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ORIGINAL REPORT

Multicenter Phase II Study of Everolimus in Patients With Previously Treated Metastatic Gastric Cancer

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ABSTRACT

Purpose
Everolimus, an oral inhibitor of the mammalian target of rapamycin, has shown antitumor activity in gastric cancer in preclinical and phase I studies. This phase II study evaluated the efficacy and safety of everolimus in pretreated patients with advanced gastric cancer.

Patients and Methods
Patients with advanced gastric cancer who experienced progression despite prior chemotherapy received everolimus 10 mg orally daily until disease progression or study discontinuation. The primary end point was disease control rate (DCR; ie, complete response, partial response, or stable disease). Secondary end points included progression-free survival (PFS), overall survival (OS), and safety.

Results
Fifty-three patients were assessable (median age, 63 years; 51% and 49% received one or two prior chemotherapy regimens, respectively). Although no complete or partial response was obtained, a decrease in tumor size from baseline was observed in 45% of patients by central review. The DCR was 56.0% (95% CI, 41.3% to 70.0%); median PFS was 2.7 months (95% CI, 1.6 to 3.0 months). At a median follow-up time of 9.6 months, median OS was 10.1 months (95% CI, 6.5 to 12.1 months). Common grade 3 or 4 adverse events included anemia, hyponatremia, increased γ-glutamyltransferase, and lymphopenia. Grade 1 or 2 pneumonitis was reported in eight patients (15.1%).

Conclusion
Everolimus monotherapy resulted in a promising DCR in patients with previously treated advanced gastric cancer. Adverse events are consistent with the reported safety profile of everolimus. These results warrant further evaluation in patients with advanced gastric cancer.

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INTRODUCTION

Gastric cancer is the fourth most common cancer worldwide, with 603,003 and 330,290 new cases among men and women, respectively, reported annually between 1993 and 2001.[1,2] Globally, it is the second most common cause of cancer death, with an estimated 700,000 deaths annually.[1,2] In Japan, gastric cancer is the second leading cause of cancer death (50,415 deaths in 2006), accounting for 15.3% of all cancer deaths.[3]

Only surgical resection is curative; however, patients with gastric cancer commonly present with unresectable disease.[4] Even after curative surgical resection, 60% of these patients eventually experience relapse.[5] Systemic chemotherapy has been evaluated extensively in patients with unresectable and recurrent gastric cancer.[4,5] At present, although fluoropyrimidine-based therapy is used worldwide, there is no globally accepted standard first-line chemotherapy for advanced gastric cancer. In randomized studies, combination chemotherapy regimens including fluorouracil (FU) or its derivatives, taxanes, irinotecan, and platinum derivatives generally achieved median overall survival (OS) times of less than 1 year in the first-line setting.[6-12] In Japan, S-1 (tegafur + gimeracil + oteracil potassium) is an established first-line agent for advanced gastric cancer. A recent phase III trial demonstrated a median OS time of 13 months with the combination of S-1 plus cisplatin as first-line therapy for patients (n = 148) with advanced gastric cancer.[13]

The poor long-term outcomes associated with chemotherapies to date strongly suggest considerable unmet needs in gastric cancer and a need for new agents, particularly targeted agents that will confer a survival benefit with acceptable tolerability. This is especially true in the second- and third-line

settings, in which to date there are no phase III studies demonstrating survival benefit for chemotherapy compared with best supportive care.

Inhibition of the mammalian target of rapamycin (mTOR) pathway represents a new therapeutic target in the treatment of various human cancers. mTOR, a key protein kinase that regulates cell growth and proliferation, cellular metabolism, and angiogenesis,[14] is mainly activated via the PI3 kinase pathway through Akt/PKB and tuberous sclerosis complex. Mutations in these components or in PTEN, a negative regulator of PI3 kinase, result in inappropriate mTOR activation.[14] The mTOR pathway has been shown to be frequently dysregulated in a variety of human cancers, including gastric cancer.[15] Oncogenic transformation maintained by a dysregulated mTOR pathway may sensitize tumor cells to mTOR inhibitors.[14] Overexpression of the mTOR downstream effectors[14] eIF4E and 4E binding protein 1 (4E-BP1) was shown in GI cancer cells. Everolimus reduced 4E-BP1 phosphorylation and attenuated production of the proangiogenic factors hypoxia-inducible factor 1α and vascular endothelial growth factor in these gastric cancer cell lines.[15]

Everolimus is an orally bioavailable mTOR inhibitor that binds with high affinity to its intracellular receptor FKBP12.[16] Everolimus has demonstrated antitumor activity in gastric cancer in preclinical studies[14,15,17] and a phase I study involving patients with advanced gastric cancer.[18] The current phase II study evaluated the efficacy and safety of everolimus monotherapy in patients with advanced gastric cancer who had experienced treatment failure with one or two prior chemotherapy regimens.

PATIENTS AND METHODS

Patient Eligibility

This open-label, single-arm, multicenter, proof-of-concept, phase II study was conducted in Japan and included patients ≥ 20 years of age with pathologically confirmed advanced gastric adenocarcinoma who had received one or two prior chemotherapy regimens (one regimen was required to contain any of the following: FU or its derivatives, platinum derivatives, taxanes, or irinotecan) and who had ≥ one measurable lesion according to Response Evaluation Criteria in Solid Tumors (RECIST). Patients were required to have documented progressive disease (PD) based on imaging during or after last prior treatment. Before study entry, prior therapies had to be completed for ≥ 2 weeks for anticancer agents and for ≥ 4 weeks for surgery or radiotherapy, and patients had to recover from adverse reactions of prior therapy. Patients were required to have Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0 or 1 and adequate organ function (bone marrow function: neutrophils ≥ 1.5×10^9/L, platelets ≥ 100×10^9/L, hemoglobin ≥ 8.5 g/dL; liver function: serum bilirubin ≤ 1.5 mg/dL and ALT and AST ≤ 2.5 × upper limit of normal [ULN] if no evidence of liver metastasis or serum bilirubin ≤ 1.5 mg/dL and ALT and AST ≤ 5.0 × ULN with liver metastases; renal function: serum creatinine ≤ 2 × ULN). Exclusion criteria were CNS metastases already detected, malignant ascites requiring invasive treatment (eg, ascites drainage), or severe or uncontrolled medical conditions (eg, impaired heart and lung function, diabetes, active infections, or liver disease).

This study was conducted according to the ethical principles of the Declaration of Helsinki and approved by the institutional review board of each center. All patients provided written informed consent.

Study Treatment and Assessment

All patients were treated with everolimus 10 mg/d orally in continuous 28-day cycles until tumor progression, unacceptable toxicity, or study discontinuation for any other reason. Two levels of dose reduction were permitted (5 mg/d and then 5 mg every other day) for tolerability. For the baseline tumor assessment, radiographic assessments (computed tomography or magnetic resonance imaging scans of the chest, abdomen, and pelvis) were performed within 2 weeks before the first dose of everolimus. Tumor response was assessed every 4 weeks from cycle 2 to cycle 4 and then every two cycles until determination of disease progression and/or at the end of the study. Disease status was assessed by a local radiologist with the investigator and reviewed by central review of radiology using RECIST criteria.

Safety assessments consisted of continuous monitoring and recording of all adverse events (AEs) and regular monitoring of hematology, serum chemistry, vital signs, weight, ECOG PS, chest computed tomography scans, and physical condition. AEs were evaluated according to the National Cancer Institute Common Terminology Criteria for Adverse Events (version 3).

Statistical Considerations

The primary efficacy objective was to assess disease control rate (DCR), which was defined as the proportion of patients with complete response, partial response, or stable disease (SD) as the best overall response according to RECIST. DCR was summarized in terms of percentage, with a 95% CI. The DCR was calculated primarily based on the assessment of the central radiologic review. All results were analyzed in the full analysis set (FAS), which included all patients who received at least one dose of everolimus. DCR as primary end point was also analyzed in the per-protocol set (PPS), which consisted of patients from the FAS who completed a minimum exposure requirement (dose-intensity ≥ 0.5) or experienced progression before the minimum exposure requirement without any major protocol deviation and was defined as the primary analysis population. This study adopted a Simon two-stage design for sample size determination,[19] which required disease control in ≥ eight of the first 21 patients enrolled onto the first stage to proceed to the second stage, in which an additional 27 patients were planned to be enrolled. The null hypothesis was a DCR of ≤ 30%. DCRs of 30% (futility rate) and 50% (targeted antitumor activity rate) were used for power setting.[20,21] If ≥ 20 of 48 patients achieved disease control, the null hypothesis would be rejected, and everolimus would be considered to have antitumor activity in this population.

The secondary end points of the study were to assess objective response rate, progression-free survival (PFS), OS, and the safety profile of everolimus. PFS and OS curves were generated using the Kaplan-Meier product-limit method. Median PFS and OS were obtained with a 95% CI. Safety analysis was performed in the safety population, which consisted of all patients who received ≥ one dose of everolimus and had ≥ one postbaseline safety assessment.

As an exploratory end point, the influence of gastrectomy on the pharmacokinetics (PKs) of everolimus was investigated. Blood samples for PK analyses were collected from patients enrolled onto the first stage before dose and at 1, 2, 3, and 4 hours after dose on day 1 of cycles 1 and 2 and from all patients before dose on day 1 of cycles 1, 2, 3, and 4. Everolimus concentrations in whole blood were determined by liquid chromatography–mass spectrometry. The PK population consisted of all patients from the safety population who had PK samples available. Noncompartmental methods with WINNonlin Pro (Version 5.2; Pharsight, St Louis, MO) were used to determine the PK parameters of area under the concentration-time curve from 0 to 4 hours after drug administration [$AUC_{(0-4)}$], observed predose concentration (C_{min}), maximum blood drug concentration (C_{max}), and time to reach maximum concentration after drug administration (T_{max}).

RESULTS

Patient Disposition

A total of 26 patients were enrolled onto the first stage to ensure 21 patients in the PPS population at week 8. Central radiologic review confirmed that ≥ eight patients achieved disease control, and an additional 28 patients were enrolled onto stage 2 (Fig 1). The FAS population included 53 patients; the remaining patient did not receive study medication because of ineligibility. Three patients were not

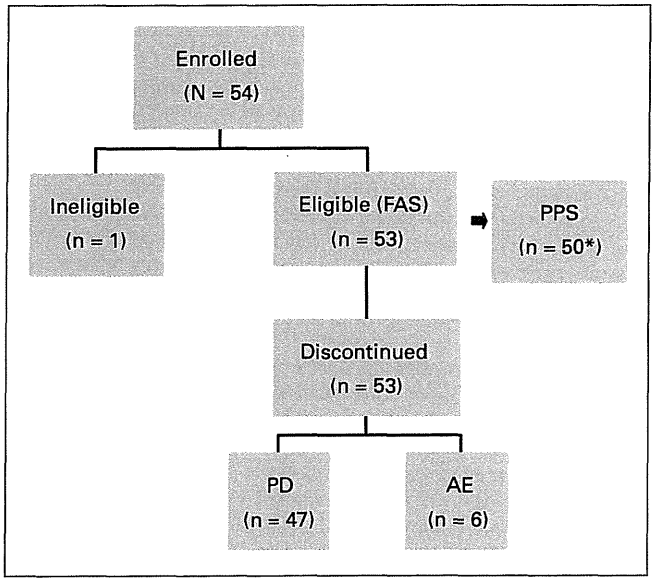

Fig 1. Patient disposition. (*) Three patients were excluded from the per-protocol set (PPS), two with unknown best response and one with dose intensity less than 50% during the first 8 weeks of treatment. FAS, full analysis set; PD, progressive disease; AE, adverse event.

included in the PPS population (n = 50); two patients were not assessable for best overall response, and one patient had a dose-intensity of less than 50% during the first 8 weeks of treatment. At study completion, 47 patients had discontinued treatment as a result of disease progression (Fig 1).

Patient Characteristics

Most patients were men (77%), and the median age was 63 years (Table 1). All treated patients had an ECOG PS of 0 or 1 (PS 0 = 60%; PS 1 = 40%). Most patients had moderately (47%) or poorly (42%) differentiated adenocarcinomas. Most patients had been previously treated; 25 of 53 patients had a gastrectomy, and all patients had received chemotherapy (51% with one prior line; 49% with two prior lines). The most commonly used prior chemotherapy agents in the study population were FU derivatives (S-1) as monotherapy (49%) and in combination with cisplatin (55%), and the most common second-line agents were paclitaxel (17%) or irinotecan (11%) as monotherapy (Table 1).

Efficacy

Best overall responses per central radiology review are listed in Table 2; 28 (56.0%) and 22 patients (44.0%) in the PPS population and 29 (54.7%) and 22 patients (41.5%) in the FAS population had SD and PD, respectively. Disease control was observed in more than 20 patients in the first 48 patients (out of 50 patients) in the PPS population, and the null hypothesis (DCR $\leq$ 30%) was rejected at the one-sided $\alpha = .05$. At the final analysis, disease control was observed in 28 patients (56.0%; 95% CI, 41.3% to 70.0%) in the PPS population. The lower limit 95% CI value (41.3%) exceeded the threshold (30%) for futility. Results in the FAS population (DCR = 54.7%; 95% CI, 40.4% to 68.4%) were consistent with the results observed in the PPS population. Although no complete or partial response was obtained, a decrease in tumor size from baseline was observed in 45% of patients by central review. The maximum best change observed was a 34% decrease in sum of longest diameters when compared with baseline (Fig 2). Subgroup analysis by number of previous chemotherapies indicated that the effect of everolimus was consistent in the second- and third-line PPS populations, with the same proportions of patients with SD (56.0%) and PD (44.0%) observed in each group.

Table 1. Patient Demographic and Clinical Characteristics

Demographic or Clinical Characteristic	No. of Patients (N = 53)	%
Age, years		
Median	63	
Range	30-77	
Asian	53	100
Male	41	77
ECOG *performance status* (0/1)		
0	32	60
1	21	40
Degree of tumor differentiation		
Well	6	11
Moderate	25	47
Poor	22	42
Gastrectomy	25	47
No. of prior chemotherapy regimens		
1	27	51
2	26	49
Contents of prior chemotherapy regimens		
FU monotherapy*	26	48
FU plus cisplatin	29	55
Paclitaxel monotherapy	9	17
Irinotecan monotherapy	6	11
Other†	9	17
Site of measurable lesion		
Abdominal lymph node	26	49
Liver	25	47
Distant lymph node	11	21
Peritoneum	4	8
Lung	3	6
Ovary	3	6
Other‡	5	9

Abbreviations: ECOG, Eastern Cooperative Oncology Group; FU, fluorouracil.
*Including FU derivatives S-1, capecitabine, and so on.
†Other includes irinotecan plus cisplatin (n = 4), FU plus paclitaxel (n = 3), FU plus irinotecan (n = 1), and FU plus methotrexate (n = 1).
‡Other measurable lesion sites include abdominal mass, adrenals, thyroid gland, pleura, pulmonary lymphangitic spread (n = 1 each).

Table 2. Best Overall Response and DCR per Central Review

Best Overall Response and DCR	PPS (n = 50)		FAS (N = 53)	
	No. of Patients	%	No. of Patients	%
Best overall response				
CR	0	0	0	
PR	0	0	0	
SD	28	56.0	29	54.7
PD	22	44.0	22	41.5
Unknown	0	0	2	3.8
DCR (CR + PR + SD)	28	56.0	29	54.7
95% CI, %	41.3 to 70.0		40.4 to 68.4	

Abbreviations: DCR, disease control rate; PPS, per-protocol set; FAS, full analysis set; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

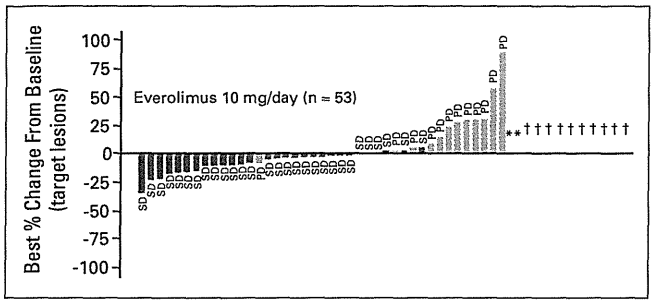

Fig 2. Maximum best change in tumor size from baseline. Decrease in best percent change from baseline = 45.28%; increase in best percent change or no percent change from baseline = 32.08%. (*) Percent change in target lesion was available but contradicted by overall lesion response = unknown 3.77%. (†) Percent change in target lesion was available but contradicted by overall lesion response = progressive disease (PD) 18.87%. SD, stable disease.

Median PFS was 2.7 months (95% CI, 1.6 to 3.0 months; Fig 3A). At 4 months, 28.3% (Kaplan-Meier estimate) of patients were progression free. Subgroup analysis did not reveal a difference in PFS stratified by number of prior chemotherapy regimens; in the second-line setting, median PFS was 2.6 months (95% CI, 1.0 to 3.0 months), and in the third-line setting, median PFS was 2.8 months (95% CI, 1.6 to 4.0 months). At a median follow-up time of 9.6 months, median OS was 10.1 months (95% CI, 6.5 to 12.1 months; Fig 3B); in the second-line setting, median OS was 9.8 months (95% CI, 6.2 to 12.3 months), and in the third-line setting, median OS was 10.7 months (95% CI, 6.3 months to not reached).

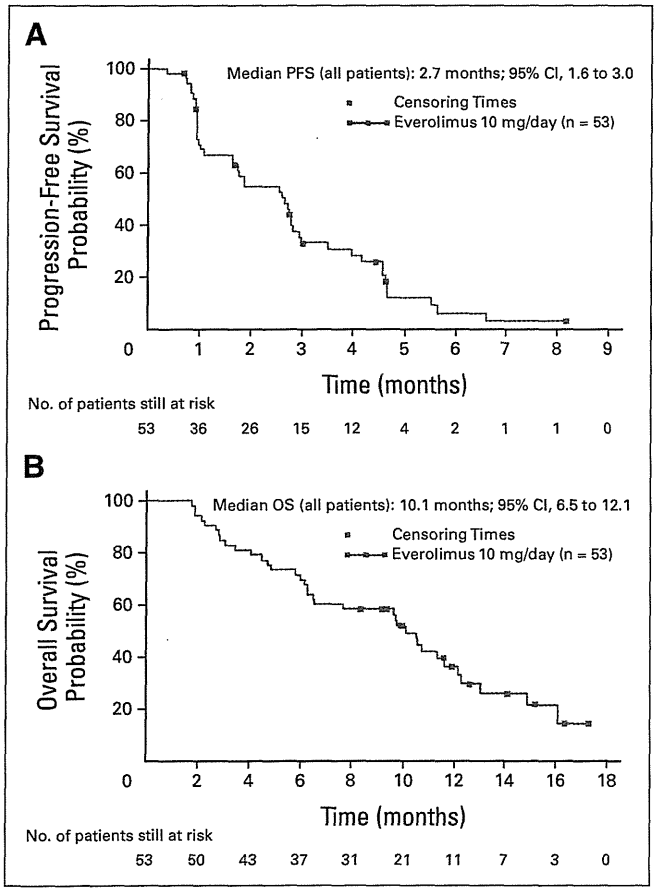

Fig 3. Kaplan-Meier plots of (A) median progression-free survival (PFS) and (B) median overall survival (OS) in all patients.

PK Analysis

On day 1 of cycle 1 and at steady-state (day 1 of cycle 2), slightly higher peak plasma concentrations (C_{max}) of everolimus were observed in patients who had undergone gastrectomy compared with patients who had not. In addition, T_{max}, C_{max}, and $AUC_{(0-4)}$ data on day 1 of cycle 1 and at steady-state also suggest that the rate of absorption of everolimus was faster in patients who had undergone gastrectomy [higher C_{max} and $AUC_{(0-4)}$ and shorter T_{max}] than in patients who had not (Table 3). However, mean C_{min} values on day 1 of cycles 1, 2, 3, and 4 were similar between patients with and without gastrectomy, as were AUC during dosing interval values at steady-state,

Table 3. PK Parameters of Everolimus

PK Parameter	No. of Patients	PK Value Mean	PK Value SD
Day 1 of cycle 1			
C_{max}, ng/mL			
No gastrectomy	14	78.3	39.7
Gastrectomy	12	122	33.2
T_{max}, hours			
No gastrectomy	14	1.5	0.92
Gastrectomy	12	1.1	0.29
$AUC_{(0-4)}$, h · ng/mL			
No gastrectomy	14	172	84
Gastrectomy	12	271	84
Day 29 (day 1 of cycle 2)			
C_{max}, ng/mL			
No gastrectomy	6	98.7	33.4
Gastrectomy	10	134	33.0
T_{max}, hours			
No gastrectomy	6	2.0	1.29
Gastrectomy	10	1.0	0.11
$AUC_{(0-4)}$, h · ng/mL			
No gastrectomy	6	254	101
Gastrectomy	10	324	94
C_{min}, ng/mL			
Day 1 (day 1 cycle 1)			
No gastrectomy	9	11.2	2.43
Gastrectomy	6	15.4	2.96
Day 29 (day 1 cycle 2)			
No gastrectomy	16	27.6	22.3
Gastrectomy	21	25.5	14.4
Day 57 (day 1 cycle 3)			
No gastrectomy	11	25.0	10.2
Gastrectomy	11	17.7	6.87
Day 85 (day 1 cycle 4)			
No gastrectomy	6	23.3	7.48
Gastrectomy	12	18.0	6.31
AUC0-τ on day 29 (day 1 of cycle 2), ng · h/mL			
No gastrectomy	6	1,080	744
Gastrectomy	10	1,100	417

Abbreviations: PK, pharmacokinetic; SD, standard deviation; C_{max}, maximum blood concentration; T_{max}, time to reach maximum plasma concentration; $AUC_{(0-4)}$, area under the concentration time curve during the first 4 hours after drug administration; C_{min}, minimum blood concentration; AUC0-τ, area under the concentration time curve during the dosing interval.

suggesting that the extent of oral absorption was similar between the two groups (Table 3).

Safety

The median duration of everolimus therapy was 57.0 days (range, 11 to 249 days), with a median cumulative dose of 540 mg (range, 110 to 1,960 mg). Although 23 patients (43.4%) had a dose reduction or interruption, the mean relative dose-intensity was 0.9.

The major AEs observed with everolimus were grade 1 or 2 in severity. The most common AEs were stomatitis (73.6%), anorexia (52.8%), fatigue (50.9%), rash (45.3%), nausea (32.1%), peripheral edema (22.6%), diarrhea (20.8%), and pruritus (18.9%). Grade 3 or 4 AEs observed during the study are listed in Table 4. Grade 3 AEs occurred in 20 patients (37.7%), including anemia (11.3%), hyponatremia (9.4%), increased γ-glutamyltransferase (7.5%), lymphopenia (7.5%), fatigue (5.7%), stomatitis (5.7%), anorexia (5.7%), abnormal hepatic function (5.7%), hyperglycemia (3.8%), hypophosphatemia (3.8%), and ileus (3.8%). Grade 4 AEs suspected to be related to treatment were reported in four patients; one patient each had tumor hemorrhage, increased γ-glutamyltransferase, lymphopenia, and cerebral infarction. Six patients discontinued the protocol treatment as a result of AEs; five of these patients had AEs suspected to be related to everolimus (grade 2 pneumonitis, n = 2; grade 3 stomatitis, n = 1; liver dysfunction, n = 1; and tumor hemorrhage, n = 1). Pneumonitis related to everolimus was observed in eight patients (15.1%); the maximum severity was grade 2.

At the time of this analysis, 36 (67.9%) of 53 patients had died; 33 of these patients died of gastric cancer, two patients died of aspiration pneumonia (not suspected to be related to everolimus), and one patient died 313 days after last dose of study drug with the cause of death unknown.

DISCUSSION

Everolimus monotherapy demonstrated a promising DCR of 56% in pretreated patients with advanced gastric cancer. In addition, 45% of patients demonstrated tumor shrinkage from baseline, the median PFS was 2.7 months, and the median OS was 10.1 months. All efficacy data except survival were judged by an independent central radiologic review committee.

The choice of DCR as the primary end point in this study was considered appropriate because it reflects clinical practice where progression usually necessitates a change of treatment; its use is also appropriate in a proof-of-concept study in the second- and third-line settings. Patients in this study were previously treated; nearly half (49%) received everolimus as a third-line therapy. The reasons for the choice of this population were the recent establishment of S-1 plus cisplatin as a standard first-line regimen in Japan and the lack of any evidence, at the time of the study, to support a survival benefit of chemotherapy over best supportive care in the second- or third-line setting in advanced gastric cancer.

The clinical evaluation of everolimus in patients with gastric cancer is supported by research regarding the mTOR pathway in preclinical models[14,15,17,22,23]; blockade of PI3 kinase signaling via mTOR inhibition has shown antitumor activity in experimental models of gastric cancer.[22,23] It is noteworthy that the efficacy results were similar in patients who had received one or two prior chemotherapy regimens. A number of other agents and combinations have been evaluated as second-line therapy in patients with advanced gastric cancer, including docetaxel, paclitaxel, irinotecan/cisplatin, paclitaxel/doxifluridine, paclitaxel/cisplatin, and S-1/mitomycin.[20,24-29] Median OS ranged from 3.5 months[24] to 7.2 months[30] in the single-agent trials and from 6 months[28] to 10.5 months[29] in the combination therapy trials. In this trial, median OS was 10.1 months (9.8 months in the second-line setting and 10.7 months in the third-line setting). These results seem to compare favorably with those observed in the other trials evaluating single-agent and combination therapy in the second-line setting. Although the number of patients in the third-line setting in this study is small, their median OS of more than 10 months is encouraging when compared with other studies in this patient population.

In earlier studies comparing FU monotherapy with FU plus cisplatin, uracil/tegafur plus mitomycin,[31] or irinotecan plus cisplatin,[32] the combinations had no survival advantage over FU monotherapy. One potential explanation for this observation is that therapy with a single agent preserved the patients' PS, allowing them to receive additional lines of chemotherapy. The same effect may have been seen in this study, where the majority of patients (n = 45) received additional chemotherapy after discontinuation of everolimus, again potentially implying that the single-agent therapy with everolimus preserved the patients' PS, making them suitable candidates for further line(s) of therapy. At the time of study discontinuation, 85% of patients (45 of 53 patients) had PS of 0 to 1, and 92% of patients (49 of 53 patients) had PS of 0 to 2.

Everolimus was generally well tolerated, and no new safety concerns were identified in the study. Grade 3 stomatitis was reported in three patients. Other major grade 3 or 4 AEs included anemia (11.3%), hyponatremia (9.4%), increased γ-glutamyltransferase (7.5%), and lymphopenia (7.5%). Pneumonitis related to everolimus was observed in eight patients (15.1%), with no grade 3 or 4 pneumonitis observed. There were no treatment-related deaths and no deaths within 28 days after discontinuation of study drug. The frequency and severity of AEs in this study, including pneumonitis, seem to be consistent with those in a large phase III placebo-controlled trial in patients with advanced renal cell carcinoma.[33] Compared with other

Table 4. Grade 3 or 4 Adverse Events > 3% Regardless of Relationship to Study Drug (N = 53)

Adverse Event	No. of Patients: Grade 3	No. of Patients: Grade 4	Total Grade 3 or 4: No. of Patients	Total Grade 3 or 4: %
Anemia	4	1	5	9.4
Hyponatremia	5	0	5	9.4
Increased GGT	2	2	4	7.5
Lymphopenia	2	2	4	7.5
Fatigue	3	0	3	5.7
Stomatitis	3	0	3	5.7
Anorexia	3	0	3	5.7
Abnormal hepatic function	2	1	3	5.7
Hyperglycemia	2	0	2	3.8
Hypophosphatemia	2	0	2	3.8
Ileus	2	0	2	3.8

Abbreviation: GGT, γ-glutamyltransferase.

second-line advanced gastric carcinoma trials,[20,25,28,30] everolimus monotherapy exhibits less bone marrow suppression in this setting. These results suggest that everolimus monotherapy is suitable for use on an outpatient basis.

PK analyses in this trial suggested that although the rate of oral absorption seems to be faster in patients who had undergone gastrectomy compared with patients who did not [as evidenced by a higher C_{max} and $AUC_{(0-4)}$ and shorter T_{max}], no differences in the extent of oral absorption were observed because the mean C_{min} and steady-state AUC over the dosing interval values were similar between patients with and without gastrectomy. Further investigation is needed because the sample size of this study is limited. In conclusion, everolimus monotherapy showed a promising rate of disease control and was well tolerated in previously treated patients with advanced gastric cancer, warranting further evaluation in a phase III trial of everolimus monotherapy in this population.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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ORIGINAL PAPER

Progression-free survival in first-line chemotherapy is a prognostic factor in second-line chemotherapy in patients with advanced gastric cancer

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Abstract
Purpose First-line chemotherapy (Cx-1) in advanced gastric cancer (AGC) provides survival benefit. However, it is unclear who should proceed to second-line chemotherapy (Cx-2).
Methods We reviewed patients who received Cx-2 for AGC following progressive disease after Cx-1 from 2000 to 2005 at the National Cancer Center Hospital, Tokyo. To evaluate the prognostic factors in Cx-2, Cox regression multivariate analysis was performed.
Results Of 995 patients who received Cx-1 in this study period, 466 met the eligibility criteria. The median progression-free survival in Cx-1 (PFS-1) was 133 days. The median survival time from the date of starting second-line chemotherapy (MST-2) was 207 days. Multivariate analysis revealed that the factors affecting short survival time in Cx-2 were poor performance status (≥2), low serum albumin level (<3.5 mg/dL), elevated C-reactive protein level (≥1.0 mg/dL), patients with bone, liver or peritoneal metastasis, and patients without previous gastrectomy ($p < 0.01$). PFS-1 was an independent prognostic factor for survival (PFS-1 <120, MST-2 133 days, PFS-1 ≥120, MST-2 258 days, hazard ratio 0.71, 95% confidence interval 0.58–0.86, $p < 0.01$). The Cx-2 regimen (irinotecan vs. taxane) did not correlate with survival.
Conclusion PFS-1 is one of the prognostic factors of Cx-2 in patients with AGC.

Keywords Advanced gastric cancer · Second-line chemotherapy · Prognostic factor · Time to progression · Survival

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Introduction

Gastric cancer is the fourth most common malignancy with approximately 940,000 new patients in the world and ranks second in all causes of death from cancer, with about 700,000 confirmed deaths annually (Kamangar et al. 2006). In 2002, there were about 100,000 gastric cancer patients in Japan and roughly half died from the malignancy in the same year.

For the first-line chemotherapy (Cx-1) of advanced gastric cancer (AGC), some randomized controlled trials have revealed survival benefit (Glimelius et al. 1997; Pyrhonen et al. 1995). Combination chemotherapy with fluorouracil (FU) (including S-1 and capecitabine) and platinum analogs (e.g., cisplatin and oxaliplatin) is presently the most widely accepted regimen for Cx-1 (Glimelius et al. 1997; Koizumi et al. 2008; Ohtsu et al. 2003; Pyrhonen et al. 1995; Ross et al. 2002; Vanhoefer et al. 2000). Although there is still no established second-line chemotherapy (Cx-2) for AGC, some promising agents such as irinotecan, taxane, and cisplatin have been used (Boku et al. 1999; Kodera et al. 2007; Lee et al. 2007; Shirao et al. 1997; Sulkes et al. 1994).

Several potential prognostic factors for short survival time in Cx-1 have been proposed. These include performance status (PS) ≥2, presence of liver or peritoneal metastasis, and

elevated alkaline phosphatase (ALP) level ≥100 U/L (Chau et al. 2004; Louvet et al. 2003; Yamamura et al. 2002). Ichikawa and Sasaki (2006) suggested that the response to Cx-1 affects progression-free survival and overall survival. However, the prognostic factors in Cx-2 remain unclarified.

In the present study, we determined the prognostic factors of survival at the start of Cx-2. Identification of these factors may assist oncologist in selecting patients for subsequent chemotherapy following progressive disease in Cx-1.

Patients and methods

Patient population

We extracted AGC cases meeting the eligibility criteria from the database of the National Cancer Center Hospital in Tokyo. The eligibility criteria were as follows (1) histologically proven gastric adenocarcinoma and received Cx-2 after Cx-1 failure due to progressive disease (PD) between January 2000 and December 2005, (2) <75 years of age, with an Eastern Cooperative Oncology Group (ECOG) PS range of 0–2 at the start of Cx-1, (3) maintained adequate main organ function for chemotherapy when Cx-1 was started. Adequate main organ function was indicated by the following: serum creatinine level <1.5 mg/dL; serum asparate aminotransferase (AST) level <100 U/L; alanine aminotransferase (ALT) level <100 U/L; white blood cell count (WBC) >3.0 × 10^9/μL but <12.0 × 10^9/μL; platelet count >100 × 10^9/μL; total bilirubin <1.5 mg/dL; able to take oral medication; no symptoms of brain metastasis or pleural effusion.

Data collection

Chart reviews for all patients were performed to obtain laboratory data on Cx-1 and Cx-2, chemotherapy regimens, treatment duration, and reason for chemotherapy discontinuation. Data included the following: WBC, hemoglobin, platelet count, ALP, lactate dehydrogenase (LDH), AST, ALT, serum creatinine, C-reactive protein (CRP), bilirubin, metastatic site (liver, peritoneum, and bone), number of metastatic sites, and tumor differentiation on histopathology. Baseline characteristics such as sex, age, PS and history of previous gastrectomy when starting Cx-2 were also evaluated. This study was conducted in accordance with Japanese ethics guidelines for clinical and epidemiological studies, which took effect in August 2007.

Chemotherapy regimens and treatment evaluation

Chemotherapy regimens were divided as follows: (1) Taxane-based: paclitaxel or docetaxel, (2) Irinotecan-based: irinotecan monotherapy, or combination with S-1, mitomycin C, or cisplatin, (3) 5FU-based: oral S-1 monotherapy or combination with cisplatin; continuous 5-FU infusion or combination with methotrexate.

In Cx-1, PD was defined as clinical progression (e.g., increased ascites or pleural effusion; deteriorated general condition due to bone metastasis; elevated tumor marker level) or radiographic progression according to response evaluation criteria in solid tumors.

Statistical analysis

Univariate and multivariate analyses were performed using the Cox regression model to determine the prognostic factors for survival. Hazard ratio (HR) and 95% confidence interval (CI) were also determined. Progression-free survival in first-line chemotherapy (PFS-1) was defined as the interval between the start of first-line chemotherapy to recognition of PD. Overall survival (OS) was defined as the interval between the start of chemotherapy to death or the date of last follow-up. Median survival time in patients who received first-line chemotherapy (MST-1) was calculated by the median interval from the start of first-line chemotherapy to death. Median survival time in patients who received second-line chemotherapy (MST-2) was the median interval from the start of second-line chemotherapy to death. PFS-1 was dichotomized according to the median PFS-1. Statistical significance was set at alpha = 0.05 for a two-sided test. OS and PFS were estimated by the Kaplan–Meier method.

Results

Baseline characteristics of patients

Of 995 patients who received Cx-1 in the study period, 466 met the eligibility criteria. Patient clinical characteristics at the start of Cx-2 are shown in Table 1. The median age of the patients was 60 years. S-1 monotherapy was the most commonly used regimen in Cx-1 of 178 patients (38.2%). Only eight patients underwent S-1 plus cisplatin combination chemotherapy (1.7%). The median PFS-1 was 129 days (95% CI 119–142 days) (Table 1). In Cx-2, taxane alone (n = 201) and irinotecan with or without cisplatin (n = 141) were the most frequently used chemotherapeutic agents.

Survival analysis

MST-1 was 371 days (95% CI 340–418 days) (Fig. 1) and MST-2 was 207 days (95% CI 182–227 days). MST-2 was significantly longer in the group with PFS-1 ≥120 than in the group with PFS <120 (Fig. 2) [i.e., 258 days (95% CI 229–287) and 133 days (95% CI 117–156), respectively (p < 0.001)].

Table 1 Baseline characteristics

Characteristic	No.	%
Age (years)		
Median	60	
Range	22–73	
Sex		
Male	305	65.5
Female	161	34.5
Performance status		
<1	396	85.0
≥2	70	15.0
Tumor differentiation		
Intestinal	313	67.2
Diffuse	145	31.1
Undetermined	8	1.7
Recurrence or metastasis		
Recurrence	132	28.3
Primary metastasis	334	71.7
Previous gastrectomy		
Yes	201	43.1
No	265	56.9
Number of metastatic site		
<3	414	88.8
≥3	52	11.2
Peritoneal metastasis		
Yes	211	45.3
No	255	54.7
Bone metastasis		
Yes	42	9.0
No	424	91.0
Liver metastasis		
Yes	169	36.3
No	297	63.7
First-line treatment		
S1 only	178	38.2
5-Fluorouracil only	97	20.8
Cisplatin/Irrinotecan	80	17.2
Methotrexate/5-Fluorouracl	90	19.3
Others	21	4.5
Second-line treatment		
Taxane base	201	43.1
Paclitaxel	182	
Docetaxel	19	
Irinotecan base	141	30.3
Irinotecan/Cisplatin	108	
Irinotecan/Mitomycin	29	
Irinotecan/S1 or Irinotecan only	4	
5-Fluorouracil base	116	24.9
S1 only	66	
S1/Cisplatin	6	
5-Fluorouracil/Methotrexate	31	
5-Fluorouracil only	9	
5-Fluorouracil/Cisplatin	4	
Others	8	1.7
Monotherapy	285	61.2
Combination chemotherapy	181	38.8
Serum albumin (mg/dL)		
Median	3.5	
Range	1.7–4.7	
Alkaline phosphatase (mg/dL)		
Median	299	
Range	34–9,656	
Total birilubin (mg/dL)		
Median	0.6	
Range	0.1–8.8	
Hemoglobin (g/dL)		
Median	10.9	
Range	5.3–108	
White blood cell count ($\times 10^3$)/μL		
Median	6.3	
Range	2.4–25.3	
Platelet count ($\times 10^4$)/μL		
Median	27.85	
Range	1.9–116.1	
Lactate dehydrogenase (IU/L) (normal range 119–229)		
Median	252	
Range	10.3–9,300	
Serum creatinine (mg/dL)		
Median	0.8	
Range	0.3–2.8	
Asparate aminotransferase (IU/L)		
Median	25	
Range	1–646	
Alanine aminotransferase (IU/L)		
Median	18	
Range	4–246	
C-reactive protein (mg/dL)		
Median	0.6	
Range	0.1–25.9	
Progression-free survival in first-line chemotherapy (days)		
Median	129	
Range	6–1,199	

Univariate and multivariate analyses

Table 2 shows the results of the univariate analysis for survival from the start of Cx-2. The following data were significantly correlated with shorter survival time: PFS-1

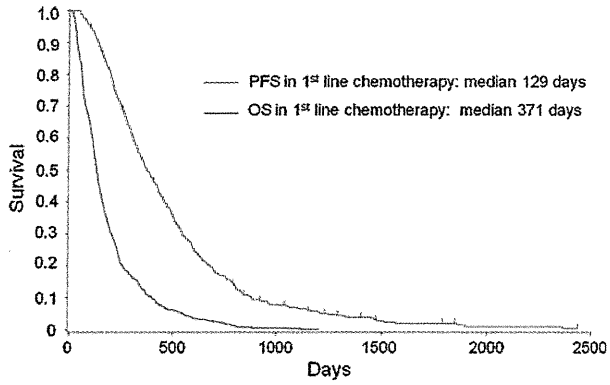

Fig. 1 Overall survival (OS) and progression-free survival (PFS) from the start of first-line chemotherapy

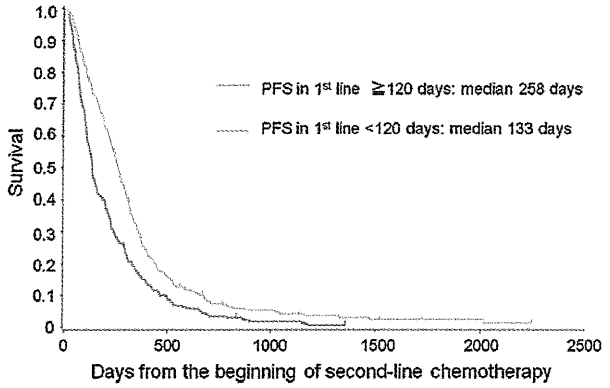

Fig. 2 Overall survival from the start of second-line chemotherapy. *Orange line* progression-free survival of first-line chemotherapy (PFS-1) ≥120 days; *green line* PFS-1 <120 days

<120 days, ECOG PS ≥2, site of metastasis ≥3, presence of peritoneal or bone metastasis, serum albumin <3.5 mg/dL, ALP ≥360 mg/dL, LDH ≥250 IU/L (above the normal range), bilirubin ≥1 mg/dL, AST >40 IU/L, ALT >40 IU/L, CRP ≥1.0 mg/dL, and WBC ≥6.5 × 10^3/μL. In the Cx-2 regimen, neither irinotecan nor taxane affected survival (irinotecan vs. taxane; HR 0.95; $p = 0.62$). We further analyzed monotherapy versus combination therapy in second-line treatment, which failed to detect the relation to survival in Cx-2.

Cox multivariate analysis included all variables that were found to have prognostic significance in univariate analysis. The results of the analysis identified the following eight independent prognostic factors that correlated with shorter survival time in Cx-2: PFS-1 <120 days (HR 0.71; $p < 0.001$); PS ≥2 (HR 0.51; $p < 0.001$); history of no previous gastrectomy (HR 0.78; $p = 0.02$); presence of peritoneal or bone metastasis (HR 0.60; $p < 0.001$ and HR 0.48; $p < 0.001$, respectively); presence of liver metastasis (HR 0.76; $p = 0.02$); serum albumin <3.5 mg/dL (HR 0.65; $p < 0.001$); CRP >1.0 mg/dL (HR 0.65; $p < 0.001$) (Table 3).

We also performed Cox regression analysis for the Cx-2 regimens to potentially identify predictive factors for survival in Cx-2; however, these factors showed no independent prognostic significance for survival (data not shown).

Discussion

We identified the following eight factors affecting the survival of AGC patients in Cx-2: PFS-1, PS, history of previous gastrectomy, peritoneal, liver, or bone metastasis, serum albumin, and CRP.

There are several studies that have described prognostic factors for the survival of AGC patients in Cx-1. These include PS, liver or peritoneal metastasis, elevated ALP level, and number of metastatic site (Chau et al. 2004; Lee et al. 2007; Louvet et al. 2003; Yamamura et al. 2002). Response to Cx-1 was previously reported to correlate with PFS-1 and survival from Cx-1 ($r = 0.56$, 0.47, respectively) (Ichikawa and Sasaki 2006). However, the prognostic factors for Cx-2 remain to be determined. In the present study, patients with longer PFS-1 had longer MST-2, resulting in longer survival from the start of Cx-1. Since gastric cancer consists of a heterogeneous population of neoplastic cells, tumor growth rate and the chemosensitivity of each tumor may vary. Longer PFS-1 in AGC may identify a chemosensitive cohort from this heterogeneous cell population.

Recently, Catalano et al. (2008) have reported five clinico-pathological factors affecting the survival of 175 AGC patients in Cx-2. These included PS, PFS-1 duration, hemoglobin, carcino embryonic antigen level, and number of metastatic sites. Their results also suggested that PFS-1 duration (HR 1.79, 95% CI 1.39–2.80, $p < 0.0001$) and PS (HR 1.79; 95% CI 1.16–2.77; $p = 0.008$) were independent prognostic factors for short survival time in Cx-2. PS has been identified as a prognostic factor in AGCs in several trials (Catalano et al. 2008; Koizumi et al. 2008) and also in other cancers (Bellmunt et al. 2002; Janisch et al. 1994; Sargent et al. 2009). PFS-1 was additionally identified as a prognostic factor in Catalano et al's analysis as well as in ours. Therefore, we conducted additional analysis focusing on two factors, namely, PS and PFS-1. Poor PS (≥2) with PFS-1 <120 days was considered to indicate dismal prognosis, with an MST-2 of only 60 days (95% CI 41–73 days) (data not shown). We consider that this cohort may not have survival benefit when using Cx-2.

In Japan, the standard Cx-1 for AGC includes S-1 and cisplatin (Koizumi et al. 2008), and most patients with good PS proceed to Cx-2. Although there is still no evidence that Cx-2 provides survival benefit in clinical practice, irinotecan

Table 2 Univariate analysis on survival

		HR	95% CI	*p* value
PFS-1	≥120 versus <120	0.64	0.53–0.77	<0.01
Age	≥60 versus <60	0.89	0.74–1.07	0.22
Sex	Male versus Female	0.95	0.78–1.16	0.61
PS	0–1 versus 2–4	0.36	0.28–0.47	<0.01
Histopathology	Intestinal versus Diffuse	1.08	0.89–1.32	0.43
	Unclassified versus Diffuse	1.32	0.64–2.69	0.45
Previous gastrectomy	Yes versus No	0.65	0.54–0.79	<0.01
Number of metastatic site	3< versus 3≥	0.54	0.40–0.72	<0.01
Peritoneal metastasis	No versus Yes	0.71	0.59–0.86	<0.01
Bone metastasis	No versus Yes	0.58	0.42–0.80	<0.01
Liver metastasis	No versus Yes	0.86	0.71–1.04	0.12
Serum albumin (mg/dL)	≥3.5 versus <3.5	0.44	0.37–0.54	<0.01
ALP (mg/dL)	<360 versus ≥360	0.60	0.49–0.72	<0.01
Total bilirubin (mg/dL)	<1 versus ≥1	0.77	0.60–0.98	0.03
Hemoglobin (g/dL)	≥11 versus <11	0.86	0.71–1.03	0.10
WBC ($\times 10^3$) (/μL)	<6.5 versus ≥6.5	0.77	0.64–0.92	<0.01
Platelet count ($\times 10^4$) (/μL)	≥15 versus <15	0.88	0.63–1.23	0.45
LDH (IU/L)	<250 versus ≥250	0.73	0.61–0.88	<0.01
Serum creatinine (mg/dL)	≥1 versus <1	0.95	0.75–1.20	0.65
AST (IU/L)	≤40 versus >40	0.63	0.51–0.78	<0.01
ALT (IU/L)	≤40 versus >40	0.71	0.55–0.90	<0.01
C-reactive protein (mg/dL)	<1 versus ≥1	0.44	0.36–0.53	<0.01
Second-line treatment	Irinotecan base versus Taxane base	0.95	0.76–1.18	0.62
	Others versus Taxane base	0.98	0.78–1.23	0.85
	Combination chemotherapy versus Monotherapy	1.11	0.92–1.34	0.29

PFS-1 progression-free survival in first-line chemotherapy, *PS* performance status, *ALP* alkaline phosphatase, *WBC* white blood cell, *LDH* lactate dehydrogenase, *AST* asparate aminotransferase, *ALT* alanine aminotransferase, *HR* hazard ratio, *CI* confidence interval

Table 3 Multivariate analysis on survival

		HR	95% CI	*p* value
PFS-1	≥120 versus <120	0.71	0.58–0.86	<0.001
Performance status	0–1 versus 2–4	0.51	0.38–0.69	<0.001
Previous gastrectomy	Yes versus No	0.78	0.64–0.96	0.02
Peritoneal metastasis	No versus Yes	0.60	0.49–0.74	<0.001
Bone metastasis	No versus Yes	0.48	0.33–0.67	<0.001
Liver metastasis	No versus Yes	0.76	0.60–0.95	0.02
Albumin (mg/dL)	≥3.5 versus <3.5	0.65	0.53–0.81	<0.001
ALP (mg/dL)	<360 versus ≥360	0.83	0.66–1.02	0.08
CRP (mg/dL)	<1 versus ≥1	0.65	0.52–0.80	<0.001

PFS-1 progression-free survival in first-line chemotherapy, *ALP* alkaline phosphatase, *CRP* C-reactive protein, *HR* hazard ratio, *CI* confidence interval

or taxane containing regimen is commonly used for Cx-2 (Boku et al. 1999; Kodera et al. 2007; Shirao et al. 1997; Sulkes et al. 1994). In our analysis, patients who received irinotecan tended to have liver metastasis but not peritoneal metastasis (data not shown); however, the prognosis of these patient groups was not significantly different. In the early 1990s when Cx-2 was not widely used, the median survival time of AGC patients was nearly 7 months (Ohtsu et al. 2003). A recent trial conducted in Japan suggested that median survival time was prolonged to almost 1 year with the same regimens of Cx-1 used previously (Koizumi et al. 2008). This prolongation may be attributed to changes in the Cx-2 regimen. In the JCOG9205 study, the median survival time of patients receiving the 5-FU arm was 7.1 months; however, this time increased to 9.0 months in the JCOG9912 study (Boku et al. 2009). These results imply that the Cx-2 regimen improved survival. Taken together, we believe that Cx-2 may show benefit for selected patients.

Because of the retrospective nature of the present analysis, some considerations must be taken into account in interpreting our findings. First, the regimens used may not be valid in other facilities or countries where S-1 is not used as standard chemotherapy for AGC. Second, PFS-1 may possibly become a substitute potential prognostic factor in Cx-1, that is, patients with poor PS or other risks when starting Cx-1 may have shorter PFS-1 than those without. To exclude this possibility, we did not include patients with poor organ function, advance age (over 75 years), and inadequate bone marrow function in Cx-1 from the cohort. Nevertheless, the results of our analysis are important from three

aspects. First, our findings can be used as a basis for excluding patients who will not likely benefit from subsequent chemotherapy following disease progression after Cx-1. Second, our results may help oncologists in providing advice to patients regarding their potential survival. Third, the present data suggest the need to stratify future studies of Cx-2 to adequately assess treatment response and survival data.

In conclusion, we demonstrated that PFS-1 is an independent prognostic factor for survival in Cx-2. Survival benefit from Cx-2 may be limited for patients with PFS-1 <120 days and other risk factors. Physicians should carefully consider PFS-1 and PS, as well as other potential variables, when considering Cx-2 and advising patients about the potential risks, harms and benefit of CX-2 for AGC.

Conflict of interest statement None declared.

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Association of *carboxylesterase 1A* genotypes with irinotecan pharmacokinetics in Japanese cancer patients

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- Association of *UDP-glucuronosyltransferase 1A1 (UGT1A1)* genetic polymorphisms **6* and **28* with reduced clearance of SN-38 and severe neutropenia in irinotecan therapy was demonstrated in Japanese cancer patients.
- The detailed gene structure of *CES1* has been characterized.
- Possible functional SNPs in the promoter region have been reported.

WHAT THIS STUDY ADDS

- Association of functional *CES1* gene number with AUC ratio [(SN-38 + SN-38G)/irinotecan], an *in vivo* index of CES activity, was observed in patients with irinotecan monotherapy.
- No significant effects of major *CES1* SNPs on irinotecan PK were detected.

AIMS

Human carboxylesterase 1 (CES1) hydrolyzes irinotecan to produce an active metabolite SN-38 in the liver. The human *CES1* gene family consists of two functional genes, *CES1A1* (*1A1*) and *CES1A2* (*1A2*), which are located tail-to-tail on chromosome 16q13-q22.1 (*CES1A2-1A1*). The pseudogene *CES1A3* (*1A3*) and a chimeric *CES1A1* variant (*var1A1*) are also found as polymorphic isoforms of *1A2* and *1A1*, respectively. In this study, roles of *CES1* genotypes and major SNPs in irinotecan pharmacokinetics were investigated in Japanese cancer patients.

METHODS

CES1A diplotypes [combinations of haplotypes A (*1A3-1A1*), B (*1A2-1A1*), C (*1A3-var1A1*) and D (*1A2-var1A1*)] and the major SNPs (−75T>G and −30G>A in *1A1*, and −816A>C in *1A2* and *1A3*) were determined in 177 Japanese cancer patients. Associations of *CES1* genotypes, number of functional *CES1* genes (*1A1*, *1A2* and *var1A1*) and major SNPs, with the AUC ratio of (SN-38 + SN-38G)/irinotecan, a parameter of *in vivo* CES activity, were analyzed for 58 patients treated with irinotecan monotherapy.

RESULTS

The median AUC ratio of patients having three or four functional *CES1* genes (diplotypes A/B, A/D or B/C, C/D, B/B and B/D; $n = 35$) was 1.24-fold of that in patients with two functional *CES1* genes (diplotypes A/A, A/C and C/C; $n = 23$) [median (25th–75th percentiles): 0.31 (0.25–0.38) *vs.* 0.25 (0.20–0.32), $P = 0.0134$]. No significant effects of *var1A1* and the major SNPs examined were observed.

CONCLUSION

This study suggests a gene-dose effect of functional *CES1A* genes on SN-38 formation in irinotecan-treated Japanese cancer patients.

Introduction

Human carboxylesterases (CESs) are members of the α/β-hydrolase-fold family and are localized in the endoplasmic reticulum of many different cell types. These enzymes efficiently catalyze the hydrolysis of a variety of ester- and amide-containing chemicals as well as drugs (including prodrugs) to the respective free acids. They are involved in detoxification or metabolic activation of various drugs, environmental toxicants and carcinogens. CESs also catalyze the hydrolysis of endogenous compounds such as short- and long-chain acyl-glycerols, long-chain acyl-carnitine, and long-chain acyl-CoA esters. The two major CES families CES1 and CES2 have been identified in human tissues. CES1 is abundant in the liver and lung but not in the intestine, while CES2 is highly expressed in the intestine and kidney but has low expression in the liver and lung [1].

Human CES1 and CES2 are involved in producing a topoisomerase I inhibitor SN-38, an active metabolite of irinotecan which is clinically used for colorectal, lung and other cancers [2]. SN-38 is further inactivated by UDP-glucuronosyltransferase 1As (UGT1As) to produce SN-38 glucuronide (SN-38G). Irinotecan is also converted by cytochrome P450 3A4 (CYP3A4) to an inactive compound 7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino]carbonyloxycamptothecin (APC) (Figure 1).

Recent pharmacogenetic studies on irinotecan have revealed significant associations of *UGT1A1* polymorphisms **28* [–54_39A(TA)$_6$TAA>A(TA)$_7$TAA or –40_39insTA] and **6* [211G>A (G71R)], the latter being specifically detected in East Asians, with reduced clearance of SN-38 resulting in severe neutropenia [3–8]. These findings have led to the clinical application of genetic testing for *UGT1A1*28* in the United States (since August 2005) and for *UGT1A1*6* and **28* in Japan (since March 2009). In addition, possible additive effects of genotypes of the transporters for irinotecan and its metabolites, such as *ABCB1*, *ABCC2*, *ABCG2* and *SLCO1B1*, have been suggested [9–12]. We previously analyzed *CES2* polymorphisms in a Japanese

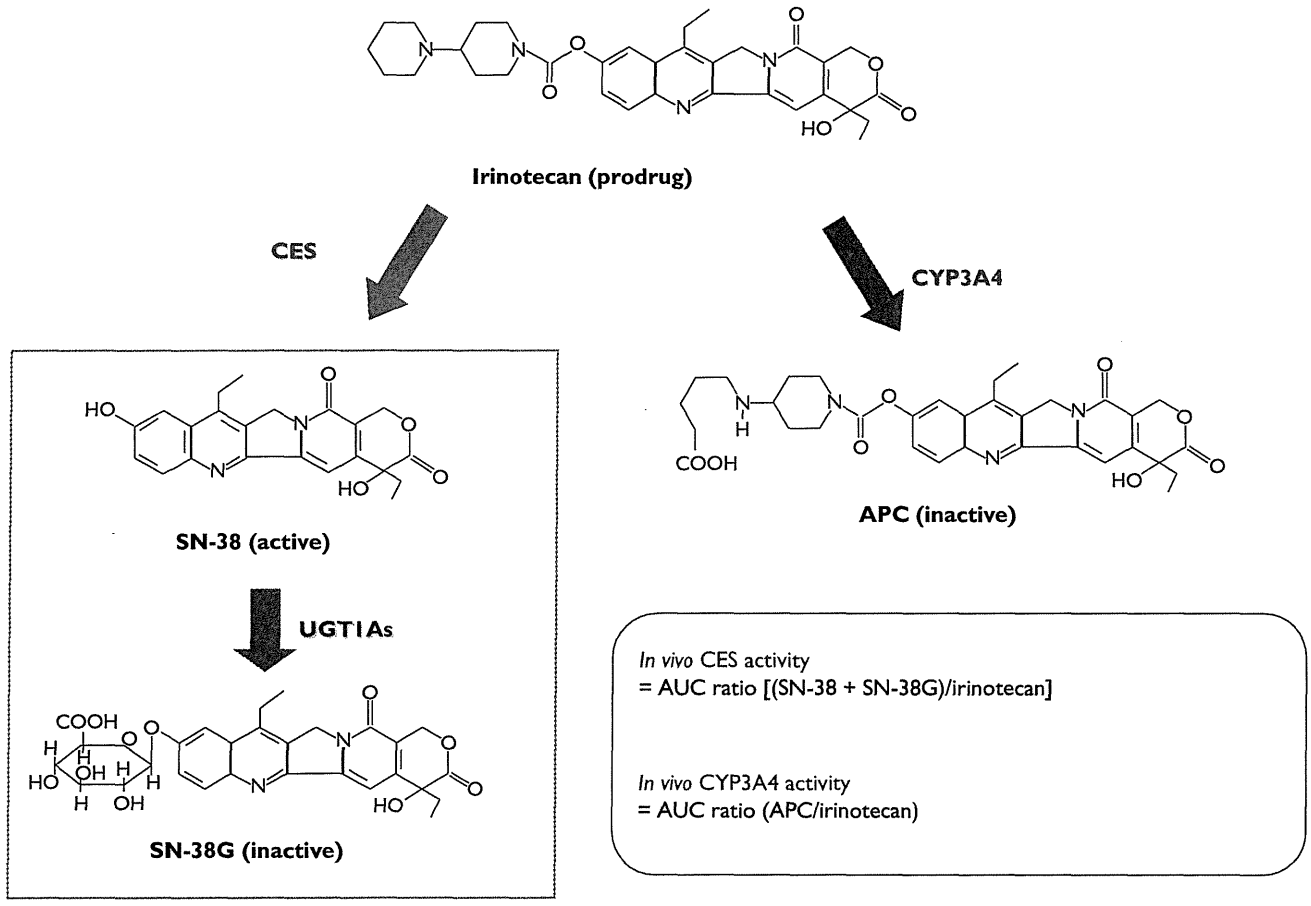

Figure 1

Metabolic pathway of irinotecan. The prodrug irinotecan is hydrolyzed by carboxylesterase (CES) to produce an active metabolite SN-38, and subsequently detoxified by UDP-glucuronosyltransferase 1As (UGT1As) to produce an inactive metabolite SN-38 glucuronide (SN-38G). Irinotecan is also metabolized by cytochrome P450 3A4 (CYP3A4) to produce another inactive metabolite APC

population and identified minor genetic variations which were associated with lower expression/function *in vitro* and *in vivo* [13, 14]. However, major *CES2* haplotypes (**1b* and **1c*) did not affect irinotecan pharmacokinetics (PK) [14]. Since CES1 is expressed at higher levels in the liver, a major organ for activating irinotecan, it is possible that *CES1* genotypes affect the plasma concentrations of irinotecan metabolites. However, their clinical relevance to irinotecan pharmacokinetics/pharmacodynamics has not yet been fully investigated.

Functional human *CES1* genes include *CES1A1* (*1A1*) and *CES1A2* (*1A2*), which are inversely located (tail-to-tail) on chromosome 16q13-q22.1 (*1A2-1A1*). Both *1A1* and *1A2* consist of 14 exons encoding 567 amino acids, and they have 98% homology with 5 nucleotide (4 amino acid) differences in exon 1, which encodes a signal peptide [1]. Recent studies also identified *CES1A1* variants (*var1A1*), in which exon 1 was replaced with exon 1 of *CES1A2*, and a pseudogene *CES1A3* (*1A3*; formerly referred to as *CES4*) replacing *CES1A2* [15, 16]. The *1A3* sequence from the promoter region to exon 1 is the same as that of *CES1A2*, but contains a stop codon in exon 3. The sequence downstream from exon 11 is highly homologous with that of *1A1* (NT_010498) [16]. Ethnic differences in these *CES1* genes (*1A1*, *var1A1*, *1A2* and *1A3*) have been reported [16].

Expression levels of CES1A2 mRNA were lower than those of CES1A1 mRNA in several tissues. This CES1A1 up-regulation could be mediated by additional Sp1 and C/EBP binding sites in the promoter region [17]. Transcript levels of CES1A2 derived from *var1A1* were reported to be higher than those from the original *1A2* [15, 16]. These findings suggest that polymorphisms in the upstream region of *CES1A1* or *var1A1* could affect their expression.

In addition to structural variations of the *CES1* gene family, several single nucleotide polymorphisms (SNPs) and small deletion/insertion variants were found. −816C in the *CES1A2* promoter region was reported to be associated with enhanced CES1A2 expression and imidapril efficacy [18]. Furthermore, −816A>C was found to be linked with several SNPs (−62T>C, −47G>C, −46G>T, −41C>G, −40A>G, −37G>C, −34del/G and −32G>T) in the proximal promoter region, leading to two additional Sp1 binding sites, and these additional sites were suggested to increase transcription of *1A2* [19].

In this context, this study investigated the clinical significance of *CES1* genotypes in irinotecan therapy. For this purpose, we analyzed the *CES1* genotypes (combinations of four *CES1A* isoforms) and major SNPs in the *CES1A1* exon 1 with its adjacent region and in the *CES1A2* and *1A3* promoter regions, which could be important for CES1 expression or function, in Japanese cancer patients treated with irinotecan, and then examined the associations of these *CES1* genotypes or SNPs with irinotecan PK.

Methods

Patients

Genetic analysis of 177 Japanese cancer patients who received irinotecan therapy at the National Cancer Center in Japan was performed. The patients were the same as those described in our previous study [7], where details on eligibility criteria for irinotecan therapy, patient profiles and irinotecan regimens were described. Since the AUC ratio [(SN-38 + SN-38G) : irinotecan], a parameter of *in vivo* CES activity, was influenced by irinotecan regimens [14], 58 patients receiving irinotecan monotherapy (100 mg m^{-2} weekly or 150 mg m^{-2} biweekly) from the 177 patients were primarily used for analysis of the association between *CES1* genotypes and irinotecan PK parameters. The patient set was the same as used in our previous study on *CES2* [14]. This study was approved by the ethics committees of the National Cancer Center and the National Institute of Health Sciences, and written informed consent was obtained from all participants.

Determination of CES1 genotypes and SNPs

For describing the *CES1* gene family, haplotypes A to D designated by Fukami *et al.* [16] were used (Figure 2): haplotype A, *CES1A3-CES1A1* (*1A3-1A1*); haplotype B, *CES1A2-CES1A1* (*1A2-1A1*); haplotype C, *CES1A3-CES1A1* variant (*1A3-var1A1*); and haplotype D, *CES1A2-CES1A1* variant (*1A2-var1A1*). To determine the diplotypes, combinations of haplotypes A to D, we sequenced *1A1/var1A1* exon 1 and its flanking region and the *1A2/1A3* promoter region of 177 patients. These regions are indicated in Figure 2, and a list of primers/probes is shown in Table 1.

For discrimination between *1A1* and *var1A1*, their exon 1s and flanking regions were sequenced (Figure 2a). Briefly, the first PCR was performed using 25 ng of genomic DNA with 0.625 units of Ex-Taq (Takara Bio. Inc., Shiga, Japan) and 0.2 μM of primers, Ces1-FP and Ces1-RP (Table 1a, first PCR). The PCR conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 2 min, and then a final extension at 72°C for 7 min. Then, the second PCR was performed with the primers, Ces1_seqF and Ces1_seqR (Table 1a, second PCR) under the same reaction conditions described above. The PCR products were treated with a PCR Product Pre-Sequencing Kit (USB Co., Cleveland, OH, USA) and directly sequenced on both strands using an ABI BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with the sequencing primers listed in Table 1a (sequencing). Excess dye was removed by a DyeEx96 kit (Qiagen, Hilden, Germany), and the eluates were analyzed on an ABI Prism 3730 DNA Analyzer (Applied Biosystems). The conditions of the PCR and sequencing procedures described in the following section were the same as described above unless otherwise noted.

1A2 and *1A3* were discriminated by the restriction fragment length polymorphism (RFLP) method for exon 5

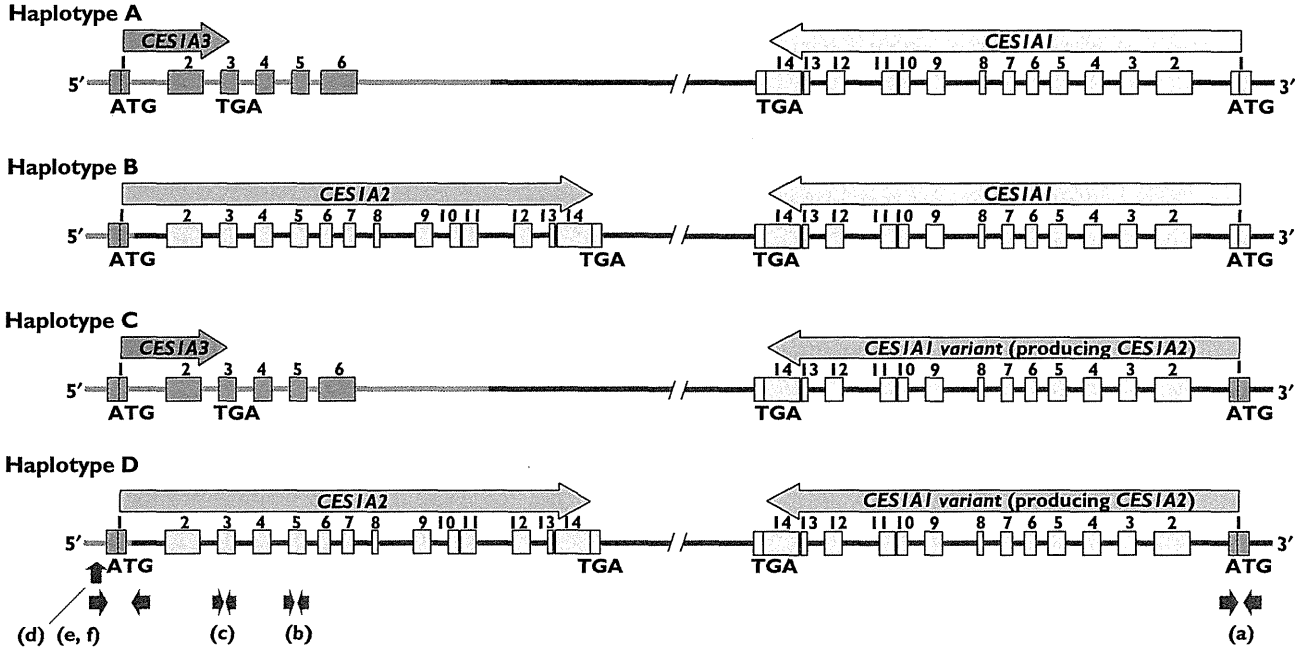

Figure 2

CES1 gene structure and haplotypes. The regions used for haplotype determination in this study are indicated with arrows (a–f)

reported by Fukami *et al.* [16] (Figure 2b). Briefly, the PCR was performed using a primer set (1A-int4F and 1A-int5AS) (Table 1b), and then the PCR products were digested with *Pvu*II to produce *CES1A3*-derived fragments (409 bp and 248 bp). UV intensity of the fragments stained with ethidium bromide was measured after electrophoresis (2% agarose gel). The number of *1A3* (0, 1 or 2) was also confirmed by direct sequencing of exon 5 using the same primer set. To verify that the *1A3* sequence is derived from the pseudogene, we confirmed the existence of a stop codon at codon 105 of *1A3* exon 3 (Figure 2c) in 11 randomly selected patients (heterozygous or homozygous) by amplification and sequencing using primers listed in Table 1c.

Genotyping for –816A>C in the *1A2* and *1A3* promoter region (Figure 2d) was conducted by the TaqMan method of Geshi *et al.* [18] (Table 1d) in all patients. We also examined attribution of –816C to *1A2* or *1A3* by specific amplifications from 5′-regions to intron 1 of the *1A2* and *1A3* (Figure 2e,f) in 23 randomly selected heterozygous patients. For specific amplifications, primers CES1A3-1A2_F1 and CES1A2 R1 for *CES1A2* (Table 1e) and primers CES1A3-1A2_F1 and CES1A3 R1 for *1A3* (Table 1f, first PCR) were used with 0.05 U μl^{-1} LA-Taq with GC buffer I (Takara Bio. Inc.); and for *1A3*, the second PCR using primers CES1A3-1A2_F2 and CES1A3 R2 (Table 1f, second PCR) was also conducted with 0.05 U μl^{-1} Ex-taq. Then, direct sequencing of the *1A2* and *1A3* PCR products was performed. Complete linkage among –816A>C and several SNPs in the proximal promoter region (between –62 to –32) [19] was confirmed for 11 randomly selected subjects.

All variations were confirmed by sequencing PCR products generated from new amplifications from genomic DNA. GenBank NT_010498.15 was used as the reference sequence for *CES1A1, CES1A3* and the promoter region of *CES1A2*, and AB119998.1 was used for exon 1 and its downstream region of *CES1A2*. The translational initiation site was designated as +1 to describe the polymorphism positions. Diplotype configuration was estimated with the LDSUPPORT software [20]. The diplotypes A/D and B/C could not be distinguished.

Pharmacokinetic data and association analysis

The area under the concentration–time curve (AUC) values for irinotecan and its metabolites, SN-38, SN-38G and APC, were previously obtained [4, 21]. The AUC ratio of SN-38 plus SN-38G to irinotecan [$AUC_{(SN\text{-}38\ +\ SN\text{-}38G)}/AUC_{irinotecan}$] was used as a parameter reflecting *in vivo* CES activity [14]. The AUC ratio of APC to irinotecan [$AUC_{APC}/AUC_{irinotecan}$] was used as a parameter for *in vivo* CYP3A4 activity [21].

Statistical significance (two-sided, $P < 0.05$) for associations between AUC ratios (or AUC/dose) and *CES1* genotypes or SNPs was determined by the Mann-Whitney test or the Jonckheere-Terpstra (JT) test using Prism version 4.0 (GraphPad Prism Software Inc. San Diego, CA, USA) and StatXact version 6.0 (Cytel Inc., Cambridge, MA). Correla-

Table 1
Primers and probes used in this study

Region (indicated in Figure 2)	Primer	Primer sequence	Reference
(a) *CES1A1* exon 1 and promoter region			This study
First PCR	Ces1-FP	5′-CCAGGCAAAACCTAGGAGTG-3′	
	Ces1-RP	5′-AGTACAGGGCGATCTCAGGA-3′	
Second PCR	Ces1_seqF	5′-GTATTTCCTTAGCCAGCGGTA-3′	
	Ces1_seqR	5′-CAGAGCCGGACCTGTTGT-3′	
Sequencing	Ces1_SF2	5′-AGAGCCTGGAAAGCTATGAAAA-3′	
	Ces1_SR	5′-TTTCTACGCATCTGCGCCCACC-3′	
(b) *CES1A1, 1A2* and *1A3* exon 5			[16]
PCR and sequencing	1A-int4F	5′-GCTCAGTAAATAGTTGCCAGTT-3′	
	1A-int5AS	5′-TCTCATCAGCATCACATCAAG-3′	
(c) *CES1A3* exon 3			This study
PCR and sequencing	CES1A3-15183F	5′-CAGGGAAGATCGTTGTATTGGTTT-3′	
	CES1A3-15974R	5′-TTCCTTCCACCACTAACATTATTG-3′	
Sequencing (additional primer)	CES1A3-15823R	5′-AAGATGTTCATTAAAGATGCACAG-3′	
(d) *CES1A2* and *1A3* -816A>C genotyping			[18]
PCR	F	5′-CCTTAATTTGGTGATTTCACATTGC-3′	
	R	5′-CAAGACATGGTTCAGCTTCTCAAG-3′	
TaqMan probe	FAM	5′-CATCACCCCTACTGC-3′	
	VIC	5′-CATCACACCTACTGCT-3′	
(e) *CES1A2* promoter region			This study
PCR	CES1A3-CES1A2_F1	5′-ATGATTTCCAGCTTCATCTACA-3′	
	CES1A2_R1	5′-GAGAGAACGTTCCCATGTCTTT-3′	
(f) *CES1A3* promoter region			This study
First PCR	CES1A3-CES1A2_F1	5′-ATGATTTCCAGCTTCATCTACA-3′	
	CES1A3_R1	5′-GCTTGAGTTTTCTTTACAGACA-3′	
Second PCR	CES1A3-CES1A2_F2	5′-AACAGTTTATAACCTGTTATTTTT-3′	
	CES1A3_R2	5′-TGCTTTGGATAAAGACAAGATGTT-3′	
Seqeuncing of *CES1A2/1A3* promoter region			
	CES1A3-CES1A2_F2	5′-AACAGTTTATAACCTGTTATTTTT-3′	
	CES1A3-CES1A2_R1	5′-CACACTTCCAATCTCAGGTAAA-3′	
	CES1A3-CES1A2_F3	5′-TTATGCCACAAGCAGTTGGGCG-3′	
	CES1A3-CES1A2_R2	5′-TCCAAGTCCAATTCCAAGTACGGA-3′	

NT_010498.15 was used as the reference sequence for *CES1A1*, *CES1A3* and the promoter region of *CES1A2*, and AB119998.1 was used for exon 1 and its downstream region of *CES1A2*.

tions between the AUC ratios [$AUC_{(SN\text{-}38 + SN\text{-}38G)}/AUC_{irinotecan}$] and [$AUC_{APC}/AUC_{irinotecan}$] were analyzed by Spearman's rank correlation test. Multiplicity adjustment was not applied to bivariate analysis, and contributions of the candidate genetic markers to the AUC ratios [$AUC_{(SN\text{-}38 + SN\text{-}38G)}/AUC_{irinotecan}$] were further determined by multiple regression analysis after logarithmic transformation of the AUC ratio. The variables examined were age, sex, body surface area, history of smoking or drinking, performance status, serum biochemistry (GOT, ALP, creatinine) at baseline, *CES1* genotypes and SNPs, *CES2*2* [100C>T(R34W)] or **5* [1A>T (M1L)] [13, 14], *UGT1A1*6* or **28* [7, 8], and the transporter haplotypes, *ABCB1*2* [2677G>T(A893A)], *ABCC2*1A* (–1774delG), *ABCG2#IIB* [421C>A (Q141K) and IVS12+49G>T] and *SLCO1A1*15·17* [521T>C (V174A)] [10]. The variables in the final models were selected by the forward and backward stepwise procedure at a significance level of 0.10 using JMP version 7.0.0 (SAS Institute, Inc., Cary, NC, USA). *UGT1A1*6 or *28* was grouped as '+' for stratifying patients: for example, homozygous *UGT1A1 *6 or *28* was depicted as *UGT+/+*.

Results

Genotypes and SNPs of CES1 gene family in Japanese

Frequencies of individual *CES1* genes and *CES1* diplotypes stratified according to the number of functional *CES1* genes are summarized in Table 2. The frequencies of the patients with two, three and four functional *CES1* genes were 44%, 47% and 9%, respectively, in all 177 patients.

By sequencing *1A1* and *var1A1* exon 1s and their flanking region, we detected four novel variations; three in the 5′-flanking region and one in the 5′-untranslated region (5′-UTR) (Table 3): –258C>T (allele frequency: 0.014), –233C>A (0.003), –161A>G (0.006) and –30G>A (0.042). Eleven nucleotide substitutions from the 5′-UTR to intron 1 at allele frequencies of 0.294–0.299 were closely linked with *var1A1* (Table 3). The SNP –816A>C found in the *1A2* and *1A3* promoter regions was genotyped by a TaqMan method [18], and the allele frequency of –816A>C in 177 subjects was 0.249 (Table 4). It was noted that –816C was detected only in patients with *1A3* (*1A3/1A2* and *1A3/1A3*),

Table 2

Frequency of *CES1* genes and diplotypes in Japanese cancer patients

CES1 diplotype	Number of *CES1* gene *1A1*	*var1A1*	*1A2*	*1A3*	Total*	Frequency (n = 177)†		Frequency (monotherapy: n = 58)†	
A/A	2	0	0	2	2	0.203	0.441	0.138	0.397
A/C	1	1	0	2		0.220		0.241	
C/C	0	2	0	2		0.017		0.017	
A/B	2	0	1	1	3	0.237	0.469	0.293	0.534
A/D or B/C	1	1	1	1		0.192		0.190	
C/D	0	2	1	1		0.040		0.052	
B/B	2	0	2	0	4	0.040	0.090	0.017	0.069
B/D	1	1	2	0		0.034		0.052	
D/D	0	2	2	0		0.017		0.000	
Frequency (n = 354)‡	0.703	0.297	0.325	0.675					
(monotherapy: n = 116)‡	0.690	0.310	0.336	0.664					

*Number of functional genes. †Number of subjects. ‡Number of chromosomes.

but not in the *1A2* homozygotes (*1A2/1A2*). In the *1A2/1A3* patients, 38 of the 39 patients having −816C were heterozygous for −816C (Table 4). These findings suggested a close association between −816C with *1A3*. Following specific amplifications of the regions from 5′-regions to intron 1 in *1A2* and *1A3* (Figure 2e,f) of 23 patients randomly selected from the 38 patients with −816A/C and *1A2/1A3*, we confirmed that −816C resided in the *1A3* gene (data not shown). Thus, −816A>C is the major SNP of *1A3* but very rare in *1A2*. In addition, the SNPs, −62T>C, −47G>C, −46G>T, −41C>G, −40A>G, −37G>C, −34del/G and −32G>T, in the proximal promoter region reported to be linked with −816A>C [19] were found to be completely linked with *1A3* (data not shown).

Association of CES1 genotypes with in vivo CES activity

CES1 diplotypes In patients treated with irinotecan monotherapy, we found the AUC ratios of patients with haplotypes A or C (having the *1A3* pseudogene) were lower than those without A or C, indicating functional *CES1* gene number dependency. The median AUC ratio of patients having three or four functional *CES1* genes was 1.24-fold of that in patients with two functional *CES1* genes [median (25th–75th percentiles): 0.31 (0.25–0.38) *vs.* 0.25 (0.20–0.32), P = 0.0134, Mann-Whitney test)] (Figure 3a). No significant differences were observed between *1A1* and *var1A1* (among *1A1/1A1*, *var1A1/1A1* and *var1A1/var1A1*). As we previously reported, the *CES2* variations, *CES2*5* [1A>T(M1L)] and *CES2*2* [100C>T(R34W)] [13, 14] showed low CES activity as indicated in Figure 3a.

Platinum-containing regimens themselves enhance renal excretion of irinotecan and its metabolites, especially SN-38G. No significant effect of *CES1* gene number on the AUC ratio was observed. However, it was noted that the median renal excretion ratio [(SN-38 + SN-38G)/irinotecan] in patients with four functional *CES1* genes was 1.37-fold higher than that in patients with two or three functional genes (P = 0.0217, Mann-Whitney test) (data not shown).

To exclude the possibility that the higher AUC ratio observed above (Figure 3a) was biased by CYP3A4, another metabolic enzyme for irinotecan, we analyzed the association between the (SN-38 + SN-38G)/irinotecan AUC ratio and the APC/irinotecan AUC ratio, an *in vivo* parameter of CYP3A4 activity [21], in patients treated with irinotecan monotherapy. The result showed no correlation between the two parameters (Spearman r = 0.126, P = 0.345).

CES1 SNPs Next, associations of the two *1A1* SNPs, −75G>T and −30G>A (Table 3) and *1A3*-816A>C with the AUC ratio [(SN-38 + SN-38G)/irinotecan] were analyzed. The effects of the SNPs were analyzed in patients stratified by the functional *CES1* gene number and also in all the patients receiving monotherapy. A −75G>T-dependent increase in the AUC ratio was observed in the whole group of patients (P = 0.027, JT test) (Figure 3b), and this trend was remarkable in patients with three or four functional *CES1* genes. No significant effect of −30G>A was observed (Figure 3c). As for −816C in *1A3*, no association between this SNP and the AUC ratio was evident in patients with two or three functional *CES1* genes (Figure 3d). In the platinum-containing regimens, no significant effects of these SNPs on the AUC ratio or the renal recovery ratio were observed (data not shown).

Multivariate analysis The contribution of *CES1* genotypes to the AUC ratio was further analyzed by multivariate analysis, using the patient background factors and polymorphisms including the haplotypes of *CES2*, *UGT1A1* and transporters as variables [7, 8, 10, 13, 14]. The final model revealed a significant association of the functional *CES1* gene number (n = 3 or 4) with the AUC ratio. Contributions of smoking history, irinotecan dose, hepatic and renal function were also detected while that of *ABCB1*2* (+/+) was