rate and longer time to progression (TTP) of FP compared with 5-FUci, associated with lower toxicity of 5-FUci than of FP (11). Therefore, 5-FUci was considered a reference regimen for the present phase III study of advanced gastric cancer patients. It has also been reported that a better response to chemotherapy contributes to longer survival and cures in some patients (12). However, severe toxicity associated with intensive chemotherapy causes deterioration of patients' quality of life, especially that of nonresponders. Thus, the ability to predict the effects of chemotherapy and to select an appropriate regimen for each patient before commencement of chemotherapy is important.

Many factors and mechanisms are involved in sensitivity and resistance to chemotherapy, of which some are clinically relevant (13–16). However, there are no reports of clinically useful biological markers for prognosis and chemotherapy regimens for advanced gastric cancer patients. In our previous phase II study of FP for advanced gastric cancer, vascular endothelial growth factor (VEGF) (+), p53 (−), bcl-2 (−), thymidylate synthase (TS) (−), and gluthathione *S*-transferase π (GST-π) (−) were shown to be favorable phenotypes for chemoresponse in 39 patients (17). Patients with VEGF (+) had significantly higher response rates than those with VEGF (−).However, there were no differences in survival times between (+) and (−) marker types. The number of favorable phenotypes was related to response rate: the 10 patients with four or five favorable phenotypes survived significantly longer than the 29 patients with three phenotypes or less.

However, because our previous study was investigational, it was considered necessary to confirm the results in a different cohort. In addition, investigation of the clinical utility of these markers is certainly warranted for selecting chemotherapy regimens in a randomized phase III trial.

In this study, we investigated relationships between expression of five biological markers and survival among patients registered for a phase III study (JCOG9205 (11)) to confirm the results of our previous study and to clarify the utility of these markers for selecting 5-FUci or FP chemotherapy regimens. The study was approved by the chair of the Japan Clinical Oncology Group.

PATIENTS AND METHODS

Patients

Two hundred and eighty patients were enrolled in the phase III study (JCOG9205 (11)); 106 patients had been treated with 5-FUci, 104 with FP and 70 with a combination of futrafur and uracil (UFT) plus mitomycin C (UFTM). Biopsy samples were obtained from 180 patients, consisting of 68 (64%) from the 5-FUci group, 67 (64%) from the FP group, and 45 (64%) from the UFTM group. Patients treated with UFTM were excluded from this study because enrollment for UFTM treatment ceased after interim analysis of the phase III study revealed that it had no survival advantage and more severe toxic effects than 5-FUci (11). Three patients in the 5-FUci group and one in the FP group were excluded because their biopsy samples were too small for immunostaining. The subjects selected for this study comprised 65 patients treated with 5-FUci and 66 treated with FP from whom sufficient amounts of pretreatment biopsy specimens had been obtained endoscopically. These patients met the eligibility criteria of JCOG9205 (11): (1) histological confirmation of gastric cancer, (2) measurable or assessable lesions, (3) ability to accept oral administration of UFT, (4) aged 75 years or younger, (5) a performance status of two or less on the ECOG scale, (6) no prior treatment except surgery, (7) fully functioning liver, kidney, and bone marrow, (8) life expectancy of eight weeks or longer, and (9) written informed consent. All the patients in the study received the protocol chemotherapy as the first line therapy.

Treatment Schedule

The treatment schedule for the 5-FUci group comprised a continuous infusion of 5-FU (800 mg/m^2 per day) on days 1–5. The FP schedule consisted of a drip infusion of CDDP (20 mg/m^2 per day) on days 1–5, together with the same dose of 5-FUci as the 5-FUci group. These two treatments were repeated every four weeks until the appearance of disease progression, unacceptable toxicity, or the patient's voluntary withdrawal from the study.

Immunohistochemistry

Biopsy samples from 180 patients were immunostained as described in our previous report (17). All immunohistochemical analyses were performed using tissue sections from formalin-fixed, paraffin-embedded biopsy material obtained endoscopically from primary tumors. Serial 3 μm thick slices were cut, deparaffinized in xylene, dehydrated with graded ethanol and then immersed in methanol containing 0.3% H_2O_2 for 20 min to inhibit endogenous peroxidase activity. Sections stained for p53 and TS were heated to 95°C by microwave irradiation for 10 min in phosphate buffered saline (PBS) or 10 mM citrate buffer, respectively. Sections stained for VEGF were treated with 0.05% pepsin in 0.01 N HCl for 20 min at room temperature. After blocking with 10% normal swine serum in PBS (blocking buffer) for 60 min, all sections were incubated overnight at room temperature with the primary antibodies diluted in blocking buffer to the following concentrations: anti-p53 antibody (Nichirei, Tokyo, Japan), 1:20000; anti-bcl-2 antibody (DAKO, Glostrup, Denmark), 1:40; anti-GST-π antibody (MBL, Nagoya, Japan), 1:24000; anti-TS antibody (TS106 (16)), 1:200; anti-VEGF antibody (Santa Cruz Biochemistry, CA, USA), 1:500. The sections were washed with PBS and then incubated for 1 h with biotinylated secondary antibody diluted 1:200. After washing with PBS, the sections were incubated with ABC reagent (Vector Laboratories, CA, USA) and the color reaction was

Table 1. Patient characteristics

		5-FUci ($n = 65$)	FP ($n = 66$)	P value
Age	Median (range)	63 (34–73)	63 (19–75)	0.6065
Gender	M/F	49/16	48/18	0.7257
Performance status (ECOG scale)	0/1/2/unknown	34/18/11/2	24/35/5/2	0.0242
Macroscopic type	Expansive/infiltrative/others	15/48/2	11/53/2	0.6520
Histological type	Intestinal/diffuse unknown	29/31/5	32/29/5	0.9019
Tumor extent	Locally advanced/ascites/metastatic	15/33/17	17/39/10	0.1196
Resection of the primary tumor	+/−	17/48	16/50	0.8011

Source: A phase III study of the Japan Clinical Oncology Group (JCOG9205); continuous infusion of 5-fluorouracil (5-FUci) versus a combination of 5-FU and cisplatin (CDDP). Pre-treatment biopsies were available in 65 patients treated with 5-FUci and 66 patients with FP.

developed in Tris buffer containing 2% 3,3′-diaminobenzidine and 0.3% hydrogen peroxide. The sections were then counterstained with hematoxylin or methyl green.

All immunostained specimens from the 180 patients were assessed by an investigator (N.B.) who was not informed of clinical information such as treatment schedules and clinical outcomes. The intensity of staining for p53 and GST-π was graded as (+) when strong, as (±) when faint, and as (−) when no staining was visible. For bcl-2, the intensity of staining was graded as (++) when stronger than that of correspondingly stained lymphocytes, as (+) when equal to that of stained lymphocytes, and as (−) when weaker than that of stained lymphocytes. The staining of VEGF was graded as (++) when the intensity of staining in cancer cells was stronger than that in stromal cells, as (+) when equal to that of stromal cells, and as (−) when weaker than that of stromal cells. TS expression was graded as (++), (+), (±), or (−) based on the intensity of staining. For all markers, patients were defined as positive when more than 20% of the cancer cells in each section were (++) or (+). VEGF (+), p53 (−), bcl-2 (−), TS (−) and GST-p (−) were defined as favorable phenotypes for chemoresponse to FP on the basis of the results of our previous phase II study.

Anti-tumor Effects

The responses of measurable metastatic lesions and of primary lesions were evaluated according to the standard World Health Organization criteria (18) and evaluation criteria proposed by the Japanese Gastric Cancer Association (19). All patients were followed up for at least 1 year after registration for the study. Survival was calculated from the date of registration to the date of death from any cause or to the last confirmation of survival. TTP was estimated from the interval between the date of registration and the date of confirmation of disease progression by image and clinical diagnosis, or the date of death for patients for whom confirmation of disease progression was absent. All clinical information was obtained from the JCOG data center.

Statistical Analysis

Survival curves were constructed using the Kaplan–Meier method and compared using the Log-rank test. Patient characteristics and response rates were compared using a χ^2 test.

RESULTS

Patient Characteristics

Patient characteristics are shown in Table 1. The subjects constituted two thirds of all patients enrolled in JCOG9205 (11). The numbers of patients treated with 5-FUci and FP were similar. The two groups were well balanced in respect of age, sex, macroscopic type, histological type, and history of resection of primary lesions, but there were more patients with poor performance status in the FP group than in the 5-FUci group ($P = 0.0242$). Seventeen patients (26%) in the 5-FU group and 10 (15%) in the FP group had distant metastases ($P = 0.1196$).

Overall Survival and Time to Progression

Figure 1 shows the overall survival times of subjects treated with 5-FUci or FP. There was no significant difference in survival between patients treated with 5-FUci or with FP;

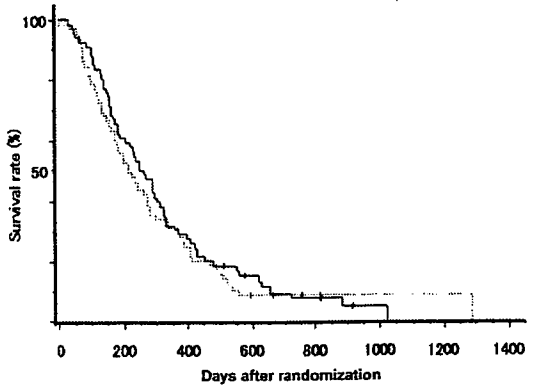

Figure 1. Overall survival of 66 patients treated with FP (solid line) and 65 patients treated with FUci (dotted line).

Table 2. Expression of biological markers in 5-FUci and FP

		5-FUci ($n = 65$)	FP ($n = 66$)
VEGF	(+)	30 (49)	32 (47)
	(−)	35 (51)	34 (53)
TS	(+)	37 (57)	21 (32)
	(−)	28 (43)	45 (68)
p53	(+)	28 (43)	28 (42)
	(−)	37 (57)	38 (58)
GST-π	(+)	38 (58)	41 (62)
	(−)	27 (42)	25 (38)
Bcl-2	(+)	7 (11)	11 (17)
	(−)	58 (89)	55 (83)

Expressions of vascular endothelial growth factor (VEGF), thymidylate synthase (TS), p53, gluthathione S-transferase π (GST-π) and bcl-2 were examined immunohistochemically. Values in parentheses are percentages.

Table 3. Expression of biological markers and response to 5-FUci and FP

		5-FUci ($n = 65$)	FP ($n = 66$)
VEGF	(+)	1/30 (3)	13/32 (41)
	(−)	7/35 (20)	16/34 (47)
TS	(+)	5/37 (14)	9/21 (43)
	(−)	3/28 (11)	20/45 (44)
p53	(+)	2/28 (7)	11/28 (39)
	(−)	6/37 (16)	18/38 (47)
GST-π	(+)	3/38 (8)	20/41 (49)
	(−)	5/27 (19)	9/25 (36)
Bcl-2	(+)	1/7 (14)	4/11 (36)
	(−)	7/58 (12)	25/55 (45)

Expressions of vascular endothelial growth factor (VEGF), thymidylate synthase (TS), p53, gluthathione S-transferase π (GST-π) and bcl-2 were examined immunohistochemically. The number of patients with complete or partial remission after treatment with 5-FUci and FP in all patients with positive or negative expression of each biological marker. Values in parentheses are percentages.

median survival times were 216 days for the 5-FUci group and 253 days for the FP group ($P = 0.6953$). TTP was longer after FP treatment than after 5-FUci treatment (median TTP: 111 days and 61 days, respectively; $P = 0.0477$).

Expression of Biological Markers and Response

The staining patterns and incidences of positive reactions for the biological markers were similar to those observed in our previous study (17) (Table 2). Table 3 shows the relationships between biological markers and chemoresponses. The overall response rates in the FP and 5-FUci groups were 44% (29/66) and 12% (8/65), respectively. While the response rates of the patients with VEGF (−) were higher than those with VEGF (+) in the 5-FUci group ($P = 0.0599$), there was no significant difference in response between patients with (+) and (−) types of VEGF, p53, bcl-2, TS or GST-π.

Eleven of the 20 patients (55%) with four or five favorable phenotypes and 18 of the 46 patients (39%) with three or less favorable phenotypes were responders ($P = 0.2326$) in the FP treatment group. The response rate of the 16 patients with four or five favorable phenotypes (13%) was similar to that (12%) of the other 49 patients with three or fewer favorable phenotypes ($P > 0.9999$) in the 5-FUci treatment group.

Number of Favorable Phenotypes, Survival and Time to Progression

In the FP treatment group, the 20 patients with four or five favorable phenotypes survived longer than the 46 patients with three or less favorable phenotypes (MST, 334 and 243 days, respectively; $P = 0.0463$) (Fig. 2A), whereas there was no difference between the two types of patient in the 5-FUci group (MST, 203 and 216 days, respectively; $P = 0.315$) (Fig. 2B). No significant differences were observed in TTP between patients with four or five favorable phenotypes and patients with three or less favorable phenotypes in the FP or 5-FUci groups (FP: favorable, 118 days; others, 102 days; $P = 0.2766$, and 5-FUci: favorable, 41 days; others, 61 days; $P = 0.6830$).

VEGF, Survival and Time to Progression

In the 5-FUci and FP groups, there were no significant differences in survival times between patients with (+) or (−) types of p53, bcl-2, TS or GST-π. The survival times of the 32 (49%) patients with VEGF (+) and the 34 (51%) patients with VEGF (−) were almost equal in the FP treatment group (MST: 269 and 253 days, respectively; $P = 0.6317$) (Fig. 3A), whereas the 30 patients with VEGF (+) had shorter survival times than the 35 with VEGF (−) in the 5-FUci treatment group (MST: 142 and 302 days, respectively; $P = 0.0043$) (Fig. 3B). In the FP group, there was no difference in TTP between patients with VEGF (+) and those with VEGF (−) (median TTP: 111 days and 123 days, respectively; $P = 0.3497$). However, the TTP of patients with VEGF (−) was significantly longer than that of patients with VEGF (+) in the 5-FUci group (median TTP: 101 days and 36 days, respectively; $P = 0.0046$).

DISCUSSION

The recruitment rates of patients from the phase III study (JCOG9205 (11)) into the present study were equal among the three regimens. Patient characteristics and rates of positive reactions for biological markers were well balanced. These data indicate that biopsy samples were collected without bias. The overall response rates, survival times and

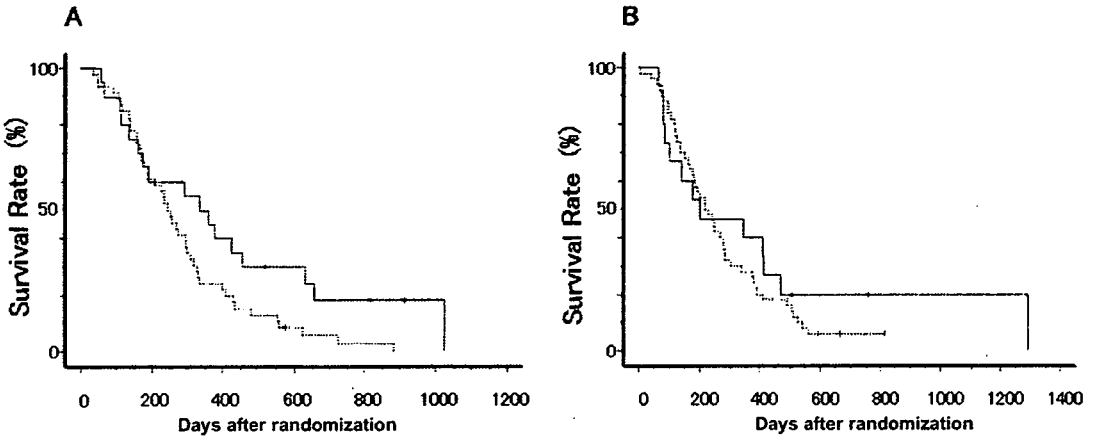

Figure 2. Overall survival of patients (solid line) with four or five favorable phenotypes out of VEGF (+), TS (−), p53 (−), bcl-2 (−), GST-π (−), and those (dotted line) with 3 or fewer, after treatment with FP (A) or 5-FUci (B).

TTPs were similar to those of patients enrolled in the phase III study (11). Although biopsy specimens were collected only from two-thirds of the patients enrolled in JCOG9205 (11), the subjects of this study were considered representative of those of the phase III study.

Biopsy samples can only be obtained from the superficial part of primary tumors and may not be representative of the biological behavior of the entire tumor. Because many patients destined to be treated with chemotherapy have unresectable tumors, only biopsy samples can be used to assess biological markers. Takiuchi (20) and our group (17) have shown that VEGF (+) is a predictive marker of chemoresponse in advanced gastric cancer patients treated with FP. Nagashima (21) reported that patients with VEGF (+) who were treated with a combination of irinotecan (CPT-11) and CDDP had a higher response rate than those with VEGF (−). These results suggest that assessment of biological markers using endoscopic biopsy samples can yield useful information and that the expression of VEGF in the biopsy samples of gastric cancer patients may be a predictor of chemotherapeutic effects in CDDP containing regimens.

The incidence of VEGF (+) was 47% (62/131), which recapitulated the result of our previous study (51%, 20/39). The incidences of other biological markers in the two studies were also similar. These results show that the method used for evaluating biological markers was reproducible.

In our previous study, expression of VEGF and the number of favorable phenotypes were significant predictors of chemoresponse to FP (17). In the present study, there was no relationship between the expression of VEGF and chemoresponse to FP. The response rate of patients with four or five favorable phenotypes was slightly but not significantly

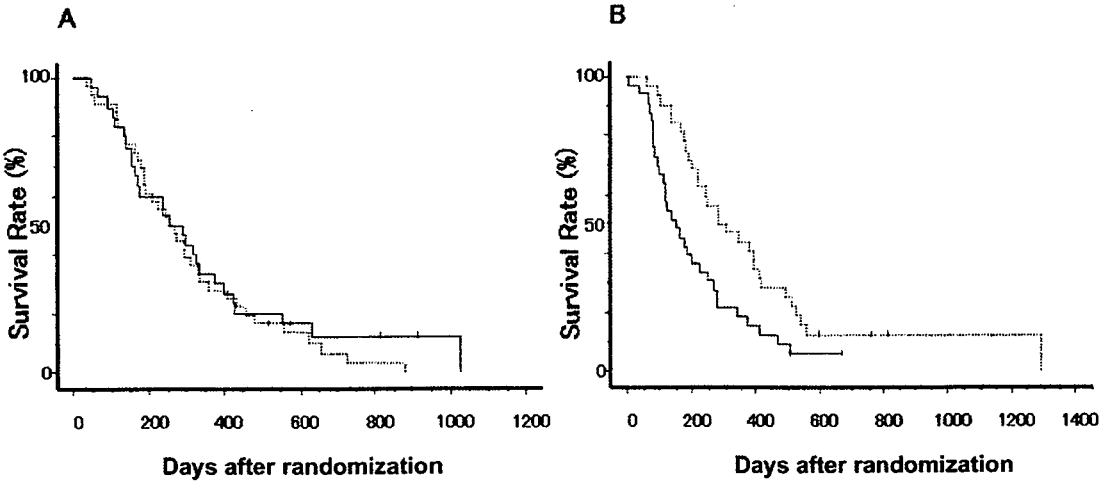

Figure 3. Overall survival of patients (solid line) with VEGF (+) and those (dotted line) with VEGF (−) after treatment with FP (A) or 5-FUci (B).

higher than that of patients with three or less favorable phenotypes. It could be argued that these discrepancies indicate that expression of VEGF and the number of favorable phenotypes are not good predictive markers of chemoresponse to FP. However, Takiuchi et al. (20) reported that immunohistochemical expression of VEGF can predict the response to FP in patients with gastric cancer. Nagashima et al. (21) reported that VEGF and the number of favorable phenotypes of similar biological markers were predictive markers of chemoresponse to irinotecan plus CDDP and of survival. Several reports have described differences in chemoresponse between phase II and III studies of the same chemotherapy regimens. It is possible that the discrepancies between our previous and present studies were caused by the difference in the method of evaluating responses between the phase II and III studies of FP.

In the FP group of this study, the 20 patients with four or five favorable phenotypes survived longer than the 46 patients with three or less favorable phenotypes. This result recapitulates our previous phase II findings on survival. In the 5-FUci treatment group, there was no difference in survival between these two phenotype groups. However, the differences in survival between the FP and 5-FUci treatments were small in patients with four or five favorable phenotypes and in those with three or less favorable phenotypes. Moreover, in the FP and 5-FUci treatment groups, there were no significant differences in TTP between patients with four or five favorable phenotypes and those with three or less favorable phenotypes. These results suggest that the presence of favorable phenotypes is a prognostic marker for patients treated with FP, but not a selective marker between FP and 5-FUci.

VEGF promotes angiogenesis and permeability of blood vessels and is associated with microvessel abundance and metastasis (22,23). It has been reported that VEGF is a marker of poor prognosis after surgical resection in various kinds of malignancies including gastric cancer (24–30). Our previous study showed no differences in survival between patients with VEGF (+) or (−) despite a higher response rate in those with VEGF (+). Similarly, in the present study, there were no differences in survival or TTP between patients with VEGF (+) or (−) after treatment with FP. However, in the 5-FUci group, patients with VEGF (+) had a significantly shorter survival time and TTP than those with VEGF (−). Thus, VEGF is considered to be a risk factor for a poor prognosis in patients treated with 5-FUci. It is suggested that CDDP in addition to 5-FUci may overcome the malignant potential of VEGF, although the relationship between VEGF and the chemoresponse to FP was not as clear in the present study as in our previous study (17).

In the phase III study (JCOG9205 (11)), FP treatment had no survival benefit over treatment with 5-FUci even though the response rate and TTP after FP treatment was significantly better than after 5-FUci treatment. This study showed that in the subset of patients with VEGF (−), 5-FUci treatment resulted in slightly longer survival times than FP treatment and the TTPs were almost equal (5-FUci 101 days, FP 123 days). In contrast, in the subset of patients with VEGF (+), survival and TTP of patients treated with FP were longer than those of patients treated with 5-FUci. From these results, it is speculated that patients with VEGF (+) may achieve longer survival and TTP after treatment with FP than with 5-FUci and that the status of VEGF expression might be a selective marker for treatment with 5-FUci versus FP.

In conclusion, the number of favorable phenotypes (≥ 4 versus ≤ 3) of markers VEGF (+), p53 (−), bcl-2 (−), TS (−), and GST-π (−) was prognostic for the outcome of advanced gastric cancer treatment with FP. Clinical outcomes such as TTP and survival differed between 5-FUci and FP treatment according to the status of VEGF expression. Although the methodology used to evaluate biological markers in this study might be considered less advanced than methods based on microarrays or proteomics, the results illustrate some important points: (i) multiple factors should be investigated to clarify prognostic markers of cytotoxic agents, (ii) confirmation of results is mandatory, (iii) comparison in a phase III study is necessary to clarify the utility of markers for selecting treatments.

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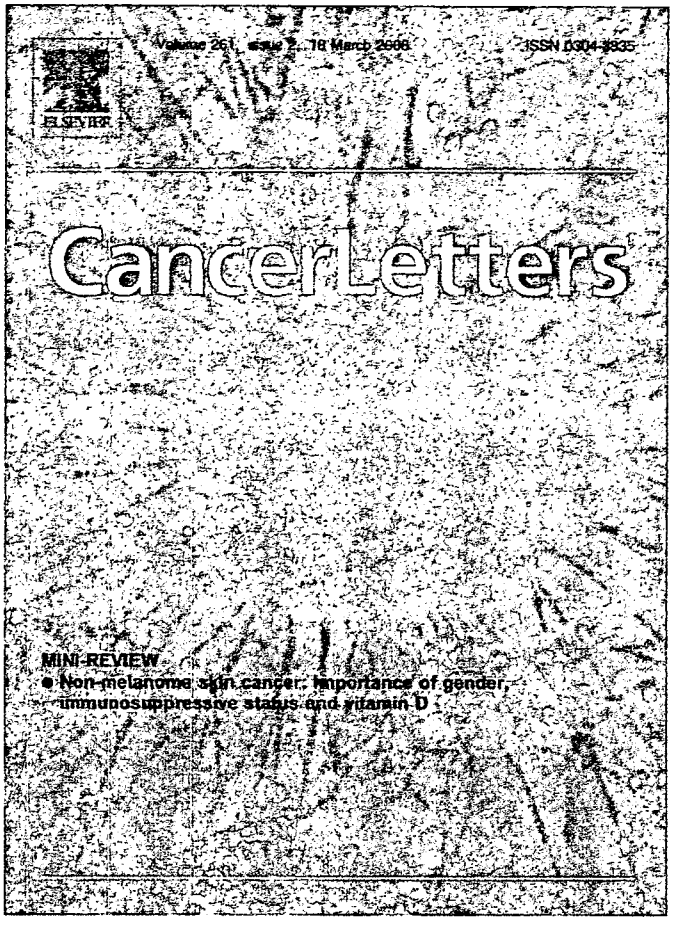
Conflict of interest statement

None declared.

References

1. Wils J, Bleiberg H, Dalesio O, Blijham G, Mulder N, Planting A, et al. An EORTC gastrointestinal group evaluation of the combination of sequential methotrexate and 5-fluorouracil, combined with adriamycin in advanced measurable gastric cancer. *J Clin Oncol* 1986;4:1799–803.
2. Moertel CG, Rubin J, O'Connel MJ, Schutt AJ, Wieand HS. A phase II study of combined 5-fluorouracil, doxorubicin, and cisplatin in the treatment of advanced upper gastrointestinal adenocarcinomas. *J Clin Oncol* 1986;4:1053–7.
3. Preusser P, Wilke H, Achterrath W, Fink U, Lenaz L, Heinicke A, et al. Phase II study with the combination etoposide, doxorubicin, and cisplatin in advanced measurable gastric cancer. *J Clin Oncol* 1989;7:1310–7.
4. Wilke H, Preusser P, Fink U, Archterrath W, Lenaz L, Stahl M, et al. High dose folinic acid/etoposide/5-fluorouracil in advanced gastric cancer phase II study in elderly patients or patients with cardiac risk. *Invest New Drugs* 1990;8:65–70.
5. Findlay M, Cunningham D, Norman A, Mansi J, Nicolson M, Hickish T, et al. A phase II study in advanced gastric cancer using epirubicin and cisplatin in combination with continuous 5-fluorouracil (ECF). *Ann Oncol* 1994;5:609–16.

6. Ohtsu A, Shimada Y, Yoshida S, Saito H, Seki S, Morise K, et al. Phase II study of protracted infusional 5-fluorouracil combined with cisplatinum for advanced gastric cancer: report from the Japan Clinical Oncology Group (JCOG). *Eur J Cancer* 1994;30A:2091–3.
7. Sakata Y, Ohtsu A, Horikoshi N, Sugimachi K, Mitachi Y, Taguchi T. Late phase II study of novel oral fluoropyrimidine anticancer drug S-1 (1 M tegafur-0.4 M gimestat-1 M otastat potassium) in advanced gastric cancer patients. *Eur J Cancer* 1998;34:1715–20.
8. Boku N, Ohtsu A, Shimada Y, Shirao K, Seki S, Saito H, et al. A phase II study of a combination of irinotecan and cisplatin against metastatic gastric cancer. *J Clin Oncol* 1999;17:319–23.
9. Cullinan SA, Moertel CG, Wieand H, O'Connell MJ, Poon MA, Krook JE, et al. Controlled evaluation of three drug combination regimen versus fluorouracil alone in the therapy of advanced gastric cancer. *J Clin Oncol* 1994;12:412–6.
10. Kim NK, Park YS, Heo DS, Suh C, Kim SY, Park KC, et al. A phase III randomized study of 5-fluorouracil and cisplatin versus 5-fluorouracil, doxorubicin, and mitomycin C versus 5-fluorouracil alone in the treatment of advanced gastric cancer. *Cancer* 1993;71:3813–18.
11. Ohtsu A, Shimada Y, Shirao K, Boku N, Hyodo I, Saito H, et al. Randomized phase III trial of fluorouracil alone versus fluorouracil plus cisplatin versus uracil and tegafur plus mitomycin in patients with unresectable advanced gastric cancer: the Japan Clinical Oncology Group Study (JCOG9205). *J Clin Oncol* 2003;21:54–9.
12. Ohkuwa M, Ohtsu A, Boku N, Yoshida S, Miyata Y, Shirao K, et al. Long-term results of patients with unresectable gastric cancer who received chemotherapy in the Japan Clinical Oncology Group (JCOG) trials. *Gastric Cancer* 2000;3:145–50.
13. Bergh J, Norberg T, Sjogren S, Lindgren A, Holmberg L. Complete sequencing of the p53 gene provides prognostic information in breast cancer patients, particularly in relation to adjuvant systemic therapy and radiotherapy. *Nat Med* 1995;1:1029–34.
14. Gasparini G, Barbareschi M, Doglioni C, Palma PD, Mauri FA, Boracchi P, et al. Expression of bcl-2 protein predicts efficacy of adjuvant treatments in operable node-positive breast cancer. *Clin Cancer Res* 1995;1:189–98.
15. Nishimura T, Newkirk K, Sessions RB, Andrew PA, Trock BJ, Rasmussen AA, et al. Immunohistochemical staining for gluthathione S-transferase predicts response to platinum-based chemotherapy in head and neck cancer. *Clin Cancer Res* 1996;2:1859–65.
16. Johnston PG, Fisher ER, Rockette HE, Fisher B, Wolkman N, Drake JC, et al. The role of thymidylate synthase expression in prognosis and outcome of adjuvant chemotherapy in patients with rectal cancer. *J Clin Oncol* 1994;12:2640–7.
17. Boku N, Chin K, Hosokawa K, Ohtsu A, Tajiri H, Yoshida S, et al. Biological markers as a predictor for response and prognosis of unresectable gastric cancer patients treated with 5-fluorouracil and cis-platinum. *Clin Cancer Res* 1998;4:1469–74.
18. World Health Organization: WHO Handbook for Reporting Results of Cancer Treatment. WHO Offset Publication no. 48 Geneva, Switzerland, World Health Organization, 1979.
19. Japanese Research Society for Gastric Cancer: Japanese Classification of Gastric Carcinoma (1st Engl. edn). Tokyo, Japan, Kanehara, 1995.
20. Takiuchi H, Hirata I, Kawabe SI, Egashira Y, Katsu K. Immunohistochemical expression of vascular endothelial growth factor can predict response to 5-fluorouracil and cisplatin in patients with gastric adenocarcinoma. *Oncol Rep* 2000;7:841–6.
21. Nagashima F, Boku N, Ohtsu A, Yoshida S, Hasebe T, Ochiai A, et al. Biological markers as a predictor for response and prognosis of unresectable gastric cancer patients treated with irinotecan and cisplatin. *Jpn J Clin Oncol* 2005;35:714–9.
22. Connolly DT, Heuvelman DM, Nelson R, Olander JV, Eppley BL, Delfino JJ, et al. Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. *J Clin Invest* 1989;84:1470–8.
23. Dvorak HF, Orenstein NS, Carvalho AC, Churchill WH, Dvorak AM, Galli SJ, et al. Induction of a fibrin-gel investment: an early event in line 10 hepatocarcinoma growth mediated by tumor secreted products. *J Immunol* 1979;122:166–74.
24. Maeda K, Chung YS, Ogawa Y, Takatsuka S, Kang SM, Ogawa M, et al. Prognostic value of vascular endothelial growth factor expression in gastric carcinoma. *Cancer* 1996;77:858–863.
25. Gasparini G, Toi M, Gion M, Verderio P, Dittadi R, Hanatani M, et al. Prognostic significance of vascular endothelial growth factor protein in node-negative breast carcinoma. *J Natl Cancer Inst* 1997;89:139–47.
26. Takahashi Y, Tucker SL, Kitadai Y, Koura AN, Bucana CD, Cleary KR, et al. Vessel counts and expression of vascular endothelial growth factor as prognostic factors in node-negative colon cancer. *Arch Surg* 1997;132:541–6.
27. Itakura J, Ishiwata T, Friess H, Fujii H, Matsumoto Y, Buchler MW, et al. Enhanced expression of vascular endothelial growth factor in human pancreatic cancer correlates with local disease progression. *Clin Cancer Res* 1997;3:1309–16.
28. Kaya M, Wada T, Akatsuka T, Kawaguchi S, Nagoya S, Shindo M, et al. Vascular endothelial growth factor expression in untreated osteosarcoma is predictive of pulmonary metastasis and poor prognosis. *Clin Cancer Res* 2000;6:572–7.
29. Shih CH, Ozawa S, Ando N, Ueda M, Kitajima M. Vascular endothelial growth factor expression predicts outcome and lymph node metastasis in squamous cell carcinoma of the esophagus. *Clin Cancer Res* 2000;6:1161–8.
30. Smith BD, Smith GL, Carter D, Sasaki CT, Haffty BG. Prognostic significance of vascular endothelial growth factor protein levels in oral and oropharyngeal squamous cell carcinoma. *J Clin Oncol* 2000;18:2046–52.



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Importance of *UDP-glucuronosyltransferase 1A1*6* for irinotecan toxicities in Japanese cancer patients

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Abstract

Recent pharmacogenetic studies on irinotecan have revealed the impact of *UDP glucuronosyltransferase (UGT) 1A1*28* on severe irinotecan toxicities. Although the clinical role of *UGT1A1*6*, which is specifically detected in East Asian patients, in irinotecan toxicities is suggested, clear evidence remains limited. To examine the impact of **6*, the association of *UGT1A1* genotypes with severe irinotecan toxicities was retrospectively investigated in Japanese cancer patients. A significant **6*-dependent increase in the incidence of grade 3 or 4 neutropenia was observed in 49 patients on irinotecan monotherapy ($p = 0.012$). This study further clarifies the clinical importance of **6* in irinotecan therapy in East Asians.

Keywords: UGT1A1; Pharmacogenetics; Irinotecan; SN-38

1. Introduction

Irinotecan, an anticancer prodrug, is widely applied for a broad range of carcinomas, including colorectal and lung cancers. The active metabolite, SN-38 (7-ethyl-10-hydroxycamptothecin), a topoisomerase I inhibitor, is generated by hydrolysis of the parent compound by carboxylesterases [1]. SN-38 is subsequently glucuronidated by uridine diphosphate glucuronosyltransferase 1As (UGT1As) such as 1A1, 1A7, 1A9 and 1A10, to form the inactive metabolite, SN-38 glucuronide (SN-38G) [2–5]. Among the UGT

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isoforms, UGT1A1 is thought to be a predominant contributor to SN-38G formation [2,6]. The dose-limiting toxicities in irinotecan therapy are severe diarrhea and leucopenia [7], and lowered UGT activity is well correlated with severe irinotecan toxicities [8]. Since Ando et al. first reported the significant relevance of *UGT1A1*28* – a repeat polymorphism in the TATA box (−40_−39insTA) – to severe neutropenia/diarrhea [9], a number of clinical studies, primarily conducted in Caucasian patients, have shown associations between *UGT1A1*28* and lowered SN-38G formation or severe neutropenia/diarrhea [10–13]. Based on these findings, the Food and Drug Administration (FDA) of the United States approved a revision of the label for Camptosar (irinotecan HCl) (NDA 20-571/S-024/S-027/S-028), recommending "a reduction in the starting dose by at least one level of irinotecan for the *UGT1A1*28* homozygous patients". Subsequently, the clinical application of *UGT1A1*28* testing was put into practice for irinotecan therapy in the United States.

To implement personalized irinotecan therapy in Asian countries, the racial differences in *UGT1A1* polymorphisms among Caucasians, African-Americans, and Asians must be taken into consideration [14]. For East Asians, the frequency of **28* is one third of that of Caucasians or African-Americans, and another low-activity allele **6* [211G>A(G71R)], which is not detected in Caucasians or African-Americans, shows the same frequency as the **28* allele. Clinical studies in Japanese cancer patients have demonstrated that significantly low area under concentration-time curve (AUC) ratios of SN-38G to SN-38 are observed in patients having **6* and/or **28* [15–17], suggesting the necessity of typing **6* in addition to **28*. A recent report on Korean lung cancer patients who received a combination therapy of irinotecan and cisplatin, showed a significant association of **6* homozygotes with severe neutropenia [18]. However, data on the role of **6* in irinotecan toxicities is still limited in terms of the various irinotecan-containing regimens. In the first study by Ando et al. on Japanese cancer patients, the association of **6* with irinotecan toxicities was not evident, but a possible enhancement of **28*-related toxicities by **6* was suggested [9]. Other studies in Japanese patients showed an additive effect of **6* on the lowered UGT activity by **28* [15–17]. A significant association of the genetic marker "**6 or *28*" with severe neutropenia was also shown in our previous study, but due to a lack of **6* homozygotes in our patient population, the effect of **6* alone was not confirmed [17].

In this study, to further demonstrate the clinical importance of **6* alone, *UGT1A1* genotypes were determined using DNA extracted from paraffin-embedded specimens (non-cancerous tissues) from 75 Japanese cancer patients by the pyrosequencing method [19,20], and the associations between *UGT1A1* genotype and severe irinotecan toxicities and serum total bilirubin levels were retrospectively analyzed.

2. Materials and methods

2.1. Patients and irinotecan treatment

In a post-marketing surveillance study conducted by Daiichi Pharmaceutical Co., Ltd. (currently Daiichi Sankyo Co., Ltd., Tokyo, Japan), irinotecan was prescribed to 297 patients with various types of cancers from 1995 to 2000 at the National Cancer Center Hospital. The patients were selected through standard clinical practice according to the drug label for indications and contraindications. Methanol-fixed, paraffin-embedded archival tissue specimens, which were necessary for high-quality extraction of DNA greater than 2 kb in size [21], were available for 75 of the 297 patients and were analyzed in this study. Irinotecan was administered by intravenous 30-min infusion as a single agent or in combination chemotherapy at a dose of 60 mg/m^2 (weekly or biweekly), 100 mg/m^2 (biweekly), or 150 mg/m^2 (biweekly). Profiles of the patients in this study, including cancer type, treatment history, and regimens, are summarized in Table 1. The pre-treatment levels of serum total bilirubin were determined by a kit (VL T-BIL, Azwell Inc., Osaka, Japan) according to an enzymatic method using bilirubin oxidase [22]. Toxicities were monitored during irinotecan therapy and graded according to the Common Toxicity Criteria version 2 of the National Cancer Institute.

Because the samples in this study were residual specimens remaining after histopathological diagnosis in the hospital and not collected specifically for research purposes, the samples and their clinical information were anonymized in an unlinkable fashion according to the Ethics Guidelines for Human Genome/Gene Analysis Research by the Ministry of Education, Culture, Sports, Science and Technology, Ministry of Health, Labour and Welfare, and Ministry of Economy, Trade and Industry of Japan. This study was approved by the ethics committees of the National Cancer Center and the National Institute of Health Sciences.

2.2. DNA extraction from paraffin-embedded tissue sections and genotyping of UGT1A1 polymorphisms

Three sections (20 μm of pathologically normal tissues around tumors) were deparaffinized twice by treat-

Table 1
Profiles of cancer patients in this study

		No. of patients
Patients genotyped (Male/female)		75 (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.

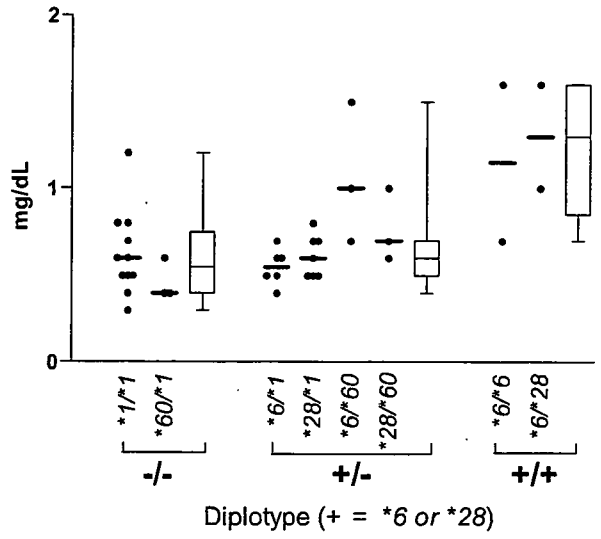

Fig. 1. Effects of *UGT1A1* genotypes on serum total bilirubin levels at baseline in Japanese cancer patients ($N = 37$). Each point represents a patient, and the median value of each diplotype is shown with a bar. All diplotypes are classified into −/−, +/−, and +/+ by the genetic marker, "*UGT1A1*6 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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References

[1] J.G. Slatter, P. Su, J.P. Sams, L.J. Schaaf, L.C. Wienkers, Bioactivation of the anticancer agent CPT-11 to SN-38 by human hepatic microsomal carboxylesterases and the in vitro assessment of potential drug interactions, Drug Metab. Dispos. 25 (1997) 1157–1164.

[2] L. Iyer, C.D. King, P.F. Whitington, M.D. Green, S.K. Roy, T.R. Tephly, et al., Genetic predisposition to the metabolism of irinotecan (CPT-11). Role of uridine diphosphate glucuronosyltransferase isoform 1A1 in the glucuronidation of its active metabolite (SN-38) in human liver microsomes, J. Clin. Invest. 15 (1998) 847–854.

[3] M. Ciotti, N. Basu, M. Brangi, I.S. Owens, Glucuronidation of 7-ethyl-10-hydroxycamptothecin (SN-38) by the human UDP-glucuronosyltransferases encoded at the UGT1 locus, Biochem. Biophys. Res. Commun. 260 (1999) 199–202.

[4] J.F. Gagne, V. Montminy, P. Belanger, K. Journault, G. Gaucher, C. Guillemette, Common human UGT1A polymorphisms and the altered metabolism of irinotecan active metabolite 7-ethyl-10-hydroxycamptothecin (SN-38), Mol. Pharmacol. 62 (2002) 608–617.

[5] T. Oguri, T. Takahashi, M. Miyazaki, T. Isobe, N. Kohno, P.I. Mackenzie, et al., UGT1A10 is responsible for SN-38 glucuronidation and its expression in human lung cancers, Anticancer Res. 24 (2004) 2893–2896.

[6] N. Hanioka, S. Ozawa, H. Jinno, M. Ando, Y. Saito, J. Sawada, Human liver UDP-glucuronosyltransferase isoforms involved in the glucuronidation of 7-ethyl-10-hydroxycamptothecin, Xenobiotica 31 (2001) 687–699.

[7] M. de Forni, R. Bugat, G.G. Chabot, S. Culine, J.M. Extra, A. Gouyette, et al., Phase I and pharmacokinetic study of the camptothecin derivative irinotecan, administered on a weekly schedule in cancer patients, Cancer Res. 54 (1994) 4347–4354.

[8] E. Gupta, T.M. Lestingi, R. Mick, J. Ramirez, E.E. Vokes, M.J. Ratain, Metabolic fate of irinotecan in humans: correlation of glucuronidation with diarrhea, Cancer Res. 54 (1994) 3723–3725.

[9] Y. Ando, H. Saka, M. Ando, T. Sawa, K. Muro, H. Ueoka, et al., Polymorphisms of UDP-glucuronosyltransferase gene and irinotecan toxicity: a pharmacogenetic analysis, Cancer Res. 60 (2000) 6921–6926.

[10] L. Iyer, S. Das, L. Janisch, M. Wen, J. Ramirez, T. Karrison, et al., UGT1A1*28 polymorphism as a determinant of irinotecan disposition and toxicity, Pharmacogenomics J. 2 (2002) 43–47.

[11] F. Innocenti, S.D. Undevia, L. Iyer, P.X. Chen, S. Das, M. Kocherginsky, et al., Genetic variants in the UDP-glucuronosyltransferase 1A1 gene predict the risk of severe neutropenia of irinotecan, J. Clin. Oncol. 22 (2004) 1382–1388.

[12] E. Marcuello, A. Altes, A. Menoyo, E. Del Rio, M. Gomez-Pardo, M. Baiget, UGT1A1 gene variations and irinotecan treatment in patients with metastatic colorectal cancer, Br. J. Cancer 91 (2004) 678–682.

[13] E. Rouits, M. Boisdron-Celle, A. Dumont, O. Guerin, A. Morel, E. Gamelin, Relevance of different UGT1A1 polymorphisms in irinotecan-induced toxicity: a molecular and clinical study of 75 patients, Clin. Cancer Res. 10 (2004) 5151–5159.

[14] N. Kaniwa, K. Kurose, H. Jinno, T. Tanaka-Kagawa, Y. Saito, M. Saeki, et al., Racial variability in haplotype frequencies of UGT1A1 and glucuronidation activity of a novel single nucleotide polymorphism 686C> T (P229L) found in an African-American, Drug Metab. Dispos. 33 (2005) 458–465.

[15] K. Sai, M. Saeki, Y. Saito, S. Ozawa, N. Katori, H. Jinno, et al., UGT1A1 haplotypes associated with reduced glucuronidation and increased serum bilirubin in irinotecan-administered Japanese patients with cancer, Clin. Pharmacol. Ther. 75 (2004) 501–515.
[16] K. Araki, K. Fujita, Y. Ando, F. Nagashima, W. Yamamoto, H. Endo, et al., Pharmacogenetic impact of polymorphisms in the coding region of the UGT1A1 gene on SN-38 glucuronidation in Japanese patients with cancer, Cancer Sci. 97 (2006) 1255–1259.
[17] H. Minami, K. Sai, M. Saeki, Y. Saito, S. Ozawa, K. Suzuki, et al., Irinotecan pharmacokinetics/pharmacodynamics and UGT1A genetic polymorphisms in Japanese: roles of UGT1A1*6 and *28, Pharmacogenet. Genomics 17 (2007) 497–504.
[18] J.Y. Han, H.S. Lim, E.S. Shin, Y.K. Yoo, Y.H. Park, J.E. Lee, et al., Comprehensive analysis of UGT1A polymorphisms predictive for pharmacokinetics and treatment outcome in patients with non-small-cell lung cancer treated with irinotecan and cisplatin, J. Clin. Oncol. 24 (2006) 2237–2244.
[19] M. Saeki, Y. Saito, H. Jinno, M. Tohkin, K. Kurose, N. Kaniwa, et al., Comprehensive UGT1A1 genotyping in a Japanese population by pyrosequencing, Clin. Chem. 49 (2003) 1182–1185.
[20] M. Saeki, Y. Saito, K. Sai, K. Maekawa, N. Kaniwa, J. Sawada, et al., A combinatorial haplotype of the UDP-glucuronosyltransferase 1A1 gene (#60-#IB) increases total bilirubin concentrations in Japanese volunteers, Clin. Chem. 53 (2007) 356–358.
[21] M. Noguchi, S. Furuya, T. Takeuchi, S. Hirohashi, Modified formalin and methanol fixation methods for molecular biological and morphological analyses, Pathol. Int. 47 (1997) 685–691.
[22] S. Otsuji, K. Mizuno, S. Ito, S. Kawahara, M. Kai, A new enzymatic approach for estimating total and direct bilirubin, Clin. Biochem. 21 (1988) 33–38.
[23] C. Kitagawa, M. Ando, Y. Ando, Y. Sekido, K. Wakai, K. Imaizumi, et al., Genetic polymorphism in the phenobarbital-responsive enhancer module of the UDP-glucuronosyltransferase 1A1 gene and irinotecan toxicity, Pharmacogenet. Genomics 15 (2005) 35–41.

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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

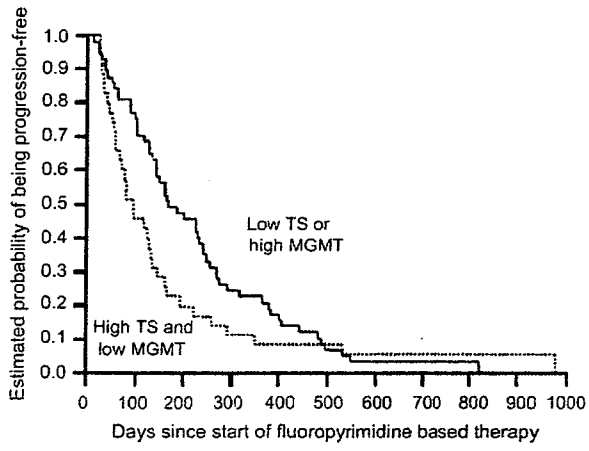

Fig. 1 – Time to progression in patients given fluoropyrimidines according to the cutoff levels of TS and MGMT expression ($p = 0.083$). TS: thymidylate synthase, MGMT: O^6-methylguanine-DNA methyltransferase.

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.