



any amino acid substitution were assigned as the \*1 group and named with small alphabetical letters in descending frequency order (\*1a to \*1x). Haplotypes with nonsynonymous variations were assigned from \*2 to \*9 groups, and their subtypes were named with small alphabetical letters. The haplotypes (\*7a to \*9a) were inferred in only one patient and described with "?" due to their ambiguity. Also, ambiguous rare haplotypes in the \*1 and \*2 groups were classified as "Others" in Figure 2. The \*1 haplotypes were further classified into the \*1A, \*1B, \*1C, \*1G and \*1H groups (capital alphabetical letters of the most frequent haplotypes were used) according to the common tagging SNPs, such as -1774delG, -24C>T, 3972C>T (Ile1324Ile), and 2937G>A (Ser978Ser).

The most frequent \*1 group, \*1A, harbors the common SNPs -1774delG and -1023G>A in the 5'-flanking region and mostly IVS29+154A>G, and the frequency of \*1A (0.331) is almost the same as that in healthy Koreans (0.323) reported by Choi et al.<sup>8</sup> They have shown that -1774delG reduced promoter activity both at the basal level and after induction by chenodeoxycholic acid (CDCA), a component of bile acids, and that the haplotype bearing -1774delG is associated with chemical-induced hepatitis (cholestatic and mixed types).<sup>8</sup> Therefore, it is possible that \*1A can affect the pharmacokinetics or pharmacodynamics of MRP2-transported drugs.

The \*1B group haplotypes (0.292 frequency) harbor no or any intronic or synonymous variations the functions of which are unknown. The functional significance of variations in the \*1B group, including the most frequent SNP IVS24+25T>C, needs further confirmation.

The third group \*1C (0.172 frequency) harbors the known common SNPs -1549G>A, -1019A>G, -24C>T, IVS3-49C>T, and 3972C>T (Ile1324Ile), except for one rare ambiguous haplotype lacking 3972C>T (Ile1324Ile). The \*1C haplotypes also harbor IVS12+148A>G, IVS15+169T>C and IVS16-105C>T. The haplotypes bearing -1549G>A, -24C>T and 3972C>T (Ile1324Ile) are commonly found in Korean populations (frequency 0.14-0.25)<sup>8</sup> and Caucasians (0.14-0.17).<sup>10,14,21</sup> The functional importance of the tagging SNP in the \*1C group, -24C>T, has been reported by several researchers; e.g., reduced promoter activity,<sup>8,11</sup> reduced mRNA expression in the kidney,<sup>11</sup> association with chemical-induced hepatitis (hepatocellular type),<sup>9</sup> and influence on irinotecan-pharmacokinetics and pharmacodynamics.<sup>12,10</sup> For other SNPs in the \*1C group, functional alterations *in vitro* have not been shown; no change in promoter activity by -1549G>A, no influence of IVS3-49C>T on splicing, and no change induced by 3972C>T (Ile1324Ile) on MRP2 expression or transporter activity.<sup>9</sup> Although -24C>T caused reduced promoter activity in the absence of the bile acid CDCA,<sup>8,11</sup> enhanced promoter activity of -24C>T under induction by CDCA has been demonstrated.<sup>9</sup> Therefore the function of this SNP

might depend on cholestatic status.

Our data demonstrated that -1019A>G was closely associated with the other \*1C SNPs (complete linkage with -1549G>A). The close linkage between -1019A>G and -1549G>A was also observed in Caucasians, but their linkages with -24C>T and 3972C>T were relatively weak.<sup>10</sup> In contrast, another study on Caucasians reported that -1019A>G was exclusive to -1549G>A, -24C>T and 3972C>T.<sup>10</sup> Although the reasons for these discrepancies are not clear, some ethnic differences might exist in the 5'-flanking region.

The \*1G group harbors 3972C>T (Ile1324Ile) but not -24C>T. Caucasians have haplotypes bearing 3972C>T (Ile1324Ile) without -24C>T at frequencies of 0.15-0.20.<sup>10,21</sup> In contrast, the frequency of the corresponding haplotype group in our study (\*1G) was much lower (0.044). Although no *in vitro* effect of 3972C>T (Ile1324Ile) was shown,<sup>8</sup> its *in vivo* association with increased area under the concentration-time curve of irinotecan and its metabolites was reported in Caucasians.<sup>13</sup>

The \*1H group (\*1h and \*1s) harbors a synonymous substitution of 2934G>A (Ser978Ser) (0.03 frequency). No influence of 2934G>A (Ser978Ser) on MRP2 expression or transport activity has been shown.<sup>8</sup>

As for haplotypes with nonsynonymous substitutions, eight haplotype groups (\*2 to \*9) were identified. The \*2 [including 1249G>A (Val417Ile)] was the most frequent among them, and its frequency (0.093) was similar to those for Asians (0.10-0.13)<sup>8,12,20</sup> and slightly lower than those for Caucasians (0.13-0.22).<sup>9,10,14,15,21</sup> The haplotype frequencies of \*3 [harboring 1457C>T (Thr486Ile)] and \*4 [2366C>T (Ser789Phe)] were 0.019 and 0.008. Other rare haplotypes with novel nonsynonymous variation, \*5 [2801G>A (Arg934Gln)], \*6 [3320T>G (Leu1107Arg)], \*7 [1177C>T (Arg393Trp)], \*8 [1202A>G (Tyr401Cys)], and \*9 [2358C>A (Asp786Glu)] were found each in only one subject as heterozygote at a 0.002 frequency. No functional significance of the marker SNP [1249G>A (Val417Ile)] of \*2 has been shown *in vitro*,<sup>8,23</sup> but its *in vivo* associations with lower MRP2 expression in the placenta<sup>20</sup> and chemical-induced renal toxicity<sup>25</sup> have been reported. The variation 2366C>T (Ser789Phe) (\*4) has been shown to cause reduced MRP2 expression and alter localization *in vitro*,<sup>23</sup> but clinical data are limited. Functional changes in \*3 [1457C>T (Thr486Ile)] and \*5 to \*9 (novel nonsynonymous variations) are currently unknown. Possible effects of these amino acid substitutions were speculated using PolyPhen analysis (<http://genetics.bwh.harvard.edu/pph/>); its prediction is based on the analysis of substitution site [e.g., a substitution in transmembrane domain is assessed by the predicted hydrophobic and transmembrane (PHAT) matrix score], likelihood of the substitution assessed by the position-specific independent count (PSIC) profile scores, and protein 3D structures. This analysis predicted a possible functional change of Leu1107Arg (\*6) due to substitution in

the transmembrane region (PHAT matrix element difference = -6), and probable functional effects of Arg393Trp (\*7) (PSIC score difference = 3.053), Tyr401Cys (\*8) (3.382) and Asp786Glu (\*9) (2.277), but no functional effects of \*3 (1.446) and \*5 (0.326).

In conclusion, the current study provided detailed information on *ABCC2* variations and haplotype structures in Japanese and also suggested a large ethnic difference in the frequencies of 3972C > T (Ile1324Ile) and 1446C > G (Thr482Thr) and their related haplotypes between Asians and Caucasians. This information would be useful for studies investigating the clinical significance of *ABCC2* alleles and haplotypes.

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# Phase I/II study of oxaliplatin with oral S-1 as first-line therapy for patients with metastatic colorectal cancer

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Two phase II studies of S-1 monotherapy have shown promising response rates (RR) of 35–40% with good tolerability in patients with untreated metastatic colorectal cancer. To investigate the usefulness of S-1 plus oxaliplatin (SOX) as an alternative to infusional 5-fluorouracil/leucovorin plus oxaliplatin, the recommended dose (RD) of SOX was determined, and its safety and preliminary efficacy were evaluated in a phase I/II study. Oxaliplatin was administered at a dose of 100 mg m<sup>-2</sup> (level 1) or 130 mg m<sup>-2</sup> (level 2) on day 1, and S-1 (80–120) was given twice daily for 2 weeks followed by a 1-week rest. This schedule was repeated every 3 weeks. Level 2 was determined to be the RD. For the 28 patients who received the RD, the median treatment course was 6.5 cycles (2–14), RR of 50% (1 CR and 13 PR; 95% CI 31–69%), with a median progression-free survival of 196 days. Survival rate (1 year) was 79%. Peripheral neuropathy was observed in all patients but with no functional disorders. Major grade 3 or 4 adverse reactions at the RD were neutropaenia (14%), thrombocytopenia (28%), and diarrhoea (3%). SOX regimen is effective and easily manageable without central vein access.

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Oral fluoropyrimidine derivatives have been developed to circumvent the problems associated with continuous infusion of 5-fluorouracil (5-FU). S-1 is an effective derivative that combines tegafur with two modulators of 5-FU metabolism, 5-chloro-2,4-dihydropyridine (CDHP), a reversible inhibitor of dihydropyrimidine dehydrogenase (DPD), and potassium oxonate in a molar ratio of 1:0.4:1 (Kato *et al*, 2001). Tegafur, an oral prodrug of 5-FU, is gradually converted to 5-FU and rapidly metabolised by DPD in the liver. The maximum concentration ( $C_{max}$ ) and area under the concentration–time curve (AUC) of 5-FU in plasma during S-1 treatment have been found to be higher than the steady-state concentration and AUC of 5-FU in plasma during protracted intravenous infusion of 5-FU at a dose of 250 mg m<sup>-2</sup> day<sup>-1</sup> (Yamada *et al*, 2003).

Potassium oxonate is an orotate phosphoribosyl transferase inhibitor that is distributed primarily to the gastrointestinal tract. This component of S-1 decreases incorporation of 5-fluorouridine triphosphate into RNA in the gastrointestinal mucosa and reduces the incidence of diarrhoea. F-β-alanine (FBAL) is the main metabolite of 5-FU. F-β-alanine and fluorocitrate are thought to cause the neurotoxic and cardiotoxic effects of 5-FU by inhibiting the tricarboxylic acid cycle (Okeda *et al*, 1990; Robben *et al*, 1993; Diasio 1998). The CDHP component of S-1 inhibits DPD, the rate-limiting enzyme in the catabolic pathway of 5-FU. Consequently, the plasma

FBAL concentration after oral administration of S-1 is significantly lower than that after continuous infusion of 5-FU (Yamada *et al*, 2003). Therefore, S-1 may decrease the incidence of neurotoxicity and cardiotoxicity. The response rate of S-1 monotherapy has been found to be 35–40% for patients with metastatic colorectal cancer (Ohtsu *et al*, 2000; Shirao *et al*, 2004), with grade 3 or 4 neutropaenia observed in 5–13%, thrombocytopenia in 0–8%, diarrhoea in 2–3%, and grade 1 hand–foot syndrome (HFS) in 5%.

Oxaliplatin is a third-generation platinum compound with less toxicity and improved convenience. The regimen of infusional 5-FU and leucovorin (LV) with oxaliplatin is the standard for first- and second-line chemotherapy in patients with metastatic colorectal cancer (de Gramont *et al*, 2000; Rothenberg *et al*, 2003; Goldberg *et al*, 2004). However, infusional 5-FU with LV has the disadvantages of increased inconvenience, cost, and morbidity related to the use of a portable infusion pump and a central venous catheter. Therefore, oral fluoropyrimidine monotherapy has been commonly used in Japan.

The primary objectives of this phase I/II study were to determine the maximum tolerated dose (MTD) of S-1 plus oxaliplatin (SOX). In the phase II study, the toxicity and antitumour activity of SOX were evaluated at the recommended dose (RD).

## MATERIALS AND METHODS

### Patient selection

Patients with histologically confirmed colorectal cancer who had measurable metastatic disease were eligible for the study. Patients

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with prior chemotherapy and radiotherapy for metastatic disease were not permitted. Patients who had received adjuvant oral fluorouracil-based therapy other than S-1 were eligible if they had remained free of disease for at least 6 months after the completion of such therapy. Other eligibility criteria included an age between 20 and 74 years; an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1; adequate baseline bone marrow function (white blood cell count more than the lower limit of normal at each hospital and less than  $12\,000\ \mu\text{l}^{-1}$ , neutrophil count more than  $2000\ \mu\text{l}^{-1}$ , and a platelet count more than  $100\,000\ \mu\text{l}^{-1}$ ), hepatic function (serum total bilirubin (T.Bil) level 1.5 times the upper limit of normal or less, and serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) 2.5 times the upper limit of normal or less), and renal function (serum creatinine level the upper limit of normal or less); and a life expectancy of at least 90 days. All patients gave written informed consent. Patients were excluded if they had symptomatic brain metastasis, pre-existing watery diarrhoea, or concomitant non-malignant disease, such as cardiac, pulmonary, renal, or hepatic disease, or uncontrolled infection. This study was approved by the institutional review board of each centre. Before enrolment, all patients underwent a physical examination (including documentation of measurable disease), a complete blood cell count (CBC) with differential count, serum chemical analysis, electrocardiography, and computed tomographic (CT) scanning or magnetic resonance imaging (MRI).

### Toxicity and response criteria

Toxicity was assessed according to the Common Terminology Criteria for Adverse Events, Version 3.0 (CTCAE v3.0) (Therasse *et al*, 2000). Neurotoxicity was assessed according to the following specific neurotoxicity grading scale: grade 1, dysesthesia or paresthesia that completely regressed within 6 days; grade 2, dysesthesia or paresthesia persisting for 7 days or longer; and grade 3, dysesthesia or paresthesia causing functional impairment. During the study, all patients were evaluated weekly for signs and symptoms of toxicity. Complete blood cell counts, including differential count, liver function tests, measurement of urea nitrogen, creatinine, and electrolyte levels, and urinalysis were performed weekly. The response of measurable and assessable disease sites was evaluated according to Response Evaluation Criteria in Solid Tumors (RECIST) (Shimoyama, 1999). Tumour dimensions were assessed by CT scanning or MRI every month to confirm response, and after RECIST efficacy was confirmed, every 2 months subsequently.

### Treatment plan

Oxaliplatin was administered as a 2-h infusion every 3 weeks. The duration of the infusion could be extended to 6 h in patients who had pharyngolaryngeal dysesthesia during infusion. S-1 was available in capsule forms containing 20 or 25 mg of tegafur. Patients received S-1 orally twice daily from the evening of day 1 to the morning of day 15 at a dose of 40 mg ( $\text{BSA} < 1.25\ \text{m}^2$ ), 50 mg ( $\geq 1.25 - < 1.50\ \text{m}^2$ ), or 60 mg ( $\geq 1.50\ \text{m}^2$ ) followed by a 7-day rest period in the 3-weekly schedule. All patients received premedication with a 5-hydroxytryptamine-3-receptor antagonist with or without dexamethasone, given as a 30 min drip infusion before chemotherapy. Treatment was routinely given on an outpatient basis. Subsequent treatment was withheld until the neutrophil and platelet counts were greater than 1500 and  $75\,000\ \mu\text{l}^{-1}$ , respectively, AST or ALT less than  $150\ \text{IU l}^{-1}$ , T.Bil less than 1.5 times the upper limit of normal, creatinine less than the upper limit of normal, and diarrhoea, stomatitis, and HFS had resolved to grade 0 or 1. Treatment was repeated until the onset of disease progression or severe toxicity. When the administration of oxaliplatin was discontinued due to oxaliplatin-induced neuropathy, S-1 was also discontinued.

### Dose-escalation schedule

The dose of S-1 was fixed and oxaliplatin was examined at doses of  $100\ \text{mg m}^{-2}$  (level 1) and  $130\ \text{mg m}^{-2}$  (level 2). A minimum of three patients were studied per dose level. Dose-limiting toxicity (DLT) was defined as any of the following findings during cycle 1: (i) a neutrophil count of less than  $500\ \mu\text{l}^{-1}$  for more than 4 days, (ii) a platelet count of less than  $50\,000\ \mu\text{l}^{-1}$ , (iii) diarrhoea of grade 3 or more that occurred despite adequate supportive therapy, (iv) grade 3 or 4 non-haematologic toxicity, excluding nausea, vomiting, anorexia, and electrolyte imbalance, or (v) a treatment delay longer than 1 week due to drug-related toxicity in the phase I portion. If DLT occurred in one of the first three patients assigned to a given dose level, three additional patients were assigned to the same dose level. The MTD was defined as the dose that induced DLT during cycle 1 in 50% or more of the subjects. The RD was defined as one dose level below the MTD. If the MTD was not achieved, even at level 2, it was regarded as the RD.

The dose was modified for each patient based on haematologic or non-haematologic toxicity. If DLT occurred, the dose of oxaliplatin in the subsequent course was reduced to 75% of the initial dose and that of S-1 was reduced by one dose level: from 80 to 50, 100 to 80, and 120 to 100. S-1 intake was interrupted mid-cycle if there was a neutrophil count less than  $1000\ \mu\text{l}^{-1}$ , a platelet count less than  $75\,000\ \mu\text{l}^{-1}$ , diarrhoea, stomatitis, or HFS occurred at grade 1 or more, AST or ALT more than  $150\ \text{IU l}^{-1}$ , T.Bil more than 1.5 times the upper limit of normal, or creatinine more than the upper limit of normal. The treatment in the subsequent cycle could be resumed if these adverse events resolved within 3 weeks after the last S-1 treatment. If peripheral neuropathy persisted between courses, the next treatment cycle was started at 75% of the previous dose of oxaliplatin. In a case with pharyngolaryngeal dysesthesia, the duration of the oxaliplatin infusion was prolonged from 2 to 6 h. Recombinant granulocyte colony-stimulating factor was subcutaneously injected if patients had grade 4 neutropaenia or grade 3 febrile neutropaenia, but prophylactic use was not allowed.

### Statistical analysis

The sample size was calculated to be at least 28 patients on the assumption of the null hypothesis of overall response rate of  $\leq 30\%$  vs the alternative hypothesis of overall response rate of  $> 60\%$ , power 80%, and  $\alpha$  2.5% (one-sided). The efficacy was analysed by the full analysis set. The primary end point was overall response rate as determined by an External Review Board. The 95% CI for response rate was calculated. Twenty-eight evaluable patients were required. Progression-free survival (PFS) and overall survival were analysed by the Kaplan–Meier method. Safety was analysed in all patients who received at least one dose of study medications. Clinical cutoff date for the study analysis was 31 May 2007.

## RESULTS

### Patient characteristics

A total of 32 patients, 23 men and 9 women, were recruited into this study between March 2005 and June 2006. The median age was 57 years. Four patients had received adjuvant oral fluorouracil-based therapy. Out of 32 patients, 31 received at least one cycle of the study treatment. The demographic data, sites of metastatic tumour, and prior adjuvant therapies are summarised in Table 1. Among the nine patients entered into the phase I study, six patients were treated at the RD. Twenty-three patients entered into the phase II study. However, one patient was excluded from the analysis of efficacy due to symptoms of brain metastasis suspected

to have existed before enrolment. All 32 patients were evaluated for toxicity and 28 patients for efficacy.

### Dose-escalation findings

The first three patients were enrolled at dose level 1 (oxaliplatin 100 mg m<sup>-2</sup>, S-1 80–120 mg day<sup>-1</sup>). No DLTs were observed, and

six patients were enrolled at dose level 2 (oxaliplatin 130 mg m<sup>-2</sup>, S-1 80–120 mg day<sup>-1</sup>). At level 1, one patient had grade 3 thrombocytopenia. At level 2, one patient had grade 3 neutropenia and one patient had grade 4 thrombocytopenia. The RD was determined to be 130 mg m<sup>-2</sup> of oxaliplatin in combination with the Japanese standard daily dose of S-1.

**Table 1** Patients characteristics

Characteristic	Level 1, n = 3	Level 2, n = 29
	L-OHP (100 mg m <sup>-2</sup> ) No. of patients (%)	L-OHP (130 mg m <sup>-2</sup> ) No. of patients (%)
Age (years)		
Median	57	57
Range	47–60	34–71
Sex		
Male	3 (100)	20 (69)
Female	0	9 (31)
ECOG performance status		
0	3 (100)	26 (90)
1	0	3 (10)
Primary tumour		
Colon	2 (67)	18 (62)
Rectum	1 (33)	11 (38)
Metastatic site		
Liver only	1 (33)	10 (35)
Lung	0	3 (10)
Liver and other lesions	1 (33)	10 (35)
Others	1 (33)	6 (21)
No. of metastatic sites		
1	1 (33)	15 (52)
≥2	2 (67)	14 (48)
Previous treatment		
Resection	2 (67)	25 (86)
Adjuvant 5-FU	0	4 (14)

ECOG = Eastern Cooperative Oncology Group; L-OHP = oxaliplatin.

### Safety assessment

After identification of tolerability at level 2 (130 mg m<sup>-2</sup>) of oxaliplatin, 29 other patients received the RD at 130 mg m<sup>-2</sup>, including the phase I part patients, to further evaluate the tolerability and toxicity of the study regimen. The median number of administered cycles was 6.5 (range: 2–14), and the total number of cycles for the 29 patients was 180. Oxaliplatin could be administered at the RD without dose reduction in 57% of 28 patients. At the RD, grade 3 neutropenia was observed in four patients (14%), and grade 3 and 4 thrombocytopenia in seven patients (24%) and one patient (3%), respectively. The median relative dose intensity was 82.8% for oxaliplatin and 74.6% for S-1 at level 2. The causes of treatment discontinuation at the RD were PD in 13 patients (36%), delayed recovery from toxicity such as neutropenia, thrombocytopenia, and slight hyperbilirubinaemia in 8 patients, discretion of the investigator in 2 patients, allergic reaction in 1 patient, and symptomatic deterioration in 1 patient. The treatment was discontinued due to prolonged thrombocytopenia in eight patients after a median of seven cycles (range: 3–8). No treatment-related death was observed.

Sensory neuropathy occurred in all patients. However, no functional impairment was observed in this study. The most common non-haematologic toxicities were anorexia, nausea, and diarrhoea. One patient had grade 3 diarrhoea at the RD. Another mild adverse event related to treatment was injection site reactions (45%). One patient had severe allergic reactions such as skin rash and fever, which are typical platinum-related reactions during the sixth cycle (Table 2).

### Response to therapy

The objective tumour response was determined by the External Review Board. One of the 28 patients given the RD at level 2 had CR and 13 patients had PR, yielding a response rate of 50% (95% CI: 30.6–69.4%). In the 28 patients studied, the median PFS was

**Table 2** Toxicity

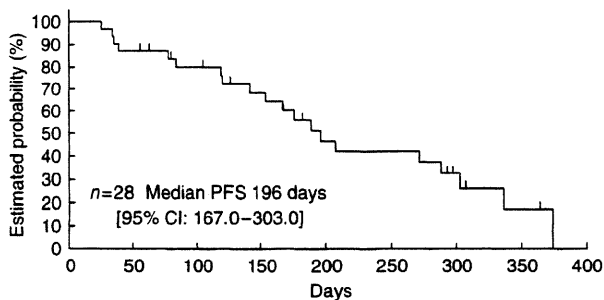
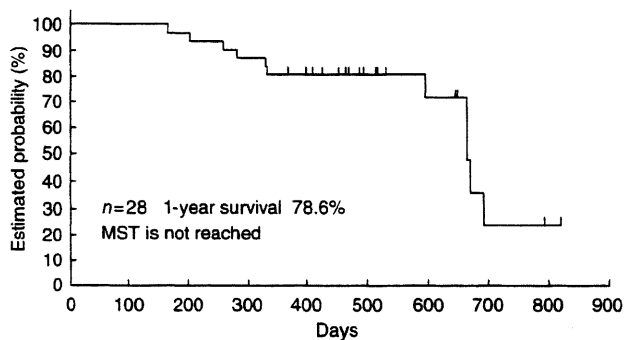
	Level 1, L-OHP (100 mg m <sup>-2</sup> ), n = 3			Level 2, L-OHP (130 mg m <sup>-2</sup> ), n = 29		
	All (%)	G3 (%)	G4 (%)	All (%)	G3 (%)	G4 (%)
<b>Non-haematologic</b>						
Nausea	1 (33)	0	0	21 (72)	0	0
Vomiting	0	0	0	7 (24)	0	0
Diarrhoea	1 (33)	0	0	17 (59)	1 (3)	0
Fatigue	1 (33)	0	0	25 (86)	0	0
Anorexia	2 (67)	0	0	26 (90)	0	0
Rash	3 (100)	0	0	13 (45)	0	0
Pigmentation disorder	1 (33)	0	0	22 (76)	0	0
Hand–foot syndrome	0	0	0	0	0	0
Peripheral neuropathy	3 (100)	0	0	29 (100)	0	0
Allergic reaction	0	0	0	0	1 (3)	0
<b>Haematologic</b>						
Neutropenia	2 (67)	0	0	18 (62)	4 (14)	0
Leukopenia	2 (67)	0	0	20 (69)	0	0
Thrombocytopenia	3 (100)	1 (33)	0	27 (93)	7 (24)	1 (3)
Anaemia	1 (33)	0	0	18 (62)	1 (3)	0

L-OHP = oxaliplatin.

**Table 3** Response rate

	No. of patients	CR	PR	SD	PD	Response rate (%)
Level 1 L-OHP ( $100 \text{ mg m}^{-2}$ )	3	0	2	1	0	67 (CI: 9.4–99.2)
Level 2 L-OHP ( $130 \text{ mg m}^{-2}$ )	28	1	13	9	5	50 (CI: 30.6–69.4)

CI = confidence interval; CR = complete response; L-OHP = oxaliplatin; PD = progressive disease; PR = partial response; SD = stable disease.

**Figure 1** Progression-free survival.**Figure 2** Overall survival.

196 days (95% CI: 167–303). The median overall survival time was not reached when 1 year passed since the last patient enrolment, namely 18 patients were alive and 10 patients were dead, and the 1-year survival rate was 78.6% by the Kaplan–Meier method (Table 3) (Figures 1 and 2).

## DISCUSSION

Our results suggest that SOX regimen is safe and effective as a first-line treatment for metastatic colorectal cancer. The RD was determined to be  $130 \text{ mg m}^{-2}$  of oxaliplatin on day 1 with 40–60 mg of S-1 twice daily from the evening of day 1 to the morning of day 15, followed by a 7-day rest period in a 3-weekly schedule. This result indicates that both oxaliplatin and S-1 could be administered at doses similar to those recommended for monotherapy for each drug. SOX regimen has demonstrated promising efficacy with a response rate of 50%, median PFS of 196 days, and a 1-year survival rate of 78.6%. Efficacy of this combination is superior to that reported for monotherapy by each drug (Diaz-Rubio *et al*, 1998; Ohtsu *et al*, 2000; Shirao *et al*, 2004; Boku *et al*, 2007). No DLTs were observed during the first cycle at levels 1 and 2. At the RD (level 2), the toxicity profile was acceptable. The

frequent non-haematologic toxicities were anorexia, nausea, and diarrhoea. Most cases of gastrointestinal toxicity were grade 1 or 2, and good oral intake was maintained. There was no grade 3 neurotoxicity observed. Although the incidence of grade 3 or 4 thrombocytopenia seems to be higher with SOX compared with the reported result of FOLFOX4 (Diaz-Rubio *et al*, 1998; Shirao *et al*, 2004), it was well managed by adequate dose modification of oxaliplatin and S-1 in subsequent cycles (Goldberg *et al*, 2004). Since the severity of thrombocytopenia is dependent on the dose of oxaliplatin, FOLFOX7 with oxaliplatin at a dose of  $130 \text{ mg m}^{-2}$  caused 9% of grade 3 thrombocytopenia (Maindault-Göebel *et al*, 2001; Tournigand *et al*, 2006).

The median time to first dose reduction was five cycles (range: 2–7) due to any reason in 16 of the 28 patients at the RD, and 4.5 cycles (range: 3–5) due to grade 3 or 4 thrombocytopenia in 6 of the 28 patients. Therapy was delayed in 22 of the 28 patients and 40 of 209 cycles, commonly due to neutropenia, thrombocytopenia, and sensory peripheral neuropathy. SOX requires only one clinic visit per 3-week cycle for a 2-h infusion of oxaliplatin. This convenience constitutes a marked advantage over regimens combining infused 5-FU/LV by ambulatory pump and oxaliplatin in terms of the impact on the daily lives of patients. In addition, very busy hospitals may have logistic issues providing pumps to all patients; therefore, oral S-1 offers an advantage over infusional 5-FU in respects of convenience and practicability.

The combination regimens of other oral fluoropyrimidine, capecitabine, and oxaliplatin have been reported in other phase II and III studies. Cassidy *et al* (2004) reported the results of a phase II study of oxaliplatin plus capecitabine (XELOX) as a first-line therapy in patients with colorectal cancer (Diaz-Rubio *et al*, 2002). Oxaliplatin ( $130 \text{ mg m}^{-2}$ ) was administered on day 1 and capecitabine ( $2000 \text{ mg m}^{-2} \text{ day}^{-1}$ ) for 14 days with a 1-week rest, every 3 weeks. The response rate, median TTP, and MST were 55%, 7.7 months, and 19.5 months, respectively. Grade 3 or 4 neutropenia according to NCI-CTC developed in 7% of patients by XELOX, and grade 3 or 4 diarrhoea developed in 16%.

The efficacy and safety of XELOX were also compared with that of 5-FU/LV plus oxaliplatin regimens (FUOX) in several phase III studies. The efficacy of XELOX was statistically not inferior to that of the FUOX regimen: median TTP 8.9 vs 9.5 months ( $P=0.153$ ), and MST 18.1 vs 20.8 months ( $P=0.145$ ) (Diaz-Rubio *et al*, 2007). Grade 3 or 4 diarrhoea was observed in 14% with both XELOX and FUOX regimens, respectively, and grade 3 or 4 HFS in 2% with XELOX. The efficacy of XELOX was also statistically not inferior to that of FOLFOX4: median TTP 8.0 vs 8.5 months, and MST 18.8 vs 17.7 months (Cassidy *et al*, 2007). Grade 3 or 4 diarrhoea was observed in 20% with XELOX and 11% with FOLFOX4, and grade 3 HFS in 6% with XELOX and 1% with FOLFOX4. Other schedules of oxaliplatin and capecitabine (CAPOX:  $70 \text{ mg m}^{-2}$  oxaliplatin on days 1 and 8 and  $2000 \text{ mg m}^{-2} \text{ day}^{-1}$  capecitabine for 14 days with a 1-week rest) were compared with FUOX. CAPOX was slightly inferior to FUOX in TTP: median TTP 7.1 vs 8.0 months ( $P=0.117$ ), and MST 16.8 vs 18.8 months ( $P=0.26$ ) (Porschen *et al*, 2007). Both regimens were generally well tolerated, although grade 2 or 3 HFS occurred more often with CAPOX (10 vs 4%) ( $P=0.028$ ). In summary, the results of these phase III studies show that the efficacy of XELOX or CAPOX was not inferior to or was slightly inferior to that of infusional 5-FU/LV plus oxaliplatin regimens. Although HFS is more commonly observed in capecitabine-combined regimens, capecitabine is expected to replace infusional 5-FU/LV.

Our limited experience of SOX regimen suggests that tri-weekly treatment with oxaliplatin and S-1 may be comparable to that of XELOX or CAPOX. The response rate of SOX was 50%, suggesting that it is worth while comparing the efficacy of SOX with that of XELOX in the phase III study. Grade 3 or 4 thrombocytopenia was observed in 28% of patients, and this incidence seems to be higher than that reported by FOLFOX4 (de Gramont *et al*, 2000).



Grade 3 or 4 thrombocytopenia with oxaliplatin monotherapy was reported in 12% of patients (Boku *et al*, 2007), and that with S-1 monotherapy in 0–8% of patients in previous phase II studies (Ohtsu *et al*, 2000; Shirao *et al*, 2004). The most commonly observed grade 3 or 4 toxicity after SOX therapy was cumulative prolonged thrombocytopenia in this phase I/II trial, which is a well-known toxicity of oxaliplatin. The protocol therapy was discontinued due to prolonged thrombocytopenia in seven patients after a median of seven cycles (range: 3–8). In cases where sudden and severe thrombocytopenia is observed, type II allergic reaction to oxaliplatin should be considered and definitive withdrawal is strongly suggested (Maindault-Göebel *et al*, 2001). A phase I study of XELOX with 130 mg m<sup>-2</sup> of oxaliplatin tri-weekly has also shown a relatively higher incidence of grade 3 thrombocytopenia in 22% (Díaz-Rubio *et al*, 2002), but only 4% during phase II with weekly assessment of CBC (Cassidy *et al*,

2004). Thrombocytopenia of SOX should be evaluated in the future phase II or III studies with a larger number of patients.

In conclusion, SOX holds promise of being a safe and effective treatment for metastatic colorectal cancer. Further evaluation is expected to examine whether SOX can be a substitute for FOLFOX.

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# Clinical Significance of Insulin-Like Growth Factor Type 1 Receptor and Epidermal Growth Factor Receptor in Patients with Advanced Gastric Cancer

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## Key Words

Advanced gastric cancer · EGFR · Gastric cancer prognosticators · HER2 · IGF-1R

## Abstract

**Objective:** To better understand the clinical implications of insulin-like growth factor type 1 receptor (IGF-1R), epidermal growth factor receptor (EGFR) and HER2 expressions in gastric cancer (GC). **Methods:** The study group comprised 86 patients who received first-line chemotherapy for advanced GC at the National Cancer Center Hospital. Using laser-captured microdissection and a real-time RT-PCR assay, we quantitatively evaluated mRNA levels of IGF-1R, EGFR and HER2 in paraffin-embedded cancer specimens of surgically removed primary tumors. **Results:** In univariate analysis of the study group as a whole, patients with low expression of both IGF-1R and EGFR ( $n = 13$ ) had a significantly longer overall survival than the other patients ( $n = 51$ ; median, 24.6 vs. 12.8 months; log-rank  $p = 0.013$ ). Multivariate survival analysis demonstrated that high EGFR expression [hazard ratio, HR: 2.94 (95% confidence interval, CI: 1.40–6.17),  $p = 0.004$ ] and poor performance status [HR: 1.96 (95% CI: 1.12–3.42),  $p = 0.018$ ] were significant predictors of poor survival. In patients given first-line S-1 monotherapy ( $n = 29$ ), low IGF-1R ( $p = 0.002$ ) and low EGFR ( $p = 0.035$ ) gene expression corre-

lated with a better response, without a significant prolongation of survival. **Conclusion:** Our data warrant further investigations on the strategy of co-targeting IGF-1R and EGFR in GC.

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## Introduction

Globally, gastric cancer (GC) is the second most common cause of cancer-related death. Even though the incidence of GC is declining, approximately 930,000 cases are newly diagnosed each year [1]. Despite the identification and development of several new classes of anticancer agents, GC remains an aggressive malignancy, with a median survival of 7–10 months in patients with metastatic or unresectable disease. However, recent advances in molecularly targeted cancer therapeutics have led to some early success, and the insulin-like growth factor (IGF) signaling axis has newly emerged as an important target for cancer therapy [2, 3].

IGF type 1 receptor (IGF-1R) is a cell membrane receptor that is activated by its ligands, IGF-1 and IGF-2. IGF-1R participates in cell proliferation, differentiation and prevention of apoptosis [2, 4]. Since IGF-1R is also involved in malignant transformation [4], development of

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IGF-1R-directed cancer therapy has been initiated. IGF-1R is frequently overexpressed in human cancers, and the association between IGF-1R expression and outcome has been assessed for breast cancer and other solid tumors [5, 6]. However, IGF-1R expression in GC remains poorly understood.

Previous studies have indicated that IGF-1R can interact with epidermal growth factor receptor (EGFR) to augment the malignant behavior of tumors [7]. EGFR and its homologues HER2 (also known as *erbB-2*) are members of the *erbB* gene family. These receptors encode for transmembrane receptor-type tyrosine kinases, for which therapeutic approaches do exist. They play a crucial role in tumor cell proliferation, resistance to apoptotic stimuli, adhesion, migration and differentiation, as well as participate in tumor angiogenesis [8]. EGF and transforming growth factor- $\alpha$  activate EGFR and HER2, which form either homodimers or heterodimers, and initiate intracellular signaling cascades. EGFR and IGF-1R regulate overlapping downstream signaling pathways and EGFR might also regulate the IGF-1R signaling pathway through IGF-binding protein-3 [9]. HER2 is the preferred co-receptor for the formation of dimers with EGFR, HER3 or HER4; the heterodimers consisting of HER2 and these other receptors have a greater capacity for translating mitogenic signals than the homodimers and act synergistically to promote cellular transformation [10]. In many cancers, the expression of these receptors may be related to patient survival [11]. Expression of EGFR or HER2 has been evaluated using several methods in GC. However, whether the expressions of EGFR and HER2 are significant predictors of survival in patients with GC remains controversial.

This study was designed to further delineate the clinical implications of IGF-1R, EGFR and HER2 mRNA expression in GC. We tested the hypothesis that the clinical outcome of patients with advanced GC, such as rate of objective response to first-line chemotherapy and overall survival, is related to the pretreatment intratumor mRNA levels of these three biomarkers. We also analyzed relationships between the study variables and clinicopathological characteristics.

## Patients and Methods

### Patient Eligibility

Patients with a diagnosis of histologically proven advanced GC were eligible for the study. Inclusion criteria were as follows: unresectable, locally advanced or metastatic disease; no prior chemotherapy and no prior adjuvant/neoadjuvant chemotherapy;

specimens of primary gastric adenocarcinomas were obtained before the start of chemotherapy by surgical resection at the National Cancer Center Hospital (Tokyo, Japan); first-line chemotherapy was administered at the National Cancer Center Hospital between July 1997 and June 2004; radiographically measurable disease, and written informed consent. Measurable disease was assessed by a standardized CT examination every 2 months. Response was evaluated according to the standard guidelines of the International Union against Cancer as complete response (CR), partial response (PR), no change (NC) or progressive disease (PD) [12]. Tumor response and survival times as of December 2006 were confirmed in all patients. Written informed consent was obtained before treatment and evaluation of tumor samples. This study was approved by the Institutional Review Board of the National Cancer Center Hospital.

### Chemotherapy

The following first-line chemotherapy regimens were administered to the patients in our study: S-1 monotherapy (n = 29), cisplatin plus irinotecan (n = 29), 5-FU monotherapy (n = 23) and other regimens (5-FU plus methotrexate, n = 2; paclitaxel, n = 2, and uracil/ftorafur, n = 1). For S-1 monotherapy, patients received S-1 (40 mg/m<sup>2</sup> twice daily) on days 1–28 of a 42-day cycle. Treatment with cisplatin plus irinotecan consisted of cisplatin (80 mg/m<sup>2</sup>) on day 1 and irinotecan (70 mg/m<sup>2</sup>) on days 1 and 15 of a 28-day cycle. For 5-FU monotherapy, patients received 5-FU (800 mg/m<sup>2</sup>/day) as a continuous infusion on days 1–5 of a 28-day cycle.

### Laboratory Methods

Ten-micrometer-thick sections obtained from identified areas with the highest tumor cell concentration were mounted on uncoated glass slides. For histologic diagnosis, representative sections were stained with hematoxylin and eosin by standard methods. Before microdissection, sections were stained with nuclear fast red (American MasterTech Scientific, Lodi, Calif., USA). The sections of interest were selectively isolated by laser-captured microdissection (PALM Microsystem; Leica, Wetzlar, Germany), according to standard procedures [13]. The dissected tissue particles were transferred to a reaction tube containing 400  $\mu$ l of RNA lysis buffer.

The samples were homogenized and heated at 92°C for 30 min. Fifty microliters of 2 M sodium acetate were added at pH 4.0, followed by 600  $\mu$ l of freshly prepared phenol/chloroform/isomyl alcohol (250:50:1). The tubes were placed on ice for 15 min and then centrifuged at 13,000 rpm for 8 min in a chilled (8°C) centrifuge. The upper aqueous phase was carefully removed. Glycogen (10  $\mu$ l) and 300–400  $\mu$ l of isopropanol were added. The tubes were chilled at –20°C for 30–45 min to precipitate the RNA. The samples were washed in 500  $\mu$ l of 75% ethanol and air-dried for 15 min. The pellet was resuspended in 50  $\mu$ l of 5 mM Tris. Finally, cDNA was prepared as described by Lord et al. [14].

Quantification of three genes of interest and an internal reference gene ( $\beta$ -actin) was done with a fluorescence-based real-time detection method (ABI PRISM 7900 Sequence detection System, TaqMan<sup>®</sup>, Perkin-Elmer Applied Biosystems, Foster City, Calif., USA) using the standard curve method. The PCR reaction mixture consisted of 1,200 nM of each primer, 200 nM of probe, 0.4 U of AmpliTaq gold polymerase, 200 nM each of dATP, dCTP, dGTP and dTTP, 3.5 mM of MgCl<sub>2</sub> and 1 $\times$  TaqMan buffer A containing

**Table 1.** Primer and probe sequences for quantitative RT-PCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	TaqMan® Probe (5'-3')
IGF-1R	TGGAGTGTGTATGCCTCTG	CACCTCCCACTCATCAGGA	CCCGGAGTACTTCAGCGCTGCTG
EGFR	TGCGTCTCTTGCCGGAAT	GGCTCACCTCCAGAAGGTT	ACGCATTCCTGCTCGGCTG
HER2	CTGAAGTGGTGTATGCAGATTGC	TTCCGAGCGGCCAAGTC	TGTGTACGAGCCGCATCCTCCA
$\beta$ -Actin	GAGCGCGGCTACAGCTT	TCCTTAATGTACGCACGATTT	ACCACCACGGCCGAGCGG

a reference dye. The final volume of the reaction mixture was 20  $\mu$ l (all reagents from Perkin-Elmer Applied Biosystems). Cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 46 cycles at 95°C for 15 s and 60°C for 1 min. The primers and probes used are listed in table 1. Gene expression values (relative mRNA levels) are expressed as ratios [differences between threshold cycle (Ct) values] between the gene of interest and an internal reference gene ( $\beta$ -actin).

For each gene, we established a usable Ct range for the data and documented the precision of the measurements within the usable range. For maximum accuracy, we demonstrated that the slopes of the plots of  $\Delta$ Ct versus log pg RNA for target genes and the housekeeping gene ( $\beta$ -actin) were parallel. Each replicate Ct data point is the average of Ct values obtained in three PCR reactions. To compare the results of two different TaqMan plates with each other, the same standardized samples are analyzed on every plate.

#### Statistical Analysis

We examined the objective tumor response to chemotherapy and overall survival. The objective response rate was calculated as the ratio of (CR + PR)/(CR + PR + NC + PD). Overall survival was calculated as the period from the start of first-line chemotherapy until death from any cause. If patients were lost to follow-up, data were censored at the date of the last evaluation.

To assess associations of gene expression levels with tumor response and overall survival, the expression levels of each gene were categorized into low and high values at optimal cutoff points. The maximal  $\chi^2$  method [15–17] was used to determine which gene expression (optimal cutoff point) best segregated patients into poor- and good-outcome subgroups (in terms of likelihood of response and survival). To determine the corrected p values based on the maximal  $\chi^2$  analysis, 2,000 bootstrap-like simulations were used in univariate analyses to estimate the distribution of the maximal  $\chi^2$  statistics under the null hypothesis of no association. Variables for multivariate analysis were selected by the stepwise method, using a significance level of  $p < 0.050$  for entering into or remaining in the model. The estimates of hazard ratios (HRs) with 95% confidence intervals (CIs) based on a Cox proportional hazard model were used to provide quantitative summaries of the gene expression data. The probability of overall survival was calculated with the Kaplan-Meier method, and differences between curves were evaluated with the log-rank test.

Correlations of three biomarker expressions with each other were examined by Spearman's rank correlation test. The Wilcoxon test was used to compare the mRNA expression levels of each growth factor receptor between two different histological types of tumors. All reported p values are two-sided, and the level of sta-

tistical significance was set at  $p < 0.050$ . All analyses were performed using the statistical software package R, version 2.4.1, and the SAS statistical package, version 9.1.3 (SAS Institute, Cary, N.C., USA).

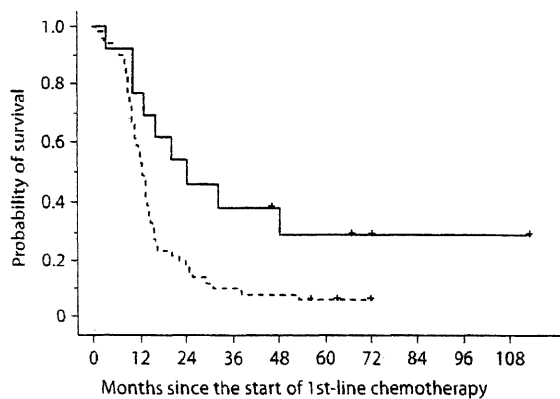
## Results

Eight hundred ninety-nine patients received first-line chemotherapy for advanced GC at our hospital between July 1997 and June 2004. A total of 86 of these patients were eligible for the present study. The demographic characteristics of the patients at the start of first-line chemotherapy are shown in table 2. There were 69 (80%) men and 17 (20%) women, with a median age of 64 years. At the time of analysis, 78 (91%) patients had died and 8 (9%) patients were alive. No patient was lost to follow up.

The chemotherapy regimens received by the patients and the response rates are also shown in table 2. The rates of response to first-line chemotherapy in our study were comparable to those reported previously [18, 19].

#### *Gene Expression Levels of IGF-1R, EGFR and HER2, Clinical Characteristics, and Overall Survival since the Start of First-line Chemotherapy in All Patients*

The gene expression levels of the three biomarkers were quantifiable in 86.0–93.0% of the 86 tumors (table 2) and their cutoff values for overall survival analyses were determined using the maximal  $\chi^2$  method. The median overall survival was 13.6 months in our study. In univariate analyses, overall survival in the study group as a whole correlated with the expression level of EGFR alone (table 3). Since the results of univariate survival analysis for HER2 expression showed a trend in an opposite direction to the results for IGF-1R and EGFR, we performed a combined analysis of IGF-1R and EGFR expressions. Patients with low mRNA expression of both IGF-1R and EGFR ( $n = 13$ ) had significantly longer overall survival than the other patients ( $n = 51$ ; median overall survival,



**Fig. 1.** Kaplan-Meier plot of overall survival for all patients according to the mRNA expression levels of IGF-1R and EGFR: — = Low IGF-1R and low EGFR expression (n = 13; median survival: 24.6 months); - - - - = other (n = 51; median survival: 12.8 months); log-rank test p = 0.013.

24.6 vs. 12.8 months; log-rank p = 0.013; fig. 1). Multivariate analysis with a Cox proportional hazard model demonstrated that high EGFR expression [HR: 2.94 (95% CI: 1.40–6.17)] and poor performance status [HR: 1.96 (95% CI: 1.12–3.42)] were significant predictors of shorter survival (table 3).

#### *Gene Expression Levels of IGF-1R, EGFR and HER2, Tumor Response, and Overall Survival in Patients Treated with S-1 Monotherapy or Cisplatin plus Irinotecan as First-Line Chemotherapy*

To better understand the relationship between the mRNA levels of the three biomarkers and treatment outcomes with each chemotherapy regimen, we performed subgroup analysis. Gene expression cutoff values that best segregated patients into poor- and good-response subgroups were defined by the same methods as those used for the analyses of overall survival. In patients given first-line S-1 monotherapy, low IGF-1R gene expression (objective response rate: 57 vs. 0% in patients with low vs. high IGF-1R, respectively; p = 0.002) and low EGFR gene expression (objective response rate: 71 vs. 26% in patients with low vs. high EGFR, respectively, p = 0.035) correlated with a better response (table 4). However, the expression level of none of the three biomarkers correlated with overall survival (table 4).

In patients treated with first-line cisplatin plus irinotecan, expressions of the three biomarkers did not show any

**Table 2.** Patient characteristics

Characteristics	Patients (n = 86)	
	n	%
Sex		
Male	69	80
Female	17	20
Age, years		
Median	64	
Range	39–84	
ECOG performance status		
0	42	48
1	41	48
2	3	3
Metastatic site		
Lymph nodes	43	49
Liver	26	30
Peritoneum	22	26
Lung	4	5
Other	4	5
Histological type		
Intestinal	38	44
Diffuse	48	56
First-line chemotherapy regimen		
S-1	29	38 <sup>1</sup>
Cisplatin + irinotecan	29	36 <sup>1</sup>
5-FU	23	4 <sup>1</sup>
Other	5	20 <sup>1</sup>
Gene expression levels <sup>2</sup>		
IGF-1R [2.03 (0.16–14.98)]	75	87.2
EGFR [3.66 (0.35–57.78)]	74	86.0
HER2 [0.19 (0.01–2.39)]	80	93.0

ECOG = Eastern cooperative oncology group.

<sup>1</sup> Response rates are shown (in percentages).

<sup>2</sup> Medians (in brackets) and ranges (in parentheses) of mRNA expression levels are given relative to the internal reference gene  $\beta$ -actin ( $\times 10^{-3}$ ).

correlation with response (table 4). However, the expression level of EGFR correlated with overall survival (table 4). At an EGFR cutoff value of  $1.39 \times 10^{-3}$ , low EGFR gene expression was associated with a trend toward a better response (60 vs. 25% in patients with low vs. high EGFR, p = 0.100), and the median overall survival was as long as 20.6 months in the low EGFR expression group, compared with 14.3 months in the high EGFR expression group (log-rank p = 0.042). Expression levels of the other two genes did not correlate with overall survival (table 4).

Other first-line regimens were not examined because the number of patients who responded to treatment was too small.

**Table 3.** Univariate analysis and Cox regression multivariate analysis of overall survival in all patients included in this study: correlation with mRNA expression levels and clinical data

Factors	Cutoff	Patients	MST months	Univariate analysis		Multivariate analysis*	
				HR (95% CI)	p	HR (95% CI)	p
EGFR	$\leq 0.77 \times 10^{-3}$	16	24.6	1	<b>0.001</b>	1	<b>0.004</b>
	$> 0.77 \times 10^{-3}$	58	13.5	1.49 (1.10-2.13)		2.94 (1.40-6.17)	
PS	continuous	86	-	-	0.055	1	<b>0.018</b>
	variable			1.48 (0.99-2.19)		1.96 (1.12-3.42)	
IGF-1R	$\leq 3.28 \times 10^{-3}$	65	13.5	1	0.055		
	$> 3.28 \times 10^{-3}$	10	10.4	1.44 (0.99-1.98)			
HER2	$\leq 0.179 \times 10^{-3}$	65	13.3	1	0.064		
	$> 0.179 \times 10^{-3}$	15	22.0	0.76 (0.55-1.02)			
Tissue type	intestinal	38	16.0	1	0.089		
	diffuse	48	12.6	1.21 (0.97-1.53)			

PS = Performance status. Factors are listed in ascending order of p values, and cutoff points were determined by the maximal  $\chi^2$  method.

\* Factors were selected by the stepwise method from among all five factors used for univariate analysis. The level of significance was set at  $p < 0.050$ . Significant values are shown in bold.

**Table 4.** Correlation of gene expression levels with tumor response and overall survival in patients with advanced GC according to first-line chemotherapy

	S-1 monotherapy (n = 29)						Cisplatin + irinotecan (n = 29)					
	cutoff $\times 10^{-3}$	patients	RR %	p	MST months	p log rank	cutoff $\times 10^{-3}$	patients	RR %	p	MST months	p log rank
IGF-1R	$\leq 1.88$	14	57	<b>0.002</b>	13.5	0.306	$\leq 0.64$	10	30	0.134	15.5	0.255
	$> 1.88$	11	0		10.4		$> 0.64$	15	60		14.6	
EGFR	$\leq 0.76$	7	71	<b>0.035</b>	24.6	0.197	$\leq 1.39$	15	60	0.100	20.6	<b>0.042</b>
	$> 0.76$	19	26		13.2		$> 1.39$	8	25		14.3	
HER2	$\leq 0.026$	6	67	<b>0.054</b>	13.5	0.741	$\leq 0.021$	7	71	0.126	12.7	0.304
	$> 0.026$	19	26		13.2		$> 0.021$	21	38		17.9	

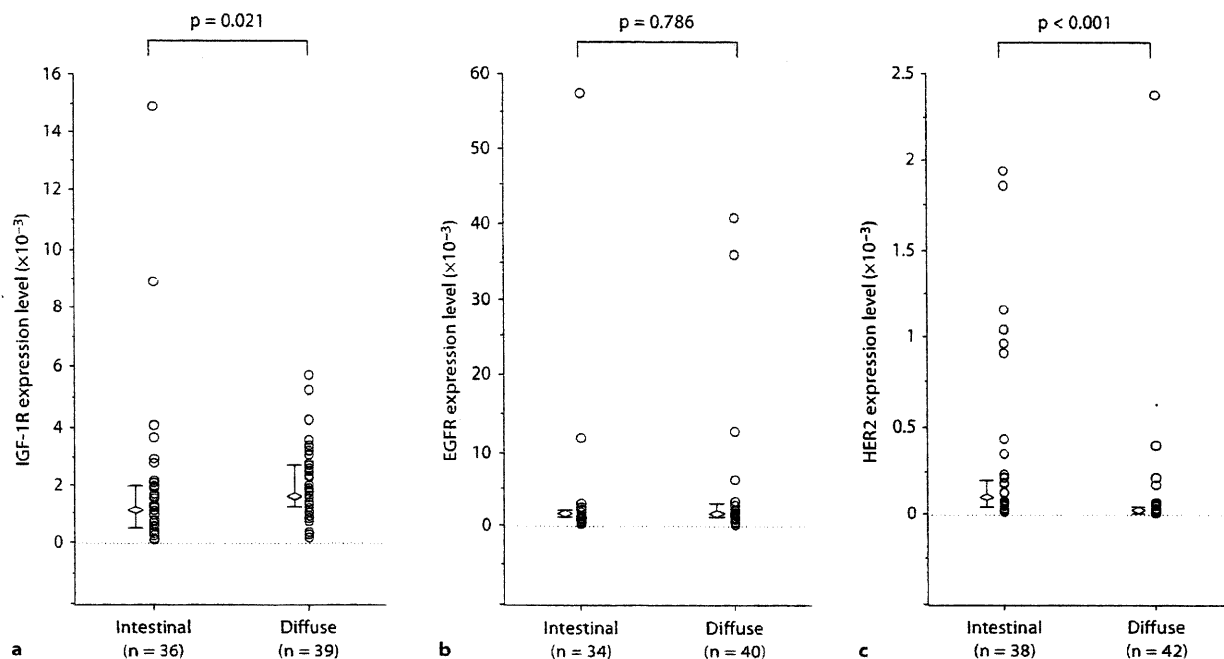
RR = Objective response rate. The cutoff point was determined by the maximal  $\chi^2$  method. The level of significance was set at  $p < 0.050$ . Significant values are shown in bold.

#### Expressions of IGF-1R, EGFR and HER2, and Histological Tumor Types

There was no correlation between the mRNA expression levels of IGF-1R and EGFR (Spearman's rank correlation coefficient:  $r = 0.180$ ), IGF-1R and HER2 ( $r = -0.088$ ), and EGFR and HER2 ( $r = -0.087$ ). Intestinal tumors had lower IGF-1R ( $p = 0.021$ , Wilcoxon test; fig. 2a) and higher HER2 ( $p < 0.001$ , fig. 2c) expression levels than diffuse tumors.

#### Discussion

Since reports that the IGF system participates in cancer progression, angiogenesis, metastasis and resistance to apoptosis, IGF-1R has received considerable attention as a potential target for cancer therapy [3, 20, 21]. Two phase I/II clinical trials of receptor-specific blocking of monoclonal antibodies are now underway [22, 23], as are many phase I and preclinical trials of other monoclonal antibodies and small-molecule tyrosine kinase inhibitors. In GC, evidence supporting an association of IGF-1R mRNA



**Fig. 2.** Expression levels of IGF-1R (a), EGFR (b) and HER2 (c) in intestinal and diffuse tumors. Every result is plotted, and median values (diamonds) and interquartile ranges from 25 to 75% (bars) are also shown. Correlations of IGF-1R, EGFR and HER2 expressions with histological tumor types were tested by the Wilcoxon test. **a** IGF-1R,  $p = 0.021$ . **b** EGFR,  $p = 0.786$ . **c** HER2,  $p < 0.001$ .

expression with survival and clinicopathological characteristics remains scant so far. Our study showed that patients with low IGF-1R expression had slightly but not significantly longer survival. Our findings suggest that anti-IGF-1R strategies with specific monoclonal antibodies or tyrosine kinase inhibitors, as well as anti-EGFR strategies, may prove valuable in patients with GC.

Targeting EGFR has been a successful approach to treat colorectal cancer [24, 25]. In GC, the results of phase II clinical trials of cetuximab (anti-EGFR monoclonal antibody) and lapatinib (a dual tyrosine kinase inhibitor of EGFR and HER2) were reported in 2007 [26, 27]. Our data suggest that anti-EGFR strategies may be beneficial in patients with GC and contribute to improved outcomes. Since EGFR antagonists will be approved for the clinical treatment of GC in the near future, there is an urgent need for a better understanding of the clinical significance of EGFR expression at the molecular level.

Cross-talk between IGF-1R and EGFR has been implicated in the development of tumor cell resistance to treat-

ment with EGFR inhibitors [28, 29]. Recently, a combination of IGF-1R-targeted therapy and anti-EGFR strategies has been shown to synergistically enhance antitumor activity in vitro [30, 31]. Our study showed that overall survival in patients with low expression of both IGF-1R and EGFR in primary GC was significantly longer than in the other patients. Although we did not use anti-IGF-1R and anti-EGFR compounds in the present study, our results suggest that the co-targeting strategy of anti-IGF-1R and anti-EGFR may be beneficial in GC, potentially leading to improved survival.

In contrast to IGF-1R and EGFR expression, high HER2 expression was associated with a longer overall survival than low HER2 expression (table 3). This finding was probably related to the facts that intestinal-type tumors had higher HER2 expression levels than diffuse-type tumors (fig. 2c) and that patients with intestinal-type tumors had longer overall survival than those with the diffuse type (table 3), as is typically the case.

Patients with low expression of IGF-1R or EGFR had higher response rates to first-line S-1 monotherapy (table 4). Given that survival signals originating at IGF-1R and EGFR limit the efficacy of cytotoxic agents designed to induce apoptosis, treatments targeting IGF-1R and EGFR might augment the efficacy of such agents [32]. However, the small sample size of our study precludes drawing any firm conclusions.

mRNA expression of IGF-1R, EGFR, and HER2 could not be measured in approximately 10% of the patients (table 2). Response and survival were similar between patients with complete data and patients with missing data (data not shown). We excluded those patients with missing data from some analyses in the present study because there would be little effect of missing data on the overall results of our study.

In conclusion, our study provides evidence that low mRNA expression of both IGF-1R and EGFR in primary GC correlates with significantly longer overall survival, whereas high EGFR expression in GC specimens and

poor performance status are significant predictors of poorer survival in patients with advanced GC. Although our results may provide a specific rationale for combining anti-IGF-1R and anti-EGFR strategies for the antineoplastic treatment of GC, our small sample size precludes drawing any firm conclusions. The potential therapeutic benefits of simultaneously co-targeting such receptors in patients with GC should be critically evaluated, especially in prospective studies.

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# Identification of prognostic biomarkers in gastric cancer using endoscopic biopsy samples

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Endoscopic biopsy prior to chemotherapy provides an opportunity for studying biomarkers to predict the overall survival in gastric cancer patients. This prospective study was performed to identify prognostic biomarkers in patients with unresected gastric cancer. Fifty-nine cases of chemotherapy-naïve metastatic gastric cancer were enrolled in this study. A microarray analysis was performed using 40 biopsy samples to identify candidate genes whose expressions might be correlated with the overall survival. After adjusting for clinical covariates based on a multivariate analysis, the identified genes were validated using real-time reverse transcription polymerase chain reaction (RT-PCR) analysis in 19 independent validation samples. Ninety-eight candidate genes whose expression levels were significantly correlated with the overall survival were identified using a microarray analysis based on a proportional hazards model ( $P < 0.005$ ). Multivariate analysis was performed to assess 10 of these genes, and the results yielded a statistical significance level for *DACH1* and *PDCD6*. We further evaluated these two genes in independent samples using real-time RT-PCR and found that lower mRNA expression levels of *PDCD6* were correlated significantly with a poor overall survival. We identified *PDCD6* as a prognostic biomarker in patients with unresected gastric cancer using endoscopic biopsy samples. Our PCR-based single gene prediction strategy successfully predicted the overall survival and may lead to a better understanding of this disease subgroup. (*Cancer Sci* 2008; 99: 2193–2199)

Over the past two decades, various anticancer agents have been examined for their efficacy against gastric cancer, including 5-fluorouracil (5-FU) and 5-FU-based drugs, taxanes, CPT-11 and cisplatin, all administered either as monotherapy or in combination regimens;<sup>(1)</sup> however, the median survival time (MST) of these patients remains at only approximately 7 months.<sup>(2,3)</sup> In a recent randomized phase III trial examining oral S-1 monotherapy and cisplatin plus irinotecan combination therapy, the response rates to both S-1 and to the cisplatin plus irinotecan combination therapy were approximately 50%, indicating that around half of the patients did not respond to chemotherapy,<sup>(4–7)</sup> and the MST in both the arms was less than 1 year.<sup>(8)</sup> Thus, the prognosis of patients with gastric cancer remains poor.

The commonly recognized prognostic factors in cases of unresectable gastric cancer are the performance status, presence/absence of liver metastases, presence/absence of peritoneal metastases and the serum levels of alkaline phosphatase.<sup>(9)</sup> Many molecular biomarkers have been also investigated for their potential to predict the outcome in hypothesis-based studies. Several studies have shown that the mRNA levels and immunohistochemical staining intensity of thymidylate synthase (TS) in

gastric cancers treated with fluorouracil are associated with the response and survival; in addition, the excision repair cross-complementing (ERCC)1 gene expression level has been shown to be associated with the clinical outcome in patients treated with cisplatin.<sup>(10,11)</sup> HER2 expression has also been reported to be a prognostic marker in cases of differentiated gastric cancer.<sup>(12,13)</sup> Mutation of p53 and high p53 protein expression, and high expression levels of urokinase-plasminogen activator, xanthine oxidoreductase, claudin-4, vascular endothelial growth factor, interleukin-8 and cyclin E have all been correlated with poor survival.<sup>(13–19)</sup> In terms of epigenetic alterations, reduced expression of acetylated histone H4 or DNA methylation of CDH1 and RAR- $\beta$  have been shown to be correlated with tumor invasiveness and the tumor metastasizing potential.<sup>(20,21)</sup>

On the other hand, the recent introduction of the microarray technology has enabled significant genes to be identified almost throughout the genome using a hypothesis-free approach. The possibility of performing genome-wide searches is a major advantage, and such searches may be the only way to discover genes that would otherwise be unlikely to even be suggested as candidates. In gastric cancer, biopsy samples of the primary lesions can be easily obtained by endoscopy prior to treatment; however, few prospective biomarker studies using endoscopic biopsy samples to predict patient outcome have been performed to date. Therefore, we conducted a prospective study to identify biomarkers for predicting survival in patients with unresected metastatic gastric cancer.

## Materials and Methods

**Patients and samples.** The eligible subjects in this study were patients with histologically confirmed, untreated and metastatic stage IV gastric cancer between 20 and 75 years of age. Additional inclusion criteria included an Eastern Cooperative Oncology Group performance status of 0–2. The exclusion criteria included history of prior chemotherapy or major surgery. All patients received chemotherapy using a 5-FU-based regimen (5-FU alone, S1 alone, 5-FU + methotrexate, 5-FU + cisplatin, or S1 + cisplatin) or a CPT-11 plus cisplatin regimen. Sixty-five gastric cancer patients were enrolled in the study. Of these, two were excluded because of insufficient RNA quantities extracted from their biopsy specimens, and four were excluded because of the poor RNA quality. Thus, samples from the remaining 59 patients were analyzed. The survival time was followed after the patients were initiated on chemotherapy. This study was approved

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by the Institutional Review Board of the National Cancer Center Hospital, and written informed consent was obtained from all the patients.

The endoscopic biopsy samples collected were immediately placed in an RNA stabilization solution (Isogen; Nippongene, Tokyo, Japan) and stored at  $-80^{\circ}\text{C}$ . Other biopsy samples obtained from the same location were reviewed by a pathologist to confirm the presence of tumor cells. The RNA extraction method and the quality check protocol have been described previously.<sup>(22)</sup>

**Study design.** This prospective study was started in July 2003 and enrollment was completed in November 2006 at the National Cancer Center Hospital. Fifty-nine gastric cancer samples were evaluated in this study. The samples were divided into a training set ( $n = 40$ ) and a validation set ( $n = 19$ ; 2:1) using computer-generated randomization (Microsoft Office Excel, Microsoft, Redmond, WA, USA). A microarray analysis was performed using the training set of 40 samples, and candidate genes whose expressions were correlated with the overall survival were identified. Multivariate analysis was performed to adjust the expression of 10 of these candidate genes for clinical features. Finally, the significant genes were evaluated in an independent set of 19 samples and survival was predicted using the results of real-time reverse transcription polymerase chain reaction (RT-PCR) analyses.

**Real-time RT-PCR.** Real-time RT-PCR was performed for 10 genes: *DACH1* (dachshund homolog 1, NM\_004392); *EGFR* (epidermal growth factor receptor, NM\_005228); *MT1X* (metallothionein 1X, NM\_005952); *YWHAE* (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide, NM\_006761); *GPX3* (glutathione peroxidase 3, NM\_002084); *PDCD6* (programmed cell death 6, NM\_013232); *WDR33* (WD repeat domain 33, NM\_018383); *C14orf43* (chromosome 14 open reading frame 43, NM\_194278); *MYLIP* (myosin regulatory light chain interacting protein, NM\_013262); and *GKAP1* (G kinase anchoring protein 1, NM\_025211). Glyceraldehyde 3 phosphate dehydrogenase (*GAPD*, NM\_002046) was used to normalize the expression levels in the subsequent quantitative analyses. RNA was converted to cDNA using a GeneAmp RNA PCR Core kit (Applied Biosystems, Foster City, CA). The transcripts were quantified using the Power SYBR Green PCR Master Mix (Applied Biosystems) and 7900HT Fast Real-time PCR system (Applied Biosystems) and reported relative to the *GAPD* expression levels. The PCR conditions were as follows: one cycle of denaturation at  $95^{\circ}\text{C}$  for 10 min, followed by 40 cycles at  $95^{\circ}\text{C}$  for 15 s and  $60^{\circ}\text{C}$  for 60 s. To amplify the target genes, the following primers were purchased from Takara (Yotsukaichi, Japan): *DACH1*-FW, 5'-AAG GGC TGC TAA AGC AAT CAG G-3', and *DACH1*-RW, 5'-CTT TGT GGC AAA GCG ACA TTA GG-3'; *EGFR*-FW, 5'-GGT GCG AAT GAC AGT AGC ATT ATG A-3', and *EGFR*-RW, 5'-AAA TGG GCT CCT AAC TAG CTG AAT C-3'; *MT1X*-FW, 5'-TTG ATC GGG AAC TCC TGC TTC T-3', and *MT1X*-RW, 5'-ACA CTT GGC ACA GCC GAC A-3'; *GPX3*-FW, 5'-ATG CCT ACA GGT ATG CGT GAT TG-3', and *GPX3*-RW, 5'-TGC AGG CAC ACA GAT GGT ACA-3'; *PDCD6*-FW, 5'-TCA AGG CCA GAC TAG ATC AGC CTA A-3', and *PDCD6*-RW, 5'-GCT GGG ATG AGG CAC ATG AC-3'; *YWHAE*-FW, 5'-GGC AGA ATT TGC CAC AGG AA-3', and *YWHAE*-RW, 5'-ACC TAA CGC AAT AGG ATG CGT TG-3'; *WDR33*-FW, 5'-ATG CAT GGG CTC TGT CAG TTT C-3', and *WDR33*-RW, 5'-GGC TGA TAC CGG GAC AAC ACT AC-3'; *C14orf43*-FW, 5'-CAG ACT GGC AAG CCT AAC TCC ATA-3', and *C14orf43*-RW, 5'-CAA GGC TGT TCC TGT GCT CTG-3'; *MYLIP*-FW, 5'-ACG TCT ATC TGC CAA CGC ACA C-3', and *MYLIP*-RW, 5'-CAG TTC ATG GAA ACA TGC CAA GTC-3'; *GKAP1*-FW, 5'-TTG CGA ATA AGT TTC GGA GCA TC-3', and *GKAP1*-RW, 5'-GCC ACT GCC ACT ATC CAC TTG TAA-3'; *GAPD*-FW, 5'-GCA

CCG TCA AGG CTG AGA AC-3', and *GAPD*-RW, 5'-ATG GTG GTG AAG ACG CCA GT-3'.

**Oligonucleotide microarray study.** The microarray procedure was performed according to the Affymetrix protocols (Santa Clara, CA). In brief, the total RNA extracted from the tumor samples was analyzed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) for quality check, and cRNA was synthesized using the GeneChip 3'-Amplification Reagents One-Cycle cDNA Synthesis Kit (Affymetrix). The labeled cRNA were then purified and used for construction of the probes. Hybridization was performed using the Affymetrix GeneChip HG-U133 Plus 2.0 array for 16 h at  $45^{\circ}\text{C}$ . The signal intensities were measured using a GeneChip Scanner3000 (Affymetrix) and converted to numerical data using the GeneChip Operating Software, ver. 1 (Affymetrix).

**Statistical analysis.** The microarray analysis was performed using the BRB Array Tools software ver. 3.3.0 (<http://linus.nci.nih.gov/BRB-ArrayTools.html>) developed by Dr Richard Simon and Dr Amy Peng. In brief, a log base 2 transformation was applied to the raw microarray data, and global normalization was used to calculate the median over the entire array. Genes were excluded if the percentage of data missing or filtered out exceeded 20%. Genes that passed the filtering criteria were then considered for further analysis. We computed a statistical significance level ( $P < 0.005$ ) for each gene based on a univariate proportional hazards model.

To adjust the expression of 10 genes (*DACH1*, *EGFR*, *MT1X*, *YWHAE*, *GPX3*, *PDCD6*, *WDR33*, *C14orf43*, *MYLIP* and *GKAP1*) for clinical features (age, sex, performance status [PS], number of metastatic sites, received chemotherapy), clinical data and the normalized microarray expression data of the 10 genes were imported into SAS software ver. 9.1.3 (SAS Institute, Cary, NC, USA) and a Cox regression model was constructed for multivariate analysis against each of the variables. The study groups were divided into two groups based on each of the clinical features: age ( $<65$  or  $\geq 65$  years), sex (male or female), PS (0 or  $\geq 1$ ), number of metastatic sites ( $<3$  or  $\geq 3$ ), chemotherapy (5-FU-based or CPT11 + CDDP) and expression levels of 10 genes).  $P < 0.05$  was considered significant.

## Results

**Identification of 98 candidate prognosis-related genes using a microarray analysis.** The univariate analysis of clinical features including age ( $<65$  or  $\geq 65$  years), sex, PS (0 or  $\geq 1$ ), number of metastatic sites (1, 2 or  $\geq 3$ ) and received chemotherapy (5-FU-based or CPT11 + CDDP) were performed for 40 microarray samples (Table 1). There were no significant differences between any of the two groups divided according to age, sex, number of metastatic sites or received chemotherapy; however, significant differences were noted between the two groups divided according to PS ( $P = 0.048$ ).

To identify the candidate prognosis-related genes from amongst over 47 000 transcripts, a microarray analysis was performed for a training set of 40 samples. A total of 21 308 genes passed the filtering criteria and were further analyzed. Ninety-eight genes were significantly correlated with survival, according to a Cox proportional hazards model ( $P < 0.005$ ) (Table 2). Fifty-nine genes were protective genes (hazard ratio,  $<1$ ), and 39 were risk genes (hazard ratio  $>1$ ).

A heat-map of the expression values of the 98 selected genes comparing the unfavorable prognosis group (survival time,  $<180$  days) and favorable prognosis group (survival time,  $\geq 180$  days) is shown in Fig. 1. Genes are plotted via hierarchical clustering.

**Multivariate analysis of prognosis-related genes.** Of the 98 candidate genes, we prioritized those that: (i) were selected by overlapping probes; (ii) were novel genes; or (iii) had a lower

**Table 1. Univariate analysis of clinical features**

Variable	No. of patients	MST (days)	P-value (log-rank test)
Age (years)			
≥65	16	235	0.454
<65	24	250	
Sex			
Male	29	243	0.926
Female	11	267	
PS			
≥1	24	182	0.048
0	16	309	
Metastasis			
1, 2	10	137	0.102
≥3	30	261	
Chemotherapy			
5-FU-based	26	245	0.594
CPT11 + CDDP	14	240	

MST, median survival time; PS, performance status.

P-value according to a Cox proportional hazards model. We selected the following 10 genes of interest for real-time RT-PCR analysis: *DACH1*, *EGFR*, *MTIX*, *YWHAE*, *GPX3*, *PDCD6*, *WDR33*, *C14orf43*, *MYLIP* and *GKAP1*.

To adjust for relevant clinical covariates against these 10 genes, we performed a multivariate analysis (Table 3). The results of the multivariate analysis revealed that high *DACH1* expression and high *PDCD6* expression were significantly correlated with the favorable outcome ( $P = 0.0134$  and  $P = 0.0015$ , respectively). We therefore considered that the *DACH1* and *PDCD6* expressions were independent prognostic markers from the results of the multivariate analysis. Results of microarray data and patient survival in the training set of 40 patients are shown in Fig. 2. The Kaplan-Meier method was used for *DACH1* and *PDCD6*. The low *PDCD6* and *DACH1* expression groups had significantly poorer outcomes ( $P < 0.0001$  and  $P = 0.0045$ ).

**Validation using real-time RT-PCR in independent samples.** The mRNA expression levels of *DACH1* and *PDCD6* were quantified using real-time RT-PCR in 19 independent samples to validate the results of the microarray. While the expression levels of *DACH1* were not correlated with survival, those of *PDCD6* in independent samples were significantly correlated with the survival ( $P = 0.007$ ) (Table 4). The Kaplan-Meier method was used to estimate the overall survival using the median value (Fig. 3a). All quantified expression levels of real time RT-PCR data are shown as Fig. 3(b). The mRNA expressions of *PDCD6* varied by approximately 25 fold (range,

0.98–25.1). The low *PDCD6* expression groups had significantly poorer outcomes ( $P = 0.0018$ ). We concluded that *PDCD6* was a valuable gene for predicting the survival in patients with gastric cancer. These results indicate that our PCR-based single gene prediction strategy using endoscopic biopsy samples could successfully predict the overall patient survival.

## Discussion

Several studies have identified prognostic biomarkers in cases of gastric cancer using microarray analysis. Hasegawa *et al.* identified 12 genes that were associated with lymph node metastasis.<sup>(23)</sup> Hippo *et al.* identified several genes associated with lymph node metastasis, including Oct-2, and genes associated with the histological type, including liver-intestine cadherin.<sup>(24)</sup> These studies introduced a novel direction in which microarray analysis could be used to predict postoperative recurrences. Inoue *et al.* selected 78 genes that were differentially expressed between aggressive and non-aggressive cancers and constructed a prognostic scoring system.<sup>(25)</sup> Leung *et al.* found that high CCL18 expression levels were associated with prolonged overall and disease-free survival.<sup>(26)</sup> They also found that phospholipase A2 group IIA expression in gastric adenocarcinoma was associated with prolonged survival and less frequent metastasis.<sup>(27)</sup> Chen *et al.* demonstrated a survival prediction model consisting of three genes (*CD36*, *SLAM*, *PIM-1*) that was capable of predicting poor or good survival in 23 (76.7%) of 30 newly enrolled patients.<sup>(28)</sup> Most of these studies used surgical specimens to predict postsurgical survival and were conducted retrospectively. Thus, we think that our present prospective study is unique in that we used endoscopic biopsy samples to predict the survival time in patients with unresectable gastric cancer. In patients with unresectable cancer, endoscopic biopsy samples may be the most appropriate specimens available non-invasively for microarray analysis. Although tumor heterogeneity may pose problems when biopsy samples are used as representative tissue specimens and further investigation is required, we believe that endoscopic biopsy samples should continue to be used for microarray analyses. Current clinical study has been confronted with a number of obstacles. Microarray analysis for clinical studies, in particular, has been hampered with bottlenecks such as RNA quality, the extremely large number of genes to be analyzed, an immature analytical tool or methodology and so on. There are two types of obstacles: controllable obstacles and uncontrollable ones. One uncontrollable obstacle is a complex chemotherapy regimen. It is easy to say that a clinical biomarker study should be performed in one particular regimen. Chemotherapy regimen has, however, progressed and become more sophisticated in a short range of time. This study was prospective clinical study and was largely followed by a guideline, Recommendations for Tumor Marker Prognostic Studies (REMARK). To minimize

**Fig. 1. Heat map of expression values for microarray identifying 98 genes whose expressions were correlated with survival. The hierarchical clustering of the 98 genes comparing the unfavorable prognosis group (survival time, <180 days) and favorable prognosis group (survival time, ≥180 days) is shown. The blue or red colors of each block represent the normalized gene expression levels. Each row represents a sample, and each column represents a gene. The 10 genes included in the multivariate analysis (Table 3) are shown.**

