15.4 months, but no significant difference was observed between the four arms. Response rates were higher in the concurrent arms than in the sequential arms.

Conclusion Our study did not show sufficient prolongation of survival with the concurrent strategy to proceed to a phase-III trial; however, the sequential arms showed survival comparable to that in the concurrent arms, with less toxicity. In patients who are ineligible for cisplatin (CDDP), sequential treatment starting with S-1 and proceeding to PTX would be a good alternative strategy, considering quality of life (QOL) and the cost-benefits of an oral agent as first-line treatment.

Keywords Advanced gastric cancer · Paclitaxel · S-1 · Sequential chemotherapy · Concurrent combination chemotherapy · Randomized phase-II trial

Introduction

Gastric cancer is the second most common cause of cancer-related death worldwide [1]. Most patients (except those from northeast Asian countries) present with advanced, inoperable, or metastatic disease, and the 5-year survival rate is approximately 10–15%. Palliative chemotherapy for advanced disease improves survival as compared with the best supportive care [2–4]. Despite the innumerable efforts of investigators in various countries to test various chemotherapeutic and immunotherapeutic agents and combination regimens, there has been little progress in the therapy for patients with advanced gastric cancer.

Probably because there is less evidence regarding the treatment of gastric cancer compared to that of other malignancies, the standard treatment for gastric cancer differs from country to country, although most of the "standard" regimens do not have sufficient evidence. Moreover, the insurance systems in most western countries approve only first-line treatment, and in these countries, doublet or triplet therapies could be the standard choice, while some countries, including Japan, approve second- and greater-line strategies, where we can choose not only concurrent but also sequential strategies. Reflecting these historical and social circumstances, "standard" treatment for gastric cancer shows wide variety, with some confusion. In Japan, the evidence-based standard regimen involved continuous infusion of 5-fluorouracil (5-FU) only (JCOG9205) before the results of the Japan Clinical Oncology Group (JCOG) 9912 and SPIRITS trials had been obtained [5–7]. After the results of SPIRITS trial were shown, S-1 plus cisplatin (CDDP) has been accepted as the standard first-line treatment for patients with good condition, but S-1 without CDDP was also widely used in general practice. This means we still need an alternative strategy, whose sequence starts from a fluoropyrimidine (infusional 5-FU or oral S-1) with or without other agents.

As for candidates as the fluoropyrimidine partner, some potent agents have been approved for gastric cancer in the past two decades. One of the promising agents was paclitaxel (PTX) [8], which had shown beneficial results in single use or concurrent use with a fluoropyrimidine [9–12]. However, these studies were conducted as single-arm phase I–II trials. Hence, the choice between sequential and concurrent strategies for fluoropyrimidine and PTX remains unclear.

We therefore planned a randomized phase-II trial to compare the following four treatment regimens: A, sequential 5-FU monotherapy followed by PTX monotherapy; B, sequential S-1 monotherapy followed by PTX monotherapy; C, concurrent 5-FU plus PTX [11]; and D, concurrent S-1 plus PTX [12]. The purpose of the study was twofold: (1) to compare S-1 with infusional 5-FU to determine which was the better partner of PTX, and (2) to compare a concurrent strategy with a sequential one, the latter strategy being the one that is widely used in Japanese general practice.

Patients and methods

The detailed study design and protocol treatment of this study has already been described by Morita et al. [13]. Below we outline a summary of the methodological issues in this study with the protocol (informed consent form) that was amended after the SPIRITS trial.

Eligibility criteria

Patients more than 20 years of age with histologically confirmed non-resectable advanced or recurrent gastric cancer were eligible. Patients who had undergone prior anti-tumor therapy (except for surgery and postoperative adjuvant chemotherapy) were excluded. Patients had to have adequate renal, hepatic, hematologic, and cardiac function, with an Eastern Cooperative Oncology Group performance status (PS) of 0–1. Patients had to be able to take food via the oral route to be considered for enrolment in the study.

The protocol was approved by the Institutional Review Board (IRB) of each institution, and written informed consent was obtained before treatment. Participating investigators were instructed to send an eligibility criteria report to the data center operated by the non-profit organization Epidemiological and Clinical Research Information Network (ECRIN). Eligible patients were registered and then randomized to receive either of the four treatment regimens (A, B, C, and D), using a centralized dynamic

randomization method with the following balancing factors: measurable disease according to criteria set by Response Evaluation Criteria in Solid Tumours (yes/no); disease type [inoperable advanced/postoperative recurrent (with postoperative chemotherapy)/postoperative recurrent (with no postoperative chemotherapy)]; PS (0/1); peritoneal metastasis based on diagnosis with images (yes/no); age (<75 years/≥75 years), and institution. Information regarding the necessary follow-up examinations and chemotherapy schedule was then sent from the ECRIN data center. The accrual started in December 2005 and was continued for 3 years.

Projected treatments

Based on previous trials, we adapted four promising regimens for this selection design trial [13]. Patients in arm A received sequential therapy with intravenous (i.v.) 800 mg/m^2 5-FU daily for 5 days every 4 weeks until progression, followed by PTX 80 mg/m^2 on days 1, 8, and 15 every 4 weeks. Patients in arm B received sequential therapy with 80 mg/m^2 of oral S-1 daily for 4 weeks and 2-week rest after the administration (total of 6 weeks per single course) until progression. This was followed by PTX, utilizing the same administration dose and schedule as that in arm A's second-line PTX. Patients in arm C received a combination therapy with 600 mg/m^2 5-FU (i.v.) daily for 5 days from day 1 and infusion of 80 mg/m^2 PTX on days 8, 15, and 22 every 4 weeks. Patients in arm D received a combination therapy with 80 mg/m^2 oral S-1 for 14 days from day 1 and infusion of 50 mg/m^2 PTX on days 1 and 8 every 3 weeks. In the sequential treatment arms A and B, the administration of 5-FU or S-1 monotherapy was discontinued if the following were observed: (1) disease progression or occurrence of new disease; (2) grade-4 nonhematological toxicities evaluated according to the Common Terminology Criteria for Adverse Events version 3.0; (3) adverse events causing patients to refuse treatment or causing a clinician to discontinue treatment; (4) increase in the tumor markers carcinoembryonic antigen (CEA) and/or cancer antigen (CA) 19-9 in two or more consecutive measurements or symptomatic progression (e.g., cancer pain and dysphagia). An irinotecan-containing regimen was recommended for use in case further lines of treatment were to be given.

Follow-up

Disease progression and occurrence of new disease were examined using radiographs, computed tomography (CT) or magnetic resonance imaging (MRI) of the abdomen, and thoracic CT and measurements of the tumor markers CEA and CA19-9. These examinations were performed at baseline and at least every 4–5 weeks during treatment. Blood tests and symptom checks were performed before treatment and at least every 2 weeks during treatment. In cases where therapy was discontinued owing to toxicity, clinicians followed up patients until they recovered from the effects of toxicity.

Study design and statistical methods

The primary aim of this study was to compare treatment regimens A–D in terms of the primary endpoint of the 10-month overall survival (OS) rate. In addition, OS and treatment failure curves were constructed as time-to-event plots using the Kaplan–Meier method [14]. Time-to-event curves were compared using log-rank tests and the hazard ratio (HR) estimated by Cox regression models [15]. The prevalence of grade-3 or grade-4 adverse events was compared between the treatment arms. Calculation of the sample size required 40 patients in each arm to assure 80% probability in order to select the best treatment arm [16] as long as the true expected 10-month OS rate exceeded that of any other arm by at least 15%. The total number of patients to be accrued was set at 160.

Protocol amendment after SPIRITS trial

After the results of the SPIRITS trial were publicized, standard first-line therapy in Japan shifted from monotherapies with 5-FU or S-1 to an S-1/CDDP combination. The protocol committee of the present trial discussed this issue and decided not to change the protocol treatments, because none of the treatment arms has actually been shown to be inferior to the S-1/CDDP combination. Instead, all patients who became candidates for accrual in the trial after the results of the SPIRITS trial were publicized were to be informed of the novel standard treatment in Japan, using a newly compiled explanatory note, and they were to be offered the alternative of receiving the combination therapy instead of participating in the trial. Each participating institution agreed on the use of the newly compiled explanatory note without correction in the study protocol itself, and case recruitment was re-started after the IRB approval of the amendment was obtained.

Results

A total of 161 patients were enrolled in the trial from December 2005 to November 2008. The numbers of patients in arms A, B, C, and D were 40, 40, 41, and 40, respectively. Two patients in arm A and two in arm C declined therapies before the start of the assigned treatment. Therefore, 38, 40, 39, and 40 patients in arms A, B,

C, and D, respectively, were considered to be eligible for evaluation (Fig. 1). Initial patient characteristics in the four arms were well matched (Table 1). The median age was 67 years (range 40–90 years).

Survival

The ten-month OS rates predetermined as the primary endpoint were 63, 65, 61, and 73% in arms A, B, C, and D, respectively. Although concurrent therapy with S-1 plus PTX demonstrated the best survival benefit among the four arms, the difference in OS rates between the arms with highest (D) and lowest (C) rates was less than the predetermined criterion (i.e., 15%). Kaplan–Meier survival curves did not show a significant difference between the four arms (Fig. 2). The survival rates in the sequential (A, B) and concurrent (C, D) arms were almost identical ($p = 0.93$) (Fig. 3a). In addition, no difference in survival was observed between the 5-FU-containing regimens (arms A and C) and the S-1-containing regimens (arms B and D) ($p = 0.83$) (Fig. 3b).

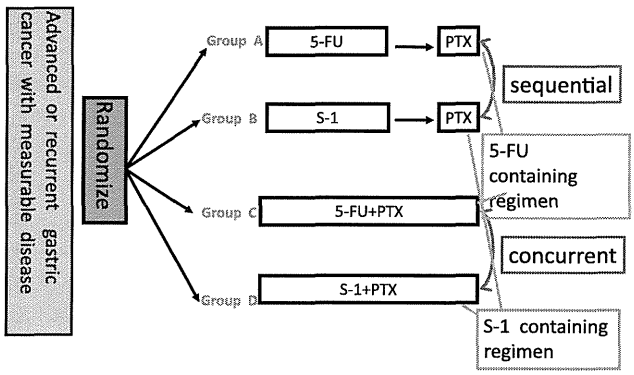

Fig. 1 CONSORT diagram that accounts for all patients. *5-FU* 5-fluorouracil, *PTX* paclitaxel

Time to treatment failure (TTF)

In arms A and B, TTF was calculated by the addition of the prior 5-FU or S-1 treatment period and the sequential PTX period. Median TTF values were 213, 222, 177, and 189 days in arms A, B, C, and D, respectively. No difference was observed between the four arms. However, Kaplan–Meier TTF curves for sequential and concurrent regimens showed better TTF in favor of sequential treatment compared with concurrent treatment (HR 0.71, 95%

Table 1 Patient characteristics

Treatment arm	Arm A 5-FU→PTX $n = 38$	Arm B S-1→PTX $n = 40$	Arm C 5-FU+PTX $n = 39$	Arm D S-1+PTX $n = 40$
Gender				
Male	25 (65.8%)	28 (70.0%)	28 (71.8%)	32 (80.0%)
Female	13 (34.2%)	12 (30.0%)	11 (28.2%)	8 (20.0%)
Age (years)				
Median	67.0	68.0	67.3	66.6
Range	48–79	51–81	40–82	47–90
74≤	31 (81.6%)	33 (82.5%)	31 (79.5%)	31 (77.5%)
≤75	7 (18.4%)	7 (17.5%)	8 (20.5%)	9 (22.5%)
Performance status				
0	29 (76.3%)	27 (67.5%)	25 (64.1%)	28 (70.0%)
1	9 (23.7%)	13 (32.5%)	14 (35.9%)	12 (30.0%)
Stage				
Non-resectable, no previous chemotherapy	31 (81.6%)	33 (82.5%)	32 (82.1%)	32 (80.0%)
Recurrent after curative surgery, adjuvant chemotherapy (+)	2 (5.3%)	1 (2.5%)	3 (7.7%)	3 (7.5%)
Recurrent after curative surgery, adjuvant chemotherapy (–)	5 (13.2%)	6 (15.0%)	4 (10.3%)	5 (12.5%)
Peritoneal metastasis				
Yes	9 (23.7%)	13 (32.5%)	5 (12.8%)	10 (25.0%)
No	29 (76.3%)	27 (67.5%)	34 (87.2%)	30 (75.0%)
Measurable disease				
Yes	19 (50.0%)	23 (57.5%)	17 (43.6%)	20 (50.0%)
No	19 (50.0%)	17 (42.5%)	22 (56.4%)	20 (50.0%)

5-FU 5-fluorouracil, *PTX* paclitaxel

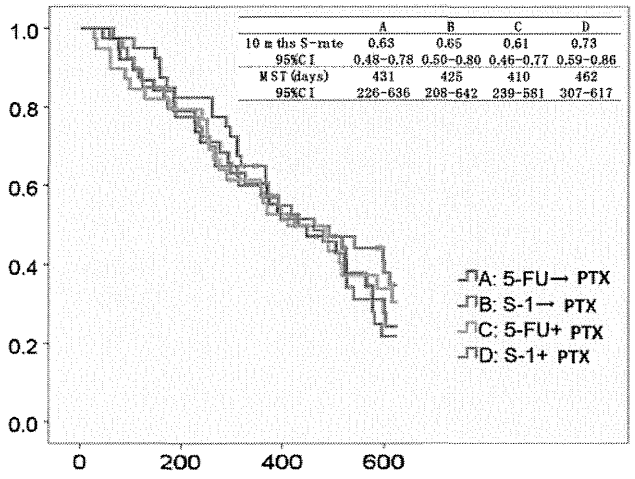

Fig. 2 Kaplan–Meier plot of overall survival in the four treatment arms. *S-rate* survival rate, *CI* confidence interval, *MST* median survival time

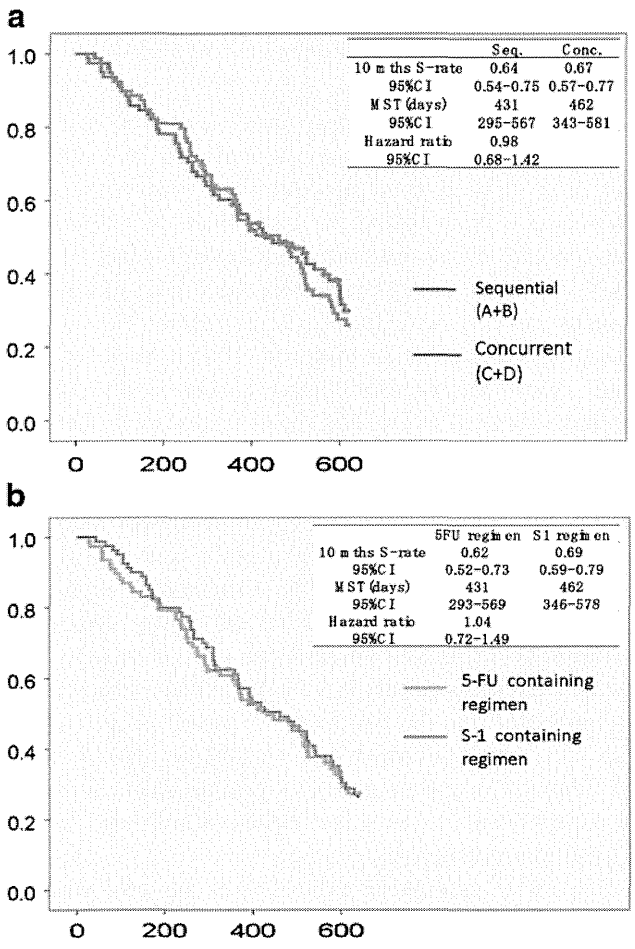

Fig. 3 Kaplan–Meier plot of overall survival by **a** sequential regimens (arms *A* and *B*) and concurrent regimens (arms *C* and *D*), **b** 5-FU-containing regimens (arms *A* and *C*) and S-1-containing regimens (arms *B* and *D*). *seq.* sequential, *conc.* concurrent

Table 2 Tumor response rates

Treatment arm/agent	*n* (With measurable lesion)	CR	PR	SD	PD	Response rate (%)
A						
5-FU	17	0	5	8	4	29.4
PTX	17	0	2	10	5	11.8
B						
S-1	20	1	4	10	5	25.0
PTX	14	1	1	10	2	14.3
C						
5-FU + PTX	13	0	9	2	2	69.2
D						
S-1 + PTX	19	1	7	11	0	42.1

CR complete response, *PR* partial response, *SD* stable disease, *PD* progressive disease

confidence interval [CI] 0.50–1.02, $p = 0.06$). A difference in TTF was not observed between the 5-FU-containing and S-1-containing regimens.

Response rates

The overall response rates in patients who had measurable disease are summarized in Table 2. Response rates were higher in the concurrent arms than in the sequential arms. The 5-FU and PTX combination regimen showed the best response rate among the four arms.

Toxicities

All patients could be assessed for hematological and non-hematological toxicities (Table 3). Ten of 78 patients (12.8%) who received sequential therapy and 26 of 79 patients (33.0%) who received concurrent therapy showed grade-3 or grade-4 neutropenia. With respect to hemoglobin decrease, 21 patients (26.2%) with the S-1-containing regimens showed grade-3 or grade-4 adverse events, whereas only 8 patients (10.4%) with the other regimens showed adverse events. No difference was observed in non-hematological toxicity.

Compliance

Compliance with S-1 treatment was inferior to that with 5-FU treatment. The median numbers of courses accomplished in the first- and second-line treatment of the

Table 3 Toxicities

	A: 5-FU→PTX (n = 38)	B: S-1→PTX (n = 40)	C: 5-FU+PTX (n = 39)	D: S-1+PTX (n = 40)
Hematological toxicities				
CTC Grade	>=3	>=3	>=3	>=3
Leucopenia (%)	7.9	7.5	10.3	7.5
Neutropenia (%)	13.2	12.5	25.6	22.5
Thrombocyte (%)	0.0	2.5	0.0	2.5
Hemoglobin (%)	10.5	32.5	10.3	20.0
Total Bil (%)	2.6	2.5	0.0	5.0
Hepatic Tox (%)	7.9	5.0	2.6	7.5
Non-hematological toxicities				
CTC Grade	>=3	>=3	>=3	>=3
Weight loss (%)	2.6	0.0	2.6	0.0
Fatigue (%)	0.0	0.0	0.0	0.0
Lassitude (%)	7.9	12.5	5.1	10.0
Anorexia (%)	10.5	12.5	7.7	10.0
Nausea (%)	2.6	5.0	5.1	2.5
Vomiting (%)	0.0	0.0	2.6	0.0
Stomatitis (%)	5.3	0.0	2.6	2.5
Diarrhea (%)	2.6	2.5	5.1	2.5
Neuropathy (%)	0.0	2.5	5.1	5.0

CTC Common Toxicity Criteria

sequential regimens were 4 (range 1–26) and 3 (range 1–8) in arm A and 6 (range 1–24) and 4 (range 1–30) in arm B, respectively. For the concurrent regimens, these numbers were 6 (range 1–24) and 7.5 (range 1–30) in arms C and D, respectively.

Discussion

The strategy for the chemotherapy of gastric cancer differs from country to country. In Japan, according to community standards, fluoropyrimidine monotherapy has been widely used as the first-line of a sequential strategy, whereas most western countries use doublet or triplet concurrent regimens without second-line treatment. In fact, little is known about whether concurrent regimens or a sequential strategy with satisfactory second- and greater-line treatments would be better. Although one trial has shown the superiority of doublet (S-1 with CDDP) treatment compared with S-1 alone even in Japan [7], other pivotal trials have failed to show the superiority of concurrent regimens [17, 18]. This suggests that sequential strategies may not be so bad if we can use adequate second- (and more)-line therapies in sequence. Thus, when we decided to evaluate PTX in a clinical trial, we created the study plan so as to evaluate whether PTX should be used in second-line (sequential) or in first-line (concurrent) treatment.

In accordance with the general rule in a randomized phase-II trial, in the present study we assumed that we should choose the best regimen in the aspect of 10-month overall survival (OS). However, as shown in the results, all four arms showed good survival times with very small differences. This finding suggests that the difference between concurrent and sequential strategies may be very small if we take enough care with the timing of regimen changes and are meticulous in surveying for clinical disease progression. Similar trends have been observed with some other malignancies; breast cancer is one of the examples. Several studies have been conducted to show the survival superiority of concurrent regimens, but superiority was seen only in TTF and the response rate (RR) [19, 20]. As a result, the sequential strategy is still used. Recently, the result of the GEST trial in pancreatic cancer showed a superior RR and a superior TTF in the combination arm. Despite this superiority, this concurrent strategy also failed to improve OS [21]. Our phase-II trial with its small sample size nevertheless suggests that the sequential strategy could be considered for the treatment of gastric cancer, along with other types of cancer, and that the sequential use of S-1 followed by paclitaxel (PTX) remains as an alternative for patients who are for some reason not indicated for the S-1/CDDP combination.

One more issue to be evaluated in our trial was the difference between infusional 5-FU and oral S-1. The results of a worldwide advanced gastric cancer trial (FLAGS trial) comparing S-1 plus CDDP (SF) versus 5-FU plus CDDP (CF) failed to show a superior effect of SF over CF [22]. The JCOG9912 trial has already shown no

inferiority of S-1 compared to infusional 5-FU in the first-line setting [6]. However, that trial did not limit the post-treatment, so the setting of PTX use in first- or second line mandatorily might show different results. The present study had started before the results of these two trials were disclosed. Consequently, it is important to check whether our results are in line with the data obtained in the JCOG9912 and the FLAGS trials. In our study, the OS, PFS, and RR for the 5-FU-containing and S-1-containing regimens were almost the same, without any significant differences, suggesting both oral and infusional fluorinated pyrimidine regimens have similar potency, a finding which would be confirmatory of the previous trials. In general, treatment with an oral agent would be more preferable both for the patients and for medical staff than a treatment requiring continuous intravenous infusion, with its risks of infection and thrombotic events.

In conclusion, our study did not show sufficient prolongation of survival with a concurrent strategy to proceed to a phase-III trial; however, the sequential arms showed survival comparable to that in the concurrent arms, with a lower incidence of neutropenia. In patients who are ineligible for CDDP, sequential treatment starting from S-1 and proceeding to PTX would be a good alternative strategy, considering the quality of life (QOL) and cost-benefits of an oral agent as first-line treatment.

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Cediranib in combination with mFOLFOX6 in Japanese patients with metastatic colorectal cancer: results from the randomised phase II part of a phase I/II study

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Background: Colorectal cancer (CRC) is the second most common malignancy in Japan. Treatment with inhibitors of the vascular endothelial growth factor (VEGF) signalling pathway has proven benefit in metastatic CRC. Cediranib is an oral highly potent VEGF signalling inhibitor that inhibits all three VEGF receptors.

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Patients and methods: In this phase II, double-blind, placebo-controlled study, 172 patients with metastatic CRC were randomised to receive once-daily cediranib (20 or 30 mg) or placebo, each combined with modified FOLFOX6 (mFOLFOX6). The primary objective was comparison of progression-free survival (PFS).
Results: The comparison of cediranib 20 mg versus placebo met the primary objective of PFS prolongation [hazard ratio = 0.70 (95% confidence interval 0.44–1.11), $P = 0.167$], which met the protocol-defined criterion of $P < 0.2$. Median PFS was 10.2 versus 8.3 months, respectively. The PFS comparison for cediranib 30 mg versus placebo did not meet the criterion. The most common adverse events (AEs) in the cediranib-containing groups were diarrhoea and hypertension.
Conclusions: Cediranib 20 mg plus mFOLFOX6 met the predefined criteria in terms of improved PFS compared with placebo plus mFOLFOX6. Cediranib 20 mg was generally well tolerated and the AE profile was consistent with previous studies.
Key words: cediranib, colorectal cancer, mFOLFOX6, placebo, progression-free survival

introduction

In Japan, the incidence of colorectal cancer (CRC) has increased nearly fivefold in the last 25 years, owing primarily to changing Japanese dietary habits, which are becoming increasingly similar to those of Western countries. In 2008, there were 101 656 new cases of CRC in Japan and 43 349 deaths attributed to this disease [1]. CRC is now the second most common malignancy in Japan and is predicted to become the most common by 2015. Fluorouracil (5-FU) was one of the first chemotherapies used for the treatment of CRC, and the combination of 5-FU with leucovorin and oxaliplatin (FOLFOX) has improved outcomes. Treatment with these components (plus irinotecan in some regimens) can provide a median overall survival (OS) of up to 20 months, compared with ~6 months with best supportive care [2]. Japanese clinical guidelines recommend FOLFOX as standard treatment of metastatic colorectal cancer (mCRC) [3]. To reduce toxicity associated with the FOLFOX regimen, a number of modifications have been tried [4, 5]; the current standard is modified FOLFOX6 (mFOLFOX6).

Inhibition of the vascular endothelial growth factor (VEGF) signalling pathway with bevacizumab has demonstrated additional clinical benefit in CRC when used with 5-FU-based regimens in the first-line setting in mCRC [6, 7]. Cediranib is an oral highly potent VEGF tyrosine kinase inhibitor (TKI) that inhibits all three VEGF receptors [8, 9]. Cediranib is suitable for once-daily dosing and has demonstrated antitumour activity during early phase clinical evaluation in patients with advanced cancer [10]. Further studies demonstrated that cediranib was generally well tolerated as monotherapy [11–15] and in combination with various anticancer agents at doses ≤30 mg/day [16–21].

The efficacy of cediranib in combination with chemotherapy has been investigated in two phase III studies—HORIZON II [22] and HORIZON III [23]—in Western patients with previously untreated mCRC. Two cediranib doses were initially selected for investigation in the HORIZON programme: 20 (lowest biologically active dose) and 30 mg/day (maximum dose suitable for chronic dosing in combination with chemotherapy). The decision to investigate cediranib 20 and 30 mg/day doses in this study was taken before an end-of-phase II decision from the HORIZON programme to proceed with only the 20 mg/day dose. As such, this two-part phase I/II study, which mirrored HORIZON II, investigated cediranib, at the same doses used initially in the Western studies, plus mFOLFOX6 in Japanese patients with previously untreated mCRC (ClinicalTrials.gov identifier NCT00494221; AstraZeneca study code D8480C00039). The phase I part of this study demonstrated that both doses of cediranib were generally well tolerated in combination with mFOLFOX6 [24]. Here, we report the results of the randomised, double-blind, phase II part of this study, which assessed the efficacy of cediranib (20 or 30 mg/day) plus mFOLFOX6 compared with mFOLFOX6 alone.

patients and methods

eligibility

Eligible patients were aged ≥18 years with histological or cytological confirmation of carcinoma of the colon or rectum. Patients required chemotherapy for stage IV (metastatic) disease, had a World Health Organisation (WHO) performance status (PS) of zero or one, and one or more measurable lesions according to the RECIST (version 1.0). Any adjuvant oxaliplatin or 5-FU therapy must have been completed >12 and >6 months, respectively, before study entry. Patients with brain or meningeal metastases were considered eligible if they were clinically stable and had not required corticosteroid treatment of 10 days. Exclusion criteria included prior systemic therapy for metastatic disease and prior therapy with monoclonal antibodies or small molecule inhibitors against VEGF or VEGF receptors, including bevacizumab and cediranib.

study design

This phase II, randomised, double-blind, placebo-controlled study assessed the efficacy of first-line treatment with cediranib plus mFOLFOX6 compared with mFOLFOX6 alone. Patients were randomised 1 : 1 : 1 to receive once-daily cediranib (20 or 30 mg) or placebo, each in combination with 14-day treatment cycles of mFOLFOX6 (oxaliplatin 85 mg/m^2 IV, day 1; leucovorin 200 mg/m^2 IV, day 1; 5-FU 400 mg/m^2 IV bolus, day 1 and then 2400 mg/m^2 continuous IV infusion over 46 h). Patients were stratified at randomisation according to a two-level liver function covariate [based on baseline albumin and alkaline phosphatase (ALP) levels] and WHO PS (0 versus 1). Randomised treatment was continued until objective disease progression (as defined by RECIST) or until the occurrence of toxicity, death, withdrawal of patient consent or other discontinuation criteria. RECIST measurements were made using computed tomography or magnetic resonance imaging scans; clinical assessment of these scans was conducted by the study investigators.

The primary objective was to determine the efficacy of cediranib plus mFOLFOX6 compared with mFOLFOX6 alone by assessment of progression-free survival (PFS). Secondary objectives included comparison of OS, objective response rate (ORR: complete response + partial response), duration of response, change in tumour size and assessment of the safety

and tolerability of cediranib plus mFOLFOX6. An exploratory end point was to investigate the effect of treatment on soluble markers of angiogenesis (VEGF and sVEGFR-2). VEGF and sVEGFR-2 were measured by enzyme-linked immunosorbent assay of plasma samples from patients who provided separate informed consent.

PFS and ORR were determined from objective tumour assessments (RECIST) carried out at weeks 6, 12, 18, 24 and then every 12 weeks until disease progression or death. Adverse events (AEs) were recorded and graded according to Common Terminology Criteria for Adverse Events version 3.0. The study was approved by each centre's institutional review board and was carried out in accordance with the Declaration of Helsinki, the International Conference on Harmonisation/Good Clinical Practice, applicable regulatory requirements and the AstraZeneca policy on Bioethics.

statistical analysis

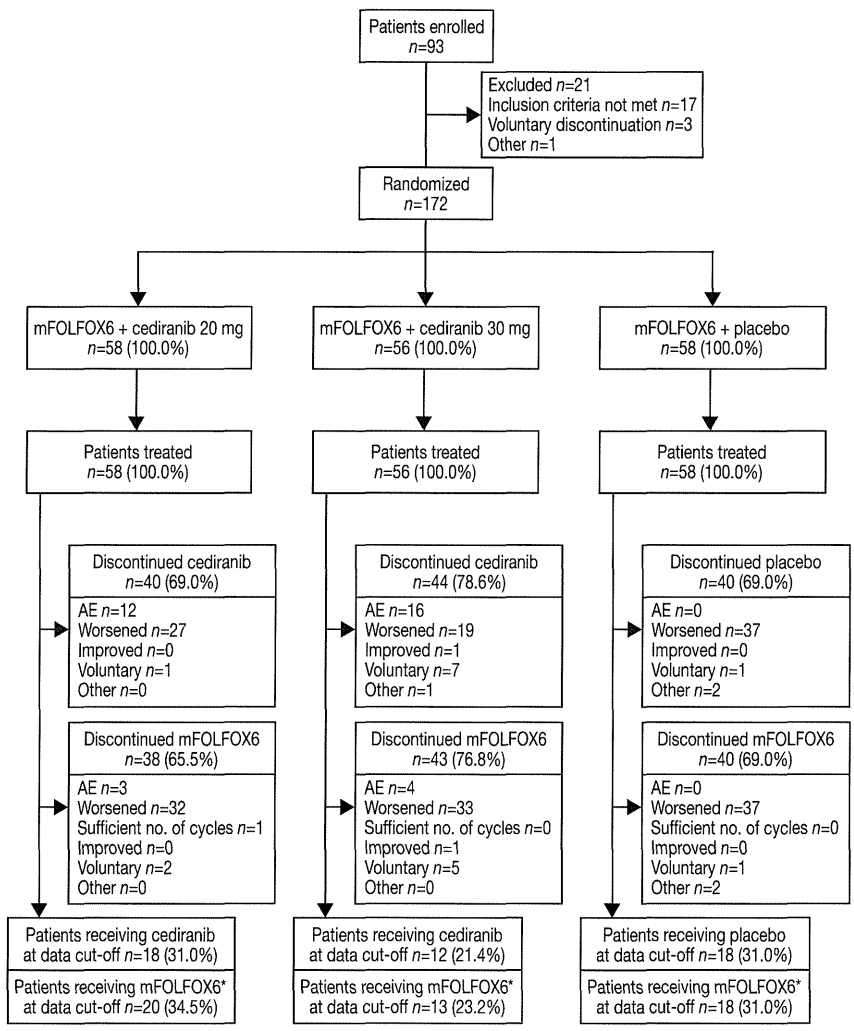
Assuming a median PFS of 9 months in the placebo group, an 18-month accrual period and a minimum 12-month follow-up, a total of 55 patients per group was required to have 80% power to detect a true PFS hazard ratio (HR) of 0.6 at two-sided significance level of $P < 0.2$ (one-sided $P < 0.1$), which was considered appropriate evidence of activity for a randomised phase II study [25]. The primary PFS analysis was conducted using a log-rank test stratified by WHO PS (0 or 1) and a two-level baseline liver function covariate (covariate 1 for baseline albumin < 3.5 g/l or ALP > 320 U/l; covariate 0 for all other values). PFS and OS were summarised by treatment group using the Kaplan–Meier method. The formal analysis was conducted when ~105 progression events had occurred across the three groups. No formal statistical analysis was carried out on safety data.

The results in the present study were relatively immature (65% of PFS events versus 81% in HORIZON II) and the HR was favourable compared with HORIZON II (HR = 0.84). Furthermore, there was a higher proportion of patients with a PS of zero. Therefore, further analysis of efficacy and safety outcomes was carried out when 81% of progression events had occurred.

results

patients

Between January 2008 and January 2009, 172 Japanese patients were randomised to treatment with cediranib 20 mg plus mFOLFOX6 ($n = 58$), cediranib 30 mg plus mFOLFOX6 ($n = 56$) or placebo plus mFOLFOX6 ($n = 58$) (Figure 1). Patient characteristics were representative of the patient population (Table 1). All patients were Japanese and 20%



*Patients may be receiving either 5-FU/leucovorin or 5-FU/leucovorin/oxaliplatin.

Figure 1. CONSORT diagram.

were receiving antihypertensive treatment at baseline. Baseline characteristics were generally well balanced across the groups, although there were more female patients in the cediranib 30 mg group. Imbalances were noted in metastases at baseline, time from initial diagnosis to randomisation, tumour grading, baseline ALP and baseline liver function (Table 1).

At the protocolled data cut-off (13 October 2009), 65% (112) of patients had progressed and 22% (38) had died. The most common reason for discontinuation of placebo/cediranib was worsened condition. At the second data cut-off (11 June 2010), 81% of patients had progressed and median OS follow-up was 19.0 months with 74 OS events.

efficacy

For the PFS comparison of cediranib 20 mg versus placebo, the HR was 0.70 [95% confidence interval (CI) 0.44–1.11], two-sided $P = 0.167$ (Figure 2A), which met the protocol-defined criterion for evidence of activity ($P < 0.2$). Median PFS was 10.2 and 8.3 months, respectively. For the PFS comparison of cediranib 30 mg versus placebo, the HR was 0.82 (95% CI 0.54–1.31), two-sided $P = 0.261$ (Figure 2B), which did not meet the predefined criterion. Median PFS was 8.9 months in the cediranib 30 mg arm. Predefined subgroup analysis of PFS for both dose groups did not identify a particular patient

Table 1. Patient demographics and baseline characteristics

Characteristic	Cediranib 20 mg + mFOLFOX6 ($n = 58$)	Cediranib 30 mg + mFOLFOX6 ($n = 56$)	Placebo + mFOLFOX6 ($n = 58$)
Median age (range), years	63.5 (33–79)	64.5 (40–82)	64.0 (36–80)
Sex, *n* (%)			
Male	38 (65.5)	30 (53.6)	39 (67.2)
Female	20 (34.5)	26 (46.4)	19 (32.8)
World Health Organisation performance status, *n* (%)			
0	44 (75.9)	43 (76.8)	47 (81.0)
1	14 (24.1)	13 (23.2)	11 (19.0)
Type of cancer, *n* (%)			
Colon	39 (67.2)	34 (60.7)	36 (62.1)
Rectal	19 (32.8)	22 (39.3)	22 (37.9)
Tumour grading, *n* (%)			
Well differentiated (G1)	11 (19.0)	14 (25.0)	16 (27.6)
Moderately differentiated (G2)	44 (75.9)	38 (67.9)	36 (62.1)
Poorly differentiated (G3)	2 (3.4)	3 (5.4)	4 (6.9)
Undifferentiated (G4)	1 (1.7)	1 (1.8)	1 (1.7)
Unassessable (GX)	0	0	1 (1.7)
Metastatic sites, *n* (%)			
1	32 (55.2)	29 (51.8)	28 (48.3)
>1	26 (44.8)	27 (48.2)	30 (51.7)
Metastases at baseline, *n* (%)			
Patients with liver only metastases at baseline	14 (24.1)	10 (17.9)	14 (24.1)
Patients with liver and other metastases at baseline	25 (43.1)	22 (39.3)	32 (55.2)
Patients with no liver involvement at baseline	19 (32.8)	24 (42.9)	12 (20.7)
Prior adjuvant therapy, *n* (%)			
Yes	13 (22.4)	9 (16.1)	8 (13.8)
No	45 (77.6)	47 (83.9)	50 (86.2)
Time from initial diagnosis to randomisation, *n* (%)			
<6 months	36 (62.1)	38 (67.9)	45 (77.6)
6 to <12 months	2 (3.4)	0	1 (1.7)
12 to <24 months	6 (10.3)	10 (17.9)	4 (6.9)
24 to <36 months	6 (10.3)	2 (3.6)	3 (5.2)
≥36 months	8 (13.8)	6 (10.7)	5 (8.6)
Baseline ALP, *n* (%)			
≤320 U/l	31 (53.4)	35 (62.5)	29 (50.0)
>320 U/l	27 (46.6)	21 (37.5)	29 (50.0)
Baseline liver function			
ALP > 320U/l or albumin < 35 g/l	29 (50.0)	22 (39.3)	30 (51.7)
Other	29 (50.0)	34 (60.7)	28 (48.3)
Baseline vascular endothelial growth factor			
n	36	37	38
Mean (standard deviation), pg/ml	146.5 (416.3)	74.3 (56.6)	96.9 (100.7)
Median (min, max), pg/ml	46.6 (31.2, 2520.5)	55.5 (31.2, 243.3)	54.6 (31.2, 508.1)

mFOLFOX6, modified FOLFOX6; ALP, alkaline phosphatase.

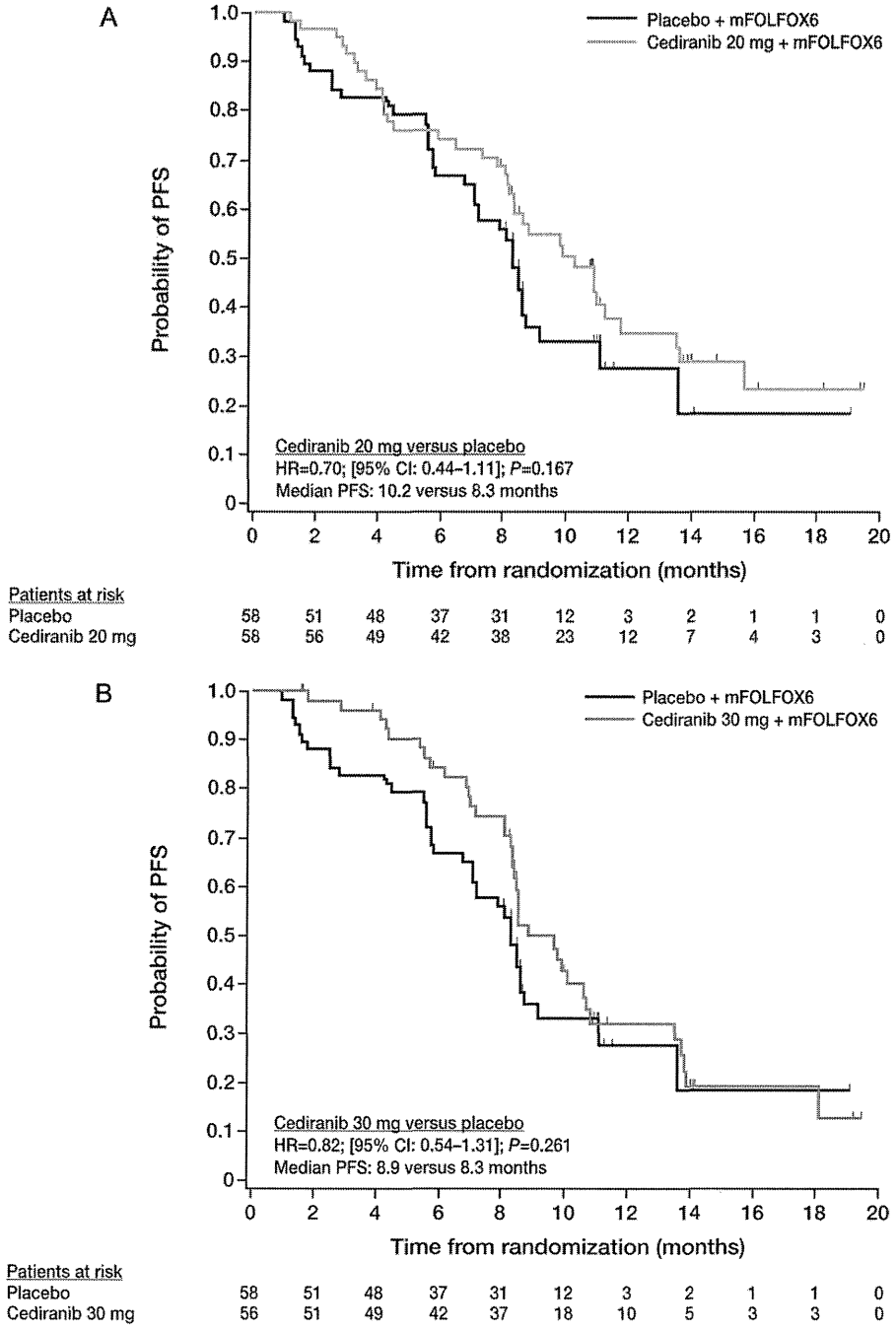


Figure 2. (A) Progression-free survival (PFS) for patients who received cediranib 20 mg + modified FOLFOX6 (mFOLFOX6) versus placebo + mFOLFOX6. (B) PFS for patients who received cediranib 30 mg + mFOLFOX6 versus placebo + mFOLFOX6.

population that derived a differential PFS benefit from cediranib versus placebo (supplemental Figure S1, available at *Annals of Oncology* online).

The ORR was 53.4%, 69.6% and 53.4% in the cediranib 20 mg, cediranib 30 mg and placebo arms, respectively; RECIST best response is summarised in Table 2. The median best percentage changes in tumour size were −37.3% (cediranib 20 mg), −43.4% (cediranib 30 mg) and −40.0% (placebo). The median duration of response was 9.2 (cediranib 20 mg), 6.7 (cediranib 30 mg) and 7.1 months (placebo) (Figure 3). At the primary analysis, there were insufficient deaths (total = 38; 15, 9 and 14 in the cediranib 20 mg, cediranib 30 mg and placebo arms, respectively) to draw conclusions on OS.

safety and tolerability

Overall, the most common AEs were diarrhoea and hypertension (Table 3); neither caused discontinuation of cediranib at the 20 mg dose. The incidence of AEs leading to discontinuation of cediranib/placebo was higher in the cediranib 30 mg group (27%) compared with the cediranib 20 mg (19%) or placebo (0%) groups; of these, only decreased

Table 2. Best RECIST response

Best response, *n* (%)	Cediranib 20 mg + mFOLFOX6 (*n* = 58)	Cediranib 30 mg + mFOLFOX6 (*n* = 56)	Placebo + mFOLFOX6 (*n* = 58)
CR	0	0	2 (3.4)
PR	31 (53.4)	39 (69.6)	29 (50.0)
Stable disease ≥6 weeks	24 (41.4)	14 (25.0)	20 (34.5)
Progressive disease	3 (5.2)	1 (1.8)	7 (12.1)
Non-evaluable	0	2 (3.6)	0

mFOLFOX6, modified FOLFOX6; CR, complete response; PR, partial response.

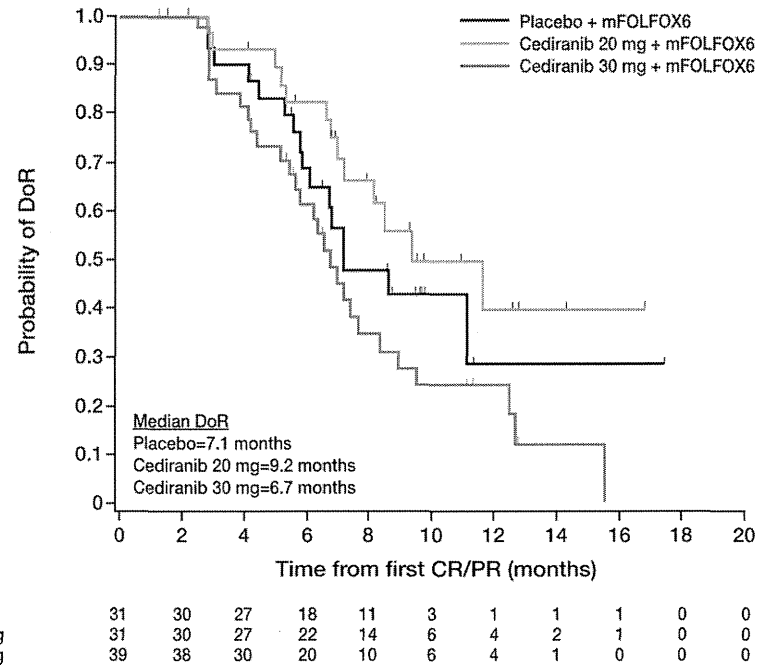


Figure 3. Duration of response for patients who received cediranib 20 mg, cediranib 30 mg or placebo, each in combination with modified FOLFOX6.

Table 3. AEs (frequency ≥30% in any group)

AE, *n* (%)	Cediranib 20 mg + mFOLFOX6 (*n* = 58)	Cediranib 30 mg + mFOLFOX6 (*n* = 56)	Placebo + mFOLFOX6 (*n* = 58)
Diarrhoea	53 (91.4)	49 (87.5)	22 (37.9)
Hypertension	47 (81.0)	48 (85.7)	18 (31.0)
Decreased appetite	43 (74.1)	43 (76.8)	39 (67.2)
Fatigue	39 (67.2)	40 (71.4)	36 (62.1)
Peripheral neuropathy	42 (72.4)	35 (62.5)	38 (65.5)
Nausea	39 (67.2)	37 (66.1)	37 (63.8)
PPES	31 (53.4)	34 (60.7)	8 (13.8)
Stomatitis	33 (56.9)	30 (53.6)	25 (43.1)
Vomiting	24 (41.4)	27 (48.2)	14 (24.1)
Dysphonia	24 (41.4)	16 (28.6)	2 (3.4)
Dysgeusia	18 (31.0)	17 (30.4)	18 (31.0)
Constipation	21 (36.2)	14 (25.0)	16 (27.6)
Alopecia	12 (20.7)	17 (30.4)	15 (25.9)
Epistaxis	15 (25.9)	19 (33.9)	9 (15.5)
Dysphonia	24 (41.4)	16 (28.6)	2 (3.4)

AE, adverse event; mFOLFOX6, modified FOLFOX6; PPES, palmar–plantar erythrodysaesthesia syndrome (hand–foot syndrome).

Table 4. CTC grade 3/4 AEs (>5% frequency in any arm)

AE, *n* (%)	Cediranib 20 mg + mFOLFOX6 (*n* = 58)	Cediranib 30 mg + mFOLFOX6 (*n* = 56)	Placebo + mFOLFOX6 (*n* = 58)
Decreased appetite	11 (19.0)	10 (17.9)	1 (1.7)
PPES	8 (13.8)	12 (21.4)	0
Diarrhoea	6 (10.3)	12 (21.4)	1 (1.7)
Hypertension	4 (6.9)	6 (10.7)	1 (1.7)
Peripheral neuropathy	5 (8.6)	3 (5.4)	2 (3.4)
Peripheral sensory neuropathy	2 (3.4)	5 (8.9)	2 (3.4)
Neutropenia	3 (5.2)	0	0
Ileus	0	0	3 (5.2)

AE, adverse event; CTC, Common Terminology Criteria; mFOLFOX6, modified FOLFOX6; PPES, palmar–plantar erythrodysaesthesia syndrome (hand–foot syndrome).

appetite, diarrhoea and pneumonia (all $n = 2$) were reported in multiple patients.

The incidence of grade 3/4 AEs was 66%, 75% and 36% in the cediranib 20 mg, cediranib 30 mg and placebo groups, respectively. The most common grade 3/4 AEs are summarised in Table 4. The incidence of serious adverse events (SAEs) was 39.7%, 39.3% and 19.0% in the cediranib 20 mg, cediranib 30 mg and placebo groups, respectively. No AEs had an outcome of death.

Clinical laboratory evaluation showed that treatment with cediranib plus mFOLFOX6 caused decreases in leucocyte, neutrophil and platelet counts and an increase in thyroid-stimulating hormone, but no new clinically important trends were observed in either cediranib group.

The median duration of exposure was 241.5, 213.0 and 223.5 days in the cediranib 20 mg, cediranib 30 mg and placebo groups, respectively. The proportion of patients experiencing a dose reduction/pause was highest in the cediranib 30 mg group (83.9%) versus the cediranib 20 mg (79.3%) and placebo (56.9%) groups (supplemental Figure S2, available at *Annals of Oncology* online). The dose intensity of cediranib/placebo was lower in the 30 mg group compared with the 20 mg and placebo groups; the mean daily dose of cediranib was 16.6 and 22.8 mg in the cediranib 20 and 30 mg groups, respectively. Exposure to mFOLFOX6 was similar in all arms; the median numbers of cycles of 5-FU, leucovorin and oxaliplatin were 17.0, 17.0 and 12.5, respectively, in the cediranib 20 mg group, 14.0, 14.0 and 11.0, respectively, in the cediranib 30 mg group and 15.0, 15.0 and 11.5, respectively, in the placebo group. However, more patients in the cediranib 30 mg group (33%) stopped oxaliplatin >12 weeks before progression compared with those in the cediranib 20 mg (14%) or placebo (8%) groups.

soluble biomarkers

Median VEGF levels ranged from 47 to 55 pg/ml at baseline; during treatment, levels remained similar to baseline in the placebo group but increased in cediranib-treated patients. In the cediranib 20 mg group, levels increased to 89 pg/ml by day 28 and to ~130 pg/ml thereafter. In the cediranib 30 mg group, levels increased to 160–170 pg/ml from days 28 to 84 before decreasing to 151 pg/ml by day 112.

Median sVEGFR-2 levels ranged from 9095 to 10 126 pg/ml at baseline. In the placebo group, median levels decreased to 7204 pg/ml on day 112. In the cediranib 20 mg group, median levels decreased to 7091 pg/ml on day 28 and 6403 pg/ml on day 112. The corresponding median levels in the cediranib 30 mg group were 5836 and 5789 pg/ml.

extended follow-up

At second data cut-off, PFS events had been observed in 47 (81%), 46 (82%) and 46 (79%) patients in the cediranib 20 mg, cediranib 30 mg and placebo groups, respectively. The PFS HR for the cediranib 20 mg group versus placebo was 0.76 (95% CI 0.51–1.15), two-sided $P = 0.0879$. Median PFS was 10.9 and 8.3 months, respectively. In the cediranib 20 mg group, 40.5% of patients were event free at 12 months compared with 28.9% in the placebo group. The PFS comparison for cediranib 30 mg versus placebo was 0.96 (95% CI 0.64–1.46), two-sided $P = 0.429$. Median PFS was 9.8 and 8.3 months, respectively, and 36.1% of patients were event free at 12 months in the cediranib 30 mg group versus 28.9% in the placebo group.

At final data cut-off, 24 (41.4%), 27 (48.2%) and 23 (39.7%) patients had died in the cediranib 20 mg, cediranib 30 mg and placebo groups, respectively. For the comparison of cediranib 20 mg versus placebo, the HR was 1.09 (95% CI 0.61–1.95), two-sided $P = 0.543$; median OS was not reached in the cediranib 20 mg group. For the comparison of cediranib 30 mg versus placebo, the HR was 1.28 (95% CI 0.73–2.24), two-sided $P = 0.706$. Median OS was 22.4 and 23.3 months in the cediranib 30 mg and placebo groups, respectively.

discussion

Patients enrolled in this study were representative of the target population of Japanese patients with previously untreated mCRC and consistent with previous studies [26, 27]. Although baseline characteristics were generally well balanced across the three groups, imbalances were noted. The imbalances in ALP and albumin levels probably occurred because the data were analysed at a central laboratory, whereas stratification according to baseline liver function was carried out in individual centres.

The median PFS of patients who received mFOLFOX6 alone in this study (8.3 months) was consistent with the SWIFT-2 (8.2 months) [27] and TREE-1 (8.7 months) [28] studies, in

which patients received mFOLFOX6 as first-line treatment of mCRC. Furthermore, the median PFS of patients in this study who received cediranib 20 mg plus mFOLFOX6 (10.2 months) compares well with the time to progression (9.9 months) for patients who received bevacizumab plus mFOLFOX6 in the TREE-2 study [28]. It is worth noting that TREE-2 was conducted in non-Japanese patients and there is a lack of phase III data for bevacizumab plus FOLFOX in the first-line setting in Japanese mCRC patients. A recent phase I/II study of first-line therapy comprising capecitabine plus oxaliplatin (XELOX) and bevacizumab in 64 Japanese patients with mCRC revealed a median PFS of 11 months, although the primary end points of this study were safety and ORR [29].

Here, the higher response rate observed in patients treated with cediranib 30 mg compared with the other arms did not translate into prolonged PFS, possibly due to differences in tolerability profiles of the cediranib arms. More patients in the cediranib 30 mg group experienced AEs (in particular, grade 3/4 diarrhoea) that led to discontinuation, dose reduction or dose interruption, than in the cediranib 20 mg or placebo groups. This appeared to impact on chemotherapy delivery—patients in the 30 mg arm received a lower dose intensity of oxaliplatin, which may reflect the differences in PFS outcomes. Due to these differences in tolerability, results from this study suggest that cediranib 20 mg is more suitable than 30 mg for long-term dosing in combination with mFOLFOX6 in Japanese patients with previously untreated mCRC. Cediranib 20 mg plus mFOLFOX6 was generally well tolerated, although the incidence of SAEs was higher compared with the placebo group. The most frequently reported AEs for the combination of cediranib 20 mg and mFOLFOX6 were diarrhoea and hypertension. The >50% incidence of palmar–plantar erythrodysaesthesia syndrome (hand–foot syndrome) in patients who received cediranib is consistent with a previous phase I study of cediranib monotherapy in Japanese patients and with studies of other targeted agents in Japanese patients with advanced cancer [30, 31]. Overall, no new safety issues were identified; no fatal AEs occurred and the AE profile was consistent with previous cediranib studies [10, 15]. With the exception of hypertension, diarrhoea, proteinuria, hypothyroidism, reversible posterior leukoencephalopathy syndrome, fatigue, hepatotoxicity, haematological toxicity and thrombocytopenia (for which specific management protocols were employed), cediranib-associated AEs were managed by dose interruption of up to 14 days or, if longer, treatment discontinuation. The incidences of grade ≥3 AEs and SAEs observed in this trial following addition of a TKI to FOLFOX therapy are consistent with those reported in trials involving vatalanib and bevacizumab in combination with a FOLFOX regimen [23, 32]. Cediranib treatment has shown a less favourable AE profile compared with bevacizumab in Western patients in the HORIZON III study [23]. In a phase I/II study in Japanese mCRC patients treated with XELOX plus bevacizumab, the most common grade 3/4 AEs were neurosensory toxicity (17%) and neutropenia (16%), both of which were managed by dose reduction of XELOX components; the incidence of grade 3/4 diarrhoea was only 3% [29]. It is not clear why the toxicity profiles of cediranib and bevacizumab differ, but it is probably related to differences in mechanism of action; cediranib is a potent inhibitor of the three VEGF receptor tyrosine kinases, whereas the activity of bevacizumab is dependent on preventing VEGF from binding to VEGF receptors, rather than blocking the receptors directly. In addition, the potential contribution of cediranib activity versus non-VEGFR kinases, e.g. c-Kit inhibition [33], cannot be excluded. Furthermore, cediranib undergoes extensive metabolism, so it is possible that one or more metabolites may add to the toxicity profile.

An assessment of the levels of the soluble biomarkers VEGF and sVEGFR-2 was conducted as an exploratory objective. Owing to the limited data, caution should be taken when drawing conclusions from these findings; however, the observed increase in VEGF levels and decrease in sVEGFR-2 levels in cediranib-treated patients are consistent with previous cediranib trials [10, 21]. The increased VEGF levels may represent an acute stress response to inhibition of VEGF signalling by cediranib, whereas changes in sVEGFR-2 levels could be a surrogate marker for biological activity.

Analysis with an additional 8 months of follow-up data revealed similar findings to the pre-specified protocol analysis in both efficacy and safety outcomes. This additional analysis confirmed that PFS in this study (HR = 0.76) is consistent with the HORIZON II study (HR = 0.84), in which significantly improved PFS was observed with the addition of cediranib 20 mg to standard chemotherapy (FOLFOX/XELOX) [22].

This study met its primary end point for improved PFS with cediranib 20 mg plus mFOLFOX6 compared with placebo plus mFOLFOX6. The outcomes from this study, and from HORIZON II [22] and HORIZON III [23], provide some understanding of the potential role of VEGFR TKIs in the management of previously untreated mCRC. In unselected patient populations, cediranib provided marginal clinical benefit when added to standard oxaliplatin-based chemotherapy. These data did not support further development of cediranib in CRC; however, further investigation may reveal a particular benefit in a more selective patient population.

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disclosure

KY has received speaker fees (Merk Serono and Chugai Pharmaceutical). XS and KF are employees of AstraZeneca and own stock. All other authors have no conflicts of interest to declare.

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Upregulation of ERCC1 and DPD expressions after oxaliplatin-based first-line chemotherapy for metastatic colorectal cancer

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BACKGROUND: The updated randomised phase 2/3 FIRIS study demonstrated the noninferiority of IRIS (irinotecan and S-1) to FOLFIRI (irinotecan, folinic acid, and 5-FU) for metastatic colorectal cancer. Meanwhile, in the subset analysis including patients who previously have undergone oxaliplatin-containing chemotherapy, the IRIS group showed longer survival than the FOLFIRI group. However, the molecular mechanism underlying this result is still unknown.

METHODS: The National Cancer Institute 60 (NCI60) cell line panel data were utilised to build the hypothesis. A total of 45 irinotecan-naive metastatic colorectal cancer patients who had undergone hepatic resection were included for the validation study. The mRNA expressions of *excision repair cross-complementing group 1* (*ERCC1*), *dihydropyrimidine dehydrogenase* (*DPD*), and *topoisomerase-I* (*TOP1*) were evaluated by quantitative RT–PCR. The expressions of ERCC1 and DPD were also evaluated by immunohistochemistry.

RESULTS: Sensitivity to oxaliplatin in 60 cell lines was significantly correlated with that of 5-FU. Resistant cells to oxaliplatin showed significantly higher *ERCC1* and *DPD* expression than sensitive cells. In validation study, *ERCC1* and *DPD* but not *TOP1* expressions in cancer cells were significantly higher in FOLFOX (oxaliplatin, folinic acid, and 5-FU)-treated patients ($N=24$) than nontreated patients ($N=21$). The ERCC1 and DPD protein expressions were also significantly higher in FOLFOX-treated patients.

CONCLUSION: The ERCC1 and DPD expression levels at both mRNA and protein levels were significantly higher in patients with oxaliplatin as a first-line chemotherapy than those without oxaliplatin. The IRIS regimens with the *DPD* inhibitory fluoropyrimidine may show superior activity against DPD-high tumours (e.g., tumours treated with oxaliplatin) compared with FOLFIRI.

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Translational Therapeutics

The combination of fluorouracil (5-FU) and folinic acid with either oxaliplatin (FOLFOX-4 and FOLFOX-6 regimens) or irinotecan (FOLFIRI and AIO regimens) has been established as the standard first-line chemotherapy for metastatic colorectal cancer (O'Neil and Goldberg, 2008). Second-line therapy for patients whose disease progresses or recurs has been investigated in several clinical studies (Cunningham *et al*, 1998; Rougier *et al*, 1998, 2002; Tournigand *et al*, 2004). Patients who are initially treated with an oxaliplatin-based regimen tend to be offered an irinotecan-based regimen as second-line therapy and vice versa. However, the basic rationale for a sequential treatment strategy has been poorly studied.

An orally administered 5-FU pro-drug, S-1, is approved for the treatment of gastric cancer, colorectal cancer, breast cancer, head and neck cancer, non-small cell lung cancer, pancreatic cancer, and hepato biliary cancer in Japan, and for gastric cancer in Europe. S-1 consists of tegafur, a pro-drug of 5-FU, 5-chloro-2,4-dihydroxypyridine (CDHP), a dihydropyrimidine dehydrogenase (*DPD*) inhibitor maintaining the serum concentration of 5-FU, and potassium oxonate, an inhibitor of orotate phosphoribosyl transferase that reduces gastrointestinal toxicities.

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We previously reported the updated results of the randomised phase 2/3 FIRIS study of 426 patients, which reconfirmed the noninferiority of IRIS (irinotecan/S-1) to FOLFIRI using progression-free survival (PFS) as the primary end point (Muro *et al*, 2010; Baba *et al*, 2011). Furthermore, we reported the pre-planned subset analysis that revealed that the median overall survival (OS) of the IRIS group in patients who previously underwent oxaliplatin-containing chemotherapy was significantly longer than that of the FOLFIRI group (adjusted HR = 0.755; 95% CI = 0.580–0.987) (Baba *et al*, 2011). Regarding this intriguing finding, Muro *et al* (2010) have speculated that S-1 might have some salvage effects in patients who previously received FOLFOX, containing oxaliplatin with bolus and infusional 5-FU. However, the mechanism underlying this interaction between the presence or absence of oxaliplatin and therapeutic effects in the FIRIS study remains unclear. The current retrospective study therefore investigated the molecular mechanisms for the superiority of IRIS to FOLFIRI in patients previously treated with oxaliplatin-based chemotherapy.

MATERIALS AND METHODS

NCI60 cell line data

The National Cancer Institute (NCI) database (http://dtp.nci.nih.gov) containing data from 60 NCI60 cell lines was used as the

source of cytotoxicity data for oxaliplatin (NSC266046), 5-FU (NSC19893), and DNA copy number. The GI_{50}, which is the concentration required to inhibit growth by 50%, was used as a parameter for cytotoxity. The DNA microarray data for gene expression were downloaded from the Genomics and Bioinformatics group website (http://discover.nci.nih.gov/). Downloaded data were processed and loaded into GeneSpring software, version 7.3 (Agilent Technologies, Santa Clara, CA, USA). Correlations were calculated using Student's *t*-tests with JMP8.0 software (SAS Institute, Tokyo, Japan).

Patient characteristics

Irinotecan-naive metastatic colorectal cancer patients, with Eastern Cooperative Oncology Group performance status (ECOG PS) 0–1, adequate organ function, and resectable liver metastases were enroled in the study. Blocks from resected tumour specimens of liver metastatic lesions were available from 24 patients who preoperatively received the FOLFOX regimen, and 21 with no prior oxaliplatin-containing chemotherapy. All patients underwent hepatic resection for colorectal liver metastasis in the Department of Gastroenterological Surgery, Kumamoto University. The study was carried out in accordance with the Declaration of Helsinki and Good Clinical Practice Guidelines. Written informed consent was obtained from all patients participating in the study. Approval of the protocol was obtained from an Independent Ethics Committee or the Institutional Review Board.

Microdissection

Representative haematoxylin and eosin-stained slides of formalin-fixed, paraffin-embedded (FFPE) blocks were reviewed by a pathologist to estimate tumour load per sample. Section slides of 10-μm thickness were then stained with nuclear fast red (Sigma-Aldrich, St Louis, MO, USA) for manual microdissection. Malignant cells were selected under microscope magnification of $\times 5$ to $\times 10$ and dissected from the slide using a scalpel as described previously (Ceppi *et al*, 2006).

Isolation of RNA and cDNA synthesis

RNA isolation from tumour tissue isolated by manual microdissection and cDNA preparation steps were accomplished as described previously (Kuramochi *et al*, 2006), with a slight modification in the extraction step using RNeasy Mini Elute spin-columns (Qiagen, Chatsworth, GA, USA).

Quantitative real-time PCR

Gene expression levels of *excision repair cross-complementing group 1* (*ERCC1*), *DPD*, and *topoisomerase-1* (*TOP1*) were determined using TaqMan real-time PCR (Life Technologies, Foster City, CA, USA) as described previously (Kuramochi *et al*, 2006). β-Actin was used (*ACTB*) as an endogenous reference gene. All genes were run on all samples in triplicate. The detection of amplified cDNA results in a cycle threshold (Ct) value, which is inversely proportional to the amount of cDNA. Universal Mix RNAs (Stratagene, La Jolla, CA, USA) were used as control calibrators on each plate. The primer sequences for *ERCC1*, *DPD*, and *ACTB* were as previously described (Schneider *et al*, 2005). The Ct was the fractional cycle number at which the fluorescence generated by cleavage of the probe exceeded a fixed level above baseline. The relative amount of tissue target mRNA standardised against the amount of *ACTB* mRNA was expressed as follows: $-\Delta Ct = -(Ct_{(\text{target gene-1})} - Ct_{(\beta\text{-actin})})$. The ratio of the number of target mRNA copies to the number of *ACTB* mRNA copies was then calculated as follows: $2^{-\Delta Ct} \times K$. Here, K is a constant (Livak and Schmittgen, 2001). Contamination with genomic DNA was limited by amplifying nonreverse-transcribed RNA.

Immunohistochemistry

The FFPE tumour tissues were sliced into 4-μm sections. The tissue specimens on the slide were then deparaffinised, and endogenous peroxidase was inactivated. For ERCC1 analysis, the slides were incubated at 4 °C overnight with the primary anti-ERCC1 monoclonal antibody (Clone D-10; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in a dilution of 1 : 100. For DPD analysis, the slides were incubated at 4 °C overnight with the primary anti-DPD monoclonal antibody (Clone OF-303, Taiho Pharmaceutical Co., Ltd, Tokyo, Japan) in a dilution of 1 : 100. They were then reacted with a reagent containing horseradish peroxidase-labelled polymer-bound anti-mouse IgG (EnVision + system; Dako Japan Inc., Tokyo, Japan). The chromogenic substrate used for detection was DAB (3,3′-diaminobenzidine). Slides were counterstained with haematoxylin.

Immunohistochemical data analysis

The staining intensities of ERCC1 (Kim *et al*, 2009) and DPD (Okabe *et al*, 2000) were evaluated on a scale from 0 to 2+, as described previously with slight modifications. In brief, the positive reaction for both antibodies was scored into three grades, according to the intensity of the staining: 0, 1+, and 2+. The percentages of ERCC1- and DPD-positive cells were also scored into three categories: 0 (0%), 1 (1–49%), and 2 (50–100%). The product of the intensity by percentage scores was used as the final score. The immunostained specimens were independently evaluated by two blinded investigators (HB and HO). There was close agreement (>90%) between the two investigators; in the case of any disagreement, final grading was determined by consensus.

Statistical analysis

Categorical data analysis was conducted using the χ^2 test. The GI_{50} of 5-FU and ERCC1, mRNA level of *ERCC1* and *DPD*, and immunohistochemical score of ERCC-1 and DPD were compared using Spearman's correlation coefficient. Either the Student's *t*-test or Wilcoxon test was performed to determine the differences between groups. Results were considered statistically significant at $P<0.05$. All statistical analyses were done with JMP version 8.01 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Data mining in the NCI database

The relationship between the cytotoxic effects of oxaliplatin (NSC266046) and 5-FU (NSC19893) in 60 NCI60 panel cell lines is shown in Figure 1A. The cytotoxic effects of oxaliplatin were significantly correlated with those of 5-FU (Spearman's Rho = 0.55, $P<0.0001$).

For elucidating the underlying mechanism of the correlations between oxaliplatin and 5-FU cytotoxicities, gene expression levels as determined by cDNA microarray analysis were also examined. The NCI60 panel cell lines were arbitrarily classified as oxaliplatin-high-sensitive and oxaliplatin-low-sensitive cell lines according to their respective GI_{50} values. The oxaliplatin-high-sensitive cell lines were those with GI_{50} values within the 15th percentile, whereas the oxaliplatin-low-sensitive cell lines were above the 85th percentile. The remaining cell lines were classified as having intermediate sensitivity.

The Student's *t*-test revealed that the gene expression level of *ERCC1* differed significantly ($P<0.05$) between oxaliplatin-high-sensitive and oxaliplatin-low-sensitive cell lines, as shown in

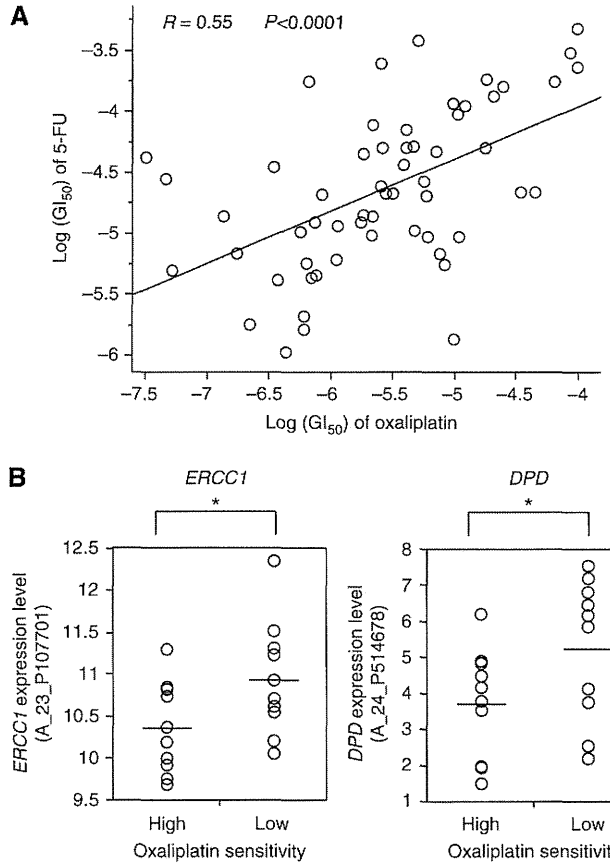

Figure 1 Oxaliplatin-resistant cells showed high *ERCC1* and *DPD* expression in *in silico* analysis. (**A**) Relationship between cytotoxic effects of oxaliplatin (NSC266046) and 5-FU (NSC19893) in 60 NCI60 panel cell lines. (**B**) Comparison of gene expression level, *ERCC1* and *DPD*, or copy number between low sensitive cells and high sensitive cells to oxaliplatin. Data expressed as $\log_2$ (per chip normalised value × 500). *$P<0.05$.

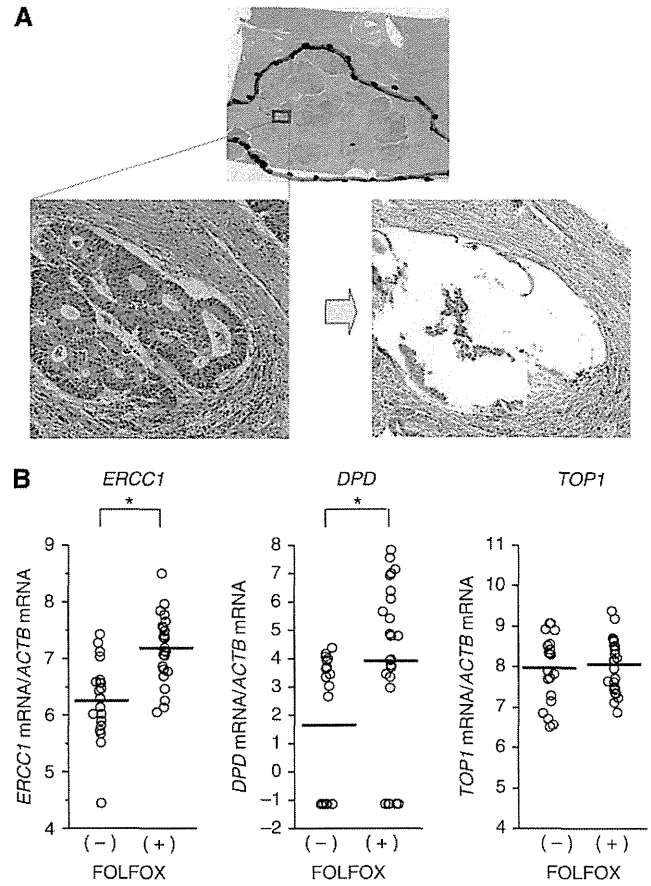

Figure 2 The *ERCC1* and *DPD* mRNAs upregulated in CRC patients with preoperative FOLFOX. (**A**) Typical slide for pathological diagnosis of FFPE tumour specimens (magnification ×2.4). Sections, 5-μm-thick, stained with haematoxylin and eosin before microdissection (magnification ×50). After staining with nuclear fast red, standard manual microdissection was performed (magnification ×50). (**B**) Comparison of gene expression levels of *ERCC1*, *DPD*, and *TOP1* in tumour cells with or without FOLFOX regimen before hepatectomy. *$P<0.001$ for ERCC1 and $P=0.005$ for DPD, respectively.

Table 1 Patient characteristics

	Oxaliplatin free, $n=21$ (%)	Oxaliplatin treated, $n=24$ (%)	*P*-value[a]
Gender, no. (%)			0.344
Male	13 (62)	18 (75)	
Female	8 (38)	6 (25)	
Age			0.715
Median, years	62	63	
Range, years	45–75	28–82	
Tumour location (%)			0.974
Proximal colon	3 (14)	3 (13)	
Distal colon	9 (43)	11 (46)	
Rectum	9 (43)	10 (42)	
Differentiation (%)			0.873
Well	10 (48)	12 (50)	
Moderate	11 (52)	12 (50)	
Prior chemotherapy (%)			—
None	19 (90)	—	
5-FU/LV	1 (5)		
S1 + CPT-11 (IRIS)	1 (5)		
mFOLFOX6	—	20 (83)	
mFOLFOX6 + bevacizumab	—	4 (17)	

Abbreviations: 5-FU/LV = fluorouracil/leucovorin; IRIS = irinotecan and S-1; mFOLFOX6 = modified FOLFOX6. [a]The *P*-values for gender were calculated using χ^2 test. The *P*-values for age, tumour location, differentiation, and prior chemotherapy were calculated using the Wilcoxon test.

Figure 1B. Interestingly, the gene expression level of *DPD* also differed significantly ($P<0.05$) between oxaliplatin-high-sensitive and oxaliplatin-low-sensitive cell lines (Figure 1B). Expression levels of *ERCC1* and *DPD* in oxaliplatin-low-sensitive cell lines were 1.5 and 2.9 times higher than those in high-sensitive cell lines, respectively.

Lower sensitivity to oxaliplatin was associated with a parallel increase in *ERCC1* and *DPD* expression. This finding may support that *ERCC1* influences cytotoxicity after oxaliplatin treatment. Based on the findings of recent clinical translational studies (Lentz *et al*, 2005), *ERCC1* was likely a predictive marker for colorectal cancer patients receiving oxaliplatin-containing therapy. Therefore, *ERCC1* was investigated using clinical specimens from patients who had received a first-line chemotherapy with or without oxaliplatin.

Patient characteristics

Table 1 summarises patient characteristics. The median patient age at the time of liver dissection was 62 years (range, 28–82 years). There were no significant differences in clinicopathological factors such as gender, age, tumour location, or differentiation between patients with and without a prior oxaliplatin regimen.

Gene expression level of tumour specimens

The FFPE tumour specimens resected from liver metastasis were subjected to manual microdissection to ensure that only tumour cells were dissected (Figure 2A). As shown in Figure 2B, *ERCC1* and *DPD*, but not *TOP1*, showed statistically significant higher expression in FOLFOX-treated patients ($n=24$) compared with the nontreated group ($n=21$). The mean expression level of *ERCC1* and *DPD* in those receiving the FOLFOX regimen was 1.8 and 4.9 times higher, respectively, than in patients without any prior oxaliplatin-containing chemotherapy (*ERCC1*, $P<0.0001$; *DPD*, $P=0.005$). The expression level of *ERCC1* was significantly correlated with that of *DPD* (Spearman's correlation coefficient = 0.519; $P=0.0003$).

Immunohistochemical results

The RT–PCR analysis revealed higher expression of *ERCC1* and *DPD* in FOLFOX-treated patients than nontreated patients. To confirm the protein expression levels of these genes, immunohistochemical examination was performed. The protein expression of ERCC1 (Figures 3A–C) was found in tumour cells, especially in the nucleus, whereas DPD protein expression was found in tumour cells and stromal cells (Figures 3D–F). For ERCC1, the mean (s.d.) expression was 0.48 (0.68) in patients without FOLFOX and 1.42 (1.41) with FOLFOX (Figure 3G). For DPD, the mean (s.d.) expression was 0.14 (0.36) in patients without FOLFOX and 0.79 (1.02) with FOLFOX (Figure 3G). In accordance with RT–PCR results, immunohistochemical analysis showed that protein expression of both ERCC1 and DPD was significantly higher in FOLFOX-treated patients than nontreated patients ($P=0.015$ and 0.0025, respectively; Figure 3G). Furthermore, a significant correlation between ERCC1 score and DPD score was shown (Spearman's correlation coefficient = 0.65; P-value <0.0001).

DISCUSSION

In the present study, gene expression levels of *ERCC1*, which were extracted by the data mining process of NCI60 screening panel data, were significantly higher in recurrent metastatic cancer cells resected from patients who had received the FOLFOX regimen than from patients with no prior oxaliplatin-containing chemotherapy. In addition, the nucleoside catabolic gene *DPD* expression level also showed significant differences between patients with and without oxaliplatin as a first-line regimen. Given that the IRIS regimens with the *DPD* inhibitory fluoropyrimidine may show superior activity against DPD-high tumours compared with FOLFIRI, our

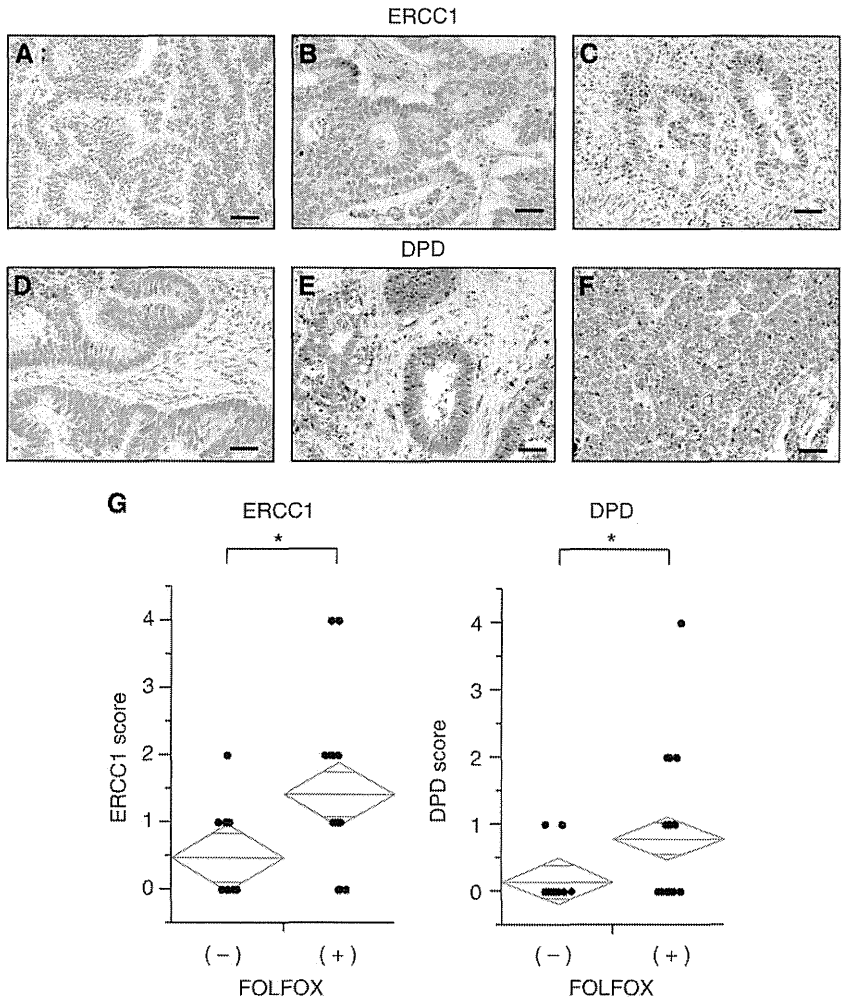

Figure 3 ERCC1 and DPD upregulated in CRC patients with preoperative FOLFOX. Representative pictures of ERCC1 and DPD in CRC patients. Cases of CRC showing weak (**A**), moderate (**B**), and strong (**C**) ERCC1 staining. Cases of CRC showing weak (**D**), moderate (**E**), and strong (**F**) DPD staining; bar = 50 μm. (**G**) The expression scores of ERCC1 and DPD were compared between patients with FOLFOX and patients without FOLFOX using Wilcoxon test. *$P=0.015$ for ERCC1 and $P=0.0025$ for DPD, respectively.

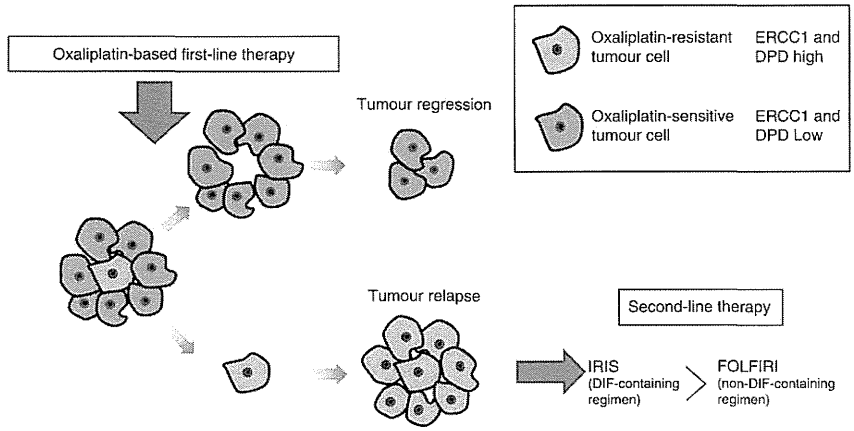

Figure 4 Hypothesis of molecular mechanism of superiority in IRIS group for prior oxaliplatin-treated patients. This study demonstrated that oxaliplatin-resistant tumour cells showed high ERCC1 and DPD, and thereby seemed to be sensitive to IRIS therapy.

findings may support the recent clinical result on the superiority of IRIS to FOLFIRI in patients previously treated with oxaliplatin-based chemotherapy.

Colon cancer is known to be a relatively heterogeneous tumour, and is characterised by a heterogenic pool of cells with distinct differentiation patterns. As an example, the *K-ras* mutation was thought to occur during early-stage tumour development; however, a recent study revealed intratumoural heterogeneity of *K-ras* mutations in 35–47% of primary colorectal carcinomas (Giaretti *et al*, 1996; Al-Mulla *et al*, 1998; Losi *et al*, 2005). Baldus *et al* (2010) also reported heterogeneity between primary tumours and lymph-node metastases in 31% (*K-ras*), 4% (*BRAF*), and 13% (*PIK3CA*) of cases. Watanabe *et al* (2011b) found intratumoral heterogeneity of *K-ras* mutations in laser-captured microdissected specimens with respect to discordant *K-ras* status between primary and metastatic colorectal tumours. Such genetic alterations, not only in *K-ras* but also in other genes, could result in intratumoral heterogeneous gene expression (Watanabe *et al*, 2011a). Recently, the concept that cancer might arise from a rare population of cells with stem cell-like properties has received support with regard to several solid tumours, including colorectal cancer (Barker *et al*, 2007; Dalerba *et al*, 2007; O'Brien *et al*, 2007; Ricci-Vitiani *et al*, 2007; Huang *et al*, 2009; Ricci-Vitiani *et al*, 2009; van der Flier *et al*, 2009). Considering the therapeutic implications of cancer stem cells, the failure of current standard therapies to eradicate tumours fully could be explained by assuming that colorectal cancer stem cells are able to survive treatments and achieve only a transitory clinical remission.

Based on our experimental results and knowledge of tumour cell biology, we propose the following hypothesis to explain why the IRIS regimen was superior to the FOLFIRI regimen for colorectal cancer patients who had been treated with oxaliplatin-based regimen. As shown in Figure 4, heterogeneous tumours were exposed to first-line oxaliplatin-containing therapy (mainly the mFOLFOX6 regimen for the FIRIS study, and partly mFOLFOX6 combined with bevacizumab). After the first-line treatment, oxaliplatin-sensitive tumour cells (i.e., *ERCC1* low; illustrated in blue in Figure 4) are killed and a small fraction of relatively oxaliplatin-resistant cells (i.e., *ERCC1* high; illustrated in yellow in Figure 4) survive, which might include cancer stem cells. In NCI60 cell line data, *ERCC1* and *DPD* gene expression is confounding; surviving cells will exhibit high *DPD* gene expression. Consequently, failure of first-line treatment might result in the proliferation of oxaliplatin-resistant tumour cells, which exhibit high levels of *DPD* gene expression. Because the IRIS (S-1/irinotecan) regimen contains S1, the *DPD* inhibitory fluoropyrimidine, it will show superior activity to FOLFIRI (5-FU/LV/irinotecan, non-*DPD* inhibitory fluoropyrimidine) against *DPD*-high tumours. This hypothesis was originally proposed when the updated results of the FIRIS study were reported at the 2011 meeting of the American Society of Clinical Oncology (ASCO) (Baba *et al*, 2011). Molecular mechanisms explaining why *ERCC1* and *DPD* gene expressions seemed to be confounding each other in cancer cells remain unclear. Recently, methylation has been recognised as an epigenetic alteration that leads to gene silencing in human cancer (Estellar, 2003). The role of aberrant methylation of the *DPD* or *ERCC1* promoter as a potential common epigenetic regulatory mechanism in tumour cells remaining after oxaliplatin-based chemotherapy warrants investigation.

A limitation of the present study was the relatively small number of patients included. Nevertheless, the phenomenon identified might be useful in selecting second-line treatments for patients who would benefit the most, and in providing a rationale for selecting therapy. To confirm our hypothesis, the study should be confirmed using an independent cohort of patients. To our knowledge, this is the first report to demonstrate a basic rationale for second-line therapy against the failures of first-line therapy containing oxaliplatin in colorectal cancer patients.

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