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Relationship between Expression of Vascular Endothelial Growth Factor in Tumor Tissue from Gastric Cancers and Chemotherapy Effects: Comparison between S-1 alone and the Combination of S-1 plus CDDP

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Relationship between Expression of Vascular Endothelial Growth Factor in Tumor Tissue from Gastric Cancers and Chemotherapy Effects: Comparison between S-1 alone and the Combination of S-1 plus CDDP

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Background: We have reported that vascular endothelial growth factor (VEGF) expression in gastric cancers might be a selective marker between 5-fluorouracil (5-FU) and a combination of 5-FU plus cisplatin (CDDP). In this study, the relationship between VEGF expression and effects of S-1 with and without CDDP is investigated.
Methods: The subjects were 44 patients treated with S-1 (40 mg/m^2, twice daily, days 1–28, repeated every 6 weeks) and 24 patients treated with S-1 plus CDDP (S-1 40 mg/m^2, twice daily, days 1–21, CDDP, 60 or 70 mg/m^2, day 8, repeated every 5 weeks). VEGF expression in pretreatment endoscopic biopsy samples was assessed immunohistochemically.
Results: Median survival times (MST) of the patients treated with S-1 and S-1 plus CDDP were 344 and 388 days. Among evaluable patients, the response rates of patients with VEGF (+) and (−) tumors to S-1 were 40% (6/15) and 54% (13/24), and to S-1 plus CDDP, 79% (15/19) and 80% (4/5). While the survival of patients with VEGF (−) tumors was slightly longer than those with VEGF (+) tumors in the S-1 group (MST, 425 versus 308 days, $P = 0.42$), patients with VEGF (+) tumors survived remarkably longer than those with VEGF (−) tumors in the S-1 plus CDDP group (MST, 570 versus 333 days, $P = 0.19$).
Conclusion: Similarly to our previous study, it is suggested that the effects of adding CDDP to S-1 might be more remarkable in gastric cancer patients with VEGF (+) tumors than in those with VEGF (−) tumors. These results should be confirmed in a large phase III study.

Key words: vascular endothelial growth factor – S-1 – gastric cancer

INTRODUCTION

The prognosis of patients with unresectable and recurrent gastric cancer is still poor. 5-fluorouracil (5-FU)-based chemotherapy has been widely used for advanced gastric cancer, showing a survival benefit compared with best supportive care (1). In randomized phase III trials, the survival benefit of additional cisplatin (CDDP) to 5-FU has not been clarified (2–4). In the phase III study of the Gastrointestinal Oncology Study Group in the Japan Clinical Oncology Group, although combination therapy of 5-FU plus cisplatin (FP) showed a higher response rate and longer time to progression than continuous infusion of 5-FU (5-FUci), the survival with these two regimens were identical and 5-FUci was less toxic than FP (4).

Recently, many chemotherapy regimens including new agents have been developed that show high response rates for advanced gastric cancer (5–8). S-1 is a new oral fluoropyrimidine, consisting of tegafur (FT), 5-chloro-2, 4-dihydropyrimidine (CDHP) and potassium oxonate. The two phase II studies of S-1 for advanced gastric cancer showed a response rate of 45% in total, with low incidences of severe toxicities (9,10). A combination of S-1 plus CDDP showed a very high response rate of 74% in a phase I/II study

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(11). In Japan, S-1-based regimens have been widely used in clinical practice for gastric cancer and a randomized phase III study comparing S-1 with S-1 plus CDDP is underway.

It has been generally considered that additional CDDP may bring a benefit to some patients with tumors sensitive to CDDP, whereas it may deteriorate the quality of life in patients with tumors refractory to it. Thus, it is necessary to differentiate patients to be treated with or without additional CDDP.

Progress in basic research has revealed many factors and mechanisms implicated in sensitivity and resistance to chemotherapy. In our first report of the phase II study of FP, patients with positive expression of vascular endothelial growth factor (VEGF) in their primary tumors showed a significantly higher response rate than those negative for VEGF (12). In our second report, patients with VEGF (+) tumors showed a higher response rate than those with VEGF (−) tumors after treatment with a combination of irinotecan plus CDDP (13). Moreover, in our third report, patients with VEGF (+) tumors showed a shorter survival than those with VEGF (−) tumors after treatment with 5-FUci, while there was no difference in survival between patients with VEGF (+) and (−) tumors after treatment with FP (14). These results suggest that patients with VEGF (+) tumors might receive a greater benefit from chemotherapy containing CDDP than those with VEGF (−) tumors. However, these results should be recapitulated in other cohorts.

In this study, we investigate the relationship between the expression of VEGF and chemotherapy effects of S-1 alone and S-1 plus CDDP in advanced gastric cancer patients to confirm our previous results that VEGF might be a selective marker for the addition of CDDP.

PATIENTS AND METHODS

Patient Population

The subjects of this study consisted of two groups. One was 24 of 25 patients enrolled in the phase I/II study of S-1 combined with CDDP (11). The other group was 44 consecutive patients recruited from 99 patients registered to the post-marketing survey of S-1 (15) from the National Cancer Center Hospital East between April in 1998 and March in 2000. The recruitment criteria for the S-1 group was the same as the eligibility criteria of the phase I/II study of S-1 plus CDDP (11): histologically proven gastric adenocarcinoma; age, 20–74 years; performance status 0–2 on the ECOG scale; no prior chemotherapy; and adequate bone marrow, liver and renal function. Most importantly, endoscopic biopsy samples taken from primary tumors before chemotherapy were available.

Treatment Schedule

The treatment schedule with S-1 was oral administration at a dose that did not exceed 40 mg/m^2 based on the patient's body surface area (BSA): BSA < 1.25 m^2, 40 mg; 1.25 m$^2 \leq$ BSA < 1.5 m^2, 50 mg; and BSA ≥ 1.5 m^2, 60 mg. This was administered twice daily for 28 consecutive days, followed by 2 weeks rest. In the S-1 plus CDDP group, the same dose of S-1 was administered for 21 consecutive days and CDDP at a dose of 60 (18 patients) or 70 (six patients) mg/m^2 was given intravenously with adequate hydration and repeated every 5 weeks. Both treatments were repeated until disease progression, unacceptable toxicity, or patient refusal.

Evaluation of Antitumor Effects

Tumor responses were evaluated according to the classification of the Japanese Research Society for Gastric Cancer (16), using endoscopy, X-ray imaging or CT scanning. The survival time was calculated from the initial date of the therapy to the date of death from any cause or last confirmation of survival.

Immunohistochemistry

Biopsy samples were immunostained as described in our previous studies (12–14). All immunohistochemical examinations were performed on tissue sections from formalin-fixed and paraffin-embedded biopsy materials from primary tumors. Serial 3-μm thick slices were cut, deparaffinized in xylene, dehydrated with graded ethanol and then immersed in methanol with 0.3% H_2O_2 for 20 min to inhibit endogenous peroxidase activity. The sections were treated with 0.05% pepsin in 0.01 N HCl for 20 min at room temperature. After blocking with 10% normal swine serum in phosphate-buffered saline (PBS; blocking buffer) for 60 min, all sections were incubated overnight at room temperature with the primary antibodies (polyclonal; Santa Cruz Biochemistry, CA, USA) diluted in blocking buffer to 1:500. The sections were washed with PBS and then incubated for 1 h with biotinylated secondary antibody diluted to 1:200. After washing with PBS, the sections were incubated with ABC reagent (Vector Laboratories, CA, USA), and the color reaction was developed in 2% 3-3′-diaminobenzidine and 0.3% H_2O_2 in Tris buffer. The sections were then counterstained with hematoxylin or methyl green.

All immunostained specimens were assessed by one investigator (N.B.) who was blinded to all clinical information. The VEGF staining (Fig. 1) was graded as (++) when the intensity of staining in cancer cells was stronger than that in stromal cells, as (+) when they were equal and as (−) when weaker. Patients were defined as positive when more than 20% of all cancer cells in each section were (++) or (+).

Statistical Analysis

Survival curves were calculated by the Kaplan–Meier method and compared with the log-rank test. Patient characteristics and response rates were compared with a χ^2 test or Fisher's exact test.

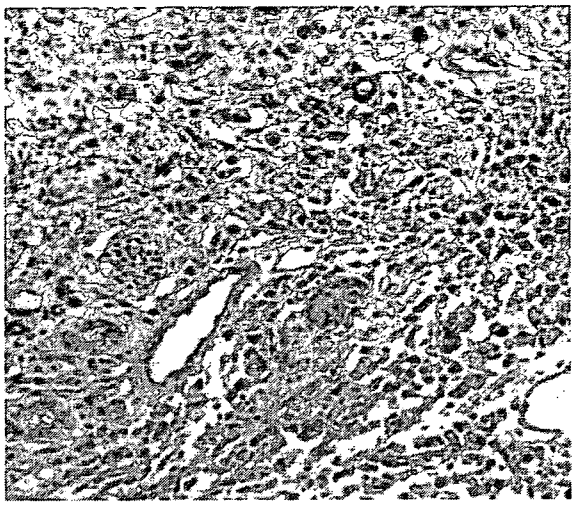

Figure 1. Expression of VEGF detected by immunohistochemistry. VEGF, vascular endothelial growth factor.

RESULTS

Subjects

Table 1 shows the clinicopathological features of the subjects. The median age was around 60 years in both treatment groups. Forty-three of the 44 patients in the S-1 group and all 24 patients in the S-1 plus CDDP group had a good performance status of one or less. Histologically, 31 patients (70%) in the S-1 group and 15 (63%) in the S-1 plus CDDP group had diffuse type adenocarcinoma. Twenty-eight patients (64%) in the S-1 group and 20 (83%) in the S-1 plus CDDP group had one or less metastatic sites.

Expression of VEGF and Clinicopathological Features

Fifteen of the 44 patients (34%) in the S-1 group had VEGF (+) tumors, while the tumor VEGF positive rate was 21% (5/24) in the S-1 plus CDDP group. Table 2 shows the clinicopathological features of patients with VEGF (−) and (+) tumors in the S-1 group and the S-1 plus CDDP group. In the S-1 group, patients with VEGF (+) tumors were significantly younger than those with VEGF (−) tumors, and other factors related to prognosis such as performance status, tumor extent and number of metastatic sites were slightly better in patients with VEGF (+) tumors than in those with VEGF (−) tumors. In the S-1 plus CDDP group, patient characteristics, except age and histological type, were well balanced between VEGF (−) and (+) tumors.

Table 1. Patient characteristics

	S-1 ($n = 44$)	S-1 + CDDP ($n = 24$)
Age (median)	59 (range 28–78)	60 (range 31–72)
Gender (M/F)	22/22 (50/50)	20/4 (83/17)
PS (0/1/2)	31/12/1 (71/27/2)	19/5/0 (79/21/0)
Histological type (intestinal/diffuse)	13/31 (30/70)	9/15 (37/63)
Resection of primary tumor (−/+)	27/17 (61/39)	19/5 (79/21)
Tumor extent (locally advanced, metastatic)	28/16 (64/36)	16/8 (67/33)
No. of metastatic sites (0/1/2/3 or more)	1/27/14/2 (2/61/32/5)	1/19/3/1 (4/79/13/4)

CDDP, cisplatin; PS, performance status.

Expression of VEGF and Response to Chemotherapy

Among the 39 patients (89%) with evaluable lesions in the S-1 group, the response rate was 49% (19/39); the response rate of all 24 patients in the S-1 plus CDDP was 79% (Table 3). In the S-1 group, the response rate of the 24 patients with VEGF (−) tumors (54%) was slightly higher than that of the 15 patients with VEGF (+) tumors (40%) ($P = 0.39$). In the S-1 plus CDDP group, the response rates of the patients with VEGF (+) and (−) were very similar.

Expression of VEGF and Survival

The median survival times (MST) for the S-1 and S-1 plus CDDP groups were 344 and 388 days, respectively (Fig. 2). Figure 3 shows the survival curves of the patients with VEGF (+) and (−) tumors in the S-1 group (A) and the S-1 plus CDDP group (B). In the S-1 group, the MST of the 29 patients with VEGF (−) tumors was 425 days and that of the 15 patients with VEGF (+) tumors was 308 days ($P = 0.42$). In the S-1 plus CDDP group, the survival of the five patients with VEGF (+) tumors was remarkably long (MST, 570 days), while the MST of the 19 patients with VEGF (−) tumors was 333 days ($P = 0.19$). In the 48 patients with VEGF (−) tumors, the 29 treated with S-1 survived relatively longer than the 19 patients treated with S-1 plus CDDP (MST, 425 days versus 333 days, $P = 0.23$). For the 20 patients with VEGF (+) tumors, five patients treated with S-1 plus CDDP showed a longer survival than the 15 patients treated with S-1 (MST, 570 days versus 308 days, $P = 0.24$).

DISCUSSION

In this study, patients were recruited from two sources; one a registry of a post-marketing survey of S-1 (15), the other a phase I/II study of S-1 plus CDDP (11). The response rate and MST of patients treated with S-1 were 49% (19/39 in evaluable patients) and 344 days, and for those treated with S-1 plus CDDP, 79% (19/24) and 384 days, respectively. Two phase II studies of S-1 showed a response rate of 45% in total, with a MST of 9 months. After the above phase I/II study of S-1 plus CDDP, subsequent

Table 2. Expression of VEGF and clinicopathological features

	S-1			S-1 + CDDP		
	VEGF (−) ($n = 29$)	VEGF (+) ($n = 15$)	P	VEGF (−) ($n = 19$)	VEGF (+) ($n = 5$)	P
Age (median)	63 (39–78)	52 (28–66)	<0.01	59 (31–72)	63 (49–72)	0.41
Gender (M/F)	13/16	9/6	0.34	16/3	4/1	>0.99
PS (0/1/2)	19/9/1	12/3/0	0.53	15/4/0	4/1/0	>0.99
Histological type (intestinal/diffuse)	9/20	4/11	>0.99	6/13	3/2	0.33
Resection of primary tumor (−/+)	18/11	9/6	>0.99	15/4	4/1	>0.99
Tumor extent (locally advanced, metastatic)	17/12	11/4	0.51	13/6	3/2	>0.99
No. of metastatic sites (0/1/2/3 or more)	1/18/8/2	0/9/6/0	0.56	1/15/2/1	0/4/1/0	0.85

VEGF, vascular endothelial growth factor.

studies of similar combination chemotherapy also reported high response rates and long MSTs (17,18). It is considered that the subjects in this study could reflect the general outcomes of gastric cancer patients treated with S-1 alone and S-1 plus CDDP.

VEGF promotes angiogenesis and the permeability of blood vessels and is associated with microvessel counts and metastasis (19–21). It has been reported that VEGF is a marker of poor prognosis after surgical resection in various kinds of malignancy, including gastric cancer (22–30). It seems that cancers producing VEGF may have a more malignant potential than those not producing VEGF. In our previous report, after treatment with 5-FUci, patients with VEGF (−) tumors showed a slightly higher response rate and significantly longer survival than those with VEGF (+) tumors (14). In this study, after treatment with S-1, the patients with VEGF (−) tumors showed a slightly higher response rate and relatively longer survival than those with VEGF (+) tumors. Comparing the characteristics between patients with VEGF (−) and (+) tumors in the S-1 group, there were more patients with favorable prognostic factors such as good performance status and local advance disease in the VEGF (+) subgroup than in the VEGF (−) subgroup. It is speculated that the difference in survival between patients with VEGF (−) and (+) tumors might be more prominent if the patient background had been well balanced. Thus, it seems that the relationship between VEGF status and chemotherapy effects such as response and survival might be common between 5-FU alone and S-1 alone.

Table 3. VEGF expression status and response

Treatment	VEGF	CR + PR	NC + PD	RR (%)	P
S-1	(−)	13	11	54	0.39
	(+)	6	9	40	
	Total	19	20	49	
S-1 + CDDP	(−)	15	4	79	>0.99
	(+)	4	1	80	
	Total	19	5	79	

CR, complete response; PR, partial response; NC, no change; PD, progressive disease; RR, response rate.

It has been reported that the response rates in the CDDP-containing regimen of the patients with VEGF (+) tumors were higher than in those with VEGF (−) tumors (12,31). In our first report on the phase II study of FP, the response rate in patients with VEGF (+) tumors was significantly higher than in those with VEGF (−) tumors, while there was no difference in survival between patients with VEGF (+) and (−) tumors (12). Then again, patients with VEGF (−) tumors survived longer than those with VEGF (+) tumors after treatment with 5-FUci (14). In this study, because the number of patients treated with S-1 plus CDDP was small, the difference was not statistically significant. The patients with VEGF (+) tumors survived remarkably longer than those with VEGF (−) tumors after treatment with S-1 plus CDDP, while the survival of the patients with VEGF (−) tumor was slightly longer than in those with VEGF (+) tumors after treatment with S-1 alone. Considering the results of our previous study (5-FU and FP) and this study (S-1 and S-1 plus CDDP), the relationship between VEGF status and the effects of CDDP additional to 5-FU-based drugs seemed to be similar. Moreover, while the patients with VEGF (−) tumors showed a slightly higher response rate than those with VEGF (+) tumors, those of S-1 plus CDDP were similar between VEGF (−) and (+). The difference in response rate between S-1 alone and S-1 plus CDDP of VEGF (+) subgroup was larger than VEGF (−) subgroup. It is speculated that there might be some unknown mechanisms related to sensitivity to CDDP in gastric cancers producing VEGF and that the addition of CDDP might overcome the malignant potential of VEGF (+) tumor patients.

In conclusion, the relationship between VEGF status and chemotherapeutic effects that had been observed in 5-FU-based chemotherapy with and without additional

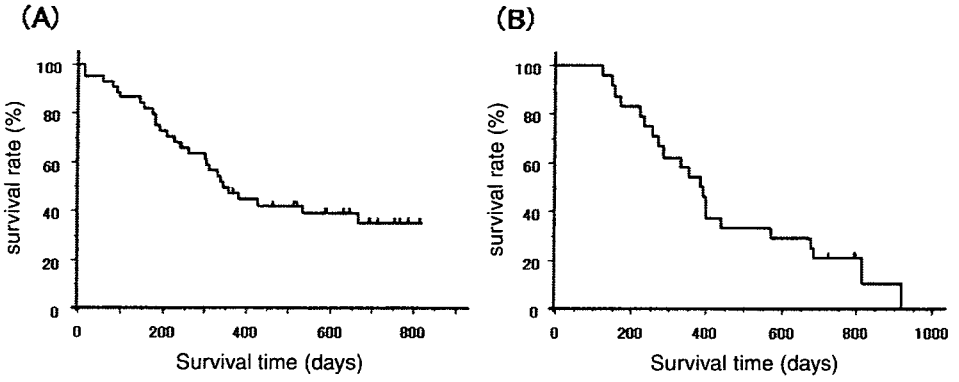

Figure 2. Overall survival of S-1 (A) and S-1 plus CDDP (B) patients. CDDP, cisplatin.

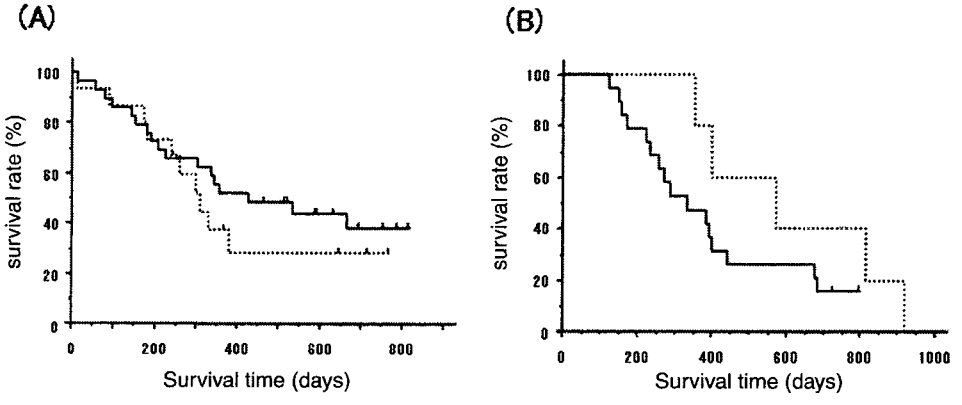

Figure 3. Survival and VEGF expression status in S-1 (A) and S-1 plus CDDP (B) groups. Solid line, VEGF (−) and dotted line, VEGF (+).

CDDP may be reproduced using S-1 and S-1 plus CDDP. It is suggested that the effects of adding CDDP to S-1 might be more remarkable in gastric cancer patients with VEGF (+) tumors than in those with VEGF (−) tumors. These results should be confirmed in a large phase III study.

Acknowledgment

The Taiho pharmaceutical company provided us with part of the clinical data from the phase I/II study of S-1 plus CDDP. We are sincerely grateful to Professor Taguchi for kind advice and arranging this study.

Conflict of interest statement

None declared.

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Haplotypes and a Novel Defective Allele of *CES2* Found in a Japanese Population

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ABSTRACT:

Human carboxylesterase 2 (hCE-2) is a member of the serine esterase superfamily and is responsible for hydrolysis of a wide variety of xenobiotic and endogenous esters. hCE-2 also activates an anticancer drug, irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxycamptothecin, CPT-11), into its active metabolite, 7-ethyl-10-hydroxycamptothecin (SN-38). In this study, a comprehensive haplotype analysis of the *CES2* gene, which encodes hCE-2, in a Japanese population was conducted. Using 21 single nucleotide polymorphisms (SNPs), including 4 nonsynonymous SNPs, 100C>T ($Arg^{34}Trp$, *2), 424G>A ($Val^{142}Met$, *3), 1A>T ($Met^{1}Leu$, *5), and 617G>A ($Arg^{206}His$, *6), and a SNP at the splice acceptor site of intron 8 (IVS8-2A>G, *4), 20 haplotypes were identified in 262 Japanese subjects. In 176 Japanese cancer patients who received irinotecan, associations of *CES2* haplotypes and changes in a pharmacokinetic parameter, (SN-38 + SN-38G)/CPT-11 area under the plasma concentration curve (AUC) ratio, were analyzed. No significant association was found among the major haplotypes of the *1 group lacking nonsynonymous or defective SNPs. However, patients with nonsynonymous SNPs, 100C>T ($Arg^{34}Trp$) or 1A>T ($Met^{1}Leu$), showed substantially reduced AUC ratios. In vitro functional characterization of the SNPs was conducted and showed that the 1A>T SNP affected translational but not transcriptional efficiency. These findings are useful for further pharmacogenetic studies on CES2-activated prodrugs.

Human carboxylesterases are members of the serine esterase superfamily and are responsible for hydrolysis of a wide variety of xenobiotic and endogenous esters. They metabolize esters, thioesters, carbamates, and amides to yield soluble acids and alcohols or amines (Satoh and Hosokawa, 1998; Satoh et al., 2002). In the human liver, two major isoforms of carboxylesterase, hCE-1 and hCE-2, have been identified (Shibata et al., 1993; Schwer et al., 1997). hCE-2 is a 60-kDa monomeric enzyme with a pI value of approximately 4.9 and shares 48% amino acid sequence identity with hCE-1 (Pindel et al., 1997; Schwer et al., 1997; Takai et al., 1997). The *CES2* gene, which encodes hCE-2, is located on chromosome 16q22.1 and consists of 12 exons. Distribution of hCE-2 is relatively limited to several tissues, such as the small intestine, colon, heart, kidney, and liver, whereas hCE-1 is ubiquitously expressed (Satoh et al., 2002; Xie et al., 2002).

Although both hCE-1 and hCE-2 show broad substrate specificities, hCE-2 is relatively specific for heroin, cocaine (benzoyl ester), 6-acetylmorphine, procaine, and oxybutynin (Pindel et al., 1997; Takai et al., 1997; Satoh et al., 2002). In addition, hCE-2 is reported to play a major role in the metabolic activation of the antitumor drug irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxycamptothecin; CPT-11). Irinotecan is a water-soluble derivative of the plant alkaloid camptothecin and is widely used for treatment of several types of cancer. Irinotecan is converted to 7-ethyl-10-hydroxy-

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ABBREVIATIONS: hCE-1, human carboxylesterase 1; hCE-2, human carboxylesterase 2 (EC 3.1.1.1); irinotecan, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxycamptothecin, CPT-11; SN-38, 7-ethyl-10-hydroxycamptothecin; SN-38G, SN-38 glucuronide; SNP, single nucleotide polymorphisms; PCR, polymerase chain reaction; LD, linkage disequilibrium; 5-FU, 5-fluorouracil; MMC, mitomycin C; AUC, area under plasma concentration curve; RT, reverse transcriptase; UTR, untranslated region; ORF, open reading frame.

camptothecin (SN-38), a topoisomerase inhibitor, by carboxylesterases (Humerickhouse et al., 2000) and further conjugated by hepatic uridine diphosphate glucuronosyltransferase to form the inactive metabolite SN-38 glucuronide (SN-38G) (Iyer et al., 1998). To a lesser extent, irinotecan is also converted to 7-ethyl-10-[4-*N*-(5-aminopentanoic acid)-1-piperidino]carbonyloxycamptothecin and 7-ethyl-10-(4-amino-1-piperidino)carbonyloxycamptothecin by cytochrome P450 3A4 (Dodds et al., 1998; Santos et al., 2000). Irinotecan and its metabolites are excreted by the efflux transporters, ABCB1 (P-glycoprotein), ABCC2 (canalicular multispecific organic anion transporter), and ABCG2 (breast cancer resistance protein), via a hepatobiliary pathway (Mathijssen et al., 2001). Although irinotecan metabolism is rather complex, hCE-2 is a key enzyme that determines the plasma levels of the active metabolite SN-38.

Hepatic hCE-2 activity toward irinotecan varies 3-fold in microsomes obtained from a panel of human livers (Xu et al., 2002). The activity loosely correlates with hCE-2 protein levels, but some microsomal samples showed unanticipated deviating activities. This result might be caused by genetic polymorphisms, such as single nucleotide polymorphisms (SNPs) in the *CES2* gene. Several SNPs and haplotypes have been reported for the *CES2* gene (Charasson et al., 2004; Marsh et al., 2004; Wu et al., 2004), and large ethnic differences in *CES2* SNP frequencies are found among Europeans, Africans, and Asian-Americans (Marsh et al., 2004).

Previously, 12 exons and their flanking regions of *CES2* were sequenced from 153 Japanese subjects, who received irinotecan or steroidal drugs, and 12 novel SNPs, including the nonsynonymous SNP, 100C>T ($Arg^{34}Trp$), and the SNP at the splice acceptor site of intron 8 (IVS8-2A>G) were found (Kim et al., 2003). In vitro functional characterization of these SNPs and an additional nonsynonymous SNP, 424G>A ($Val^{142}Met$), suggested that the ^{34}Trp and ^{142}Met variants were defective, and that IVS8-2G might be a low-activity allele (Kubo et al., 2005). In the present study, the same regions were sequenced from an additional 109 subjects (a total of 262 patients), and their haplotypes/diplotypes were determined/inferred. Then, associations between the haplotypes and pharmacokinetic parameters of irinotecan and its metabolites were analyzed for 177 cancer patients who were given irinotecan. Functional characterization of novel SNPs 1A>T and 617G>A, which were found in this study, was also performed by using a transient expression system with COS-1 cells.

Materials and Methods

Chemicals. Irinotecan, SN-38, and SN-38G were kindly supplied by Yakult Honsha Co. Ltd. (Tokyo, Japan).

Patients. A total of 262 Japanese subjects analyzed in this study consisted of 85 patients with allergies who received steroidal drugs and 177 patients with cancer who received irinotecan. The ethical review boards of the National Cancer Center, National Center for Child Health and Development, and National Institute of Health Sciences approved this study. Written informed consent was obtained from all participants.

DNA Sequencing. Total genomic DNA was extracted from blood leukocytes or Epstein-Barr virus-transformed lymphocytes and used as a template in the polymerase chain reaction (PCR). Sequence data of the *CES2* gene from 72 patients and 81 cancer patients were described previously (Kim et al., 2003). In addition, the *CES2* gene was sequenced from 13 allergic patients and 96 cancer patients. Amplification and sequencing of the *CES2* gene were performed as described previously (Kim et al., 2003). Rare SNPs found in only one heterozygous subject were confirmed by sequencing PCR fragments produced by amplification with a high-fidelity DNA polymerase KOD-Plus (Toyobo, Tokyo, Japan). GenBank accession number NT_010498.15 was used as the reference sequence.

Linkage Disequilibrium and Haplotype Analyses. LD analysis was performed by the SNPAlyze software (version 5.1; Dynacom Co., Yokohama, Japan), and a pairwise two-dimensional map between SNPs was obtained for the D' and rho square (r^2) values. All allele frequencies were in Hardy-Weinberg equilibrium. Some haplotypes were unambiguously assigned in the subjects with homozygous variations at all sites or a heterozygous variation at only one site. Separately, the diplotype configurations (combinations of haplotypes) were inferred by LDSUPPORT software, which determines the posterior probability distribution of the diplotype configuration for each subject on the basis of estimated haplotype frequencies (Kitamura et al., 2002). The haplotype groups were numbered according to the allele nomenclature systems suggested by Nebert (2000). The haplotypes harboring nonsynonymous or defective alleles were assigned as haplotype groups **2* to **6*. The subgroups were described as the numbers plus small alphabetical letters.

Administration of Irinotecan and Pharmacokinetic Analysis. The demographic data and eligibility criteria for 177 cancer patients who received irinotecan in the National Cancer Center Hospitals (Tokyo and Chiba, Japan) were described elsewhere (Minami et al., 2007).

Each patient received a 90-min i.v. infusion at doses of 60 to 150 mg/m^2, which varied depending on regimens/coadministered drugs: i.e., irinotecan dosages were 100 or 150 mg/m^2 for monotherapy and combination with 5-FU, 150 mg/m^2 for combination with mitomycin C (MMC), and 60 (or 70) mg/m^2 for combination with platinum anticancer drugs. Heparinized blood was collected before administration of irinotecan and at 0 min (end of infusion), 20 min, 1 h, 2 h, 4 h, 8 h, and 24 h after infusion. Plasma concentrations of irinotecan, SN-38, and SN-38G were determined as described previously (Sai et al., 2002). The AUCs from time 0 to infinity of irinotecan and its metabolites were calculated as described (Sai et al., 2004). Associations between genotypes and pharmacokinetic parameters including the AUC ratio (SN-38 + SN-38G)/CPT-11 were evaluated in 176 patients in whom pharmacokinetic parameters were obtained.

Construction of Expression Plasmids. The coding region of CES2L (long form) cDNA starts at an additional ATG translation initiation codon located 192 nucleotides upstream of the conventional ATG codon (Wu et al., 2003) and encodes a 623-amino acid protein found in the National Center for Biotechnology Information database (NP_003860.2). The wild-type CES2L cDNA was amplified by PCR from Human Liver QUICK-Clone cDNA (Clontech, Mountain View, CA) using *CES2*-specific primers, 5′-CACCCACCTATGACTGCTCA-3′ and 5′-AGGGAGCTACAGCTCTGTGT-3′. The PCR was performed with 1 unit of the high-fidelity DNA polymerase KOD-Plus and a 0.5 μM concentration of the *CES2* specific primers. The PCR conditions were 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 68°C for 3 min and then a final extension at 68°C for 5 min. The PCR products were cloned into the pcDNA3.1 vector by a directional TOPO cloning procedure (Invitrogen, Carlsbad, CA), and the sequences were confirmed in both directions. The resultant plasmid was designated pcDNA3.1/CES2L-WT. The 1A>T variation was introduced into pcDNA3.1/CES2L-WT by using a QuikChange Multi site-directed mutagenesis kit (Stratagene, La Jolla, CA) with the 5′-phosphorylated oligonucleotide, 5′-phospho-GAGACCAGCGAGCCGACCTTGCGGCTGCACAGACTTCG-3′ (the substituted nucleotide is underlined). The sequence of the variant cDNA was confirmed in both strands, and the resultant plasmid was designated pcDNA3.1/CES2L-A1T. Expression plasmids for the short-form wild-type (CES2S) and $Arg^{206}His$ variant CES2 were prepared and introduced into COS-1 cells according to the method described previously (Kubo et al., 2005).

Expression of Wild-Type and Variant CES2 Proteins in COS-1 Cells. Expression of wild-type and variant CES2 proteins in COS-1 cells was examined as described previously (Kubo et al., 2005). In brief, microsomal fractions (30 μg of protein/lane) or postmitochondrial fractions (0.4 μg of protein/lane) were separated by 8% SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Immunochemical detection of each type of CES2 protein was performed using rabbit anti-human CES2 antibody raised against a peptide antigen (residues 539–555, KKALPQKIQELEEPEER) (diluted 1:1500). To verify that the samples were evenly loaded, the blot was subsequently treated with a stripping buffer and reprobed with polyclonal anti-calnexin antibody (diluted 1:2000; Stressgen Biotechnologies Corp., San Diego, CA). Visualization of these proteins was achieved with horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:4000) and the Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life and Analytical Sciences, Boston, MA). Protein band densities were quantified with Diana III

and ZERO-Dscan software (Raytest, Straubenhardt, Germany). The relative expression levels are shown as the means ± S.D. of three separate transfection experiments.

Determination of CES2 mRNA by Real-Time RT-PCR. Total RNA was isolated from transfected COS-1 cells using the RNeasy Mini Kit (QIAGEN, Tokyo, Japan). After RNase-free DNase treatment of samples to minimize plasmid DNA contamination, first-strand cDNA was prepared from 1 μg of total RNA using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) with random primers. Real-time PCR assays were performed with the ABI7500 Real Time PCR System (Applied Biosystems) using the TaqMan Gene Expression Assay for *CES2* (Hs01077945_m1; Applied Biosystems) according to the manufacturer's instructions. The relative mRNA levels were determined using calibration curves obtained from serial dilutions of the pooled wild-type CES2 cDNA. Samples without reverse transcriptase were routinely included in the RT-PCR reactions to measure possible contributions of contaminating DNA, which was usually less than 1% of the mRNA-derived amplification. Transcripts of β-actin were quantified as internal controls using TaqMan β-Actin Control Reagent (Applied Biosystems), and normalization of CES2 mRNA levels were based on β-actin concentrations.

Enzyme Assay. CPT-11 hydrolyzing activity of the postmitochondrial supernatants (microsomal fraction plus cytosol) was assayed over the substrate concentration range of 0.25 to 50 μM as described previously (Kubo et al., 2005), except that the hydrolysis product, SN-38, was determined by the high-performance liquid chromatography method of Hanioka et al. (2001).

Statistical Analysis. Statistical analysis of the differences in the AUC ratios among *CES2* diplotypes, coadministered drugs. or irinotecan dosages was performed using the Kruskal-Wallis test, Mann-Whitney test, or Spearman rank correlation test (Prism 4.0, GraphPad Software, Inc., San Diego, CA). The *t* test (Prism 4.0) was applied to the comparison of the average values of protein expression and mRNA levels between wild-type and variant CES2.

Results

***CES2* Variations Detected in a Japanese Population.** Previously, the promoter region, all 12 exons, and their flanking introns of the *CES2* gene were sequenced from 72 allergic patients and 81 cancer patients and resulted in the identification of 12 novel SNPs (Kim et al., 2003). Additionally, the same region of *CES2* was sequenced from 13 allergic patients and 96 cancer patients. A total of 21 SNPs were found in 262 Japanese subjects (Table 1). Novel SNPs found in this study were −1233T>C, 1A>T, IVS2-71C>G, IVS7 + 27G>A, and IVS9 + 78C>T, but their frequency was low (0.002, identified in a single heterozygous subject for each SNP). The SNP 1A>T is nonsynonymous (M1L) and results in a substitution of the translation initiation codon ATG to TTG in the *CES2* gene. The other novel SNPs were located in the introns or the 5′-flanking region.

The nonsynonymous SNP 424G>A (V142M) reported by our group (Kubo et al., 2005) and another nonsynonymous SNP 617G>A (R206H) published in the dbSNP (rs8192924) and JSNP (ssj0005417) databases were found at a frequency of 0.002. Recently, several noncoding SNPs in *CES2* were also reported (Kim et al., 2003; Charasson et al., 2004; Marsh et al., 2004; Wu et al., 2004). Among them, the three SNPs, −363C>G in the 5′-UTR, IVS10-108(IVS10 + 406)G>A in intron 10, and 1749(*69)A>G in the 3′-UTR of exon 12, were found at frequencies of 0.031, 0.269, and 0.239, respectively, in this study.

LD and Haplotype Analysis. Using the detected SNPs, LD analysis was performed, and the pairwise values of r^2 and D' were obtained. A perfect linkage (r^2 = 1.00) was observed between SNPs −363C>G and IVS10-87G>A. A close association (r^2 = 0.85) was found between SNPs IVS10-108G>A and 1749A>G. Other associations were much lower (r^2 < 0.1). Therefore, the entire *CES2* gene was analyzed as one LD block. The determined/inferred haplotypes are summarized in Fig. 1 and are shown as numbers plus small

TABLE 1

Summary of SNPs in the CES2 gene in a Japanese population

SNP Identification			Location	Position		Nucleotide Change and Flanking Sequences (5′ to 3′)	Amino Acid Change	Allele Frequency
This Study	NCBI (dbSNP)	JSNP		NT_010498.15	From the Translational Initiation Site or from the Nearest Exon			
MPI6_CS2001			5′-Flanking	20582067	−1671[a]	CTGGAACAACTCG/CCTCCCCTCGGAA		0.010
MPI6_CS2002			5′-Flanking	20582484	−1254[a]	AACCACCACCGCT/CGATCCTAGCAGG		0.002
MPI6_CS2016[b]			5′-Flanking	20582505	−1233[a]	CAGGCGTGCGCTT/CCGCTCCAACCC		0.002
MPI6_CS2003			Exon 1 (5′-UTR)	20582979	−759[a]	AAATGTTTGTCAA/GGTGGATAAATGA		0.006
MPI6_CS2004	rs11075646		Exon 1 (5′-UTR)	20583375	−363[a]	CCTCCTATCGATC/GCCCCAGCGCGCT		0.031
MPI6_CS2017[b]			Exon 1	20583738	1[a]	AGCGAGCCGACCA/TTGCGGCTGCACA	Met[1]Leu	0.002
MPI6_CS2005			Exon 2	20586162	100[a]	GCCAGTCCCATCC/TGGACCACACACA	Arg[34]Trp	0.002
MPI6_CS2021[b]			Intron 2	20587248	IVS2–71	GGTGGCTGGGAGC/GACCTCTGAACCC		0.002
MPI6_CS2015			Exon 4	20588325	424[a]	TGATTCCCCAGG/ATGATGGTGTGGA	Val[142]Met	0.002
MPI6_CS2006			Intron 4	20588486	IVS4 + 29	GCTGGGCAACCCG/AGGCTGAGCGGGG		0.002
MPI6_CS2007			Exon 5	20588560	579[a]	CAAGCACGCAACC/TGGCAACTGGGGC	Thr[193]Thr (silent)	0.002
MPI6_CS2018	rs8192924	ssj0005417	Exon 5	20588598	617[a]	TGGCTGCACTACG/ACTGGGTCCAGCA	Arg[206]His	0.002
MPI6_CS2008			Exon 5	20588746	765[a]	CATGGAGACTGGC/TGTGGCCCTCCTG	Gly[255]Gly (silent)	0.002
MPI6_CS2009			Intron 5	20589157	IVS5–69	CCTGTTCTTGGCC/TAGGGCCTTGGGC		0.017
MPI6_CS2019[b]			Intron 7	20589775	IVS7 + 27	AAGCCCACAAGTG/ACCTGGGAGACCC		0.002
MPI6_CS2010			Intron 7	20589845	IVS7–25	CCCATCCCCAGCT/AACAGACTCTCTC		0.002
MPI6_CS2011			Intron 8	20590205	IVS8–2	TCCACCTGGGGTA/GGATGTTGCCTCC	Splicing defect	0.002
MPI6_CS2020[b]			Intron 9	20590429	IVS9 + 78	ACCTGCTGCTTC/TCGGGTCAGCACT		0.002
MPI6_CS2012	rs2241409	IMS-JST1013275	Intron 10	20591293	IVS10–108	GGAAGAAAAGCG/AGAGAAGCAGGAC		0.269
MPI6_CS2013	rs28382825		Intron 10	20591314	IVS10–87	GGACTGGGACCG/AAGGTCTCGGGGG		0.031
MPI6_CS2014	rs8192925	ssj0005418	Exon 12 (3′-UTR)	20592196	1749 (*69)[a]	GTGCCCACACACA/GCCCACTAAGGAG		0.239

[a] A of the conventional translation initiation codon ATG in *CES2* (GenBank Y09616) is numbered 1, and the number in the parentheses indicates the position from the termination codon TGA.

[b] Novel variations detected in this study.

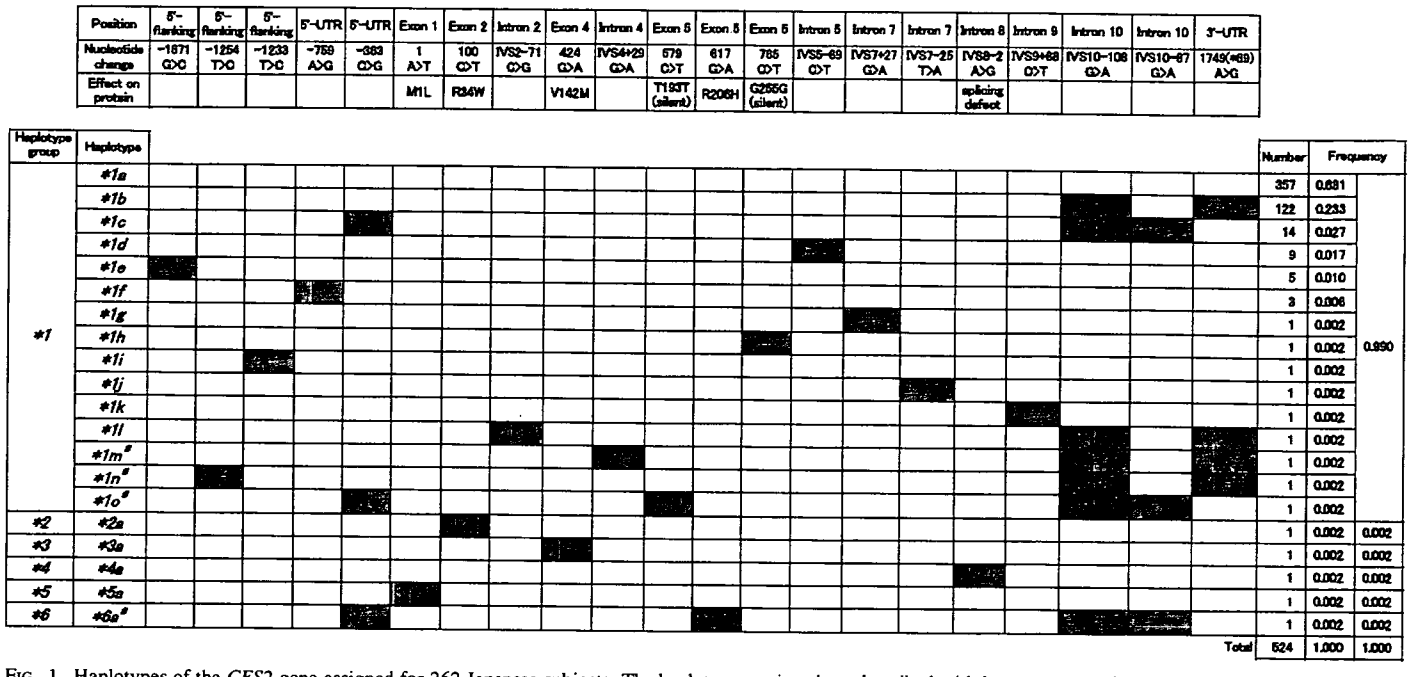

FIG. 1. Haplotypes of the *CES2* gene assigned for 262 Japanese subjects. The haplotypes assigned are described with lower case numbers and alphabetical letters. #, this haplotype was inferred in only one patient and is thus ambiguous.

alphabetical letters. Our nomenclature of haplotypes is distinct from those of previous studies (Charasson et al., 2004; Marsh et al., 2004; Wu et al., 2004). In this study, the haplotypes without amino acid changes and splicing defects were defined as the **1* group. The haplotypes harboring the nonsynonymous SNPs, 100C>T ($Arg^{34}Trp$), 424G>A ($Val^{142}Met$), 1A>T ($Met^{1}Leu$), and 617G>A ($Arg^{206}His$), were assigned as haplotypes **2, *3, *5*, and **6*, respectively. In addition, the haplotype harboring a SNP at the splice acceptor site of intron 8 (IVS8-2A>G) was assigned as haplotype **4*. Several haplotypes were first unambiguously assigned by homozygous variations at all sites (**1a* and **1b*) or heterozygous variation at only one site (**1d* to **1l, *2a, *3a, *4a*, and **5a*). Separately, the diplotype configurations (combinations of haplotypes) were inferred by LDSUPPORT software. The additionally inferred haplotypes were **1c* and **1m* to **1o*. The most frequent haplotype was **1a* (frequency, 0.681), followed by **1b* (0.233), **1c* (0.027), and **1d* (0.017). The frequencies of the other haplotypes were less than 0.01.

Association between *CES2* Genotypes and Irinotecan Pharmacokinetics. Next, the relationships between the *CES2* genotype and AUC ratio [(SN-38 + SN-38G)/CPT-11], a parameter of in vivo CES activity (Cecchin et al., 2005), in irinotecan-administered patients were investigated. The diplotype distribution of 176 patients, who received irinotecan and were analyzed for the AUC ratio, was similar to that of the 262 subjects. We examined preliminarily the effects of irinotecan dosage and comedication on the AUC ratio and obtained significant correlations of irinotecan dosage (Spearman $r = -0.559$, $p < 0.0001$) and comedication ($p < 0.0001$, Kruskal-Wallis test) with the AUC ratios. Because irinotecan dosages also depended on the drugs coadministered (see *Materials and Methods*), we finally stratified the patients with the coadministered drugs. As shown in Fig. 2, no significant differences in the median AUC ratios were observed among the **1* diplotypes in each group (p values in the Kruskal-Wallis test among **1a/*1a, *1a/*1b*, and **1b/*1b* were 0.260, 0.470, 0.129, and 0.072 for irinotecan alone, with 5-FU, with MMC and with platinum, respectively.). The relatively rare haplotype **1c*, which harbors −363C>G, did not show any associations with altered AUC ratio ($p = 0.756$ for irinotecan alone and $p = 0.230$ for irinotecan with platinum, Mann-Whitney test).

To estimate the effects of nonsynonymous SNPs on the metabolism of irinotecan, the AUC ratios in the patients carrying nonsynonymous SNPs were compared with the median AUC ratio of the **1/*1* patients. Three nonsynonymous SNPs, 100C>T ($Arg^{34}Trp$, **2*), 1A>T ($Met^{1}Leu$, **5*), and 617G>A ($Arg^{206}His$, **6*), and a SNP at the splice acceptor site of intron 8 (IVS8-2A>G, **4*) were found in 177 patients who received irinotecan. These SNPs were single heterozygotes. The AUC ratios of the patients with **2a/*1a* (0.17) and **5a/*1a* (0.10) in the monotherapy group were 60 and 36%, respectively, of the median value for the **1/*1* group (0.28) and substantially lower than the 25th percentile of the **1/*1* group (0.23) (Fig. 2). It must be noted that the **5a/*1a* patient had an extremely low AUC ratio. The AUC ratio of the **6* heterozygote who received cisplatin (0.25) was lower than the median value (0.37) but within the range for the **1/*1* group treated with platinum-containing drugs (Fig. 2). Regarding the effect of the heterozygous **4*, the AUC ratio (0.40) was not different from the median AUC ratio of the **1/*1* treated with platinum-containing drugs. To elucidate the effects of two novel amino acid substitutions, $Met^{1}Leu$ (**5*) and $Arg^{206}His$ (**6*), the functional analysis was conducted in vitro.

In Vitro Functional Analysis of the $Met^{1}Leu$ Variant. To clarify the functional significance of the novel variant $Met^{1}Leu$ (**5*), the protein expression level of CES2 carrying the nonsynonymous SNP 1A>T was examined. Wu et al. (2003) reported that transcription of CES2 mRNA was initiated from several transcriptional start sites, resulting in the expression of three *CES2* transcripts. Two longer transcripts carry a potential inframe translational initiation codon ATG at −192 that can encode an open reading frame (ORF) extending 64 residues at the amino terminus, as shown in the reference sequence in the National Center for Biotechnology Information database (NP_003860.2). Therefore, the expression of the CES2 protein from the long *CES2* ORF (CES2L), which encodes a potential 623 residue protein, was analyzed. Western analysis of membrane fraction proteins obtained from COS-1 cells

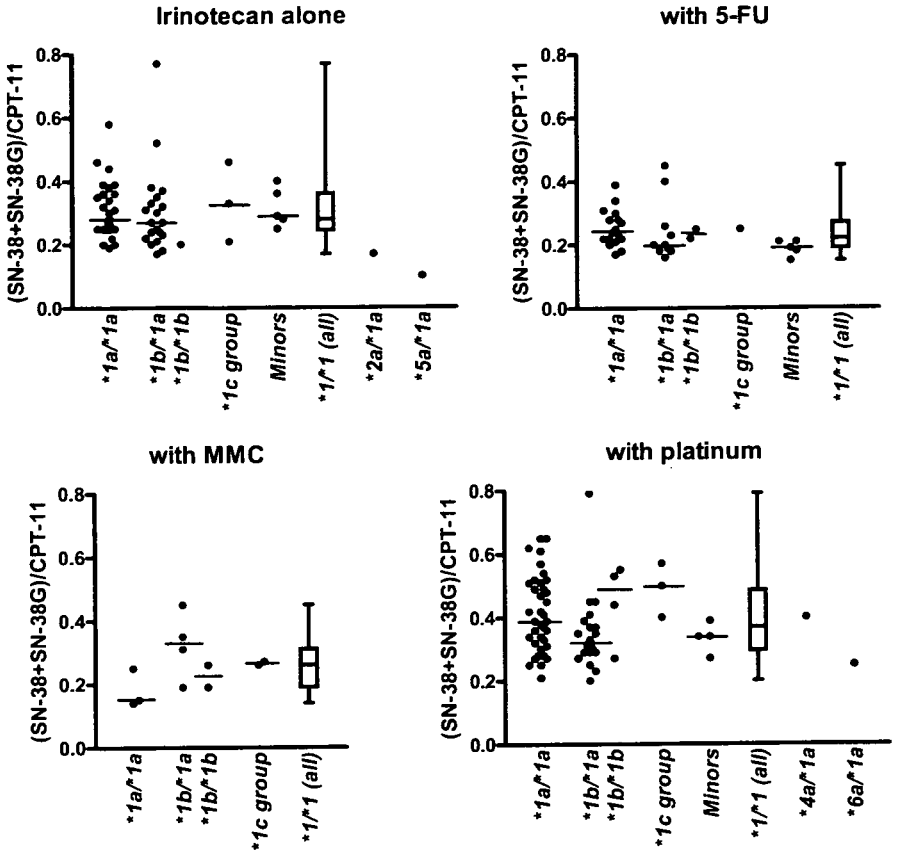

FIG. 2. Relationship between the *CES2* diplotypes and (SN-38 + SN-38G)/CPT-11 AUC ratios in Japanese cancer patients who received irinotecan. Each point represents an individual patient, and the median value in each genotype is shown with a horizontal bar. Distribution of the **1* group is shown by a box representing the 25th to 75th percentiles with a line at the median and bars representing the highest and lowest values. The **1c* group consists of **1c/*1a* and **1c/*1b*. "Minors" represents the heterozygous patients bearing minor **1* haplotypes (**1d, *1e, *1f, *1g, *1k,* and **1m*). Irinotecan alone, irinotecan monotherapy (n = 58); with 5-FU, combination therapy with 5-FU including tegafur (n = 35); with MMC, combination therapy with mitomycin C (n = 11); with platinum, combination therapy with either cisplatin (n = 62), cisplatin plus etoposide (n = 2), or carboplatin (n = 8).

transfected with the expression plasmid pcDNA3.1/CES2L-WT showed that the mobility (approximately 60 kDa) of the protein product from the CES2L cDNA was the same as that from the CES2S cDNA, which encodes a 559 residue protein (Kubo et al., 2005), and the CES2 protein in the human liver microsome (Fig. 3A). Western blot analysis of whole cell extracts also showed that CES2L yielded a single 60-kDa protein product (data not shown), indicating that translation of *CES2* was initiated from the second ATG codon of the *CES2L* ORF but not from the inframe translation initiation codon located at −192.

When the effect of the 1A>T SNP on the expression of the CES2 protein was examined by Western blotting (Fig. 3A), the relative expression levels of CES2 protein from cells transfected with plasmid pcDNA3.1/CES2L-A1T were 11.7 ± 2.4% (p = 0.0003) of the wild type. The mRNA expression levels determined by the TaqMan real-time RT-PCR assay were similar between the wild-type and variant CES2L cDNAs in COS-1 cells (Fig. 4A), indicating that the 1A>T SNP affects translational but not transcriptional efficiency. Thus, the $Met^{1}Leu$ variant was functionally deficient.

In Vitro Functional Analysis of the $Arg^{206}His$ Variant. The known nonsynonymous SNP 617G>A changes an arginine to a histidine at residue 206. Western blot analysis of the postmitochondrial supernatant (including microsomes and cytosol) fractions obtained from COS-1 cells transfected with wild-type (CES2S) and $Arg^{206}His$ variant CES2-expressing plasmids showed that the protein expression level of the $Arg^{206}His$ variant was approximately 82 ± 7% (p = 0.017) of the wild-type (Fig. 3B). No significant differences in the mRNA expression levels determined by the TaqMan real-time RT-PCR assay were observed between the wild-type and 617G>A variant CES2s (82 ± 7%, p = 0.06) (Fig. 4B). Table 2 summarizes the apparent kinetic parameters for CPT-11 hydrolysis of wild-type and $Arg^{206}His$ variant CES2.

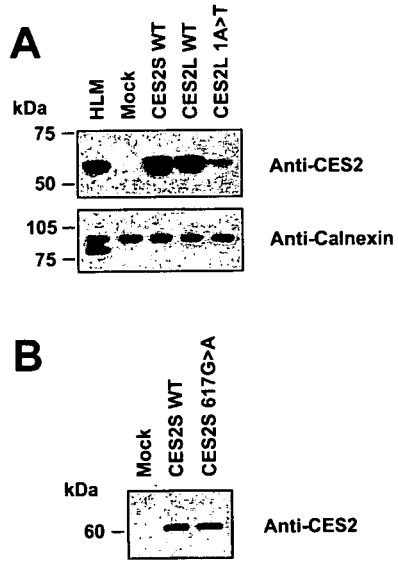

FIG. 3. Expression of CES2 protein from the wild-type and 1A>T (A) and 617G>A (B) variant *CES2* genes in COS-1 cells. Membrane fraction (A) or the postmitochondrial supernatant (B) from the cDNA-transfected cells was subjected to SDS-polyacrylamide gel electrophoresis, followed by transfer to the nitrocellulose membrane. Detection of CES2 and calnexin was performed with rabbit anti-human CES2 antiserum (A and B) and a rabbit anti-human calnexin antiserum (A) and horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody as described under *Materials and Methods*. A representative result from one of three independent experiments is shown. HLM, human liver microsomes.

Although a slight difference in the K_m values was obtained with statistical significance ($p < 0.01$), the kinetic parameters (V_{max} and V_{max}/K_m) were not significantly different when normalized by protein expression levels.

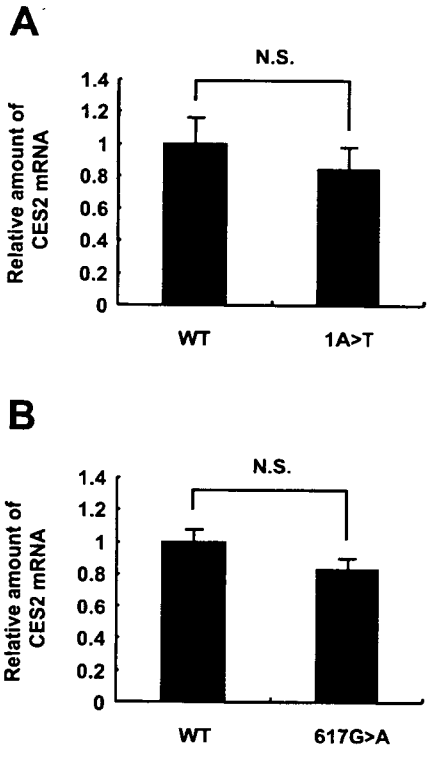

FIG. 4. Quantification of CES2 mRNA by TaqMan real-time RT-PCR in COS-1 cells transfected with wild-type (WT) and 1A>T (A) and 617G>A (B) variants. CES2 mRNA expression levels after 48 h were normalized with β-actin mRNA levels, and the mean level of the wild-type was set as 1.0. The results indicate the mean ± S.D. from three independent preparations. No significant difference in mRNA level was observed between the wild-type and variants ($p = 0.21$ an 0.06 in A and B, respectively).

Discussion

The present study provides comprehensive data on the haplotype analysis of the *CES2* gene, which encodes human carboxylesterase 2. From additional sequence analysis, a total of 21 SNPs including 4 nonsynonymous SNPs, 100C>T ($Arg^{34}Trp$), 424G>A ($Val^{142}Met$), 1A>T ($Met^{1}Leu$), and 617G>A ($Arg^{206}His$), and a SNP at the splice acceptor site of intron 8 (IVS8-2A>G) were found in 262 Japanese subjects. Among the nonsynonymous SNPs, in vitro functional analysis of the two nonsynonymous SNPs, 100C>T ($Arg^{34}Trp$) and 424G>A ($Val^{142}Met$), has already been performed to identify effects of these SNPs on expression levels and carboxylesterase activity. Kubo et al. (2005) showed that $Arg^{34}Trp$ and $Val^{142}Met$ variants had little carboxylesterase activity toward irinotecan, *p*-nitrophenyl acetate, and 4-methylumbelliferyl acetate, whereas expression levels of these variants were higher than those of the wild-type. An in vitro splicing assay using the *CES2* minigene carrying SNP IVS8-2A>G showed that IVS8-2A>G yielded mostly aberrantly spliced transcripts, resulting in the production of truncated CES2 proteins. These results have suggested that 100C>T ($Arg^{34}Trp$), 424G>A ($Val^{142}Met$), and IVS8-2A>G are functionally defective SNPs.

A novel SNP 1A>T found in this study changes the translation start codon ATG to TTG. Wu et al. (2003) identified three transcription start sites of *CES2*, resulting in the synthesis of three transcripts with either 78, 629, or 1187 nucleotides in the 5′-UTR. Another inframe ATG codon is present 192 nucleotides upstream of the conventional translational initiation codon, and two longer transcripts with 629 and 1187 nucleotides in the 5′-UTR can encode an ORF with 64 additional residues at the amino terminus (NP_003860.2). However, as shown in Fig. 3A, our in vitro experiment for the expression of CES2 showed that translation of CES2 mRNA started from the previously reported ATG codon but not from the inframe ATG codon at −192, when transiently expressed from the wild-type *CES2L* cDNA encoding a potential 623-amino acid CES2 protein in COS-1 cells. In vertebrate mRNAs, a purine residue in position −3 (A of the translational start codon is +1) is highly conserved and required for efficient translation (Kozak, 1991). The surrounding sequences of both ATG codons were accATGc for the functional ATG codon and cctATGa for the potential inframe ATG codon at −192. Thus, it is likely that their efficiencies of translation initiation depend on the flanking sequences of the translational start codon ATG.

When the expression levels between the wild-type and 1A>T variant were compared, the protein level of 1A>T was drastically reduced without changes in the mRNA levels, suggesting that the reduced protein level of the 1A>T variant might have been caused by its reduced translation initiation. It has been reported that alterations of the translational start codon ATG to TTG diminish or reduce the translation of growth hormone receptor (Quinteiro et al., 2002), protoporphyrinogen oxidase (Frank et al., 1999), low-density lipoprotein receptor (Langenhoven et al., 1996), and mitochondrial acetoacetyl-CoA thiorase (Fukao et al., 2003). Thus, it is likely that the 1A>T variation is a low-activity variation.

The functional effect of the known nonsynonymous SNP 617G>A ($Arg^{206}His$) was also investigated. The Arg^{206} residue is located in the α-helix within the catalytic domain and conserved among human carboxylesterases (Bencharit et al., 2002). However, no significant differences were found between the intrinsic enzyme activities of the wild-type and $Arg^{206}His$ variant for irinotecan hydrolysis.

In this study, 20 haplotypes of the *CES2* gene were identified. The most frequent haplotype was **1a* (frequency, 0.681), followed by **1b* (0.233), **1c* (0.027), and **1d* (0.017). Haplotype **1b* includes the polymorphisms IVS10-108G>A and 1749A>G, and haplotype **1c* harbors −363C>G, IVS10-108G>A, and IVS10-87G>A. The haplotype corresponding to **1b* in this study was found in Caucasians with a frequency of 0.086 (haplotypes 3 and 7 in Wu et al., 2004). Our **1c* corresponds to haplotypes 2 and 12 in Wu et al. (2004) and genotypes **1* and **6* in Charasson et al. (2004). Among the SNPs consisting of haplotype **1b* and **1c*, the three SNPs, −363C>G in the 5′-UTR, IVS10-108(IVS10 + 406)G>A in intron 10, and

TABLE 2

Kinetic parameters of CPT-11 hydrolysis by wild-type and $Arg^{206}His$ variant CES2 expressed in COS-1 cells

Results are expressed as the mean ± S.D. from four independent transfection experiments.

CES2	Apparent K_m	V_{max}	V_{max}/K_m	Normalized V_{max}[a]	Normalized V_{max}/K_m[a]
	μM	*pmol/min/mg protein*	*nl/min/mg protein*	*pmol/min/mg protein*	*nl/min/mg protein*
Wild-type	0.46 ± 0.01	3.45 ± 0.29	7.43 ± 0.54	3.46 ± 0.23	7.45 ± 0.50
$Arg^{206}His$	0.51 ± 0.02‡	2.81 ± 0.22†	5.53 ± 0.52‡	3.44 ± 0.16	6.77 ± 0.46

[a] V_{max} values were normalized by the relative protein expression level of the $Arg^{206}His$ variant (0.82 ± 0.07).
† Significantly different from that of the wild-type at $P < 0.05$.
‡ $P < 0.01$.

1749(*69)A>G in the 3′-UTR of exon 12, were previously reported, and their frequencies varied among several ethnic groups (Marsh et al., 2004; Wu et al., 2004). The frequency (0.269) of the **1b/*1c*-tagging SNP in Japanese, IVS10-108G>A, was comparable to that in African-Americans (0.263), but much higher than that in Asian-Americans (0.06) and European-Americans (0.063) (Wu et al., 2004). However, the **1b*-tagging SNP 1749A>G (0.239 in this study) was detected only in Asian-Americans with a low frequency (0.03) (Wu et al., 2004). The frequency of the **1c*-tagging SNP, −363C>G, also showed marked ethnic differences between Japanese (0.031) and Europeans (0.12) or Africans (0.33) (our data; Marsh et al., 2004). These findings indicate the existence of large ethnic difference in haplotype structures among African, European, and Japanese populations.

In this study, the relationship between the *CES2* genotypes and the (SN-38 + SN-38G)/CPT-11 AUC ratios of irinotecan-administered patients was analyzed. First, the relationship between the genotypes and the AUC ratios among the **1/*1* diplotypes in the patient group with or without coadministered drugs was assessed, and no significant differences in the AUC ratios were observed among the **1/*1* diplotypes in each group (Fig. 2). Wu et al. (2004) reported that the haplotype harboring SNP −363C>G that was homozygous appeared to have lower mRNA levels than the other haplotype groups. In this study, the haplotype having the SNP −363C>G was assigned haplotype **1c*. However, no functional differences were found between haplotype **1c* and the other **1* group haplotypes. Marsh et al. (2004) reported that IVS10-88C>T was associated with reduced RNA expression in colon tumor tissues. However, this SNP was not found in the present study with Japanese subjects.

The major **1* group haplotypes, **1a*, **1b*, and **1c*, account for 94% of Japanese *CES2* haplotypes. The current study revealed no association between the major *CES2* genotypes and changes in the AUC ratio, indicating that the variability in AUC ratio could not be interpreted by these haplotypes alone.

In irinotecan-administered patients, three nonsynonymous SNPs, 100C>T ($Arg^{34}Trp$, *2), 1A>T ($Met^{1}Leu$, *5), and 617G>A ($Arg^{206}His$, *6), and a SNP at the splice acceptor site of intron 8 (IVS8-2A>G, *4) were found as single heterozygotes. The patients heterozygous for $Arg^{34}Trp$ or $Met^{1}Leu$ showed substantially reduced AUC ratios. These results were consistent with in vitro functional analysis for the nonsynonymous SNPs by Kubo et al. (2005).

In the case of haplotype *6 harboring the nonsynonymous SNP, 617G>A ($Arg^{206}His$), the AUC ratio of the patient who received cisplatin was lower than the median value but within the range for the **1/*1* group treated with platinum-containing drugs. The protein expression level of the 206His variant was 82 ± 4%, and the $Arg^{206}His$ substitution itself showed no functional differences in intrinsic enzyme activity by in vitro functional analysis. Thus, the impact of the 617G>A ($Arg^{206}His$) SNP on irinotecan pharmacokinetics might be small.

On the other hand, the AUC ratio of the patient carrying the haplotype **4* was not different from the median value of the **1/*1* group treated with platinum-containing drugs. It is possible that other genetic factors might have increased the AUC ratio in this patient.

The patients with **4*, **5*, or **6* were found as single heterozygotes. Thus, further studies are needed to elucidate in vivo importance of the three haplotypes.

In conclusion, we have identified a panel of haplotypes of the *CES2* gene in a Japanese population using 21 genetic polymorphisms detected in this study and found that some rare haplotypes with nonsynonymous SNPs show a decreasing tendency toward enzymatic levels or activity. In vitro functional analysis for nonsynonymous SNPs showed that the 1A>T ($Met^{1}Leu$) SNP was a defective allele. These findings will be useful for further pharmacogenetic studies on efficacy and adverse reactions to CES2-activated prodrugs.

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SNP Communication

*Genetic Variations and Frequencies of Major Haplotypes in SLCO1B1 Encoding the Transporter OATP1B1 in Japanese Subjects: SLCO1B1*17 is More Prevalent Than *15*

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Summary: A liver-specific transporter organic anion transporting polypeptide 1B1 (OATP1B1, also known as OATP-C) is encoded by *SLCO1B1* and mediates uptake of various endogenous and exogenous compounds from blood into hepatocytes. In this study, 15 *SLCO1B1* exons (including non-coding exon 1) and their flanking introns were comprehensively screened for genetic variations in 177 Japanese subjects. Sixty-two genetic variations, including 28 novel ones, were found: 7 in the 5′-flanking region, 1 in the 5′-untranslated region (UTR), 13 in the coding exons (9 nonsynonymous and 4 synonymous variations), 5 in the 3′-UTR, and 36 in the introns. Five novel nonsynonymous variations, 311T>A (Met104Lys), 509T>C (Met170Thr), 601A>G (Lys201Glu), 1553C>T (Ser518Leu), and 1738C>T (Arg580Stop), were found as heterozygotes. The allele frequencies were 0.008 for 1738C>T (Arg580Stop) and 0.003 for the four other variations. Arg580Stop having a stop codon at codon 580 results in loss of half of transmembrane domain (TMD) 11, TMD12, and a cytoplasmic tail, which might affect transport activity. In addition, novel variations, IVS12-1G>T at the splice acceptor site and -3A>C in the Kozak motif, were detected at 0.003 and 0.014 frequencies, respectively. Haplotype analysis using -11187G>A, -3A>C, IVS12-1G>T and 9 nonsynonymous variations revealed that the haplotype frequencies for **1b*, **5*, **15*, and **17* were 0.469, 0.000 (not detected), 0.037, and 0.133, respectively. These data would provide fundamental and useful information for pharmacogenetic studies on OATP1B1-transported drugs in Japanese.

Key words: ***SLCO1B1*; direct sequencing; novel genetic variation; amino acid change**

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Introduction

Organic anion transporting polypeptide 1B1 (OATP1B1, also known as OATP-C, OATP2 and LST-1) is a liver-specific transporter expressed on the sinusoidal membrane and mediates uptake of various endogenous and exogenous compounds from blood into hepatocytes.[1,2] Exogenous compounds include several 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (such as pravastatin), an active metabolite of irinotecan SN-38, methotrexate, and rifampicin; endogenous substrates include bilirubin and bilirubin glucuronide, cholate, leukotriene C_4, and estradiol-17β-glucuronide.[1,2]

OATP1B1 protein (691 amino acid residues) is encoded by *SLCO1B1*, which consists of 15 exons (including non-coding exon 1) and spans approximately 109 kb on chromosome 12p12.2-p12.1. Similar to other OATP family members, this transporter is predicted to have 12 transmembrane domains (TMDs).[1,3]

Several genetic polymorphisms and haplotypes with functional significance are already known in *SLCO1B1*. In Japanese, two haplotypes with nonsynonymous variations **1b* and **15* have been frequently reported. The *SLCO1B1*1b* haplotype with 388A>G (Asn130Asp) has been shown to have no altered transport activity from *in vitro* expression systems.[2,4-7] Recently, however, an *in vivo* study has suggested that the area under the concentration-time curve (AUC) of pravastatin is significantly lower in **1b/*1b* subjects than in **1a/*1a* subjects, suggesting increased transport activity possibly through increased protein expression.[8] Another major haplotype, *SLCO1B1*15* harboring both 388A>G (Asn130Asp) and 521T>C (Val174Ala), has been reported to show impaired plasma membrane expression[6] and reduced transport activity *in vitro*,[6,7] probably due to the Val174Ala substitution.[2,4,6] The association of the **15* haplotype with significant increases in AUC was reported for pravastatin,[2,9] and irinotecan and SN-38.[10] The haplotype frequencies of **1b*, **5* (with 521T>C, Val174Ala), and **15* were reported to be 0.46-0.54, 0.00-0.01, and 0.10-0.15, respectively, in Japanese.[5,11]

Recently, the *SLCO1B1*17* haplotype having 388A>G (Asn130Asp), 521T>C (Val174Ala), and -11187G>A was also shown to increase the AUC of pravastatin[9] and likely reduces the pravastatin efficacy on cholesterol synthesis,[12] although the effect of -11187G>A on transcriptional activity has not been clarified *in vitro*. The frequency of **17* has not, however, been reported in Japanese.

In this study, all 15 exons and their surrounding introns were resequenced for comprehensive screening of genetic variations in *SLCO1B1*. Sequence analysis detected 62 variations including 5 novel nonsynonymous ones from 177 Japanese subjects. Haplotype frequencies of **1b*, **5*, **15*, and **17* were also estimated.

Materials and Methods

Human genomic DNA samples: One hundred seventy-seven Japanese cancer patients administered irinotecan participated in this study and provided written informed consent. The ethical review boards of the National Cancer Center and the National Institute of Health Sciences approved this study. Whole blood was collected from the patients prior to the administration of irinotecan, and genomic DNA was extracted from blood leukocytes by standard methods.

PCR conditions for DNA sequencing and haplotype analysis: First, two sets of multiplex PCR were performed to amplify all 15 exons of *SLCO1B1* from 100 ng of genomic DNA using 1.25 units of *Z-Taq* (Takara Bio. Inc., Shiga, Japan) with 0.2 μM each of the mixed primers (Mix 1 and Mix 2) designed in the intronic regions as listed in **Table 1** (1st PCR). Mix 1 contained primers for amplifying exons 1 and 2, and 12 to 14, and Mix 2 contained primers for exons 3 to 7, 8 to 11, and 15. The first PCR conditions consisted of 30 cycles of 98 °C for 5 sec, 55°C for 10 sec, and 72°C for 190 sec. Next, each exon was amplified separately by *Ex-Taq* (0.625 units, Takara Bio. Inc.) with appropriate primers (0.5 μM) designed in the introns (**Table 1**, 2nd PCR). The conditions for the second round PCR were 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 1 min, and 72°C for 2 min, and then a final extension at 72°C for 7 min. For amplification of exons 10 and 13, PCR was carried out under the following conditions: 94°C for 5 min followed by 33 cycles of 94°C for 30 sec, 55°C for 1 min, and 72°C for 30 sec, and then a final extension at 72°C for 7 min. Following PCR, the 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 1** (Sequencing). Excess dye was removed by a DyeEx96 kit (Qiagen, Hilden, Germany), and the eluates were analyzed on an ABI Prism 3700 DNA Analyzer (Applied Biosystems). All variations were confirmed by sequence analysis of PCR products generated by new amplification of the original genomic DNA templates.

As of July 18, 2007, the novel variations reported here are not found in the database of Japanese Single Nucleotide Polymorphisms (http://snp.ims.u-tokyo.ac.jp/), dbSNP in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP/), or PharmGKB Database (http://www.pharmgkb.org/).

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Table 1. Primer sequences used in this study

		Amplified or sequenced region	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Amplified region[a]	Length (bp)
1st PCR	Mix 1	Exons 1 and 2	ACTCTGGGGCTAAAACCTATTGGAC	CTGCTTGCCATAACATCTTGAGGGT	14041049_14055679	14,631
		Exons 12 to 14	CTAGGGCTTTTATTGATAGGCAGGT	AAACTTCCAGACTGTCTTCTACCAT	14127745_14137752	10,008
	Mix 2	Exons 3 to 7	TTTGTTGAGAAGAGACTGTTAGGCA	GGAAAATGGATGAAGAAGCACTGGA	14083429_14092020	8,592
		Exons 8 to 11	AAGGACAGCACCAAGCAATGAAGGA	CATTCAACTCAGCAATCCCACTACC	14106917_14119592	12,676
		Exon 15	CTGAGGAGAACTGTAATTTGATGTC	TTTCCAGAGGCAAGCATTTACAACT	14148708_14152868	4,161
2nd PCR		Exon 1	TAACAGGCATAATCTTTGGTCT	AAGGGCTCAGAATGTAAGCG	14041915_14043281	1,367
		Exon 2	TCCTTAGGCTAGAATTTGTGT	CAAAGTGAGTCTCAAGACATT	14053227_14053950	724
		Exon 3	TGGCTGAGTAGTAGTACCTG	ATCCTCACTATCAACATTTTCA	14084394_14084961	568
		Exon 4	TGAGTGGTCTAATGTAGGTGA	AGGTGTAAGTGTTGAGGTCTT	14086324_14086946	623
		Exon 5	ATCTTTCTTGCTGGACACTTC	TATTAAGGAATTTGTTACAGGG	14088485_14089158	674
		Exons 6 and 7	CATAAGAATGGACTAATACACC	GGGAGACATTTTACATTTGGTT	14090310_14091202	893
		Exon 8	TTCCTAGACAGTATCTGTTGC	CTTCCACTTGTTATGTGCTCA	14108658_14109289	632
		Exon 9	AGTTACAAAACAGCACTTACG	TCAGGAACTCATCTAAAATAAG	14112156_14112798	643
		Exon 10	CAGGGGTTAAAACCTAGATGA	ATCCATGTATTTCTCTAAGCC	14114169_14114904	736
		Exon 11	TGGCAAAGATGGAGAGCGTA	AGTCAAATGAGGTGCTTCTTA	14117563_14118256	694
		Exon 12	TTGTCCAAAAGAGTATGTGCT	CAGCCTTGAGAGTTCATAGT	14128668_14129375	708
		Exon 13	TTGACCCAGCAATCCAACTAT	CCTTTTTTTTTTCATCATACCTAGT[b]	14133788_14134290	503
		Exon 14	ATATTAACCAACATAACTTCCA	CCTTGAATCACAGTTTCTTCG	14136297_14136980	684
		Exon 15	GATGGCTTAACAGGGCTTGA	TGCGGCAAATGATCTAGGAA	14150619_14151844	1,226
Sequencing		Exon 1	TAACAGGCATAATCTTTGGTCT	AAGGGCTCAGAATGTAAGCG		
			TATGTGAGAGAAGGGTCTGTA	CTACAGGTTACATTGGCATTT		
			AAATGCCAATGTAACCTGTAG	CTGAAATAAAGTACAGACCCT		
		Exon 2	TCCTTAGGCTAGAATTTGTGT	CAAAGTGAGTCTCAAGACATT		
		Exon 3	TGGCTGAGTAGTAGTACCTG	ATCCTCACTATCAACATTTTCA		
		Exon 4	TGAGTGGTCTAATGTAGGTGA	AGGTGTAAGTGTTGAGGTCTT		
		Exon 5	ATCTTTCTTGCTGGACACTTC	TATTAAGGAATTTGTTACAGGG		
		Exon 6	TTAAGAGTTTACAAGTAGTTAAA	AAGCAATTTTACTAGATGCCAA		
		Exon 7	CTTCTTTGTATTTAGGTAATGTA	ATAGTATAAATAGGAGCTGGAT		
		Exon 8	TTCCTAGACAGTATCTGTTGC	CTTCCACTTGTTATGTGCTCA		
		Exon 9	AGTTACAAAACAGCACTTACG	TCAGGAACTCATCTAAAATAAG		
		Exon 10	TTGATAGGTGCAGCAAACCAC	GGAATAAAAGAATGTGTTTGAG		
		Exon 11	TCTTTTTGATATATGTCTATCAT	AGTCAAATGAGGTGCTTCTTA		
		Exon 12	TTGTCCAAAAGAGTATGTGCT	CAGCCTTGAGAGTTCATAGT		
		Exon 13	GTTCTAACCACTTCCTCATAG	CCTTTTTTTTTTCATCATACCTAGT		
		Exon 14	TCCTTTTTACCATTCAGGCTTA	ACTAAAATGAGATACGAGATTG		
		Exon 15	GATGGCTTAACAGGGCTTGA	TGCGGCAAATGATCTAGGAA		
			CACATCTTTTATGGTGGAAGT	AGGCTTATTTATACTTCCACC		

[a]The reference sequence is NT_009714.16.
[b]Mismatched nucleotides at the 5′ end are underlined.

Furthermore, rare SNPs found in single patients as heterozygotes were confirmed by sequencing the PCR fragments produced by the amplification with a high fidelity DNA polymerase KOD-Plus- (TOYOBO, Tokyo, Japan).

Hardy-Weinberg equilibrium was analyzed by SNPAlyze version 3.1 (Dynacom Co., Yokohama, Japan). Estimation of *SLCO1B1* haplotypes was performed by an expectation-maximization based program, LDSUPPORT software.[13]

Results and Discussion

Sequence analysis from 177 Japanese subjects resulted in the identification of 62 genetic variations, including 28 novel ones (**Table 2**). Of these variations, 7 were located in the 5′-flanking region, 1 in the 5′-untranslated region (UTR), 13 in the coding exons (9 nonsynonymous and 4 synonymous variations), 5 in the 3′-UTR, and 36 in the introns. All detected variations were in Hardy-Weinberg equilibrium ($p > 0.05$).

Of the 9 nonsynonymous variations, 5 variations were novel: 311T>A (Met104Lys), 509T>C (Met170Thr), 601A>G (Lys201Glu), 1553C>T (Ser518Leu), and 1738C>T (Arg580Stop). All of these variations were found as heterozygotes with frequencies of 0.008 for 1738C>T (Arg580Stop) and 0.003 for the four other variations. Arg580, residing in TMD11, is conserved among human, rat and mouse OATP families.[1] The change from arginine residue to the immature termination codon leads to loss of this conserved amino acid

Table 2. Summary of *SLCO1B1* variations detected in this study

SNP ID					Position				
This Study	dbSNP (NCBI)	JSNP	Reference	Location	NT_009714.16	From the translational initiation site or from the end of the nearest exon	Nucleotide change	Amino acid change	Allele frequency (n=354)
MPJ6_SB1_001[a]				5'-flanking	14042128	-11355 (-1078)[b]	ccaatactctcaA/Gtaattaaccaag		0.003
MPJ6_SB1_002	rs4149015	ssj0003132	9, 15	5'-flanking	14042296	-11187 (-910)[b]	tatgtgtatacaG/Agtaaaagtgtgt		0.153
MPJ6_SB1_003[a]				5'-flanking	14042494	-10989 (-712)[b]	atctctactcaaG/Aaaaacttttaac		0.076
MPJ6_SB1_004[a]				5'-flanking	14042530	-10953 (-676)[b]	cttcctttctccA/Tcaagtcaagtca		0.003
MPJ6_SB1_005	rs11835045			5'-flanking	14042793	-10690 (-413)[b]	atttgtcttaaaT/Ctatttcctattt		0.076
MPJ6_SB1_006[a]				5'-flanking	14042860	-10623 (-346)[b]	ttaaagaaaaaaA/-tcttatgccacc		0.003
MPJ6_SB1_007[a]				5'-flanking	14043018	-10465 (-188)[b]	aactaggttttaT/Catgtttgactag		0.003
MPJ6_SB1_008[a]				Intron 1	14043209	IVS1+65	ttcacggaagaaG/Catttgatggtc		0.014
MPJ6_SB1_009	rs2010668	ssj0003141		Intron 1	14053267	IVS1-155	tcctacttttgtG/Ttccagcattgac		0.113
MPJ6_SB1_010[a]				5'-UTR	14053480	-3	atctatatttcaA/Ctcatggaccaaa		0.014
MPJ6_SB1_011[a]				Intron 2	14053635	IVS2+69	tagaaaagcaagT/Ctgttaaaaagaa		0.003
MPJ6_SB1_012[a]				Intron 2	14053648	IVS2+82	tgttaaaaagaaC/Tattatgtttcaa		0.003
MPJ6_SB1_013[a]				Intron 2	14053734	IVS2+168	aaaccagtcttT/Caatctgattaag		0.008
MPJ6_SB1_014	rs4149021	ssj0003142	9	Intron 2	14053759	IVS2+193	tatttctttggcG/Aaaatttttgatg		0.153
MPJ6_SB1_015	rs12812795		9	Intron 2	14053769	IVS2+203	gcgaaatttttgA/Ttgcttaatagtt		0.003
MPJ6_SB1_016[a]				Intron 2	14053807	IVS2+241	aatttagaaaataT/Ctttgatagcttc		0.006
MPJ6_SB1_017	rs12303784			Intron 2	14053814	IVS2+248	aaatattttgatA/Ggcttctctttgg		0.003
MPJ6_SB1_018[a]				Intron 2	14084429	IVS2-129	aaagggaaaactA/Gagtatggtttttt		0.003
MPJ6_SB1_019[a]				Intron 2	14084478	IVS2-80	aaagaagaaagcT/Cattataattcca		0.008
MPJ6_SB1_020	rs2291073	JST-043317		Intron 3	14084788	IVS3+89	actggggtaaatT/Gtatctctcacag		0.271
MPJ6_SB1_021	rs2291074	JST-043318		Intron 3	14084923	IVS3+224	attctataatgcA/Gcaaagaatgatg		0.243
MPJ6_SB1_022[a]				Exon 4	14086569	311	gttgtttcattaT/Agggaattggagg	Met104Lys	0.003
MPJ6_SB1_023	rs4149036	ssj0003160		Intron 4	14086714	IVS4+97	ataggcagttacC/Atttgagaagat		0.427
MPJ6_SB1_024[a]				Intron 4	14088523	IVS4-161	cacttttacccaT/Ccacatctcttaa		0.017
MPJ6_SB1_025	rs2306283	JST-063865	4, 5, 9, 11, 15	Exon 5	14088712	388	gaaactaatatcA/Gattcatcagaaa	Asn130Asp	0.667
MPJ6_SB1_026	rs2306282	JST-063864	11	Exon 5	14088776	452	ttttatcactcaA/Gtagagcatcacc	Asn151Ser	0.034
MPJ6_SB1_027	rs4149044	ssj0003170	9	Intron 5	14088970	IVS5+165	cacagttcgcccA/Tttaacaacacag		0.427
MPJ6_SB1_028	rs4149045	ssj0003171	9	Intron 5	14088994	IVS5+189	ggtttaaactacG/Acgttttcactic		0.429
MPJ6_SB1_029	rs4149046	ssj0003172	9	Intron 5	14088996	IVS5+191	tttaaactacgcG/Attttcacttcta		0.331
MPJ6_SB1_030	rs4149096	ssj0003230	9	Intron 5	14090372_14090377	IVS5-107_112	aaattactgtaCTTGTA/-aatttaaaaaaaa		0.427
MPJ6_SB1_031[a]				Intron 5	14090469	IVS5-15	aaatgaaacactC/Gtcttatctacat		0.003
MPJ6_SB1_032[a]				Exon 6	14090511	509	ctgggtcatacaT/Cgtggatatatgt	Met170Thr	0.003
MPJ6_SB1_033	rs4149056	ssj0003182	4, 5, 9, 11, 15	Exon 6	14090523	521	tgtggatatatgT/Cgttcatgggtaa	Val174Ala	0.175
MPJ6_SB1_034	rs4149057	ssj0003183	9, 11, 15	Exon 6	14090573	571	cccatagtaccaT/Ctggggctttctt	Leu191Leu	0.333
MPJ6_SB1_035[a]				Exon 6	14090578	576	agtaccattgggG/Actttcttacatt	Gly192Gly	0.003
MPJ6_SB1_036	rs2291075	JST-043319	9, 11, 15	Exon 6	14090599	597	cattgatgatttC/Tgctaaagaagga	Phe199Phe	0.427
MPJ6_SB1_037[a]				Exon 6	14090603	601	gatgatttcgctA/Gaagaaggacatt	Lys201Glu	0.003
MPJ6_SB1_038	rs2291076	JST-043320	9	Intron 7	14090961	IVS7+33	gtaccatgataaC/Tgtctttctaagc		0.336
MPJ6_SB1_039			11	Exon 9	14112452	1007	tccttactaatcC/Gcctgtatgttat	Pro336Arg	0.006
MPJ6_SB1_040[a]				Intron 9	14114331	IVS9-68	ttgacatacattG/Ctgtttcatctat		0.003
MPJ6_SB1_041	rs4149099	JST-080069	9	Intron 10	14117669_14117670	IVS10-106_-107	tttatctacttt-/CTTttttcccctttt		0.647
MPJ6_SB1_042			11	Intron 10	14117728_14117730	IVS10-46_-48	cttctttgtcttTTT/-cttctctctctc		0.003
MPJ6_SB1_043	rs4149070	ssj0003204	9	Intron 11	14128857	IVS11-170	gaaagaaatccaC/Gaaaactatttta		0.280
MPJ6_SB1_044	rs4149071	ssj0003205	9	Intron 11	14128938	IVS11-89	agtttgaacaagT/Cgagacttcacta		0.280
MPJ6_SB1_045	rs4149100	ssj0003234	9	Intron 11	14128952	IVS11-75	agacttcactaaA/-tataatgcaatg		0.395
MPJ6_SB1_046	rs4149072	ssj0003206	9	Intron 11	14128959	IVS11-68	actaaatataatG/Acaatgtatttgc		0.280
MPJ6_SB1_047			11	Intron 11	14129015	IVS11-12	catattttatacA/Gcaacgcttaagg		0.014
MPJ6_SB1_048[a]				Exon 12	14129082	1553	acagaaattactC/Tagcccatttggg	Ser518Leu	0.003
MPJ6_SB1_049	rs987839			Intron 12	14133812	IVS12-396	tccaactattggG/Atatctaccaaaa		0.316
MPJ6_SB1_050[a]				Intron 12	14134097	IVS12-111	ggggccattcaaC/Ttgtgagcttaat		0.020
MPJ6_SB1_051[a]				Intron 12	14134207	IVS12-1	tgtcttgtttcaG/Taattgttcaacc		0.003
MPJ6_SB1_052[a]				Exon 13	14134263	1738	tcaatggttataC/Tgagcactaggta	Arg580Stop	0.008
MPJ6_SB1_053	rs4149080	ssj0003214	9	Intron 13	14136533	IVS13-97	ctccaaatttttG/Caacttttattta		0.395
MPJ6_SB1_054	rs11045875		11	Intron 14	14136797	IVS14+50	gactatattaatT/Gcctaaaaaatat		0.011
MPJ6_SB1_055[a]				Intron 14	14150655	IVS14-232	tatatttttctcG/Attttatgaagaa		0.006
MPJ6_SB1_056[a]				Intron 14	14150656	IVS14-231	atatttttctcgT/Ctttatgaagaag		0.251
MPJ6_SB1_057[a]				Exon 15	14151004	1983	tgcatcagaaaaT/Cggaagtgtcatg	Asn661Asn	0.006
MPJ6_SB1_058[a]				3'-UTR	14151137	2116 (*40)[c]	tgtgtttccaaaC/Gagcattgcattg		0.011
MPJ6_SB1_059	rs4149085	ssj0003219		3'-UTR	14151264	2243 (*167)[c]	acaaactgtaggT/Cagaaaaaatgag		0.251
MPJ6_SB1_060	rs4149086	ssj0003220		3'-UTR	14151425	2404 (*328)[c]	aaacaaatgagtA/Gtcatacaggtag		0.025
MPJ6_SB1_061	rs4149087	ssj0003221	15	3''-UTR	14151536	2515 (*439)[c]	gaactataatacG/Taaggcctgaagt		0.333
MPJ6_SB1_062	rs4149088	ssj0003222		3'-UTR	14151560	2539 (*463)[c]	tctagcttggatG/Atatgctacaata		0.333

[a]Novel variations detected in this study.
[b]Intron 1 is skipped for counting.
[c]Positions are shown as * and bases from the translational termination codon TAA.