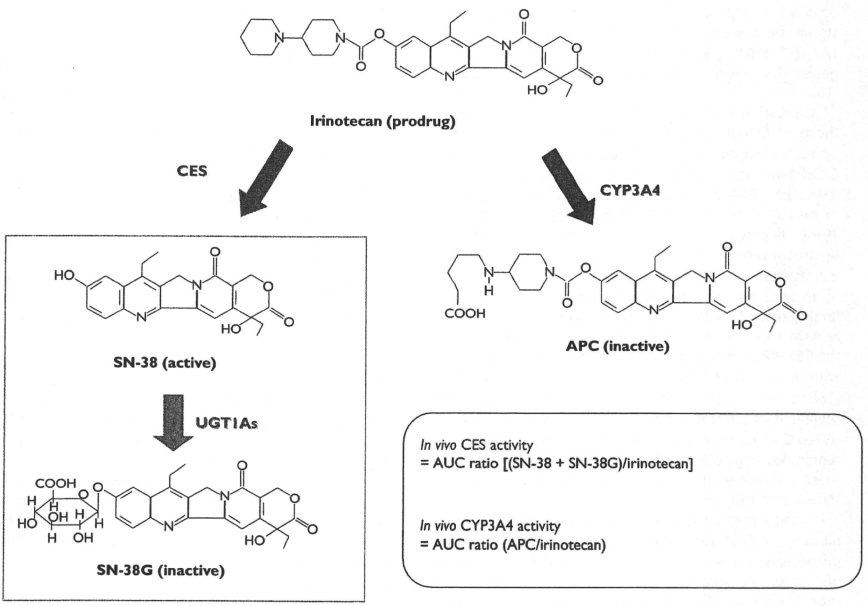

Figure 1

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

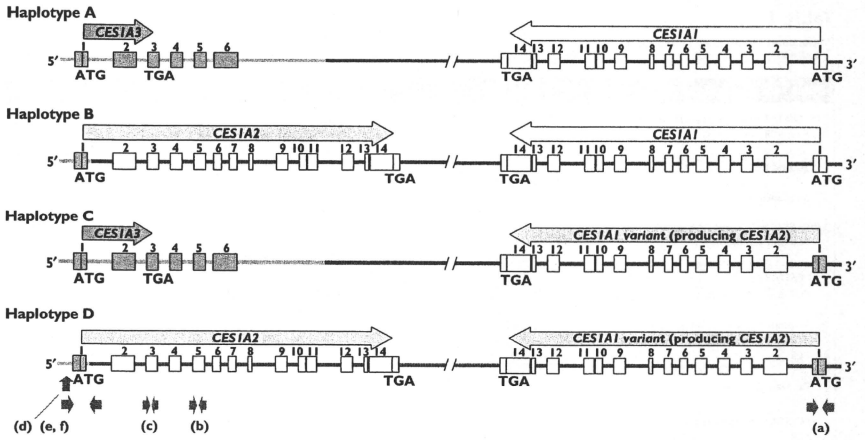

Figure 2

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 amplification 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**ggaaagggagggctttttctg		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**gatgtggtccgtgccttta		0.299	*var1A1*
MPJ6_CS1011			Exon 1	9481156	11	cccttccacgatgtggctcc**G/C**tgcctttatcctggccactc	Arg4Pro	0.297	*var1A1*
MPJ6_CS1012			Exon 1	9481152	15	tccacgatgtggctccgtgc**C/T**tttatcctggccactctctc	Ala5Ala	0.297	*var1A1*
MPJ6_CS1013			Exon 1	9481151	16	ccacgatgtggctccgtgcc**T/C**ttatcctggccactctctct	Phe6Leu	0.297	*var1A1*
MPJ6_CS1014			Exon 1	9481148	19	cgatgtggctccgtgcctttt**A/G**tcctggccactctctctgct	Ile7Val	0.297	*var1A1*
MPJ6_CS1015	rs28563878		Exon 1	9481133	34	tgcctttatcctggccactctc**T/G**tgcttccgcggctttggggt	Ser12Ala	0.297	*var1A1*
MPJ6_CS1016	rs12149359		Intron 1	9481099	IVS1+16	tttggggtgagtccttctgaaa**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

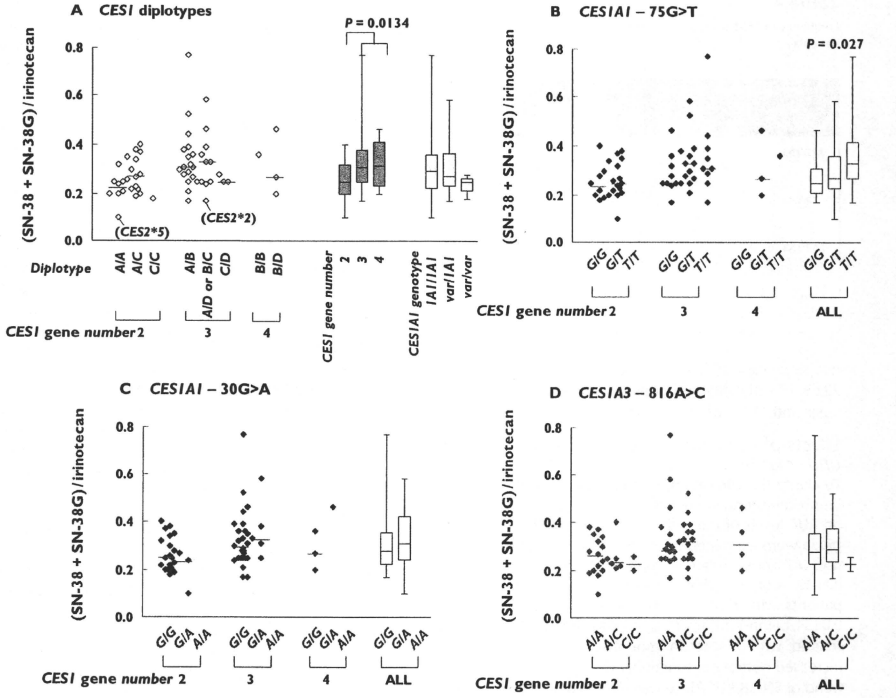

Figure 3

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