Table 1
Profiles of cancer patients in this study

		No. of patients
Patients genotyped		75
(Male/female)		(51/24)
Age		
Mean/range (y)	50.7/34–75	
Performance Status[a]		
	0/1/2	18/48/8
Previous treatment		
Surgery[a]	+/−	71/3
Chemotherapy[b]	+/−	63/10
Radiotherapy[b]	+/−	9/64
Combination therapy and tumor type [dose of irinotecan (mg/m^2)/(w or 2w)[c]]		
Irinotecan monotherapy	Lung (60/w or 100/2w)	4
	Stomach (100/2w or 150/2w)	5
	Colon (100/2w or 150/2w)	40
With cisplatin	Lung (60/w or 100/2w)	4
	Stomach (60/2w)	11
With mitomycin C (MMC)	Stomach (150/2w)	8
	Breast (120/2w)	1
With 5-fluorouracil (5-FU)	Colon (150/2w)	2
Available data on serum bilirubin levels		37

[a] Data from one patient is lacking.
[b] Data from two patients are lacking.
[c] Weekly or biweekly.

ment with 1.5 ml of xylene at room temperature. After centrifugations, the residual pellet was then washed twice with 1.5 ml of ethanol. Finally, the pellet was dried at 37 °C for 15 min. DNA extraction was performed using a QIAamp tissue kit (QIAGEN K.K., Tokyo, Japan) according to the manufacturer's instructions with some modifications. Briefly, 540 μl of ATL lysis buffer and 60 μl of proteinase K (Qiagen) were added to each pellet, mixed thoroughly, and incubated at 56 °C for 3 h with a rotator. Any remaining tissue debris was removed by centrifugation, and the resulting supernatant was used for the extraction. Twelve microliters of RNase A (100 mg/ml) was added to the supernatant and incubated for 2 min at room temperature. Next, 600 μl of buffer AL was added and mixed thoroughly, and the mixture was incubated at 70 °C for 10 min. Six-hundred microliters of ethanol was added to the solution and mixed well, followed by extraction of DNA using a Qiagen DNA extraction column. The DNA was eluted in a final elution volume of 150 μl. The yield was determined using a NanoDrop spectrophotometer (NanoDrop Technology, Inc, Rockland, DE, USA) and the size of the extracted DNA was checked by agarose gel electrophoresis.

Genotyping of *UGT1A1*6* (211G>A, G71R), **28* (−364C>T, which is perfectly linked with −40_−39insTA in Japanese), and **60* (−3279T>G) were performed by pyrosequencing as described previously [19,20].

2.3. Association analysis and statistics

For association analysis, we focused on incidences of severe diarrhea and neutropenia (grade 3 or greater) observed during irinotecan-therapy. The incidence of severe diarrhea was very low, and the incidence of neutropenia was higher in combination therapy. Therefore, the association of neutropenia with *UGT1A1* genotypes was primarily evaluated in 49 patients with irinotecan monotherapy. As a parameter for in vivo UGT1A1 activity, serum total bilirubin levels taken at baseline from 37 patients were also used.

Statistical analysis for evaluation of the relationship between *UGT1A1* genotypes and severe neutropenia was performed using the chi-square test for trend using Prism version 4.0 (GraphPad Prism Software Inc., San Diego, CA). The gene-dose effect of the genetic marker "**6 or *28*" on serum total bilirubin levels was analyzed using the Jonckheere–Terpstra (JT) test in the SAS system (version 5.0, SAS Institute, Inc., Cary, NC). The *P*-value of 0.05 (two-tailed) was set as a significant level. Multivariate logistic regression analysis on neutropenia (grade 3 or greater) was performed using JMP software (version 6.0.0, SAS Institute, Inc., Cary, NC), including variables for age, sex, body surface area, performance status, concomitant disease, history of adverse reaction, irinotecan dosage, dosing interval, and *UGT1A1* genotypes. The variables in the final model for neutropenia were chosen using the forward and backward stepwise procedure at the significance level of 0.1.

3. Results

3.1. UGT1A1 diplotypes/haplotypes

The diplotypes and haplotypes (**1, *60, *6* and**28*) of *UGT1A1* exon 1 were analyzed in 75 Japanese cancer patients (Table 1) and their frequencies were summarized (Table 2). The haplotypes were assigned according to our previous definition [15]. It should be noted that the **60* haplotype does not harbor the **28* allele (−40_−39insTA), but most of the **28* haplotype does harbor the **60* allele (−3279T>G). In this study, the **28* homozygote was not present, and the frequency of haplotype **28* (0.113) was slightly lower than that found in our previous study (0.138) [17]. In contrast, the frequency of haplotype **6* (0.213) was higher than that found in the previous study (0.167) [17].

Table 2
Frequencies of *UGT1A1* diplotypes (A) and haplotypes (B) for cancer patients in this study

		Frequency
(A) Diplotype	No. of patients (N = 75)	
**1/*1*	21	0.280
**1/*60*	9	0.120
**60/*60*	2	0.027
**6/*1*	14	0.187
**6/*60*	8	0.107
**6/*6*	4	0.053
**28/*1*	12	0.160
**28/*60*	3	0.040
**28/*6*	2	0.027
**28/*28*	0	0.000
(B) Haplotype[a]	No. of chromosomes (N = 150)	
**1*	77	0.513
**60*	24	0.160
**6*	32	0.213
**28*	17	0.113

[a] Haplotype definition follows the previous report [15]; **60*, −3279T>G without −40_−39insTA; **6*, 211G>A(G71R); **28*, −40_−39insTA.

3.2. Association of UGT1A1 genotypes with serum total bilirubin levels

Serum total bilirubin levels at baseline, a parameter of in vivo UGT1A1 activity, were available from 37 patients (treated by various regimens), and we analyzed their association with *UGT1A1* genotypes (Fig. 1). The median values of total bilirubin in **60/*1*, **28/*1* and **6/*1* heterozygotes were not significantly different from that of the wild type (**1/*1*). Higher median values were observed for the **6* homozygotes (**6/*6*) and the double heterozygotes of **6* and **28* (**6/*28*) than that of the wild type (**1/*1*), with increases of 1.9-fold and 2.2-fold, respectively. Since **6* and **28* are mutually independent and their reducing effects on UGT activity are equivalent [15,17], diplotypes were classified by the presence of "**6 or *28*" (indicated by "+" in Fig. 1). As shown in Fig. 1, a significant "**6 or *28*"-dependent increase in total bilirubin levels was observed ($p = 0.0088$, Jonckheere–Terpstra test).

3.3. Severe toxicities observed in this study

Incidences of severe diarrhea and neutropenia (grade 3 or greater) are shown in Table 3 for each irinotecan-containing regimen. Grade 3 diarrhea was observed in only 4 of the 75 subjects, and since the incidence of diarrhea was low (5.3%), an association analysis on diarrhea was not conducted. Regarding neutropenia, 26 patients experienced grade 3 or 4 neutropenia. Of these 26 patients, 90% experienced neutropenia within 2 months after starting irinotecan-therapy, and 70% within 2 weeks. Significant differences in neutropenia incidences were observed among the regimens used, and considerably high incidences were observed in the combination therapies. Accordingly, association of the *UGT1A1* genotypes with severe neutropenia was analyzed primarily in the patients who received irinotecan-monotherapy.

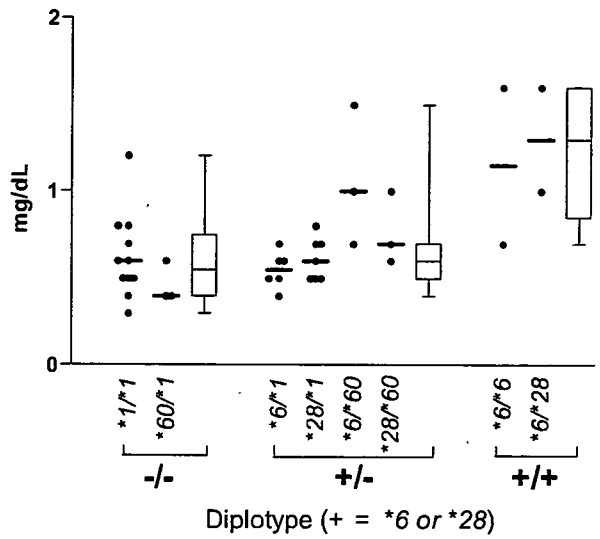

Fig. 1. Effects of *UGT1A1* genotypes on serum total bilirubin levels at baseline in Japanese cancer patients ($N = 37$). Each point represents a patient, and the median value of each diplotype is shown with a bar. All diplotypes are classified into −/−, +/−, and +/+ by the genetic marker, "*UGT1A1*6 or *28*", indicated by "+", and their distributions are shown by a box representing the 25–75 percentiles with a bar at the median and lines representing the highest and lowest values. A significant "**6 or *28*"-dependent increase in total bilirubin levels was observed ($p = 0.0088$, Jonckheere–Terpstra test).

Table 3
Severe toxicities observed in Japanese cancer patients

Treatment	Diarrhea[a]/total (%)	Neutropenia[b]/total (%)
Total patients	4/75 (5.3)	26/75 (34.7)
Irinotecan alone	1/49 (2.0)	6/49 (12.2)
With CDDP	2/15 (13.3)	11/15 (73.3)
With MMC	1/9 (11.1)	8/9 (88.9)
With 5-FU	0/2 (0.0)	1/2 (50.0)
P-value[c]	NS	<0.0001

[a] Grade 3.
[b] Grade 3 or 4.
[c] Chi-squre test.

3.4. Association of UGT1A1 genotypes with neutropenia

Since significant associations of *UGT1A1*6* and **28* with increased total bilirubin levels (decreased UGT-activity) were once again confirmed in this study, we assessed the clinical relevance of these haplotypes, focusing on the effect of **6* on severe neutropenia. In the 49

patients who received irinotecan monotherapy, the incidence of grade 3 or 4 neutropenia was *6-dependently increased ($p = 0.012$ in the chi-square test for trend). Namely, incidences of severe neutropenia in the *6 heterozygotes (*6/*1,*6/*60, and *6/*28) and homozygotes (*6/*6) were 2.3-fold and 15-fold higher, respectively, than that seen in the non-*6 bearing patients (*1/*1, *60/*1, *28/*1, and *28/*60) (Table 4). In this study, no *28 heterozygotes (*28/*1 and *28/*60) experienced any severe neutropenia, and there were no *28 homozygotes enrolled. Therefore, the effect of *28 could not be determined. For the *60-bearing patients without *6 or *28 (only heterozygote, *60/*1), one patient among six experienced severe neutropenia, and no significant *60-dependent increase was observed (data not shown). Although no statistically significant association of the *28 heterozygotes with severe neutropenia was confirmed in this study, the incidence of discontinuation of irinotecan monotherapy was higher in the *28-bearing patients (91%, $N = 11$) than that in the non-*28 subjects (79%, N = 38), while *60- or *6-dependent increased discontinuation rates were not found (data not shown). For the patients with cisplatin-combination therapy, a higher incidence of severe neutropenia was observed in the *6-bearing patients (*6/*1, *6/*60, and *6/*6) (100%, $N = 3$) than that in the non-*6 bearing subjects (*1/*1, *60/*1, *60/*60, and *28/*1) (66.7%, $N = 12$).

3.5. Multivariate analysis of neutropenia

In order to further clarify the clinical impact of *6 on irinotecan toxicities, multivariate logistic regression analysis on grade 3 or 4 neutropenia was conducted using variables, including *UGT1A1* genotypes and patient background factors, described in Section 2. The final model revealed a significant association of *6 with the incidence of grade 3 or 4 neutropenia at an odds ratio of 5.87 (Table 5).

4. Discussion

The clinical application of the genetic test for *UGT1A1*28* prior to irinotecan therapy has been in practice in the United States since 2005, which was based on cumulative evidence supporting the significant association of *28 with severe irinotecan toxicity [9–13]. Most of the evidence was obtained in Caucasian patients, where *28 is relatively frequent (30–40%) [14]. Although additive effects of another low activity allele, *6, which is specific for East Asians, has been also suggested [9,15–17], direct evidence in Japanese patients has remained limited. In this study, we clearly showed the significant correlation of *6 to grade 3 or 4 neutropenia in Japanese cancer patients who received irinotecan monotherapy. An increased incidence of severe neutropenia was also observed in the *6-bearing patients using cisplatin combination therapy. This finding is in accordance with a report on Korean lung cancer patients who received a combination therapy of irinotecan and cisplatin, which showed a significant association of *6 homozygotes with grade 4 neutropenia [18]. Since combination therapies using irinotecan may cause higher incidences of severe toxicities, the *UGT1A1* polymorphisms should be carefully considered in regimens that include irinotecan.

Since the alleles *6 and *28 are mutually independent [15] and their effects on the UGT activities were shown to be equivalent, the usefulness of the genetic marker "*6 or *28" for personalized irinotecan therapies has been suggested [17]. This was also supported in the current study, which showed a "*6 or *28"-dependent increase in serum total bilirubin levels (Fig. 1). Because of the low frequency of *28 without homozygotes among our subjects, the influence of *28 on toxicities was not clearly demonstrated, as in the case of the Korean patients where the allele frequency of *1A1*6* (23.5%) was much higher than that of *1A1*28* (7.3%) [18]. However, in the current study, the double heterozygotes of *6 and *28 (*6/*28) showed increases in serum total bilirubin levels (Fig. 1). Moreover, a higher incidence of severe neutropenia in the *6/*28 patients was observed, although the patient number was small ($N = 2$) (Table 4). This finding also indi-

Table 4
Association of *UGT1A1* genotypes with severe neutropenia (grade 3 or 4) in irinotecan monotherapy

Diplotype[b]	Neutropenia[a]/total (%)	Effect of *6 (%)	
–/–	1/20 (5.0)	non-*6/non-*6	(3.4)
*28/–	0/9 (0.0)		
*6/–	3/16 (18.8)	*6/non-*6	(22.2)
*6/*28	1/2 (50.0)		
*6/*6	1/2 (50.0)	*6/*6	(50.0)
P-value[c]		0.012	

[a] Grade 3 or 4.
[b] "–" represents "*1 or *60".
[c] Chi-square test for trend.

Table 5
Multivariate logistic regression analysis of severe neutropenia (grade 3 or 4) in irinotecan monotherapy

Variable	Coefficient	SE	*P*-value	Odds ratio	(95% Confidence limit)
*UGT1A1*6*	1.77	0.809	0.0289	5.87	(1.37–39.6)

$R^2 = 0.157$, Intercept = 3.15, $N = 49$.

cates the importance of "*6 or *28" in severe neutropenia, and in fact, a gene-dose effect of "*6 or *28" ($p = 0.04$ in the chi-square test for trend) and its significant contribution in multivariate analysis ($p = 0.0326$) were also confirmed (data not shown).

For the *60 haplotype (-3279T>G without -40_-39insTA), no association of *60 with severe neutropenia was observed in this study, which coincides with reports of other studies on Japanese cancer patients [17,23]. As for the *27 allele [686C>A(P229Q)], it was linked with the *28 allele and the haplotype was defined as the *28 subtype,*28c [15]. One *28c-heterozygous patient with irinotecan monotherapy showed no severe neutropenia, suggesting a small contribution of the *27 allele (data not shown).

In this study, the association between *UGT1A1* genotypes and antitumor activity was difficult to evaluate because of the small number of subjects stratified into each tumor type. Further clinical studies are needed to establish methods for selection of the appropriate regimen or dosage based on the *UGT1A1* genotypes, where a balance between toxicity and antitumor effect should be considered.

In conclusion, this study demonstrated the significant association of *UGT1A1*6* with severe irinotecan-mediated neutropenia. The current data also supported the usefulness of the genetic marker "*6 or *28" for personalized irinotecan therapy in Japanese, and likely East Asian, patients.

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Combination of O^6-methylguanine-DNA methyltransferase and thymidylate synthase for the prediction of fluoropyrimidine efficacy

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ABSTRACT

We investigated the correlation between the response to fluoropyrimidines as first-line therapy and the expressions of genes in patients with primary colorectal cancer (CRC). The study group comprised 92 patients with metastatic CRC. Total RNA was isolated from laser-captured tumour cells in surgically resected primary lesions, and gene expression was quantitatively evaluated by real-time RT-PCR assay. Low thymidylate synthase (TS), low γ-glutamyl hydrolase, high reduced folate carrier 1, high O^6-methylguanine-DNA methyltransferase (MGMT) and low cyclin E expressions were associated with a good response ($P = 0.0030$, 0.0250, 0.0120, 0.0030 and 0.0020, respectively) on univariate analysis. On multivariate logistic regression analysis, TS and MGMT remained independent predictors of the response. The clinical response rates were 63.2% in the low TS or high MGMT group and 14.3% in high TS and low MGMT group ($P < 0.0001$). The combination of high TS and low MGMT expression is a significant predictor of a poor response to fluoropyrimidine treatment.

1. Introduction

The median survival time of patients with colorectal cancer (CRC) has improved in the past 10 years because of the development of new agents with advantages over 5-fluorouracil (5-FU), including irinotecan hydrochloride (CPT-11) and oxaliplatin. CPT-11 or oxaliplatin monotherapy, however, was not shown to be more effective than bolus 5-FU/leucovorin (LV) in terms of response and median survival time. CPT-11 or oxaliplatin plus bolus or infusional 5-FU/LV regimens were found to be clearly more effective than 5-FU/LV, resulting in a doubling of the tumour response rate and prolongation of median survival time by 2–3 months. Regimens combining CPT-11 or oxaliplatin with fluoropyrimidines are now key first- and second-line chemotherapies for CRC. Response rates with these regimens, however, remain around 40–50%, prompting investigations of molecular predictors of the response to specific chemotherapeutic regimens. In this study, we evaluated molecular markers that could be used to predict the clinical outcomes of treatment with fluoropyrimidine-based

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regimens, now widely used to treat CRC. Because data on CPT-11-based regimens not including fluoropyrimidines will be difficult to obtain in the future, we also investigated such regimens used for second-line treatment in this study.

Evaluations of regimens including fluoropyrimidines alone as well as those including CPT-11 without fluoropyrimidines are required to produce benchmarks for predicting the efficacy of combined treatment with fluoropyrimidines and CPT-11.

Many potential predictors of the response to fluoropyrimidines have been reported. Several enzymes involved in the targeting, metabolism and catabolism of fluoropyrimidines have been extensively studied, including thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD).[1–3] The enzymes concerning folinic acid metabolism and transport are also the important factors involved in the efficacy of biochemical modulation of 5-FU by LV; among these being folylpolyglutamate synthetase (FPGS), γ-glutamyl hydrolase (GGH) and reduced folate carrier 1 (RFC1).[4,5]

The role of molecular markers in predicting the response to CPT-11-based chemotherapy remains largely unclear, as compared with oxaliplatin-based chemotherapy for CRC, for which several promising markers have been identified.[6] Recently, comprehensive analysis based on the microarray gene expression also have been performed to clarify the predictive markers for CPT-11/5-FU/LV treatment.[7] DNA topoisomerase I (TOPO I) may be a useful predictor of the response to CPT-11-based treatments in colon cancer cell lines as well as in patients with metastatic CRC.[8] Factors involved in DNA-repair systems, such as excision repair cross-complementing 1 (ERCC1) and O^6-methylguanine-DNA methyltransferase (MGMT), have also been investigated recently with respect to their role in resistance to CPT-11.[9,10] The relations between response and factors involved in drug detoxification, such as glutathione S-transferase pi (GSTpi), have been studied for many chemotherapeutic agents, including CPT-11.[9,10] On the other hand, epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF) and cell-cycle-regulation genes, such as cyclin E, have been reported to be related to the outcomes of patients with CRC.[11]

To gain further insight into potentially useful markers, we investigated the correlation between clinical response and the expressions of genes involved in the targeting, metabolism or catabolism of fluoropyrimidines, drug detoxification, cell cycles and DNA-repair systems in patients with metastatic or recurrent CRC who received first-line fluoropyrimidine-based regimens with or without LV or second-line CPT-11-based regimens.

2. Patients and methods

2.1. *Patient selection and chemotherapy*

This clinical–biological correlative study was performed retrospectively in a consecutive series of patients who underwent surgery for primary CRC at our hospital between 1996 and 2003 and received first-line fluoropyrimidine-based regimens for metastatic or recurrent CRC. Their responses to treatment and outcomes were confirmed. Patients who received second-line CPT-11-based chemotherapy were analysed as a subgroup. Approval for this study was obtained from the institutional review board of the National Cancer Center Hospital, Tokyo.

Fluoropyrimidines included 5-FU/l-LV (5-FU 600 mg/m^2 bolus and l-LV 250 mg/m^2 div, weekly × 6, q 8 weeks), continuous infusion of 5-FU (5-FU 250 mg/m^2/day), uracil-tegafur (UFT)/LV (UFT 300 mg/day and LV 75 mg/day per os, 4 weeks on and 1 week off), UFT alone (UFT 300 mg/day per os, 4 weeks on and 1 week off) and TS-1 (TS-1 80, 100 or 120 mg/day per os, 4 weeks on and 2 weeks off). CPT-11-based chemotherapy included CPT-11 alone (CPT-11 150 mg/m^2 div, biweekly) and CPT-11/mitomycin C (CPT-11 150 mg/m^2 div and mitomycin C 5 mg/m^2 bolus, biweekly).

2.2. *Clinical evaluation and response criteria*

Clinical response was evaluated every 6–8 weeks by CT imaging. Responders to treatment were classified as those patients whose tumours shrank by 50% or more, as estimated on two observations not less than 6 weeks apart. More precisely, a complete response (CR) was defined as the complete disappearance of all evidence of tumour, while a partial response (PR) was defined as a greater than 50% decrease in the sum of the products of the largest perpendicular diameters of all measurable lesions, without the occurrence of new lesions. Amongst those classified as non-responders, stable disease (SD) was defined as a change of less than 25% in tumour size, and progressive disease (PD) was defined as an increase of greater than 25% in the area of the measurable tumour deposits or the appearance of new lesions. Time to progression (TTP) during first-line or second-line chemotherapy was defined as the period from the starting date of chemotherapy to the date on which progression was confirmed.

2.3. *Laboratory methods*

Ten-micrometre-thick sections of resected primary CRC tumours were obtained from identified areas with the highest tumour concentration and were then mounted on uncoated glass slides. For histologic diagnosis, representative sections were stained with haematoxylin and eosin by standard methods. Before microdissection, sections were stained with nuclear fast red (NFR, American MasterTech Scientific, Lodi, CA). The sections of interest were selectively isolated by laser capture microdissection (P.A.L.M. Microsystem, Leica, Wetzlar, Germany), according to standard procedures.[12] The dissected particles of tissue were transferred to a reaction tube containing 400 μl of RNA lysis buffer.

The samples were homogenised and heated at 92 °C for 30 min. Fifty microlitres of 2 M sodium acetate was added at pH 4.0, followed by 600 μl of freshly prepared phenol/chloroform/isoamyl alcohol (250:50:1). The tubes were vortexed for 15 s, placed on ice for 15 min, and then centrifuged at 13,000 rpm for 8 min in a chilled (8 °C) centrifuge. The upper aqueous phase was carefully removed and placed in a 1.5-mL centrifuge tube. Glycogen (10 μl) and 300–400 μl of isopropanol were added and the samples were vortexed for 10–15 s. The tubes were chilled at −20 °C for 30–45 min to precipitate the RNA. The samples were then washed in 500 μl of 75% v/v ethanol and air-dried for 15 min. The pellet was

resuspended in 50 μl of 5 mM Tris. Finally, cDNA was prepared as described by Lord and colleagues.[13]

Quantification of the 12 genes of interest and an internal reference gene (β-actin) was done using a fluorescence-based real-time detection method (ABI PRISM 7900 Sequence Detection System, TaqMan®, Perkin–Elmer [PE] Applied Biosystems, Foster City, CA). The PCR reaction mixture consisted of 1200 nM of each primer, 200 nM of probe, 0.4 U of AmpliTaq gold polymerase, 200 nM each of dATP, dCTP, dGTP and dTTP, 3.5 mM of $MgCl_2$ and 1 × Taqman buffer A containing a reference dye. The final volume of the reaction mixture was 20 μl (all reagents from PE Applied Biosystems, Foster City, CA). Cycling conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 46 cycles of 95 °C for 15 s and 60 °C for 1 min. The primers and probes used are listed in Table 1. Gene expression values (relative mRNA levels) are expressed as ratios (differences between Ct values) between the gene of interest and the internal reference gene (β-actin).

2.4. *Statistical analysis*

To evaluate the association of gene expressions with response and TTP, gene expression levels were categorised into low and high values. To determine cutoff values, the maximally selected χ^2 method was employed.[14–16] For each observed value, patients were classified as falling below or equal to that value, or above that value. The maximally selected χ^2-test statistic was used to compare the response rates of the two resulting groups of patients (below or equal to the value versus above the value). The value that yielded the largest χ^2-test statistic (the maximal χ^2 statistic) was selected as the optimal cutoff point. To determine the P-value associated with the maximal χ^2 statistic, we performed 2000 bootstrap-like simulations. For each simulation, a randomly selected value was drawn (with replacement) from the set of observed values and assigned to each of the observed responses; the maximal χ^2 statistic was calculated based on this set of randomly matched values and responses. The corrected P-value was calculated as the proportion of the 2000 simulated maximal statistics that was larger than the original maximal χ^2 statistic. This analysis was repeated using the log-rank test to compare TTP. If promising significant predictive variables were found on this analysis, multivariate logistic regression analysis was preformed for the response to fluoropyrimidines. Stepwise variable selection was done using a significance level of 0.01 for entering into or remaining in the model.

All reported P-values are two-sided, and the level of significance was set at $P < 0.05$, except for stepwise variable selection. All analyses were performed using the statistical software package R, version 2.4.1 and the SAS statistical package, version 9.1.3 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. *Demographics and patients assessed for response and TTP*

A total of 92 Japanese patients (54 men and 38 women; median age, 61 years; range 27–77 years) were evaluated (Table 2). Seventy of these patients (43 men and 27 women; median age, 62 years; range 27–77 years) had received 5-FU/LV regimens, and 63 had received CPT-11 as second-line chemotherapy; 43 patients with CPT-11 and 20 patients with CPT-11/ mitomycin C. Gene expression levels of TS, DPD, FPGS, GGH, RFC1, TOPO I, ERCC1, MGMT, GSTpi, EGFR, VEGF and cyclin E were assessed in all patients, and the relations of these levels to response and TTP were examined.

The response to first-line fluoropyrimidine-based chemotherapy was CR in 2 patients (2%), PR in 39 (42%), NC in 30 (33%) and PD in 21 (23%). The median TTP was 5.1 months. The response to first-line 5-FU/LV treatment was CR in 2 patients (3%), PR in 31 (44%), NC in 21 (30%) and PD in 16 (23%). The median TTP was 5.0 months. For second-line CPT-11-based chemotherapy, the response was PR in 9 patients (14%), NC in 32 (50%) and PD in 22 (36%). The median TTP was 3.5 months.

Table 1 – Primers and probes

Gene	GenBank Accession	Forward primer (5′–3′)	Reverse primer (5′–3′)	Taqman probe (5′–3′)
β-Actin	NM_001101.2	GAGCGCGGCTACAGCTT	TCCTTAATGTCACGCACGATTT	ACCACCACGGCCGAGCGG
TS	NM_001071.1	GCCTCGGTGTGCCTTTCA	CCCGTGATGTGCGCAAT	TCGCCAGCTACGCCCTGCTCA
DPD	NM_000110.2	AGGACGCAAGGAGGGTTTG	GTCCGCCGAGTCCTTACTGA	CAGTGCCTACAGTCTCGAGTCTGCCAGT
FPGS	M98045	GGCTGGAGGAGACCAAGGAT	CATGAGTGTCAGGAAGCGGA	CAGCTGTGTCTCCATGCCCCCCTAC
GGH	NM_003878	GCGAGCCTCGAGCTGTCTA	AATATTCCGATGATGGGCTTCTT	ACCCCACGGCGACACCGC
RFC1	NM_194255.1	CATCGCCACCTTTCAGATT	TGGCAAAGAACGTGTTGAC	CCCGAAGACCAGGGCACAGA
TOPO I	NM_003286	TGTAGCAAAGATGCCAAGGT	TGTTATCATGCCGGACTTCT	CCTTCTCCTCCTCCAGGACATAAGTGGA
ERCC1	NM_001983.2	GGGAATTTGGCGACGTAATTC	GCGGAGGCTGAGGAACAG	CACAGGTGCTCTGGCCCAGCACATA
MGMT	NM_002412	CGTTTTCCAGCAAGAGTCGTT	GAAATCACTTCTCCGAATTTCACA	TCAGCAGCTTCCATAACACCTGTCTGG
GSTpi	X06547	CCTGTACCAGTCCAATACCATCCT	TCCTGCTGGTCCTTCCCATA	TCACCTGGGCCGCACCCTTG
EGFR	X00588	TGCGTCTCTTGCCGGAAT	GGCTCACCCTCCAGAAGGTT	ACGCATTCCCTGCCTCGGCTG
VEGF	NM_003376.4	AGTGGTCCCAGGCTGCAC	TCCATGAACTTCACCACTTCGT	TGATTCTGCCCTCCTCCTTCTGCCAT
Cyclin E	NM_001238	CAGCTTATTGGGATTTCATCTTT	ATACGCAAACTGGTGCAACT	TGCAGCCAAACTTGAGGAAATCTATCC

TS: thymidylate synthase, DPD: dihydropyrimidine dehydrogenase, FPGS: folylpolyglutamate synthetase, GGH: γ-glutamyl hydrolase, RFC1: reduced folate carrier 1, TOPO I: DNA topoisomerase I, ERCC1: excision repair cross-complementing 1, MGMT: O^6-methylguanine-DNA methyltransferase, GSTpi: glutathione S-transferase pi, EGFR: epidermal growth factor receptor, VEGF: vascular endothelial growth factor.

Table 2 – Characteristics of 92 patients treated with first-line fluoropyrimidine

Characteristic	Frequency
Median age, years (range)	61 (27–77)
Gender	
Male	54
Female	38
PS	
0	65
1	26
2	1
Metastatic site	
Liver	64
Lung	44
Lymph node	27
Peritoneum	19
Ovary	2
Bone	1
Regimens	
5-FU/l- LV	70
5-FU continuous infusion	10
UFT/LV	9
UFT	1
TS-1	2
Clinical response	
Complete response	2
Partial response	39
Stable disease	30
Progressive disease	21

PS: Performance status of Eastern Cooperative Oncology Group; 5-FU: 5-fluorouracil; it l-LV: *l*-leucovorin; UFT, uracil-tegafur; LV: leucovorin.

3.2. *Gene expression levels and clinical outcome of patients receiving first-line fluoropyrimidine-based treatment*

Median gene expression levels relative to the level of the house-keeping gene β-actin, used as an internal reference, are shown in Table 3. For descriptive purposes, we call gene expressions below the designated cut-point 'low' while those above the designated cut-point are called 'high'. The results of univariate analysis for response and TTP are shown in Table 3. Low TS, low GGH, high RFC1, high MGMT and low cyclin E expression levels were significantly associated with a good response to fluoropyrimidines on univariate analysis ($P = 0.0030$, 0.0250, 0.0120, 0.0030 and 0.0020, respectively). Low TS, low GGH, high RFC1, low TOPO I, high MGMT, low GSTpi and low cyclin E expression levels significantly correlated with a long TTP in patients given fluoropyrimidine on univariate analysis ($P = 0.027$, 0.023, 0.045, 0.025, 0.039, 0.002 and 0.009, respectively).

Seventy of the 92 patients given fluoropyrimidines had received 5-FU/LV regimens. Low TS, high FPGS, low GGH, high RFC1, high MGMT and low cyclin E expression levels were significantly associated with a good response to 5-FU/LV on univariate analysis ($P = 0.0060$, 0.0350, 0.0355, 0.0415, 0.0030 and 0.0015, respectively). Low GGH, low GSTpi, high VEGF and low cyclin E expression levels significantly correlated with a long TTP in patients given 5-FU/LV on univariate analysis ($P = 0.016$, 0.045, 0.032 and 0.003, respectively).

3.3. *Multiple logistic regression analysis of clinical response in patients receiving first-line fluoropyrimidines*

Among the expressions of TS, GGH, RFC1, MGMT and cyclin E, which were significantly associated with response as well as TTP in patients given fluoropyrimidines, TS and MGMT expressions continued to be independent predictors of the response to fluoropyrimidines on multiple logistic regression analysis. The clinical responses of patient groups divided according to the cutoff values of TS and MGMT expressions are shown in Table 4. The sensitivity of low TS or high MGMT for the response to fluoropyrimidines (responding patients in the low TS and high MGMT groups/responding patients in all groups) was 0.88, and the specificity of high TS and low MGMT for the response to fluoropyrimidines (non-responding patients in the high TS and low MGMT groups/non-responding patients in all groups) was 0.59. The positive predictive value of low TS or high MGMT for the response to fluoropyrimidines was 0.63, and the negative predictive value of high TS and low MGMT was 0.86. The shortest TTP was observed in the group of patients with TS above and MGMT below the respective cutoff values ($P = 0.083$) (Fig. 1). The median TTP was 5.7 months in patients with low TS or high MGMT and 3.3 months in those with both high TS and low MGMT.

3.4. *Gene expression levels and clinical outcome of patients receiving second-line CPT-11-based treatment*

The results of univariate analyses for response and TTP are shown in Table 5. High TS, high FPGS, low ERCC1, high MGMT, high GSTpi and low VEGF expressions significantly correlated with a good clinical response to second-line CPT-11 treatment ($P = 0.0085$, 0.0145, 0.0015, 0.0215, 0.0155 and 0.0165, respectively). No significant correlation was demonstrated between any gene expression and TTP for second-line chemotherapy.

4. Discussion

Our primary end-point was to clarify the gene expression levels of enzymes involved in the targeting, metabolism or catabolism of fluoropyrimidines, the metabolism or transport of folinic acid, DNA-repair systems and drug detoxification systems and thereby identify predictors of clinical outcomes in patients with CRC who receive fluoropyrimidines. The presence of both high TS and low MGMT expression levels was found to be a significant predictor of a poor response to fluoropyrimidine therapy. Clinically, this combination would have an important role in the selection of first-line treatment for CRC with regimens such as FORFIRI (CPT-11/5-FU/LV) or CPT-11 alone.

MGMT is a DNA-repair enzyme that removes alkyl adducts from O^6-methylguanine. Since the MGMT gene is usually not mutated or deleted in human cancers, loss of MGMT function is probably due mostly to epigenetic changes.[17] Abnormal MGMT activity causes O^6-methylguanine to accumulate in cellular DNA, potentially resulting in the activation of oncogenes or inactivation of tumour suppressor genes, followed

Table 3 – Univariate analysis of gene expression levels and clinical outcome (A: response, B: time to progression) in 92 patients treated with first-line fluoropyrimidine-based regimens

Gene	Number of patients	mRNA expression levels relative to β-actin × 10^{-3}, Median (range)	Cut-point	Bootstrap P-value	RR (%) in low group	RR (%) in high group
A: Correlation between response and gene expression						
TS	92	1.4 (0–10.9)	1.37	0.0030	60.0	29.8
DPD	87	0.22 (0–1.16)	0.48	0.0995	41.3	66.7
FPGS	92	0.49 (0.04–1.69)	0.49	0.1035	36.0	54.8
GGH	91	2.17 (0–9.94)	1.21	0.0250	68.4	38.9
RFC1	92	1.67 (0–8.79)	0.87	0.0120	18.8	50.0
TOPO I	92	1.71 (0–4.93)	1.59	0.1845	53.5	36.7
ERCC1	92	0.41 (0–2.95)	0.38	0.1680	36.4	52.1
MGMT	92	1.75 (0–64.73)	2.59	0.0030	34.3	72.0
GSTpi	92	2.19 (0.48–7.6)	3.71	0.0815	48.2	18.2
EGFR	92	0.86 (0–5.31)	0.48	0.2140	57.9	41.1
VEGF	92	3.86 (0.88–24.3)	4.83	0.2890	40.0	55.6
Cyclin E	92	0.51 (0–2.28)	0.99	0.0020	50.7	13.3

Gene	Number of patients	Cut-point	Bootstrap P-value	Median TTP (day) in low group	Median TTP (day) in high group
B: Correlation between time to progression (TTP) and gene expression					
TS	92	1	0.027	230	132
DPD	87	0.31	0.128	141	232
FPGS	92	0.75	0.293	141	165
GGH	91	4.87	0.023	151	64
RFC1	92	0.87	0.045	68	155
TOPO I	92	2.68	0.025	155	105
ERCC1	92	0.6	0.296	137	165
MGMT	92	3.22	0.039	134	253
GSTpi	92	2.47	0.002	169	86
EGFR	92	0.6	0.161	148	141
VEGF	92	7.06	0.108	141	232
Cyclin E	92	1.09	0.009	155	57

Table 4 – Predictive value of TS, MGMT and their combination for the response to fluoropyimidne (TS and MGMT were selected as independent variables in multiple logistic regression analysis)

Gene expression status	Number of non-responding patients	Number of responding patients	RR (%)	P-value (χ^2-test)
All	51	41	44.6	–
Low MGMT	44	23	34.3	0.0012
High MGMT	7	18	72.0	
Low TS	18	27	60.0	0.0036
High TS	33	14	29.8	
High MGMT or low TS	21	36	63.2	<.0001
Low MGMT and high TS	30	5	14.3	

TS: thymidylate synthase, MGMT: O^6-methylguanine-DNA methyltransferase.

by carcinogenesis.[17–20] In previous studies, the significance of the correlation between MGMT promoter hypermethylation or loss of MGMT expression and patients' prognosis was controversial,[21–25] and its prognostic value for patients treated with specific regimens of anticancer agents remains a matter of debate.

As for CRC, abnormal MGMT expression has been examined in many studies in connection with microsatellite instability (MSI) or CpG island methylator phenotype (CIMP).[25–27] Kohonen-Corish and colleagues reported that low-MSI characterised a distinct subgroup of patients with stage C colon cancer who had poor outcomes.[25] They also found that loss or reduced MGMT protein expression was associated with the low-MSI phenotype, but was not a prognostic factor for overall survival in colon cancer.[25] Recent studies have shown that 5-FU-based adjuvant chemotherapy improves overall

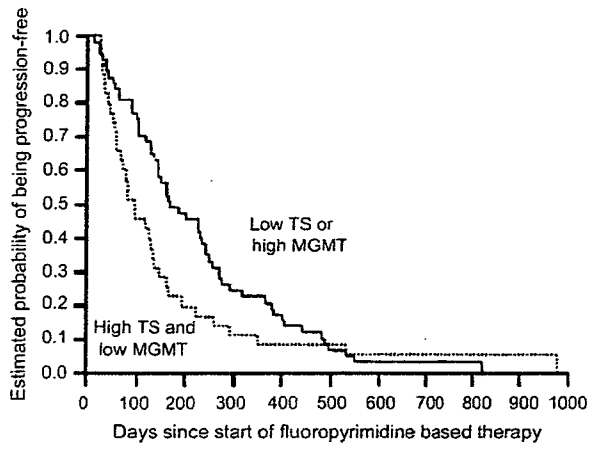

Fig. 1 – Time to progression in patients given fluoropyrimidines according to the cutoff levels of TS and MGMT expression ($p = 0.083$). TS: thymidylate synthase, MGMT: O^6-methylguanine-DNA methyltransferase.

survival in patients who have non-high-MSI CRC as compared with those who have high-MSI CRC.[28,29] On the other hand, Nagasaka and colleagues reported that the later the tumour stage at diagnosis, the less likely MGMT promoter will be methylated; in addition, the recurrence rate associated with oral fluoropyrimidine-based adjuvant chemotherapy was significantly higher in patients with unmethylated MGMT than in those with methylated MGMT (not adjusted for MSI status).[27] To date, the value of MGMT as a prognostic or predictive marker for fluoropyrimidines remains controversial. In this study, MGMT expression was found to be the most significant biomarker for the response to fluoropyrimidines. Furthermore, MGMT combined with TS, one of the most promising enzymes for predicting clinical outcomes of fluoropyrimidine treatment, was shown to be a powerful predictor of the response to fluoropyrimidines.[1,30]

In the subset analysis of 70 patients who received 5-FU/LV as first-line chemotherapy, three enzymes involved in the formation, degradation and transfer into cells of folates, i.e. FPGS, GGH and RFC1, were significantly related to response and TTP. It suggests that high FPGS, low GGH and high RFC1 activity promotes optimal modulation of 5-FU by LV, probably by augmenting the retention of high levels of reduced polyglutamated folates in tumours. We also investigated the predictive value of gene expression levels in patients who received second-line CPT-11-based chemotherapy. Ideally, predictive markers should be evaluated by large prospective randomised trials in patients receiving first-line chemotherapy, and our results suggested that TS, FPGS, ERCC1, MGMT,

Table 5 – Univariate analysis of gene expression levels and clinical outcome (A: response, B: time to progression) in 63 patients treated with second-line CPT-11-based regimens

Gene	Number of patients	Cut-point	Bootstrap *P*-value	RR (%) in low group	RR (%) in high group
A: Correlation between response and gene expression					
TS	63	1.43	0.0085	5.7	25.0
DPD	60	0.42	0.4735	11.5	25.0
FPGS	63	0.83	0.0145	10.9	37.5
GGH	62	1.1	0.2390	0.0	15.4
RFC1	63	0.77	0.3420	28.6	12.5
TOPO I	63	2.13	0.1630	10.6	25.0
ERCC1	63	0.11	0.0015	44.4	9.3
MGMT	63	1.74	0.0215	6.3	22.6
GSTpi	63	2.43	0.0155	5.3	28.0
EGFR	63	0.39	0.0810	33.3	11.1
VEGF	63	1.97	0.0165	37.5	10.9
Cyclin E	63	1.02	0.056	11.1	33.3
Gene	Number of patients	Cut-point	Bootstrap P- value	Median TTP (day) in low group	Median TTP (day) in high group
B: Correlation between time to progression (TTP) and gene expression					
TS	63	1.38	0.089	97	103
DPD	60	0.14	0.231	99	102
FPGS	63	0.35	0.096	151	98
GGH	62	1.04	0.058	57	102
RFC1	63	1.32	0.221	103	98
TOPO I	63	2.71	0.100	98	113
ERCC1	63	0.11	0.465	119	99
MGMT	63	1.64	0.086	87	110
GSTpi	63	2.89	0.060	92	155
EGFR	63	0.39	0.119	118	98
VEGF	63	2.78	0.243	151	92
Cyclin E	63	0.78	0.321	87	113

TS: thymidylate synthase, DPD: dihydropyrimidine dehydrogenase, FPGS: folylpolyglutamate synthetase, GGH: γ-glutamyl hydrolase, RFC1: reduced folate carrier 1, TOPO I: DNA topoisomerase I, ERCC1: excision repair cross-complementing 1, MGMT: O^6-methylguanine-DNA methyltransferase, GSTpi: glutathione S-transferase pi, EGFR: epidermal growth factor receptor, VEGF: vascular endothelial growth factor.

GSTpi and VEGF are candidate genes for the prediction of the response to CPT-11.

On the other hand, the methodologies in this study to evaluate mRNA levels in the primary tumours with real time RT-PCR offered several advantages. It can easily overcome problems associated with sample volume and tumour heterogeneity by obtaining specimens from primary tumours by laser-captured microdissection. Furthermore, formalin-fixed, paraffin-embedded specimens of the primary tumours are obtained from nearly all patients with CRC. However, only a few studies have examined the relation between levels of molecular markers in primary colorectal tumours and associated metastases.[10,31,32] Since we analysed samples from primary tumours to predict the response of metastatic lesions to chemotherapy, the clinical value of our technique must be validated in larger prospective studies. In addition, we should bear in mind that all patients in our study were Japanese. The potential importance of ethnicity in studies of gene expressions should be taken into account in prospective clinical trials in the future.

Our findings suggest that the presence of both high TS and low MGMT expression is a significant predictor of a poor response to fluoropyrimidine treatment, and the diagnostic value of these predictive markers should be validated in larger cohorts of patients. Furthermore, future studies should also evaluate predictive markers for chemotherapy in patients who receive oxaliplatin-based or CPT-11-based regimens as first-line treatment. A combined analysis of these results might provide new insights into the optimal design for randomised clinical trials.

Conflicts of interest statement

Yoshihiro Okayama and Toshinori Oka are employees of Optimal Medication Research Laboratory, Taiho Pharmaceutical Co., Ltd., Tokushima, Japan.

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Phase II Study of Oxaliplatin in Japanese Patients with Metastatic Colorectal Cancer Refractory to Fluoropyrimidines

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Background: Although oxaliplatin (L-OHP) combined with infusional 5-fluorouracil (5-FU) and leucovorin (LV) is one of the standard chemotherapy regimens for metastatic or recurrent colorectal cancer, its introduction to Japan has been delayed. Phase I studies of L-OHP monotherapy in Japan showed no dose-limiting toxicity at the internationally recommended dose of 130 mg/m^2 every 3 weeks, as well as no racial differences in pharmacokinetics as compared with Western subjects. This study aimed to clarify the efficacy and safety of L-OHP monotherapy in patients with metastatic colorectal cancer refractory to fluoropyrimidines.
Methods: Patients with metastatic colorectal cancer who had failed to respond to fluoropyrimidine-based chemotherapy received L-OHP at a dose of 130 mg/m^2 every 3 weeks.
Results: Sixty patients were enrolled. Two ineligible patients and one untreated patient were excluded from analysis. The median number of treatment cycles was 4 (range, 1–6). The overall response rate was 9% (5/57, 95% CI: 4–19%). The median time to progression was 2.7 months, and the median survival time was 11.1 months. Grade 3 major toxicity comprised thrombocytopenia (12%) and nausea (11%). There was no grade 4 toxicity. All patients experienced mild to moderate sensory neurotoxicity without functional impairment interfering with activities of daily living.
Conclusions: The efficacy and toxicity of L-OHP in Japanese patients with metastatic colorectal cancer refractory to fluoropyrimidines is apparently similar to those in Western patients.

Key words: oxaliplatin – monotherapy – colorectal cancer – phase II

INTRODUCTION

Oxaliplatin (L-OHP) is a platinum analogue that differs from cisplatin or carboplatin by having a diaminocyclohexane moiety that is retained after drug aquation (1,2). This bulky side chain is believed to contribute to its distinct spectrum of activity as demonstrated in preclinical models and clinical trials (3). Whereas other platinum antitumor agents show little or no activity against colorectal cancer, preclinical studies have shown that L-OHP was significantly active against six of the eight colorectal cancer cell lines in the National Cancer Institute's Human Tumor Cell Line Screen panel and inhibited tumor-colony formation in one third of explanted human colorectal cancers (4).

As first-line monotherapy in patients with colorectal cancer, L-OHP produced response rates of 12–24%, with a median progression-free survival time (PFS) of approximately 4 months and a median survival time (MST)

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of 13–15 months (5,6). Randomized controlled studies have shown that regimens combining L-OHP with infusional 5-fluorouracil (5-FU) plus leucovorin (LV) (FOLFOX) have significantly higher response rates and longer PFS with acceptable toxicity, as compared with infusional 5-FU plus LV regimens (FL) (7,8). The efficacy of FOLFOX4 as first-line treatment for metastatic colorectal cancer is supported by the results of the North American Inter-group study N9741. In that study, patients treated with FOLFOX4 had significantly longer PFS, better overall survival, a higher response rate, and lower toxicity than patients treated with a bolus FL plus irinotecan (CPT-11) regimen (IFL) (9). These results have established L-OHP as a key drug for the treatment of metastatic colorectal cancer.

The clinical development of L-OHP in Japan has been delayed. A phase I study in which Japanese patients received L-OHP in doses of 90 mg/m^2 ($n = 3$) or 130 mg/m^2 ($n = 6$) showed no dose-limiting toxicity and no racial differences in pharmacokinetics as compared with Western patients. These results suggested that the internationally recommended dose of L-OHP 130 mg/m^2 every 3 weeks was feasible for Japanese patients (10). However, the small number of patients in the phase I study precluded conclusions about dosage. To further clarify the efficacy and safety of L-OHP monotherapy, we conducted a phase II study in Japanese patients with metastatic colorectal cancer refractory to previous treatment with 5-FU.

PATIENTS AND METHODS

Study Design

The primary endpoint was response rate. The expected response rate was 15%, and the required sample size was estimated to be 49 patients, so that the lower limit of the 95% confidence interval (CI) would not be less than 5%. Thus, 60 patients were scheduled to be recruited. If none of the first 20 patients responded to treatment, the study would be terminated.

Eligibility Criteria

Eligibility criteria were as follows: (i) histologically confirmed colorectal cancer; (ii) unresectable or metastatic disease; (iii) a history of treatment with one prior fluoropyrimidine-based regimen, excluding adjuvant therapy; (iv) radiologically confirmed progressive disease (PD) during the prior chemotherapy; (v) 4 weeks' rest from the last dose of prior chemotherapy; (vi) a performance status of ≤ 2 on the Eastern Cooperative Oncology Group scale; (vii) an age of ≥ 20 to ≤ 75 years; (viii) a life expectancy of ≥ 12 weeks; (ix) at least one measurable lesion according to Response Evaluation Criteria in Solid Tumors (RECIST); (x) adequate organ functions, leukocytes $\geq 3000/mm^3$ and $\leq 10\,000/mm^3$, neutrophils $\geq 1500/mm^3$, platelets $\geq 100\,000/mm^3$, total bilirubin ≤ 2-fold the upper limit of normal, aspartate aminotransferase and alanine aminotransferase ≤ 2.5-fold the upper limit of normal and creatinine ≤ 1.5-fold the upper limit of normal; and (xi) written informed consent. Exclusion criteria were as follows: (i) a history of blood transfusion or treatment with G-CSF within 7 days before entry; (ii) a history of severe drug allergy; (iii) prior therapy with platinum-containing chemotherapy; (iv) prior hepatic arterial infusion of antitumor agents; (v) symptomatic brain metastasis; (vi) massive ascites or pleural effusion; (vii) no measurable lesions besides bone metastasis; (viii) poorly controlled hypercalcemia; (ix) poorly controlled hypertension; (x) poorly controlled diabetes; (xi) active infection; (xii) positive for hepatitis B surface antigen, hepatitis C virus antibody, or human immunodeficiency virus antibody; (xiii) severe diarrhea; (xiv) heart disease such as congestive heart failure, symptomatic coronary artery disease, or poorly controlled arrhythmias; (xv) severe lung disease such as interstitial pneumonitis, pulmonary fibrosis, or emphysema; (xvi) mental disease or a history of central nervous system disorders; (xvii) peripheral sensory neuropathy; (xviii) women who refuse to use contraception or are pregnant or nursing; and (xix) patients whom the investigators considered unsuitable for this study.

Treatment Schedule

L-OHP was administered at a dose of 130 mg/m^2 (diluted in 500 ml of 5% glucose) as an intravenous infusion over the course of 2 h. Treatment was repeated every 3 weeks and continued until the completion of six cycles or the confirmation of tumor progression or unacceptable toxicity. A 5-HT$_3$ receptor antagonist (40 μg/kg of granisetron hydrochloride) was given intravenously before treatment with L-OHP.

Before each dose of L-OHP, the following conditions had to be met: leukocyte count $\geq 2500/mm^3$, platelets $\geq 75\,000/mm^3$, diarrhea $\leq$ grade 1, and other toxicity (except for neurotoxicity and alopecia) $\leq$ grade 2. If these conditions were not satisfied, treatment was postponed until recovery. The dose of L-OHP was reduced to 90 mg/m^2 if grade 4 leukopenia, neutropenic fever, or other grade 3 adverse events (except nausea, alopecia, or electrolyte imbalance) occurred during the preceding cycle. If treatment was not possible within 22 days after the day scheduled according to the protocol, the patient was withdrawn from the study. If neurotoxicity remained on the day scheduled for treatment, administration of L-OHP was delayed until the disappearance of such toxicity. If the patient did not recover from neurotoxicity within 7 days, the dose was reduced to 90 mg/m^2 and given 7 days after the originally scheduled treatment day, regardless of the presence or absence of neurotoxicity. In patients in whom the dose of L-OHP had already been reduced to 90 mg/m^2, treatment was continued without further dose reduction, even if a lower dose was indicated because of neurotoxicity.

Evaluations

Tumor lesions were assessed by computed tomographic scanning, magnetic resonance imaging, or both every 4 weeks. Response was evaluated according to RECIST by an independent panel of diagnostic radiologists. Laboratory tests, physical examinations, and symptom assessments were performed weekly. Toxic effects other than neurotoxicity were evaluated according to the National Cancer Institute-Common Toxicity Criteria (NCI-CTC), version 2.0. Neurological toxicity was assessed according to the Neurotoxicity Criteria of Debiopharm (DEB-NTC; see Table 4). PFS was calculated from the date of initiating therapy to the date of radiologically confirming PD according to RECIST. Survival time was calculated from date of initiating therapy to the date of death. For patients lost to follow-up, data were censored on the date on which the patient was last known to have stable disease to calculate PFS; to calculate survival time data were censored on the date on which the patient was last known to be alive.

Ethics

This trial was approved by the institutional review board at each participating hospital and was conducted in accordance with Japanese Good Clinical Practice guidelines.

RESULTS

Subjects

Sixty patients were enrolled in this study from April 2001 through July 2002. In all patients, disease progression during previous treatment with 5-FU-based regimens was confirmed by an independent panel of diagnostic radiologists. Two patients were ineligible: 1 had no target lesion and the other had neuropathy caused by cervical spondylosis. L-OHP was not administered to another patient, who was suspected to have double cancers. Data from the remaining 57 patients were analyzed to assess efficacy and safety.

Patient characteristics are summarized in Table 1. The median age was 61 years (range, 23–74). All patients had a performance status of 0 or 1 at baseline. The number of organs involved by metastatic lesions was 1 (74%) in 42 patients and 2 or more in 15 (26%). Metastases were present in the liver alone in 23 patients (40%), in the lung alone in 12 (21%), in the liver plus other sites in 12 (21%) and in other sites in 10 (18%). Previous chemotherapy was 5-FU ± *l*-LV in 39 patients, CPT-11 + 5-FU ± *l*-LV in 17 and UFT/LV in one. Six patients had received radiation therapy.

Administration of L-OHP

The total number of cycles administered was 166. The median number of cycles per patient was four (range, 1–6).

Table 1. Patient characteristics ($n = 57$)

Characteristics	No. of patients	
Gender	Male/Female	34/23
Age	Median (range)	61 (23–74)
Performance status (ECOG)	0/1	36/21
Primary site	Colon/Rectum/Colon and Rectum	30/26/1
Histology	W/M/P/Unknown*	15/38/3/1
Metastatic sites of target lesions	Liver/Lung/Others	35/16/20
Number of metastatic sites	1 /≥2	42/15
Prior treatment		
Chemotherapy 5-FU based	With/without irinotecan	40/17
Surgery	+/−	51/6
Radiation	+/−	6/51

*W/M/P: well/moderately/poorly differentiated adenocarcinoma; ECOG, Eastern Cooperative Oncology Group scale.

L-OHP was administered on the scheduled day or within a 3-day delay in 148 cycles (89%). Nine patients completed the planned six cycles. The dose of L-OHP was reduced during 17 cycles (8%) in nine patients (16%). Persistent neurotoxicity necessitated dose reduction during two cycles (1.2%) in two patients (4%) and delayed treatment during two cycles (1.2%) in two patients (4%). The median dose intensity was 129 mg/m^2 every 3 weeks (range, 93–130), corresponding to 99% (range, 72–100%) of the planned dose. Treatment was stopped because of disease progression in 45 patients (79%). One patient (2%) discontinued treatment because of grade 3 vomiting, persisting even after dose reduction. Two patients (4%) refused to continue treatment for other reasons than toxicity.

Antitumor Effects

Table 2 shows the response to therapy. Five patients (9%) had partial responses and 27 (47%) had stable disease. The response (CR + PR) rate was thus 9% (95% CI: 3–19%), and the disease-stabilization (CR + PR + SD) rate was 56% (95%

Table 2. Response

	No. of patients (%)
Complete response	0 (0)
Partial response	5 (9)
Stable disease	27 (47)
Progressive disease	25 (44)
Response rate (95% C.I.)	9 (3–19)
Disease-stabilization rate (95% C.I.)	56 (42–69)

C.I., Confidence Interval.

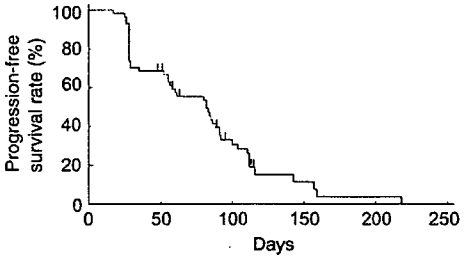

Figure 1. Progression-free survival.

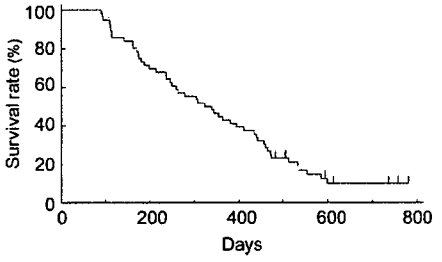

Figure 2. Overall survival.

CI: 42–69%). The median time to response was 1.6 months (95% CI: 0.9–2.1 months), and the median duration of response was 3.0 months (95% CI: 1.8–4.3 months). The median PFS was 2.7 months (Fig. 1), and the MST was 11.1 months with a 1-year survival rate of 43% (Fig. 2). The MST calculated from the date of initiating the previous regimen of chemotherapy was 20.2 months (95% CI: 16.4–22.0 months).

Table 3. Incidences (%) of toxic effects other than neurotoxicity (National Cancer Institute-Common Toxicity Criteria, vers. 2.0)

	No. of patients			
	Grade 1	Grade 2	Grade 3	Grade 4
Hematologic				
Hemoglobin	19	12	2	0
Leukocytes	32	11	2	0
Neutropenia	19	16	4	0
Lymphopenia	2	21	2	–
Platelets	26	12	12	0
Nonhematologic				
Anorexia	33	49	7	0
Nausea	37	35	7	–
Vomiting	28	23	11	0
Diarrhea	18	21	0	0
Constipation	14	2	0	0
Fatigue	33	14	2	
Injection site reaction	12	12	0	0
Fever	9	7	0	0
Headache	18	2	0	0

Table 4. Incidences (%) of neurotoxicity (Neurotoxicity Criteria of Debiopharm)

Paresthesias, dysesthesias	No. of patients		
	Grade		
	1	2	3
Cold-related transient paresthesias/ dysesthesias	21	79	–
Paresthesias/dysesthesias without pain	26	35	–
Paresthesias/dysesthesias with pain	23	11	–
Functional impairment interfering with activities of daily living	–	–	0

Grade 1, within 7 days; Grade 2, more than 7 days; Grade 3, functional impairment interfering with activities of daily living.

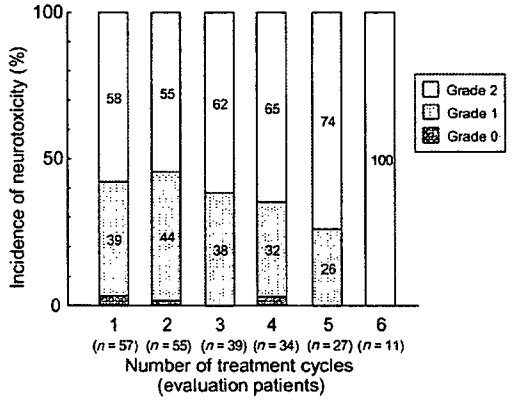

Figure 3. Treatment cycles and neurotoxicity grade.

Toxicity

Table 3 summarizes the incidences of toxic effects other than neurotoxicity. There was no grade 4 toxicity. The most common types of severe toxicity were gastrointestinal reactions and thrombocytopenia. No grade 3 toxic effect had an incidence above 12%. Table 4 shows the incidences of neurotoxicity. Cold-related transient paresthesia/dysesthesia occurred in all patients. Cold-related paresthesia/dysesthesia was grade 1 in 12 patients (21%) and grade 2 in 45 (79%). Persistent paresthesia/dysesthesia without pain was grade 1 in 15 patients and grade 2 in 20 (35%). Persistent paresthesia/dysesthesia with pain was grade 1 in 13 patients (23%) and grade 2 in 6 (11%).

Figure 3 shows the relation between the number of treatment cycles and the incidence of neurotoxicity according to grade. Nearly all patients had neurotoxicity in all cycles, and the incidence of grade 2 neurotoxicity increased gradually

with increasing numbers of treatments cycles. The median time to the resolution of neurotoxicity after the last dose of L-OHP was 9 days for cold-related transient paresthesia/dysesthesia ($n = 57$), 7 days for persistent paresthesia/dysesthesia without pain ($n = 24$), and 4 days for persistent paresthesia/dysesthesia with pain ($n = 11$).

DISCUSSION

L-OHP is a key drug for the treatment of metastatic colorectal cancer and FOLFOX regimens are recognized as one standard regimen for first-line chemotherapy (7,9). However, the introduction of L-OHP to Japan has been delayed. It was unclear whether the efficacy and safety of L-OHP in Japanese patients would be similar to those in Western patients. Therefore, initial clinical trials were conducted to examine the feasibility of using L-OHP alone or in combination with other drugs in Japan. A phase I study of L-OHP monotherapy showed no dose-limiting toxicity or racial differences in pharmacokinetics (10). That study recommended the internationally used dosage of L-OHP 130 mg/m^2 every 3 weeks as monotherapy for the further Japanese trials.

Neurotoxicity is the major drawback of L-OHP. The incidence of grade 3 neurotoxicity increases rapidly when the cumulative dose of L-OHP exceeds 800–1000 mg/m^2 or higher (11). In our study, no patient had grade 3 neurotoxicity. The median number of treatment cycles administered per patient was four (range, 1–6). This relatively short duration of treatment might have resulted in the absence of grade 3 neurotoxicity. Nonetheless, the incidence of grade 2 neurotoxicity increased with increasing numbers of treatment cycles. Although there was no grade 4 toxicity in this study, major grade 3 gastrointestinal toxic effects such as nausea, vomiting and appetite loss occurred in 7–11% of the patients. The major hematologic toxicity was thrombocytopenia, and the incidence of grade 3 thrombocytopenia was 12%. Both hematologic and nonhematologic toxic effects, including neurotoxicity, were generally mild. The relative dose intensity of L-OHP was 99%. These results suggest that monotherapy with L-OHP at a dose of 130 mg/m^2 every 3 weeks is feasible for Japanese patients.

Two phase II studies of single-agent L-OHP as second-line therapy for patients with metastatic colorectal cancer previously treated with 5-FU ± LV have been performed in Europe. The objective response rates in those studies were 10 and 11%, respectively (12). Our study, in which L-OHP was given as second-line treatment similar to recent Western trials, yielded a comparable response rate of 9% (8/57).

Although one limitation of our study is the possibility of selection bias, MSTs calculated from the date of starting treatment with L-OHP and from the date of initiating the previous regimen of chemotherapy were 11.1 and 20.2 months, respectively. Recently, the MST of patients with metastatic colorectal cancer has been reported to be about 20 months (9,13). This improvement in survival has been attributed to the increased use of three key drugs, 5-FU, CPT-11 and L-OHP (14,15). The MST in our study suggests that the inclusion of L-OHP in the therapeutic strategy for Japanese patients with colorectal cancer may further prolong survival, resulting in results comparable to those of recent Western trials of regimens including L-OHP.

All of our subjects had received first-line chemotherapy with fluoropyrimidine-based regimens, including those containing CPT-11. Progressive disease during these prior regimens was strictly confirmed by an independent panel of diagnostic radiologists. The median PFS in our study was 2.7 months. In previous studies of second-line chemotherapy with CPT-11 after failure to 5-FU, median PFS was 4 months, with MST ranging from 10 to 14 months (16,17). In contrast, monotherapy with L-OHP as second-line treatment has resulted in an MST of 8.2 months (12). Although there are limitations in comparing the results of different studies, available evidence suggests that monotherapy with L-OHP may not be as effective as irinotecan or other combination chemotherapy regimens when used for second-line therapy. In Europe, monotherapy with L-OHP had been approved in the second-line setting at first. Thereafter, in a phase III study for the patients in whom IFL had failed, monotherapy with L-OHP showed a lower response rate and a shorter progression-free survival time than combination with 5-FU (18), so monotherapy with L-OHP cannot be recommended after failure of 5-FU and irinotecan at present.

Clinically, L-OHP is often combined with infusional FL. L-OHP in combination with infusional 5-FU and *l*-LV was approved in Japan in March 2005, but the dosage of L-OHP is limited to 85 mg/m^2 every 2 weeks. In Western countries, several regimens including various dose levels of L-OHP once every 2 or 3 weeks, such FOLFOX6 (13) and FOLFOX7 (19), have been developed. Our findings suggest that a dosage of L-OHP similar to that used in Western trials may be feasible in Japan.

In conclusion, our results suggest that the efficacy and toxicity of monotherapy with L-OHP at a dose of 130 mg/m^2 every 3 weeks in Japanese patients with metastatic colorectal cancer refractory to fluoropyrimidines are similar to those reported in Western trials.

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Conflict of interest statement
None declared.

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A phase I and pharmacokinetic study of NK105, a paclitaxel-incorporating micellar nanoparticle formulation

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This phase I study was designed to examine the maximum tolerated dose (MTD), the dose-limiting toxicities (DLTs), the recommended dose (RD) for phase II, and the pharmacokinetics of NK105, a new polymeric micelle carrier system for paclitaxel (PTX). NK105 was administered as a 1-h intravenous infusion every 3 weeks, without antiallergic premedication. The starting dose was 10 mg m^{-2}, and the dose was escalated according to the accelerated titration method. Nineteen patients were recruited. The tumour types treated included pancreatic ($n = 11$), bile duct ($n = 5$), gastric ($n = 2$), and colonic ($n = 1$) cancers. Neutropenia was the most common haematological toxicity. A grade 3 fever developed in one patient given 180 mg m^{-2}. No other grades 3 or 4 nonhaematological toxicities, including neuropathy, was observed during the entire study period. DLTs occurred in two patients given 180 mg m^{-2} (grade 4 neutropenia lasting for more than 5 days). Thus, this dose was designated as the MTD. Grade 2 hypersensitivity reactions developed in only one patient given 180 mg m^{-2}. A partial response was observed in one patient with pancreatic cancer. The maximum concentration (C_{max}) and area under the concentration (AUC) of NK105 were dose dependent. The plasma AUC of NK105 at 150 mg m^{-2} was approximately 15-fold higher than that of the conventional PTX formulation. NK105 was well tolerated, and the RD for the phase II study was determined to be 150 mg m^{-2} every 3 weeks. The results of this phase I study warrant further clinical evaluation.

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Paclitaxel (PTX), an antimicrotubule agent, has a wide spectrum of antitumour activity including ovarian, breast, stomach, lung, and head and neck cancers (Rowinsky *et al*, 1990; Carney, 1996; Crown and O'Leary, 2000). The clinically used PTX preparation is a mixture of Cremophor EL and ethanol because of PTX's poor water solubility. However, the use of Cremophor EL is known to be associated with acute hypersensitivity reactions (Weiss *et al*, 1990; Rowinsky and Donehower, 1995; Kloover *et al*, 2004). Other PTX preparations that have been categorised as drug delivery systems (DDS) have also been developed. These preparations include Xyotax (polyglutamate-conjugated PTX; Singer *et al*, 2003; Boddy *et al*, 2005), Abraxane (PTX coated with albumin; Ibrahim *et al*, 2002; Deisai *et al*, 2003; Nyman *et al*, 2005), and Genexol-PM (a PTX micelle in which PTX has been simply solubilised; Kim *et al*, 2004). The common advantage shared by these formulations is that they are injectable intravenously without the mixture of Cremophor EL and ethanol. Among them, Abraxane has been approved for metastatic breast cancer by the Food and Drug Administration in the USA based on the results of a randomised phase 3 trial. In this trial, Abraxane demonstrated significantly higher response rates, compared with standard PTX, and a significantly longer time to progression (Gradishar *et al*, 2005). In addition, the incidence of grade 4 neutropenia was significantly lower for Abraxane than for PTX. However, peripheral sensory neuropathy was more common in the arm (Gradishar *et al*, 2005).

NK105 is a PTX-incorporating 'core-shell-type' polymeric micellar nanoparticle formulation (Hamaguchi *et al*, 2005). This particle can be injected intravenously without the use of Cremophor EL or ethanol as a vehicle. Therefore, NK105 is expected to possess a clinical advantage similar to that of the above-mentioned PTX formulations. The difference between NK105 and the other PTX dosage forms is that NK105 is expected to yield a markedly higher plasma and tumour area under the concentration (AUC), compared with those for the other PTX formulations. Moreover, regarding the toxic profiles, the repeated administration of NK105 to rats at 7-day intervals produced significantly fewer toxic effects on peripheral nerves than free PTX. Macromolecular drugs, including NK105, have been developed based on the characteristic macroscopic features of solid tumours, such as hypervasculature, the presence of vascular permeability factors stimulating extravasation within cancer, and the suppressed lymphatic clearance of macromolecules. These characteristics, which are unique to solid tumours, constitute the basis of the enhanced permeability and retention (EPR) effect (Matsumura and Maeda, 1986; Maeda *et al*, 2000; Duncan, 2003). The *in vivo*

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