Review

Problems with Registration-Directed Clinical Trials for Lung Cancer in Japan

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SEKINE, I., NOKIHARA, H., YAMAMOTO, N., KUNITOH, H., OHE, Y., SAIJO, N. and TAMURA, T. *Problems with Registration-Directed Clinical Trials for Lung Cancer in Japan.* Tohoku J. Exp. Med., 2007, **213** (1), 17-23 —— New anticancer agents against lung cancer are needed because efficacy of chemotherapy is limited. The long time required, low quality, and considerable costs of registration-directed clinical trials in Japan ("Chiken") have been pointed out. The quality of 24 phase I and 41 phase II trials of an anticancer drug for lung cancer were analyzed according to the approval year of the drug. The human resources and infrastructure to support oncology clinical practice and clinical trials were compared between Japan and the USA. A maximum tolerated dose was not defined in any of seven phase I trials before 1989, and was determined in two of six trials between 1989 and 1996 and in seven of 10 trials thereafter. Before 1989, 29 (20%) of 142 patients registered in two trials were ineligible, and the number of ineligible patients was not reported in the five trials. Sample size calculations were not performed in any of seven phase II trials before 1989 and were performed in only four of 10 trials between 1989 and 1996 and in all 23 trials conducted thereafter. The shortage of human resources, including medical oncologists, oncology nurse practitioners and clinical research coordinators, is serious and acute. The infrastructure to support clinical trials also remains insufficient in Japan. In conclusion, registration-directed clinical trials of anticancer agents have advanced significantly during last three decades but remain unsatisfactory. The development of infrastructure and human resources is an urgent task to ensure high-quality clinical trials without unnecessary delays. —————— clinical trials; medical oncologists; nurse practitioners; lung cancer; anticancer agents

Lung cancer is one of the most common malignancies and the leading cause of cancer-related deaths in many countries. In the year 2000, the annual number of deaths from lung cancer was estimated to be 1.1 million worldwide, and global lung caner incidence is increasing at a rate of 0.5% per year (Schottenfeld and Searle 2005). About 80% of patients with lung cancer have already developed distant metastases or pleural effusion, either by the time of the initial

diagnosis or by the time recurrence is detected after surgery for local disease. These patients can be treated with systemic chemotherapy, but the efficacy of currently available anticancer agents is limited to the extent that patients with advanced disease rarely live long. Therefore, new chemotherapeutic agents continue to be developed against lung cancer (Sekine and Saijo 2000).

The Japanese Pharmaceutical Affairs Law (PAL) was enacted in 1948, and was first amended in 1960 to provide for regulations to ensure the maintenance of the quality, efficacy, and safety of drugs and medical devices, and to promote research and development of these medical and pharmaceutical products. Good Clinical Practice (GCP) was enforced by the Bureau Notification of the Ministry of Health and Welfare of Japan ("Kyokuchou-Tsuuchi") in 1989 (the former GCP). In 1996, the PAL and its related laws were amended to strengthen GCP (the new GCP), Good Laboratory Practice, Good Post-Marketing Surveillance Practice, and standard compliance

reviews, conforming to the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use. In contrast to the laws prevailing in the US and EU, marketing approval for anticancer agents in Japan has been granted based on reports of the anti-tumor effects of the new agents in phase II trials (Fujiwara and Kobayashi 2002).

Under this Japanese drug approval system regulated by the PAL, 23 anticancer drugs have been approved for use against lung cancer during the last five decades (Fig. 1). Of these, 9 drugs are original to Japan, some of which are routinely used all over the world. Several problems, however, have been pointed out in registration-directed clinical trials in Japan ("Chiken"), including the long time required, low quality, and considerable cost (The Ministry of Health, Labour and Welfare of Japan 2002; The Ministry of Education, Science and Culture and the Ministry of Health, Labour and Welfare 2003). As a result, Japanese cancer patients must wait for a long time
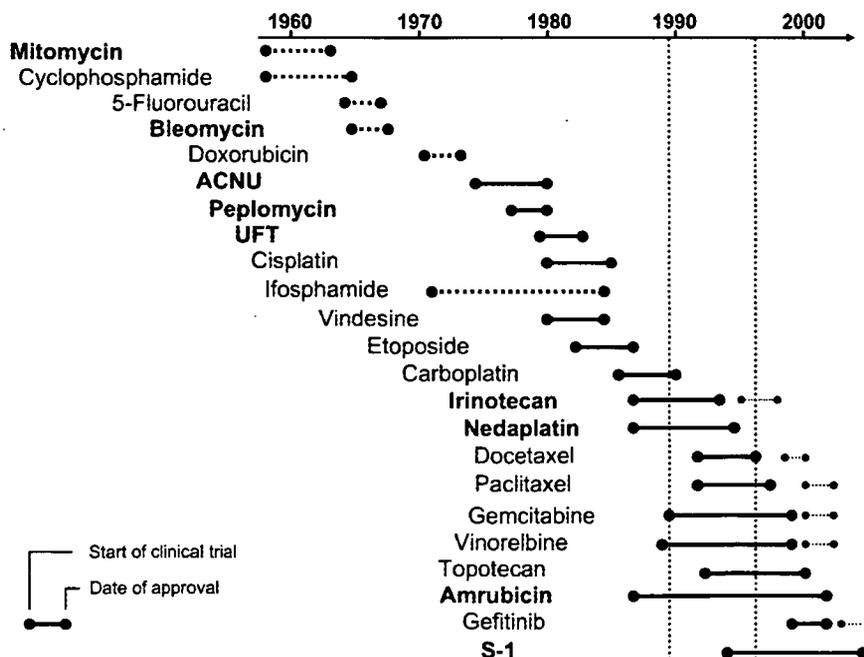

Fig. 1. Anticancer drugs approved for lung cancer in Japan.
Bold: original to Japan. Dotted line: case series studies, solid thick line: investigational new drug phase I-II trials for approval, and dotted thin line: post-marketing sponsored phase III trials. Vertical dotted lines indicate the year when the former and new GCP were issued.

until they receive new anticancer drugs which have been approved long before in other countries (The Ministry of Health, Labour and Welfare of Japan 2005). We discuss the aspects and issues of registration-directed trials in Japan by reviewing such trials for the 23 anticancer drugs.

Review of registration-directed clinical trials for the 23 anticancer drugs

A total of 65 phase I and II trials of an anticancer drug for approval were reviewed in terms of definition of eligibility criteria, maximum tolerated dose (MTD), sample size, response criteria, and extramural review for tumor responses. The MTD is the dose associated with seriouis but reversible toxicities in a sizeable proportion of patients and the one that offers the best chance for a favorable therapeutic ratio (Piantadosi 1997). The number of patients accrued in a trial, percentage of ineligible patients, number of participant hospitals in a trial, and the study period defined as the months between the first and last patient accrual were also analyzed. They were obtained from a published paper for 53 trials, from a meeting abstract and in-company resource for one trial, and from in-company resource alone for the remaining 11 trials. The clinical developmental period of an anticancer drug was defined as years between the start month of the first phase I trial and the month of the approval for lung cancer.

These parameters are compared according to the approval year of the drug. We categorized three periods of approval: 1) before 1989, 2) between 1989 and 1996, and 3) between 1997 and 2004, because the former GCP was enforced in 1989, and the new GCP in 1997 (Fujiwara et al. 2002).

Of the 23 anticancer drugs, six drugs whose clinical development started before 1974 were approved on the basis of the clinical experience of the use of the drug without clinical trials (Fig. 1). A total of 24 phase I trials were identified (Table 1). The MTD was not defined in the protocol of any trials before 1989, but was defined in 33% of trials between 1989 and 1996, and in 70% of trials after 1996. Instead of the MTD, maximum acceptable dose, defined as the dose associated with grade 2 or severer toxicity in two thirds or more patients, was used in a trial after 1996. About twice more patients were registered in a trial before 1989 than thereafter, but 20% of the registered patients before 1989 were ineligible. The study period of a phase I trial got longer as the number of participant hospitals decreased, from 7 months and 11 hospitals before 1989 to 13 months and 4 hospitals after 1996, respectively.

In this review, 41 phase II trials for approval were analyzed (Table 2). Calculation of the sample size was not made in any trials before 1989, was seen in 40% of trials between 1989 and 1996, and in all trials thereafter. Response criteria were

TABLE 1. Investigational new drug phase I trials for approval.

	Before 1989	1989-1996	1997 or thereafter
Total number of trials	7	6	11
Defined, number (%) of trials			
Eligibility criteria	4 (57)	6 (100)	11 (100)
Maximum tolerated dose*	0 (0)	2 (33)	7 (70) ‡
Results of trials, median (range)			
Number of patients**	61 (32-170)	24 (18-54)	29 (9-43)
% of ineligible patients	20 (20-21) †	8 (0-33)	6 (0-22)
Number of hospitals	11 (1-21)	9 (1-18)	4 (1-17)
Study period in months	7 (5-30)	10 (5-11)	13 (8-24)

*Statistically significant difference obtained ($p = 0.014$ by the chi-square test); **Statistically significant difference obtained ($p < 0.01$ by the Kruskal Wallis test); †Data were available in 2 trials only; ‡Data were available in 10 trials only.

TABLE 2. Investigational new drug phase II trials for approval.

	Before 1989	1989-1996	1997 or thereafter
Total number of trials	7	11	23
Defined, number (%) of trials			
Eligibility criteria	4 (57)	11 (100)	23 (100)
Sample size calculation*	0 (0)	4 (40) [‡]	23 (100)
Response criteria	6 (86)	11 (100)	23 (100)
Extramural review	3 (43)	9 (82)	23 (100)
Results of trials, median (range)			
Number of patients	71 (10-127)	68 (18-153)	61 (11-102)
% of ineligible patients	18 (0-29) [†]	3 (0-22)	3 (0-12)
Number of hospitals	27 (3-103)	17 (1-30)	20 (5-46)
Study period in months	18 (12-36)	12 (6-34)	26 (4-48) [§]

*Statistically significant difference obtained ($p < 0.01$ by the chi-square test); [†]Data were available in 5 trials only; [‡]Data were available in 10 trials only; [§]Data were available in 22 trials only.

defined in almost all studies, but an extramural review was conducted only after 1989. The median number of registered patients in a trial was constant through the three periods, but the percentage of ineligible patients was high in trials conducted before 1989. The number of patients. in a trial, and the number of hospitals in a trial were similar regardless of the year. The median study period in recent trials was 26 months.

The clinical development period was evaluated in the 23 drugs. Cisplatin was approved for germ cell tumors in 1983 and additionally approved for non-small cell lung cancer (NSCLC) in 1986. S-1 was firstly approved for gastric cancer in 1999, and additionally approved for NSCLC in 2004. The other drugs were approved for lung cancer for the first time. The median (range) clinical development period was 5.2 (3.2-14.5) years before 1989, 6.0 (4.8-9.1) years between 1989 and 1996, and 9.0 (3.9-15.4) years in 1997 or thereafter.

Development and recent problems of phase I and phase II trials in Japan

The concept of the "clinical trial" was not widely followed in Japan until 1974, when a phase I trial of nimustine hydrochloride (ACNU) was launched as one part of the United States-Japan Cooperation Cancer Research Program on

the basis of the agreement between the National Cancer Institute and Japan Society for the Promotion of Science (Sugano 1982; Niitani 1999). Phase I trials before 1989 required the accrual of many patients, because 1) the maximum tolerated dose was not defined, 2) many patients were treated at unnecessary dose levels because the modified Fibonacci dose escalation schedule was not applied, and 3) the percentage of ineligible patients was high. Some of these issues were improved in 1997 or thereafter, but the maximum tolerated dose is still not defined in as many as 40% of trials. Recently, oncology phase I trials came to be conducted among fewer hospitals than before, as more participants were recruited in each hospital. This facilitated communication among phase I investigators, which is important to complete phase I trials safely.

Phase II trials play the central role in anti-cancer agent approval in Japan, because the approval can be granted based on the response rate in these trials. The quality of protocols for phase II trials suggested by eligibility criteria, sample size calculation, response criteria, and extramural review has been improved significantly. The study period of phase II trials, however, was and is still too long, as long as 4 years in recent trials. To increase participant hospitals, however, is not necessarily a desirable solution,

because a certain number of patients per hospital are needed to maintain the quality of trials by training doctors in the application of a new drug. Thus, enhancing patient recruitment in each hospital participating in the trial is the most important consideration.

A high standard of oncology clinical practice as the basis for clinical trials

Since a high standard of clinical practice is the basis for all clinical trials, the infrastructure for oncological clinical practice should be promptly advanced. The shortage of human resources including medical oncologists and oncology nurse practitioners in Japan is serious and acute. In the United States, medical oncology was established as a separate discipline by the American Board of Internal Medicine in 1971, and approximately 8,000 certified internists as of 2003 have been further certified by the Board in the subspecialty of medical oncology (Holland et al. 2003). In contrast, medical oncology has not been established as an academic unit or a regular university course in many medical schools in Japan. The Japanese Society of Medical Oncology was launched as an association in 1993, and framed the system of cancer medical specialists in 2003. A total of 1,479 doctors were certified as a tentative medical oncology supervisor between 2003 and 2005, and 47 doctors as a medical oncology specialist in 2005 (Table 3) (Japanese Society of Medical Oncology 2005).

To deal with complex cancer care, oncology nurse practitioners in the United States have become an integral part of the multidisciplinary team in the care of patients. As of 2002, more than 19,000 oncology nurse practitioners have been certified by the Oncology Nursing Society in the United States (Rieger 2003). In contrast, the number of oncology nurse practitioners registered in the Japanese Nursing Association was only 44 as of 2005 (Table 3) (Japanese Nursing Association 2005). Introduction of oncology nurse practitioners in clinical practice should lessen the burden on oncologists significantly and help them to have the incentive to take part in registration-directed clinical trials.

The infrastructure and human resources to support clinical trials

The infrastructure to support in-house clinical trials remains insufficient and even lacking in almost all institutes in Japan, while it has been advanced systematically in the United States. In the 1960s, General Clinical Research Centers were founded with the support of National Institutes of Health in 80 universities and academic institutions to provide the primary resources and optimal environment necessary for investigators to conduct clinical research. They include experienced nursing, laboratory, computer system, and biostatistical staff (Robertson and Tung 2001; General Clinical Research Centers 2005). To carry out a multicenter trial, a central data center

TABLE 3. Medical oncology professionals in Japan and the USA.

Professionals	n of medical oncology professionals	
	Japan	USA
Medical oncologists	47 [1]	8,000 [2]
Oncology nurse practitioners	44 [3]	19,000 < [4]
Clinical research coordinators	335 [5]	10,723 [6]

[1] Certified by the Japanese Society of Medical Oncology in 2005.
[2] Certified by the American Board of Internal Medicine as of 2003.
[3] Certified by the Japanese Nursing Association as of 2005.
[4] Certified by the Oncology Nursing Society as of 2002.
[5] Certified by the Japanese Society of Clinical Pharmacology and Therapeutics as of 2005.
[6] Certified by the Association of Clinical Research Professionals as of 2005.

is needed to deal with the increased administrative difficulties and quality assurance problems associated with this type of trial (Pollock 1994). The quality control and quality assurance system of the Japan Clinical Oncology Group has been significantly developed during the last two decades (Japan Clinical Oncology Group 2005). Using Internet resources may facilitate developing national and regional networks for clinical trials by reducing the burden associated with the extensive research time and considerable cost of all these processes (Paul et al. 2005).

The new GCP demands more of the clinical researchers in time, resources and money to enhance the science, credibility, and ethics of clinical trials for approval (Sweatman 2003). The clinical research coordinator (CRC) plays a key role in the clinical trial process by supporting investigators. The CRCs are involved in every aspect of registration-directed clinical trials, including protocol development, checking eligibility criteria, informed consent, organizing study schedules, checking clinical tests, filling in case report forms, and providing support for monitoring and auditing the trials (Rico-Villademoros et al. 2004; Sakamoto 2004). Association of Clinical Research Professionals in the USA has offered the CRC certification since 1992, and there are 10,723 CRCs to date (Association of Clinical Research Professionals 2006). The Japanese Society of Clinical Pharmacology and Therapeutics launched the certified CRC system in 2003, and there were 335 certified CRCs as of 2005 (Table 3) (The Japanese Society of Clinical Pharmacology and Therapeutics 2005).

In conclusion, clinical trials of anticancer agents for approval have been developing significantly, but still remain at an unsatisfactory level. Development of the infrastructure and human resources for clinical trials is an urgent task to complete good quality clinical trials for approval without delay.

Acknowledgments

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Phase II trial of carboplatin and paclitaxel in non-small cell lung cancer patients previously treated with chemotherapy

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Summary The purpose of this phase II trial was to evaluate the efficacy and toxicity of carboplatin plus paclitaxel in the treatment of advanced non-small cell lung cancer (NSCLC) previously treated with chemotherapy. Patients with a performance status (PS) of 0 or 1 who had received one or two previous chemotherapy regimens for advanced NSCLC were eligible. Paclitaxel 200 mg/m^2 was infused over 3 h and followed by carboplatin (area under the curve 6) infusion over 1 h, once every 3 weeks. Thirty patients were enrolled. A complete response was observed in 1 patient and a partial response in 10 patients, for an overall response rate of 36.7%. The median time to progression was 5.3 months. The median survival time was 9.9 months, and the 1-year survival rate was 47%. Hematological toxicity in the form of grade 3/4 neutropenia occurred in 54%, but grade 3 febrile neutropenia developed in only 3%. Non-hematological grade 3 toxicities were less frequent. There were no treatment-related deaths. The combination of carboplatin plus paclitaxel is an active and well-tolerated regimen for the treatment of NSCLC patients who have previously been treated with chemotherapy and have a good PS.
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1. Introduction

Lung cancer remains a major cause of death from cancer in many countries. More than half of all patients diagnosed with non-small cell lung cancer (NSCLC) have advanced stage

IIIB or IV disease at presentation, and patients with advanced NSCLC are candidates for systemic chemotherapy. Platinum-based chemotherapy is considered the standard first-line treatment for patients with advanced NSCLC, and prolongs survival, palliates symptoms, and improves quality of life [1,2]. Many patients with good performance status (PS) when progression occurs after first-line chemotherapy are suitable candidates for second-line chemotherapy [3].

The taxanes are an important class of new agents for the treatment of advanced NSCLC. Paclitaxel, in combination with carboplatin, is the most common regimen

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used as first-line chemotherapy for advanced NSCLC, and this combination has a more favorable toxicity profile and is more convenient to administer than other platinum-based regimens [4,5]. Docetaxel has been investigated more extensively than any other agent for second-line treatment of advanced NSCLC, and the results of two randomized phase III trials of second-line chemotherapy in patients with advanced NSCLC demonstrated that docetaxel monotherapy significantly improved survival compared with best supportive care or other single agents (vinorelbine or ifosfamide) [6,7].

Belani et al. recently reported that results of a phase III trial comparing a carboplatin plus paclitaxel regimen with a cisplatin plus etoposide regimen for first-line treatment of advanced NSCLC [8]. Carboplatin plus paclitaxel yielded a higher response rate (23% versus 15%), time to progression (121 days versus 111 days), and overall quality of life benefit than cisplatin plus etoposide, but the median survival time was better in the cisplatin plus etoposide arm than in the carboplatin plus paclitaxel arm (274 days and 233 days, respectively [$P = 0.086$]). The authors reported that a substantially greater proportion of patients in the cisplatin plus etoposide arm received second-line chemotherapy with a taxane-containing regimen than in the carboplatin plus paclitaxel arm, and suggested that treatment with taxanes in a second-line setting may have had an impact on the survival in their study. Remarkably, more than half of the regimens that were used in the second-line setting of their study consisted of paclitaxel alone or carboplatin plus paclitaxel, not docetaxel. While the efficacy of paclitaxel-containing regimens as first-line chemotherapy for advanced NSCLC has been established in many randomized phase III trials [9], the data on the efficacy of paclitaxel-containing regimens in second-line settings are limited [10,11].

Based these considerations we conducted a phase II trial to evaluate the efficacy and toxicity of carboplatin plus paclitaxel in the treatment of advanced NSCLC previously treated with chemotherapy.

2. Patients and methods

2.1. Eligibility criteria

The inclusion criteria were: pathologically confirmed advanced NSCLC patients with measurable disease who had received one or two previous chemotherapy regimens for their disease. Patients were required to submit evidence of failure of prior chemotherapy. Patients who were previously treated with carboplatin or paclitaxel were excluded if the best response was progressive disease (PD). Patients who had received prior radiotherapy were eligible provided that at least 30 days had elapsed between the completion of radiotherapy and entry into the study. Patients were also required to be 20–75 years of age, have an Eastern Cooperative Oncology Group PS of 0 or 1, and have adequate organ function as indicated by the following parameters: absolute neutrophil count $\geq 1500\,mm^{-3}$, platelet count $\geq 100,000\,mm^{-3}$, hemoglobin $\geq 9.0\,g/dl$, AST and ALT $\leq 2.0 \times$ the institutional upper normal limits, total bilirubin $\leq 1.5\,mg/dl$, creatinine $\leq 1.5\,mg/dl$, PaO$_2$ ≥ 65 Torr.

Exclusion criteria were: uncontrolled pleural or pericardial effusion, active concomitant malignancy, prior irradiation to areas encompassing more than a third of the pelvis plus spine, active infection, myocardial insufficiency or myocardial infarction within the preceding 6 months, uncontrolled diabetes mellitus or hypertension, any other condition that could compromise protocol compliance, pregnancy and/or breast-feeding. All patients were required to provide written informed consent before entry into the study. The study was approved by the institutional review board of our instituion.

2.2. Treatment plan

Treatment was started within a week of entry into the study. Patients received paclitaxel 200 mg/m^2 diluted in 500 ml of 0.9% saline as a 3-h intravenous infusion followed by carboplatin (area under the curve [AUC] 6; Calvert formula) diluted in 250 ml of 5% glucose as a 1-h intravenous infusion, every 3 weeks. All patients were premedicated with dexamethasone (24 mg i.v.), famotidine (20 mg i.v.), and diphenhydramine (50 mg orally) 30 min before the paclitaxel infusion to prevent a hypersensitivity reaction. A 5-HT3-receptor antagonist was intravenously administered as an antiemetic before carboplatin. Therapy was continued for at least two cycles unless the patient experienced unacceptable toxicity or had PD. The maximum number of cycles of chemotherapy was six. In the event of grade 4 leukopenia or thrombocytopenia or of grade 3 neutropenic fever, the dose of carboplatin and paclitaxel was reduced to AUC 5 and 175 mg/m^2, respectively, in the following cycle of chemotherapy. The next cycle of chemotherapy was started if the neutrophil count was $\geq 1500\,mm^{-3}$, the platelet count $\geq 100,000\,mm^{-3}$, AST and ALT $\leq 100\,IU/l$, total bilirubin $\leq 2.0\,mg/dl$, creatinine $\leq 1.5\,mg/dl$, PS 0 or 1, and the patient was afebrile.

Pretreatment evaluation included a medical history, a physical examination, vital signs, height and body weight, PS, complete blood count, biochemical studies, arterial blood gas analysis, electrocardiogram, chest radiograph and computed tomography scan (CT), abdominal ultrasound or CT, and brain magnetic resonance imaging or CT. A complete blood count, biochemical studies, and chest radiograph were performed weekly during the first cycle of chemotherapy, and 2 weekly starting with the second cycle.

2.3. Response and toxicity assessment

Objective tumor response was assessed as complete response (CR), partial response (PR), stable disease ≥ 8 weeks (SD), or PD according to the Response Evaluation Criteria in Solid Tumors. Measurable lesions were defined as lesions whose longest diameter was ≥ 2 cm. Imaging studies were repeated every 4 weeks until the objective tumor response was confirmed. All responses were reviewed by an independent radiologist. Toxicity was graded using National Cancer Institute-Common Toxicity Criteria version 2.0.

2.4. Statistical analysis

The primary endpoint of this study was the response rate, defined as the proportion of patients whose best response was CR or PR among all enrolled patients in the intent-to-treat analysis. The secondary end points were toxicity and overall and progression-free survival (PFS) from the date of enrollment in this study.

According to Simon's minimax two-stage phase II study design, the treatment program was designed for a minimal response rate of 5% and to provide a significance level of 0.05 with a statistical power of 80% in assessing the activity of the regimen according to a 20% response rate. The upper limit for first-stage drug rejection was no response in 13 evaluable patients. The upper limit for second-stage drug rejection was three responses in 27 evaluable patients. Overall survival time was defined as the interval between enrollment in this study and death or the most recent follow-up visit. PFS was defined as the interval between enrollment in this study and the first documented PD, death, or the most recent follow-up visit. Survival was estimated by the Kaplan—Meier analysis method. All comparisons between proportions were performed by Fisher's exact test.

3. Results

3.1. Patient characteristics

Between October 2002 and November 2003, 30 patients were enrolled in this study, and their characteristics are shown in Table 1. Twenty-six (87%) patients were men, and 21 (70%) patients had adenocarcinoma. Median age was 60 years. The majority of the patients (93%) had received prior platinum-based chemotherapy, and seven (23%) patients had received two prior chemotherapy regimens. The platinum-based chemotherapy regimens that had been used were: cisplatin plus vinorelbine ($n = 26$), cisplatin plus gemcitabine ($n = 1$), and carboplatin plus gemcitabine ($n = 1$). There were 15 (50%) responders to any of the prior chemotherapy regimens and 12 of them had experienced a response (CR/PR) to cisplatin-based chemotherapy. Twenty-one (70%) patients had a treatment-free interval of 3 or more months since the final dose of the prior chemotherapy regimen.

A total of 94 cycles of chemotherapy were administered, and the median number of cycles per patient was three (range, 1—6). Four patients had received only one cycle of treatment either because of toxicity (two patients, grade 3 rash), the patient's refusal (one patient), or PD (one patient).

3.2. Response and survival

Two patients were not evaluable for response because the protocol treatment had been terminated because of toxicity (grade 3 rash) during the first cycle of chemotherapy, and they subsequently received further chemotherapy without PD. There was 1 CR and 10 PRs among the 30 patients, and the objective response rate in the intent-to-treat analysis was 36.7% (95% confidence interval [CI], 19.9—56.1%) (Table 2). Treatment outcomes of all patients are listed in

Table 1. Patient characteristics

Characteristic	No. of patients (%)
Patients enrolled	30
Sex	
Male	26
Female	4
Age, years	
Median	60
Range	39—75
ECOG performance status	
0	7
1	23
Stage	
IIIB	11
IV	19
Histology	
Adenocarcinoma	21
Squamous cell carcinoma	7
Large cell carcnioma	2
Prior treatment	
Platinum-based chemotherapy	28 (93)
Docetaxel	5 (16)
Chest radiotherapy	4 (13)
No. of prior chemotherapy regimens	
1	23
2	7

Table 3. The response rate of patients who experienced a response (CR/PR) to prior cisplatin-based chemotherapy was 43% (6/14), as opposed to 23% (3/13) among the non-response patients ($P = 0.41$). The response rate of the patients who had received one prior chemotherapy regimen was 39% (9/23), as opposed to 28% (2/7) among the patients who had received two regimens ($P > 0.99$). According to the treatment-free interval since the final dose of the prior chemotherapy regimen, the response rate of patients whose interval was 3 months or more was 33% (7/21), com-

Table 2. Treatment efficacy ($n = 30$)

	No. of patients	%
Response		
Overall response rate	11	36.7
Complete response	1	3.3
Partial response	10	33.3
Stable disease	12	40
Progressive disease	5	16.7
Not evaluable	2	6.7
Survival		
Median (months)	9.9	
1 year (%)	47	
Progression-free survival		
Median (months)	5.3	

Table 3 Treatment outcomes of all patients

Patient No.	Prior first-line therapy		Prior second-line therapy		Time from last therapy (months)	CBDCA + PTX, best response	PFS (months)	Survival (months)
	Regimen	Best response	Regimen	Best response				
1	CDDP + VNR	SD	DOC	PD	1.8	SD	1.4	25.2
2	CBDCA + GEM	NE	Gefitinib	PD	0.8	PR	3.8	8.8
3	CDDP + VNR	SD	—	—	6.8	SD	7.6	18.1
4	CDDP + GEM	PR	—	—	9.5	PR	7.5	33.8+
5	CDDP + VNR	SD	—	—	4.8	SD	2.8	7.0
6	CDDP + VNR + DOC + RT	PR	—	—	6.0	PR	8.0	21.6
7	GEM + VNR	SD	—	—	23.0	PD	1.2	7.8
8	CDDP + VNR + RT	PR	—	—	13.6	SD	6.7	25.0+
9	CDDP + VNR	SD	—	—	5.0	SD	2.1	3.7
10	CDDP + VNR	SD	—	—	5.0	PD	1.2	6.7
11	CDDP + VNR	PR	—	—	8.9	NE	1.1	3.3
12	CDDP + VNR	SD	Gefitinib	CR	1.9	SD	6.3	6.3
13	CDDP + VNR	PR	—	—	5.4	NE	1.0	13.4
14	CDDP + VNR	PR	—	—	1.7	SD	4.8	5.7
15	CDDP + VNR + RT	PR	—	—	9.3	SD	5.0	15.7
16	CDDP + VNR	SD	—	—	2.8	PR	3.7	15.8
17	CDDP + VNR	SD	DOC + GEM	SD	3.8	SD	5.3	21.6+
18	CDDP + VNR + DOC + RT	PR	—	—	3.9	SD	4.5	9.0
19	CDDP + VNR	PR	—	—	12.9	PR	9.4	16.0
20	CDDP + VNR	PR	—	—	11.5	CR	24.8+	24.8
21	CDDP + VNR	PD	—	—	1.1	PR	9.2	23.6+
22	CDDP + VNR	SD	DOC	SD	4.5	PD	2.3	5.5
23	Gefitinib	SD	—	—	0.9	PR	8.8	12.7
24	CDDP + VNR	PR	—	—	11.1	PR	5.3	10.2
25	CDDP + VNR	PR	Gefitinib	PR	4.4	PR	5.5	9.9
26	CDDP + VNR	NE	—	—	11.7	PR	7.0	12.2
27	CDDP + VNR	PR	—	—	5.4	SD	6.2	9.4
28	CDDP + VNR	SD	—	—	0.8	PD	1.4	2.5
29	CDDP + VNR	PR	—	—	4.4	PD	0.2	8.4
30	Gefitinib	PD	CDDP + VNR	PD	0.9	SD	3.1	3.3

CBDCA, carboplatin; PTX, paclitaxel; PFS, progression-free survival; CDDP, cisplatin; VNR, vinorelbine; GEM, gemcitabine; DOC, docetaxel; RT, chest radiotherapy; SD, stable disease; NE, not evaluable; PR, partial response; PD, progressive disease; CR, complete response.
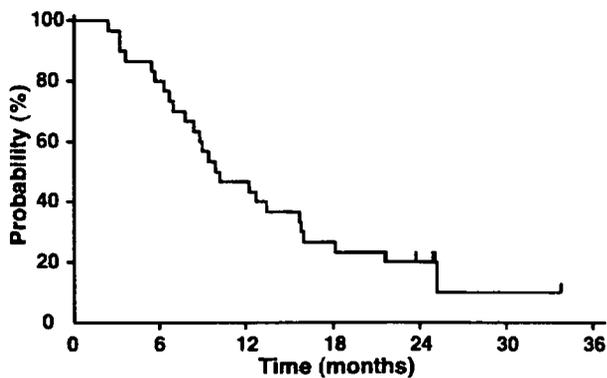
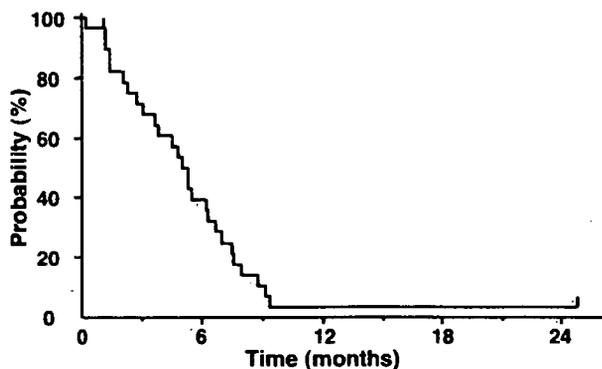Fig. 1   Kaplan—Meier curve for overall survival.

Fig. 2   Kaplan—Meier curve for progression-free survival.

pared with 44% (4/9) in patients in whom it was less than 3 months ($P = 0.68$).

The median follow-up time was 24 months. The median survival time (MST) was 9.9 months (range, 2.5—33.8 months), and the 1-year survival rate was 47% (95% CI, 29—65%). The median PFS was 5.3 months. The Kaplan—Meier curve for overall survival and for PFS is shown in Figs. 1 and 2, respectively. Nineteen patients (63%) received at least one subsequent chemotherapy regimen, and their regimens are shown in Table 4. Fourteen of them were treated with gefitinib, and a PR was achieved in three of them.

3.3. Toxicity

The common toxicities associated with carboplatin plus paclitaxel are listed in Table 5. Grade 3/4 neutropenia occurred in 54% of the patients in our study, but grade 3 febrile neutropenia developed in only 3%. Grade 3/4 anemia and thrombocytopenia were observed in five patients (16%)

Table 4   Post-study chemotherapy

Regimen	No. of patients	Responder (%)
Gefitinib	14	3 (21)
Docetaxel	9	0
Gemcitabine plus viborelbine	1	0

and two patients (13%), respectively. Non-hematological grade 3 toxicities were less frequent. Grade 3 hyponatremia was observed in five (16%) patients, but they were all asymptomatic. Grade 2 neuropathy occurred in 33% of the patients. There were no treatment-related deaths.

4. Discussion

Docetaxel, pemetrexed, and erlotinib have been approved for second-line treatment of advanced NSCLC on the basis of the results of phase III trials [6,7,12,13]. Hanna et al. reported a phase III study comparing 3-weekly pemetrexed 500 mg/m² with 3-weekly docetaxel 75 mg/m² as second-line treatment for advanced NSCLC. The overall response rate with pemetrexed and docetaxel was 9.1% and 8.8%, respectively, and MST was 8.3 months and 7.9 months, respectively. Although efficacy in terms of the outcome as measured by survival time and response rate was similar for both treatments, the pemetrexed group experienced less grades 3—4 hematological toxicity and alopecia of all grades [12]. In the trial reported by Shepherd et al. 731 NSCLC patients previously treated with chemotherapy were randomized to receive either erlotinib at a dose of 150 mg daily or placebo, and the response rate in the erlotinib group was 8.9%. MST was 6.7 months in the erlotinib group and 4.7 months in the placebo group ($P < 0.001$). The results of their trial showed that erlotinib significantly prolonged the survival of patients with advanced NSCLC who had previously been treated with chemotherapy [13]. Despite the positive results of these phase III trials, the response rate of advanced NSCLC to second-line chemotherapy remains low, and the life expectancy of advanced NSCLC patients remains short. Alternative effective chemotherapy option is needed for second-line treatment of advanced NSCLC.

The combination of carboplatin plus paclitaxel has proved effective as one of the standard platinum-based doublet regimens for first-line treatment of advanced NSCLC [4,5,14]. However, since the efficacy of carboplatin plus paclitaxel used in a second-line setting had hardly been assessed, in the present study we evaluated the efficacy and toxicity of carboplatin plus paclitaxel in the second- or third-line treatment of advanced NSCLC. The results in the 30 patients with advanced NSCLC previously treated with chemotherapy indicated that the combination of carboplatin plus paclitaxel yielded an objective response rate of 36.7% and an MST of 9.9 months, with a 1-year survival rate of 47%. Our study had not included patients who were treated with the platinum/taxane combination chemotherapy. Most of the toxicity observed in our study was hematological. Grade 3/4 neutropenia, anemia, or thrombocytopenia occurred in 54, 16, or 13% of the patients in our study, respectively. Hematological toxicity of carboplatin plus paclitaxel used in first-line treatment for Japanese patients with advanced NSCLC has been reported that grade 3/4 neutropenia, anemia, or thrombocytopenia occurred in 88, 15, or 11% of the patients [15]. The toxicity observed in our study appeared similar to that of carboplatin plus paclitaxel, which was administered as the first-line treatment, although the number of patients in our study was not large. The combination of carboplatin plus paclitaxel seems to be effective and tolerable, not only as first-line therapy for advanced NSCLC but

Table 5. Hematological and non-hematological toxicity (n = 30)

Toxicity	NCI-CTC Version 2.0, grade							
	0–1		2		3		4	
	n	%	n	%	n	%	n	%
Leukopenia	11	37	10	33	9	30	0	0
Neutropenia	10	33	4	13	14	47	2	7
Anemia	7	23	18	60	3	10	2	7
Thrombocytopenia	27	90	1	3	2	7	0	0
Febrile neutropenia	29	97	—		1	3	0	0
Nausea	27	90	3	10	0	0	—	
Fatigue	30	100	0	0	0	0	0	0
Neuropathy	20	67	10	33	0	0	0	0
Arthralgia	21	70	8	27	1	3	0	0
Rash	28	93	0	0	2	6	0	0
Infection	29	97	0	0	1	3	0	0
Arrhythmia	29	97	0	0	1	3	0	0
Alopecia	21	70	9	30	—		—	
AST/ALT	29	97	1	3	0	0	0	0
Hyponatremia	25	83	—		5	17	0	0

as second-line therapy as well if the patients had not been previously treated with the platinum/taxane combination chemotherapy.

Hotta at al. reported a meta-analysis based on abstracted data to compare the effect of carboplatin-based chemotherapy with that of cisplatin-based chemotherapy on overall survival, response rate, and toxicity in the first-line treatment of patients with advanced NSCLC [16]. The results indicated that combination chemotherapy consisting of cisplatin plus a third generation agent produced a significant survival benefit compared with carboplatin plus a third generation agent, although the toxicity profiles of the two modalities were quite different. Recently, Pignon et al. reported a pooled analysis from five randomized clinical trials of cisplatin-based chemotherapy in completely resected NSCLC patients [17]. Their analysis suggested that adjuvant cisplatin-based chemotherapy improved survival in patients with NSCLC. Based on the results of their meta-analysis, cisplatin-based chemotherapy should be recommended as first-line therapy for patients with advanced NSCLC. Moreover, in view of the results of our own study, we speculate that the combination of carboplatin plus paclitaxel may be suitable as second-line treatment for advanced NSCLC patients who had experienced progression after first-line cisplatin-based chemotherapy.

Care must be exercised in interpreting the favorable outcome in our study. One concern is that it was a single-institution phase II study, and therefore patient selection may have influenced the outcome. The responders to any of the prior chemotherapy regimens accounted for 50% of the 30 patients enrolled in this study, and about 80% of the patients had received only one prior chemotherapy regimen. The selection criteria, such as an ECOG PS of 0 or 1, may also have contributed to this favorable outcome. Another concern is that our study had included only five patients who were previously treated with chemotherapy using taxanes. Therefore, the efficacy of carboplatin plus paclitaxel as the

secondary therapy after chemotherapy using taxanes is not clear. A further randomized study is warranted to be able to draw definitive conclusions about our results.

Conflict of interest statement

None declared.

Acknowledgement

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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]-carbonyloxy-camptothecin; 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-

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'-CACCCAC-CTATGACTGCTCA-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-GAGAC-CAGCGAGCCGACCTTGCGGCTGCACAGACTTCG-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

This Study	SNP Identification NCBI (dbSNP)	JSNP	Location	Position From the Translational Initiation Site or from the Nearest Exon	Position NT_010498.15	Nucleotide Change and Flanking Sequences (5' to 3')	Amino Acid Change	Allele Frequency
MPJ6_CS2001			5'-Flanking	−1671[a]	20582067	CTGGAACAACTCG/CCTCCCCTCGGAA		0.010
MPJ6_CS2002			5'-Flanking	−1254[a]	20582484	AACCACCACCGCT/CGATCCTAGCAGG		0.002
MPJ6_CS2016[b]			5'-Flanking	−1233[a]	20582505	CAGCGTGCGCTT/CCCGCTCCAACCC		0.002
MPJ6_CS2003			Exon 1 (5'-UTR)	−759[a]	20582979	AAATGTTTGTCAA/GGTGGATAAATGA		0.006
MPJ6_CS2004			Exon 1 (5'-UTR)	−363[a]	20583375	CCTCCTATCGATC/GCCCCAGCGCGCT		0.031
MPJ6_CS2017[b]	rs11075646		Exon 1	1[a]	20583738	AGCGAGCCGACCA/TTGCGGCTGCACA	Met[1]Leu	0.002
MPJ6_CS2005			Exon 2	100[a]	20586162	GCCAGTCCCATCC/TGGACCACACACA	Arg[34]Trp	0.002
MPJ6_CS2021[b]			Intron 2	IVS2-71	20587248	GGTGCCTGGGAGC/GACCTCTGAACCC		0.002
MPJ6_CS2015			Exon 4	424[a]	20588325	TGATTTCCCCAGG/ATGATGGTGTGGA	Val[142]Met	0.002
MPJ6_CS2006			Intron 4	IVS4 + 29	20588486	GCTGGGCAACCCG/AGGCTGAGCGGGG		0.002
MPJ6_CS2007			Exon 5	579[a]	20588560	CAAGCACGCAACC/TGGCAACTGGGGC	Thr[193]Thr (silent)	0.002
MPJ6_CS2018	rs8192924		Exon 5	617[a]	20588598	TGGCTGCACTACG/ACTGGGTCCAGCA	Arg[206]His	0.002
MPJ6_CS2008			Exon 5	765[a]	20588746	CATGGAGAGTGGC/TGTGGCCCTCCTG	Gly[245]Gly (silent)	0.002
MPJ6_CS2009			Intron 5	IVS5-69	20589157	CCTGTTCTTGGCC/TAGGGCCTTGGGC		0.017
MPJ6_CS2019[b]		ssj0005417	Intron 7	IVS7 + 27	20589775	AAGCCACAAGTG/ACCTGGGGAGCCC		0.002
MPJ6_CS2010			Intron 7	IVS7-25	20589845	CCCATCCCCAGCT/AACAGACTCTCTC		0.002
MPJ6_CS2011			Intron 8	IVS8-2	20590205	TCCACCTGGGGTA/GGATGTTGCCTCC	Splicing defect	0.002
MPJ6_CS2020[b]		IMS-JST1013275	Intron 9	IVS9 + 78	20590429	ACCTGCTGCTGTC/TCGGGTCAGCACT		0.002
MPJ6_CS2012	rs2241409		Intron 10	IVS10-108	20591293	GGAAGAAAAGCG/AGAGAAGCAGGAC		0.269
MPJ6_CS2013	rs28382825		Intron 10	IVS10-87	20591314	GGACTGGGACCCG/AAGGTCTCGGGGG		0.031
MPJ6_CS2014	rs8192925	ssj0005418	Exon 12 (3'-UTR)	1749 (*69)[a]	20592196	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.

Position	5'-flanking	5'-flanking	5'-flanking	5'-UTR	5'-UTR	Exon 1	Exon 2	Intron 2	Exon 4	Intron 4	Exon 5	Exon 6	Exon 5	Intron 5	Intron 7	Intron 7	Intron 8	Intron 9	Intron 10	Intron 10	3'-UTR
Nucleotide change	-1871 G>C	-1254 T>C	-1233 T>C	-759 A>G	-383 C>G	1 A>T	100 C>T	IVS2-71 C>G	424 G>A	IVS4+23 G>A	579 C>T	817 C>T	785 C>T	IVS5-69 C>T	IVS7+27 C>T	IVS7-25 G>A	IVS8-2 T>A	IVS9+82 C>T	IVS10-108 G>A	IVS10-87 G>A	1749+69? A>G
Effect on protein						M1L	R34W		V142M		T193T (silent)	R206H	G255G (silent)				splicing defect				

Figure — haplotype chart (haplotype group, haplotype, number, frequency):

Haplotype group	Haplotype	Number	Frequency
*1	*1a	357	0.681
	*1b	122	0.233
	*1c	14	0.027
	*1d	9	0.017
	*1e	5	0.010
	*1f	3	0.006
	*1g	1	0.002
	*1h	1	0.002
	*1i	1	0.002
	*1j	1	0.002
	*1k	1	0.002
	*1l	1	0.002
	1m	1	0.002
	1n	1	0.002
	1o	1	0.002
*2	*2a	1	0.002
*3	*3a	1	0.002
*4	*4a	1	0.002
*5	*5a	1	0.002
*6	*6a*	1	0.002
	Total	524	1.000

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 (Arg34Trp), 424G>A (Val142Met), 1A>T (Met1Leu), and 617G>A (Arg206His), 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 (Arg34Trp, *2), 1A>T (Met1Leu, *5), and 617G>A (Arg206His, *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, Met1Leu (*5) and Arg206His (*6), the functional analysis was conducted in vitro.

In Vitro Functional Analysis of the Met1Leu Variant. To clarify the functional significance of the novel variant Met1Leu (*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

Irinotecan alone
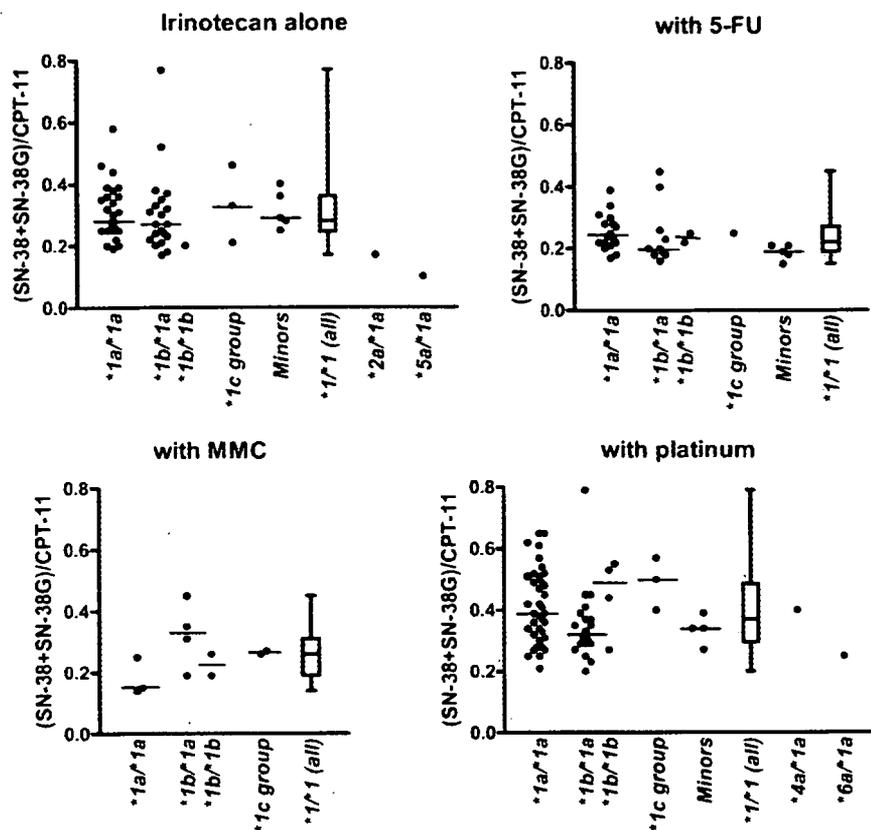

with 5-FU

with MMC

with platinum

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.
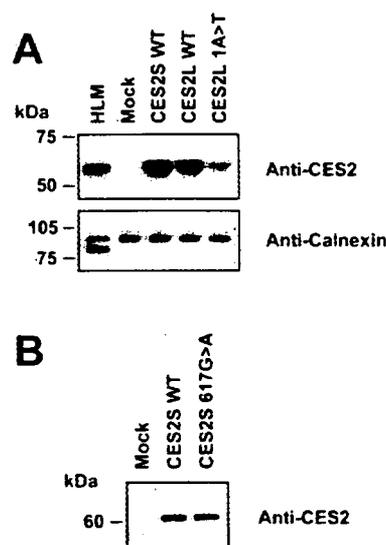

Fig. 3. Expression of CES2 protein from the wild-type and 1A>T (A) and 617G>A (B) variant CES2 genes in COS-1 cells. Membrane fraction (A) or the postmitochondrial supernatant (B) from the cDNA-transfected cells was subjected to SDS-polyacrylamide gel electrophoresis, followed by transfer to the nitrocellulose 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.
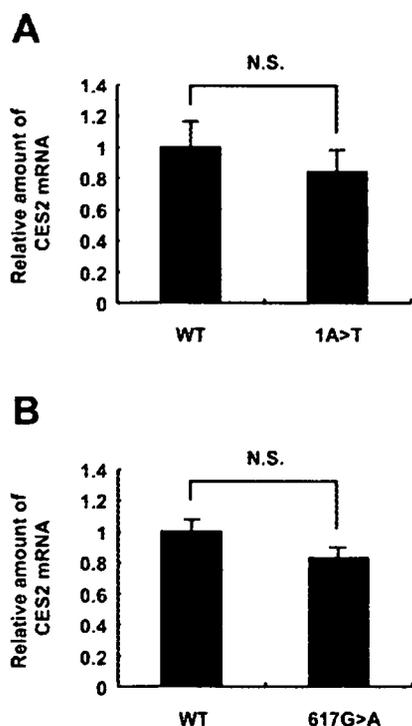
A

N.S.

B

N.S.

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 $\pm$ 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 $\pm$ 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.