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Relationships of Insulin-Like Growth Factor-1 Receptor and Epidermal Growth Factor Receptor Expression to Clinical Outcomes in Patients with Colorectal Cancer

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Key Words
Insulin-like growth factor-1 receptor · Epidermal growth factor receptor · Colorectal cancer

Abstract
Objectives: The present study evaluated the prognostic implications of insulin-like growth factor-1 receptor (IGF-1R), epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor (HER)-2 in patients with colorectal cancer (CRC). ***Methods:*** Our subjects were 91 patients who underwent surgery and subsequently received fluoropyrimidines. Expressions of IGF-1R, EGFR and HER-2 in primary lesions were analyzed immunohistochemically to determine the prognostic significance of these biomarkers. ***Results:*** Overexpression was found for IGF-1R in 48 tumors (53%), EGFR in 57 (63%) and HER-2 in 2 (2%). Overexpression of IGF-1R was significantly correlated with shorter survival from the start of first-line chemotherapy ($p = 0.033$). Overexpression of EGFR was a significant predictor of clinical response to fluoropyrimidines ($p = 0.032$). Multivariate analysis of potential prognostic factors showed that IGF-1R expression and worsened performance status were independent predictors of poor outcomes. ***Conclusions:*** Our results suggest that anti-IGF-1R strategies may offer a useful approach in molecular therapy for CRC, which has the potential to improve outcomes.

Introduction

Colorectal cancer (CRC) is the second greatest cause of cancer death in both the United States and the European Union. In recent years, with the increasing westernization of lifestyles, CRC mortality has also been increasing in Japan.

In Japan in 2003, there were approximately 37,000 deaths caused by CRC (12.4% of all deaths from malignant neoplasm), while morbidity occurred in approximately 90,000 individuals [1]. Current projections indicate that by 2015 there will be approximately 190,000 CRC patients in Japan. This would make CRC the most common form of cancer, being more prevalent than gastric and lung cancers. Surgical resection is the treatment of choice, but many patients present with distant metastasis or recurrence after curative resection. Decreases in the mortality rate require improvements in treatment

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outcomes for inoperable or recurrent CRC, for which the prognosis is currently poor.

Insulin-like growth factor-1 receptor (IGF-1R), epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor (HER)-2 are transmembrane receptors which are overexpressed in many cancers, including CRC [2, 3]. IGF-1 and IGF-2 are ligands for IGF-1R, while ligands for EGFR include EGF and transforming growth factor (TGF)-α. The binding of ligands to their receptors activates downstream signaling pathways involving molecules such as Akt, PI3K and MAPK and contributes to cancer cell proliferation, differentiation, angiogenesis, metastasis and interference with apoptosis [2, 3].

IGF-1R is frequently expressed in a wide range of tumors. The association between IGF-1R expression and clinical outcome is currently best defined in breast cancer [4, 5], with 39–90% of breast cancer patients reportedly expressing IGF-1R. Although correlations remain controversial, Railo et al. [5] reported that IGF-1R expression in breast cancers that were estrogen receptor negative correlated with unfavorable outcomes due to poorer relapse-free survival. IGF-1R inhibition has been shown to inhibit the growth of tumor cells that express IGF-1R (e.g. breast cancer, lung cancer, multiple myeloma) and to enhance the response to therapy [6, 7].

EGFR has also been shown to be expressed in various types of tumor, including CRC. Anti-EGFR monoclonal antibodies such as cetuximab and panitumumab have already shown antitumor activity against metastatic CRC. Although the prognostic value of EGFR expression remains unclear, Vallböhmer et al. [8] suggested that the expression of EGFR was associated with good response in patients with metastatic CRC who received first-line irinotecan-based chemotherapy. Moreover, EGFR might also regulate the IGF-1R signaling pathway through IGF-binding protein-3, which usually regulates IGF-1 and IGF-2 to activate IGF-1R [9]. Expression of IGF-1R has been associated with resistance to anti-EGFR or anti-HER-2 therapies in experimental settings [10, 11]. These findings support the possibility that IGF-1R represents a useful molecular target agent, along with EGFR. However, no clear associations have yet been identified between IGF-1R, EGFR or HER-2 expression and tumor response to conventional chemotherapy or outcomes in patients with CRC.

The present study aimed to immunohistochemically examine IGF-1R, EGFR and HER-2 expressions in primary lesions from patients with metastatic CRC who were receiving conventional chemotherapy, and to evaluate the responses and clinical outcomes for chemotherapy in patients receiving fluoropyrimidines as first-line treatment. Correlations between response and IGFR, EGFR and HER-2 expression were also studied.

Patients and Methods

Patient Information

Subjects in this retrospective study comprised 91 patients with primary CRC who underwent surgery and subsequently received fluoropyrimidines as first-line chemotherapy for recurrent or residual tumors at the National Cancer Center Hospital (Tokyo, Japan) between August 1996 and June 2003. Every patient displayed measurable lesions and patients with no follow-up information or an incomplete histology were excluded. Ethical approval was obtained from the research and development committee of the National Cancer Center Hospital. Detailed clinicopathological information was available for each patient. Patients who stopped chemotherapy due to adverse events were also excluded.

Clinical Evaluation and Response Criteria

Clinical response was evaluated every 6–8 weeks by CT imaging. A complete response was defined as the complete disappearance of all evidence of tumor, while partial response was defined as a >50% decrease in the sum of the products of the largest perpendicular diameters of all measurable lesions, without the occurrence of new lesions. Stable disease was defined as a change of <25% in tumor size and progressive disease was defined as an increase of >25% in the area of the measurable tumor deposits or appearance of new lesions.

Immunohistochemistry

Serial 4-μm sections were made from formalin-fixed paraffin-embedded tissue. Sections were dewaxed in xylene and rehydrated through graded alcohol. Antigen retrieval was performed by incubating sections in target retrieval solution (Dako, Tokyo, Japan) for 40 min in a 95°C water bath, then cooling for ≥20 min. After quenching endogenous peroxidase with peroxidase-blocking reagent (Dako) for 5 min and washing with Tris-buffered saline containing Tween 20, sections were incubated with IGF-1R monoclonal antibody (1:50 dilution, clone 24-31; Lab Vision Corporation, Fremont, Calif., USA) for 30 min at room temperature. Bound primary antibody was then detected by the addition of biotinylated rabbit anti-mouse secondary antibody (Dako, Carpinteria, Calif., USA) followed by avidin/biotin/horseradish peroxidase complex (Dako) for 30 min each at room temperature. Immunostaining was visualized using liquid diaminobenzidine, diluted 1:50 in horseradish peroxidase substrate buffer (Dako), and sections were counterstained with Mayer's hematoxylin before mounting. Visualization was performed using an Envision+ kit (Dako, Tokyo, Japan) with 3,3′-diaminobenzidine as chromogen, according to the instructions of the manufacturer.

Immunostaining Scoring System

The entire specimen was examined at low magnification (×40), then positive cells in areas with strong immunoreactivities were counted under high magnification (×200). The number of

Table 1. Patient characteristics (n = 91)

Factor	Patients	%
Median age, years	62 (range 27–77)	
Sex		
Male	53	58
Female	38	42
ECOG performance status (0/1/2)	64/26/1	
Location		
Colon	60	66
Rectum	31	34
Differentiation		
Good	21	23
Moderate	63	69
Other	7	8
Metastatic site		
Liver	63	69
Lung	44	48
Lymph node	27	30
Peritoneum	18	20
Ovary	2	2
Bone	1	1
First-line chemotherapy		
5-FU/LV	69	76
5-FU c.i.	10	11
UFT/LV	9	10
S-1	2	2
UFT	1	1

ECOG = Eastern Cooperative Oncology Group; FU = Fluorouracil; LV = leucovorin; c.i. = continuous infusion; UFT = uracil-tegafur.

immunoreactive cells was counted in 3 fields of view, and the mean ratio of immunoreactive cells to the total number of cancer cells per field was calculated.

Sections were scored by the percentage of positive cells (membranous staining) based on the intensity of immunostaining. Immunostaining in ≥10% of tumor cells was considered positive and staining in ≥50% of tumor cells was considered to show overexpression. Immunostaining was scored by 3 independent observers who were blinded to the sample's clinicopathological parameters.

Statistical Analysis

Associations between immunohistochemical scores and clinicopathological data were assessed using the Pearson χ^2 test. Univariate analysis of survival was performed using Kaplan-Meier survival plots and evaluation of differences between groups was performed with the log-rank test. For multivariate analysis, the Cox proportional hazards regression model was used to detect the impact of patient clinicopathological parameters and receptor expression on overall survival. Significance levels were set at $p < 0.05$, and all statistical analyses were performed using StatView version 5 for Windows software (SAS Institute, Cary, N.C., USA).

Table 2. Distribution of IGF-1R, EGFR and HER-2 protein expression

	IGF-1R	EGFR	HER-2
Negative	10 (11)	14 (15)	88 (97)
Positive	81 (89)	77 (85)	3 (3)
Low expression[1]	33 (36)	20 (22)	1 (1)
Overexpression[2]	48 (53)	57 (63)	2 (2)

The data are numbers of patients with percentages in parentheses.

[1] Positive cells ≥10% but <50%. [2] Positive cells ≥50%.

Results

Clinicopathological Features

Patient clinicopathological characteristics are detailed in table 1. All 91 patients displayed pathologically confirmed CRC. All patients received chemotherapy for recurrent or residual tumors, with first-line chemotherapy comprising 5-fluorouracil and leucovorin in 69 patients and other fluoropyrimidines in the remaining 22 patients. Furthermore, 62 patients received Camptothecin-11 (irinotecan) and 2 patients received oxaliplatin as second-line chemotherapy. Median follow-up was 28.5 months (range 5.5–88.1 months).

Distribution of IGF-1R, EGFR and HER-2 Protein Expression in CRC

All 91 samples displayed positive immunohistochemistry compared with a negative control reaction in which the primary antibody was omitted. IGF-1R expression was positive in 81 of 91 CRC patients (89%), and was overexpressed in 48 patients (53%). Conversely, 77 (85%) were EGFR-positive and 3 (3%) were HER-2 positive, with EGFR overexpression in 57 patients (63%) and HER-2 overexpression in 2 patients (2%; table 2). Patterns of IGF-1R, EGFR and HER-2 immunostaining were predominantly membranous. Typical examples of positive staining are shown in figure 1.

Association between IGF-1R and EGFR Expression and Response to First-Line Fluoropyrimidines

Expressions of IGF-1R and EGFR were evaluated with respect to response to first-line fluoropyrimidines (table 3). No correlation was identified between IGF-1R overexpression and fluoropyrimidine efficacy (nonprogressive disease vs. progressive disease, $p = 0.67$). How-

Table 3. Relationship of IGF-1R and EGFR expression to response to first-line fluoropyrimidines

	Non-PD (CR+PR+SD)	PD	p
IGF-1R			
Negative or low	33 (2+15+16) (77%)	10 (23%)	0.67
Overexpression	37 (0+24+13) (77%)	11 (23%)	
EGFR			
Negative or low	22 (1+12+9) (67%)	12 (33%)	0.032*
Overexpression	48 (1+27+20) (84%)	9 (16%)	

All p values were calculated using 2-sided χ^2 tests. * Significant p value. PD = Progressive disease; CR = complete response; PR = partial response; SD = stable disease.

ever, a significant association existed between EGFR overexpression and fluoropyrimidine efficacy (p = 0.032).

Associations between IGF-1R and EGFR Expression and Time to Progression and Overall Survival

Survival from initiation of first-line chemotherapy was assessed for relationships with IGF-IR and EGFR. No significant associations were noted between overexpression of EGFR and the time to progression after chemotherapy, which was 4.4 months for IGF-1R-overexpressing cases and 5.1 months for other cases (p = 0.60), and 5.1 months for EGFR-overexpressing cases and 4.0 months for other cases (p = 0.59).

Conversely, overexpression of IGF-1R correlated significantly with shorter median survival from the start of first-line chemotherapy (16.8 months for patients overexpressing IGF-1R vs. 22.1 months for the others, p = 0.033; fig. 2a). No significant association was noted between overexpression of EGFR and overall survival (median survival time was 18.8 months for patients overexpressing EGFR vs. 16.8 months for the others, p = 0.96; fig. 2b). Multivariate Cox regression analysis showed that positive IGF-1R expression [hazard ratio (HR) 1.81, 95% confidence interval (CI) 1.10–2.98, p = 0.019] and performance status 1 or 2 (HR 1.89, 95% CI 1.13–3.15, p = 0.015) were independent predictors of poor outcomes (table 4). Rates of co-expression and co-overexpression of IGF-1R and EGFR for CRC were 77 and 34%, respectively. The 57 patients with EGFR overexpression were divided into 31 patients with IGF1-R overexpression and 26 patients without IGF1-R overexpression, and the overall survival for the 2 subgroups was 17.1 and 21.6 months, respectively

Fig. 1. Representative tumor findings of positive immunohistochemical staining for IGF-1R (**a**), EGFR (**b**) and HER-2 (**c**). Original magnification ×100.

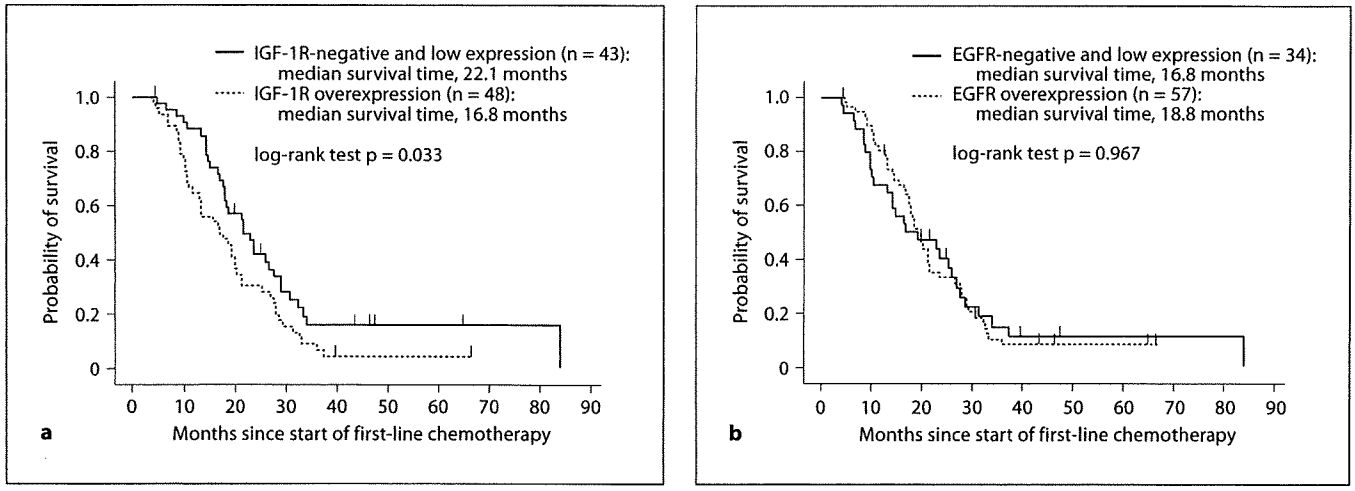

Fig. 2. Kaplan-Meier plots illustrating associations between protein expressions and overall survival since the start of first-line chemotherapy. Survival curves are plotted as graphs according to expression of IGF-1R (**a**) and EGFR (**b**).

(p = 0.062). Hence, while no significant difference was apparent, prognosis tended to be poorer for patients with co-overexpression.

In subgroup analysis, no significant association was identified between the types of 5-fluorouracil-based chemotherapy and clinical outcomes. In addition, the correlations of protein expression with the clinical outcome did not differ in the study groups.

Among the patients with colon cancer (n = 60), 32 (53%) overexpressed IGF-1R and 39 (65%) overexpressed EGFR. Among patients with rectal cancer (n = 31), 16 (52%) overexpressed IGF-1R and 18 (58%) overexpressed EGFR. The rate of overexpression was almost the same as that seen in the overall patient group. As was seen for all patients, there was a significant association between EGFR overexpression and fluoropyrimidine efficacy in patients with colon cancer (nonprogressive disease vs. progressive disease, p = 0.009). However, there were no other significant associations with respect to time to progression and overall survival in the 2 groups.

Table 4. Multivariate survival analysis by Cox proportional-hazards model

	HR	95% CI	p
IGF-1R overexpression	1.81	1.10–2.98	0.019*
Performance status 1, 2 (vs. 0)	1.89	1.13–3.15	0.015*
Metastatic sites ≥2 (vs. 1)	1.57	0.97–2.53	0.066

HR = Hazard ratio. * Significant p value.

Discussion

The present results show positive IGF-1R expression in 81 (89%) and positive EGFR expression in 77 (85%) of the 91 CRCs, with overexpression in 48 patients (53%) and 57 patients (63%), respectively. HER-2 was rarely observed. Overexpression of EGFR predicts a good clinical response to fluoropyrimidines. This study provides the first evidence by uni- and multivariate analysis that overexpression of IGF-1R is predictive of poor outcome in patients with CRC, along with poor performance status.

In recent years, chemotherapeutic treatment of CRC has advanced markedly and monoclonal antibodies are now standard components of treatment for metastatic CRC. Some antibodies, such as bevacizumab, are agents against vascular endothelial growth factor, while others, such as cetuximab and panitumumab, act against EGFR.

Preliminary results from tumor models have shown that the inhibition of IGF-1R activation suppresses tumor growth and increases tumor sensitivity to anticancer therapies [11]. Hailey et al. [7] reported that neutralizing antibodies against IGF-1R would provide a valid approach to inhibiting cancer cell growth. Such findings suggest IGF-IR as a candidate molecular target for novel

anticancer therapies. Recently, monoclonal antibodies and small molecule tyrosine kinase inhibitors targeting IGF-1R have been made and phase I/II trials and preclinical development are now underway [7, 12, 13].

Expression of IGF-1R has previously been reported in tumors such as breast cancer [4, 5], prostate cancer [14], Ewing's sarcoma [15], osteosarcoma [16], multiple myeloma [17] and CRC [18–20]. Significantly higher levels of IGF-1R (51–99%) have been found in malignant colorectal tissues [18–20]. In particular, Cunningham et al. [18] reported IGF-1R overexpression (>50% positive cells) as common in tumor specimens from CRC patients. However, no clear association between IGF-1R expression/overexpression and clinicopathological parameters or prognosis has previously been identified. According to Cunningham et al. [18], 62% of patients expressing IGF-1R showed positive reactions, but only 12% had positive membranous reactions. The reason for this might have been antibody accuracy or assay methods, and the lack of significant differences might have been attributable to false-positive cases. In our study, a new anti-IGF-1R antibody was used and positive cytoplasmic reactions were seen in 24% of patients. Subsequently, the results showed that 53% of CRC patients overexpressed IGF-1R, and IGF-1R overexpression in CRC is a predictor for poor outcomes. These findings suggest that, in the treatment of CRC, the expression of IGF-1R appears to be a prognosticator with very important clinical significance. Furthermore, EGFR overexpression in CRC has been reported in 25–82% of patients [21–23], supporting the present results (63%).

As with most published series [21–23], we found no significant association between EGFR overexpression and prognosis. However, a significant correlation between EGFR overexpression and fluoropyrimidine efficacy was apparent. The reason for this may be that, because the incidence of EGFR mutations in CRC is extremely low [24], EGFR-positive results in immunohistochemistry may represent ligand-dependent EGFR overexpression. A recent study found that prognosis was poor for patients with *KRAS* mutations [25]. As a result, patients in whom CRC proliferated dependent on such ligands as EGF might have been more chemosensitive. Khambata-Ford et al. [26] recently reported that gene expression profiles showed that patients with tumors expressing high levels of the EGFR ligands epiregulin and amphiregulin are more likely to achieve disease control with cetuximab. The relationships between *KRAS* mutations and EGFR-positive results in immunohistochemistry using the present antibody have yet to be clarified. In any case, our results indicate that EGFR overexpression is not directly related to unfavorable prognosis.

Furthermore, HER-2 expressions were conflicting. As with Schuell et al. [27], who used the same antibody (Hercep test kit), expression was very low in the present study and was not examined in subsequent analyses.

Conversely, previous studies have shown that IGF-1R is able to interact with other receptor systems to enhance the malignant behavior of tumors [10, 28]. IGF-1R signaling may thus be responsible for resistance to therapy with EGFR inhibitors and anti-HER-2 monoclonal antibodies [29, 30]. Recently, the combination of IGF-1R-targeted therapy with an anti-EGFR or anti-HER-2 therapeutic strategy has been shown to enhance the antitumor activity of these agents [10, 11]. Goetsh et al. [11] showed that the combination of cetuximab and humanized anti-IGF-1R antibody can inhibit growth in the MCF-7 human breast cancer cell line and A549 non-small cell lung cancer cell line. We showed IGF-1R and EGFR co-expression in 77% of CRC patients, similar to the findings of Cunningham et al. [18]. Furthermore, IGF-1R and EGFR co-overexpression was not rare, appearing in 34% of patients. The prognosis tended to be poorer, albeit not significantly, for patients with IGF-1R and EGFR co-overexpression compared to patients with overexpression of EGFR but not IGF-1R. In the future, treatments need to be developed for CRC patients with IGF-1R and EGFR co-overexpression.

In conclusion, we have shown that rates of IGF-1R and EGFR expression are high in patients with CRC and offer the first evidence that overexpression of IGF-1R predicts poor outcome and overexpression of EGFR predicts good clinical response to fluoropyrimidines. We also showed that co-expression of IGF-1R and EGFR is common in CRC. Our results suggest that anti-IGF-1R strategies may offer a useful molecular therapeutic approach to CRC in monotherapy and combination chemotherapy, potentially improving outcomes even if the disease is refractory to anti-EGFR monoclonal antibody, since IGF-1R can cross-talk with EGFR to enhance the malignant behavior of tumors.

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A Phase 2 Clinical Trial of Panitumumab Monotherapy in Japanese Patients with Metastatic Colorectal Cancer

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Objective: Panitumumab, a fully human monoclonal antibody targeting epidermal growth factor receptor (EGFR), has antitumor activity and an acceptable safety profile in patients with metastatic colorectal cancer (mCRC). This Phase 2 study evaluated efficacy, pharmacokinetics and safety of panitumumab in Japanese patients with mCRC who developed progressive disease during or after fluoropyrimidine, irinotecan and oxaliplatin chemotherapy.
Methods: Eligible patients had histologically proven colorectal adenocarcinoma and EGFR tumor expression in ≥1% of tumor cells by immunohistochemistry. Patients received panitumumab 6 mg/kg every 2 weeks until disease progression or unacceptable toxicity. The primary endpoint was objective response rate (ORR) per modified Response Evaluation Criteria in Solid Tumors (RECIST) by independent central review. Secondary endpoints included progression-free survival (PFS), overall survival (OS), pharmacokinetic parameters and incidence of adverse events.
Results: Fifty-two patients received at least one dose of panitumumab. Seven patients had partial responses for a confirmed ORR of 13.5% (95% CI: 5.6, 25.8). Median PFS was 8.0 weeks (95% CI: 7.4, 11.4) and median OS was 9.3 months (95% CI: 7.1, 12.8). Panitumumab pharmacokinetics were consistent with prior studies in Japanese and non-Japanese patients. The most common treatment-related adverse events (all, worst grade 3) were acne (81%, 2%), dry skin (62%, 0%), rash (46%, 2%), paronychia (33%, 2%), pruritus (33%, 0%) and hypomagnesemia (33%, 0%). No adverse event of infusion reaction was reported by the investigators.
Conclusions: Panitumumab monotherapy was active in Japanese patients with chemotherapy-refractory mCRC, with pharmacokinetic and safety profiles similar to those seen in prior studies.

Key words: panitumumab – pharmacokinetics – colorectal neoplasms – metastases – drug toxicity

INTRODUCTION

Cancer is one of the leading causes of death in Japan, and the incidence of colorectal cancer (CRC) has been increasing (1). In Japan, 131 438 new cases (87 825 colon cancers and 43 613 rectal cancers) and 55 070 deaths are anticipated in 2010 (2). Of newly diagnosed patients with CRC, 15–25% of the patients have metastatic disease (3) and 50% or more of the patients who are initially diagnosed with localized disease ultimately develop metastatic CRC (mCRC) (4).

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Expression of the epidermal growth factor receptor (EGFR) is frequently associated with malignant transformation in carcinomas, including CRC (5,6). Currently, there are two anti-EGFR monoclonal antibodies, panitumumab and cetuximab, that are approved in the USA and Europe for the treatment of mCRC. Tumor expression of EGFR is a labeling requirement for both drugs (7,8).

Panitumumab is a fully human, monoclonal antibody with high affinity ($K_D = 5 \times 10^{-11}$ M) for EGFR (7). Efficacy, pharmacokinetics and safety of panitumumab have been tested in Japanese patients with solid tumors (9). In that study, patients received panitumumab at doses of 2.5 mg/kg

once weekly, 6.0 mg/kg every 2 weeks (Q2W) and 9.0 mg/kg every 3 weeks as monotherapy. All responders in that study (4 patients of 18 enrolled) had advanced CRC. The study described here extends the evaluation of panitumumab in Japanese patients with mCRC.

PATIENTS AND METHODS

Study Design and Eligibility Criteria

This was a multicenter, open-label, single-arm, Phase 2 clinical study in Japanese patients with mCRC who developed disease progression during or after prior fluoropyrimidine, irinotecan and oxaliplatin chemotherapy. The study was approved by Institutional Review Boards at all study sites.

Panitumumab (derived from Chinese hamster ovary cells on a 12 kl production scale) was supplied at a concentration of 20 mg/ml in 10 ml vials (Amgen Inc., Thousand Oaks, CA, USA). Panitumumab was administered by intravenous infusion using a 0.22 μm in-line filter at a dose of 6.0 mg/kg Q2W over approximately 60 min. This dose of panitumumab was selected based on pharmacokinetic modeling, which indicated that 6.0 mg/kg Q2W would maintain a trough serum concentration at or above that necessary to achieve EGFR saturation levels while providing a convenient Q2W dosing. This dose has also been tested and deemed to be tolerable in a small study of Japanese patients (9).

Patients received panitumumab until they developed progressive disease or were unable to tolerate treatment. Patients who discontinued treatment for any reason underwent a safety follow-up visit. All patients were followed for 24 months after the first panitumumab infusion at approximately 3-month intervals to assess survival.

Eligible patients were men and women >20 years old who provided written informed consent. All patients had a pathologic diagnosis of colorectal adenocarcinoma with documented radiographic evidence of progressive disease during or after the most recent regimen with fluoropyrimidine, irinotecan and oxaliplatin. To ensure adequate exposure to prior chemotherapy, average dose-intensity of irinotecan ($\geq$50 mg/m^2/week) and oxaliplatin ($\geq$30 mg/m^2/week) was required. Patients were also required to have: unidimensionally measurable disease ($\geq$20 mm in at least one dimension); an Eastern Cooperative Oncology Group (ECOG) performance status of 0–2; and EGFR expression on $\geq$1% of evaluated tumor cells. EGFR expression was determined by immunohistochemistry using the EGFR pharmDX™ kit (DakoCytomation, Carpenteria, CA, USA) and all tests were performed at a central laboratory (Esoterix Clinical Trials Services BVBA, Mechelen, Belgium).

Study Endpoints

Efficacy endpoints included the objective response rate (ORR; primary endpoint), time to response, duration of response, duration of stable disease, time to treatment failure, progression-free survival (PFS) time and overall survival (OS; secondary endpoints). Time to treatment failure was calculated as the time from the date of enrollment to the date a decision was made to end the treatment period for any reason. Panitumumab pharmacokinetic endpoints included the area under the concentration–time curve (AUC); maximum (C_{max}) and minimum (C_{min}) observed concentrations; and half-life during the dosing interval ($t_{1/2}$). Safety endpoints included incidence of all adverse events; changes in laboratory values and vital signs; and the incidence of anti-panitumumab antibody formation and infusion reactions.

Assessments

Patients were evaluated for tumor response according to the modified Response Evaluation Criteria in Solid Tumors (RECIST) at weeks 8, 12, 16, 24, 32, 40 and 48 and every 8 weeks thereafter until disease progression. Tumor responses were confirmed no less than 4 weeks after the criteria for response were first met. In addition to investigators' assessments, radiographic scans of all patients were reviewed by a central reviewer.

Predose serum samples for pharmacokinetic analyses were collected from a subset of patients and at 0.5, 24, 96, 168 and 240 h after completion of the first infusion and within 0.5 h before and 0.5 h after completion of infusions at Weeks 3, 5 and 7 and every 8 weeks thereafter. An additional sample was collected during the safety follow-up visit. A validated immunoassay with electrochemiluminescence detection was used to measure panitumumab concentrations in the serum samples (10). Pharmacokinetic assays were performed by Amgen Inc.

Serum samples to test for anti-panitumumab antibodies were collected before panitumumab infusion at Weeks 1, 7 and 23, and during the safety follow-up visit. This study utilized the same validated assays to detect the potential presence of anti-panitumumab antibodies as has been used in previous panitumumab clinical trials (11). An ELISA assay and a Biacore™ assay were used for screening, and a cell-based bioassay was used to detect neutralizing antibodies. Anti-panitumumab antibody assays were performed by Amgen Inc.

A retrospective analysis of tumor *KRAS* status was performed. DNA extracted from archived paraffin-embedded tumor tissue was analyzed for mutant *KRAS* sequences using a K-RAS Mutation Test Kit (DxS Ltd, Manchester, UK) that used allele-specific real-time polymerase chain reaction (12). *KRAS* assessments were performed by HistoGeneX (Antwerpen, Belgium).

Adverse events were graded using the National Cancer Institute Common Toxicity Criteria (NCI-CTC) version 2.0 with the exception of skin or nail toxicities, which were graded using the modified Common Terminology Criteria for Adverse Events (CTCAE) version 3.0.

Statistical Analyses

Efficacy and safety analyses were conducted on the Full Analysis Set, which comprised all patients who had received at least one infusion of panitumumab. The Pharmacokinetics Analysis Set included all patients who received panitumumab and had evaluable serum data.

Mean values and standard deviations (SDs) are provided for continuous endpoints and frequency and percentage distributions are provided for discrete data. The ORR and its two-sided 95% confidence interval (CI) (13) were calculated. Survival time (progression-free and overall), time-to-event and duration endpoints are summarized with Kaplan–Meier curves and two-sided 95% CIs.

RESULTS

Demographics

Of 98 patients who were screened, 53 patients were enrolled in six study sites in Japan. The most common reasons for screen failure were: tumor was EGFR-negative, EGFR expression was not diagnostic, and insufficient dose-intensity of irinotecan or oxaliplatin. One patient did not receive panitumumab because of disease progression clinically judged by the investigator, and 52 patients received at least one dose of panitumumab and were included in the Full Analysis Set. Forty-nine (94%) patients ended treatment because of disease progression, one (2%) patient withdrew consent and no patient withdrew from the study because of adverse events. Two patients remained with the study at the time of data cut-off (12 April 2007). Baseline demographic and clinical characteristics are summarized in Table 1.

Table 1. Patient demographics and disease characteristics at baseline

Characteristic	All patients ($N = 52$)
Male sex, *n* (%)	34 (65)
Age, median years (minimum, maximum)	59.0 (23, 77)
Primary diagnosis, *n* (%)	
Colon cancer	30 (58)
Rectal cancer	22 (42)
ECOG performance status, *n* (%)	
0	29 (56)
1	23 (44)
2	0
Prior chemotherapy, *n* (%)	
1 line	0
2 lines	27 (52)
≥3 lines	25 (48)
Cells with EGFR membrane staining, *n* (%)	
1% to <10%	30 (58)
10–35%	16 (31)
>35%	6 (12)
Highest membrane staining intensity, *n* (%)	
1+ (weak)	29 (56)
2+ (moderate)	14 (27)
3+ (strong)	9 (17)

ECOG, Eastern Cooperative Oncology Group; EGFR, epidermal growth factor receptor.

Panitumumab Exposure

The median number of infusions per patient was 6 (range: 2–20 infusions). The median average weight-adjusted dosage of panitumumab was 6.04 mg/kg and the median weight-adjusted cumulative dosage was 35.5 mg/kg (range: 12.1–113.8 mg/kg).

Efficacy

Objective responses were observed in seven patients for a rate of 13.5% (95% CI: 5.6, 25.8) by central radiographic image review. All seven responders had a partial response and no patient had a complete response. Seventeen (33%) and 26 (50%) patients had stable disease and progressive disease, respectively. Two (4%) patients could not be evaluated for objective response because of withdrawn consent for one patient and lack of confirmation of response in the other patient. Similar to results from central assessment, the ORR was 15.4% (8 of 52 patients; 95% CI: 6.9, 28.1) based on the investigators' assessments. Twenty-three (44%) and 19 (37%) patients had stable disease and progressive disease, respectively.

The median time of follow-up for all 52 patients was 26.1 weeks (range: 5.4–42.0 weeks). The mean time to response in the seven responders was 7.6 weeks (95% CI: 6.1, 9.1) and the median duration of response was 16.2 weeks (95% CI: 16.1, 24.1). The median duration of stable disease was 15.0 weeks (95% CI: 11.4, 16.3). The median time to treatment failure was 11.4 weeks (95% CI: 8.4, 15.0). At the time of data cut-off, the median PFS was 8.0 weeks (95% CI: 7.4, 11.4) (Figure 1). The median OS was 9.3 months (95% CI: 7.1, 12.8).

Objective responses were also analyzed by EGFR expression (percentage of EGFR-positive cells and staining intensity) and grade of skin-related toxicity (Table 2). No correlation was observed between panitumumab efficacy and percentage of tumor cell membrane staining. All responders had grade 2 or 3 skin-related adverse events.

Tumor samples for retrospective *KRAS* testing were available for testing from only 16 patients in this study; however, data for 8 Japanese patients with mCRC enrolled in a prior Phase 1 panitumumab study (9) were also available. In a pooled analysis of these 24 patients, 14 (58%) had wild-type *KRAS* and 10 (42%) had mutated *KRAS*. Four (17%) patients

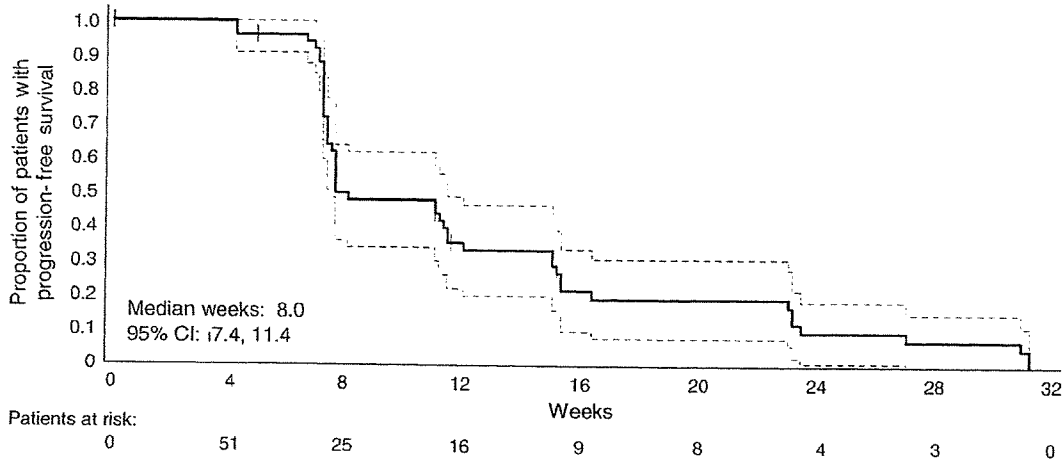

Figure 1. Progression-free survival (PFS) based on central assessment. The Kaplan–Meier curve of PFS for all patients ($N = 52$) is shown. Dashed lines represent 95% CI and censored patients are designated by vertical lines.

Table 2. Objective response and progression-free survival by EGFR tumor cell membrane staining and skin toxicity per central independent radiographic review

Category		Objective response		Progression-free survival Median time weeks (95% CI)[c]
		Patients responding,[a] *n* (%)	Rate % (95% CI)[b]	
EGFR staining				
1% to <10%	30	4 (13)	13.3 (3.8, 30.7)	8.0 (7.4, 11.4)
10% to 35%	16	3 (19)	18.8 (4.1, 45.7)	7.4 (7.1, 23.0)
>35%	6	0	0 (0, 45.9)	11.2 (7.6, 15.0)
EGFR intensity				
1+ (weak)	29	4 (14)	13.8 (3.9, 31.7)	7.6 (7.3, 11.1)
2+ (moderate)	14	1 (7)	7.1 (0.2, 33.9)	12.0 (7.1, 15.3)
3+ (strong)	9	2 (22)	22.2 (2.8, 60.0)	11.4 (7.6, 23.4)
Maximum grade[d] of skin toxicity				
Grade 1	8	0	0 (0, 36.9)	7.6 (4.1, 7.6)
Grade 2	41	6 (15)	14.6 (5.6, 29.2)	11.0 (7.4, 12.0)
Grade 3	3	1 (33)	33.3 (0.8, 90.6)	15.0 (7.1, 23.0)

CI, confidence interval; *n*, number of patients; EGFR, Epidermal growth factor receptor.
[a]Patients with a complete response or partial response.
[b]CIs for objective response rate were calculated based on the *F* distribution method (13).
[c]CIs for progression-free survival were calculated based on a sign test (25).
[d]Grades based on the National Cancer Institute Common Toxicity Criteria version 2.0 with the exception of skin or nail toxicities, which were graded using the modified Common Terminology Criteria for Adverse Events (CTCAE) version 3.0.

had a partial response to panitumumab therapy; all of these patients had tumors expressing wild-type *KRAS*. Median (95% CI) PFS was 13.2 (8.0, 23.1) weeks in patients with wild-type *KRAS* and 7.3 (7.1, 7.6) weeks in patients with mutated *KRAS* in their tumors.

Pharmacokinetics

Serum samples for pharmacokinetic analyses were collected from 20 patients who received panitumumab 6 mg/kg Q2W. After the first panitumumab dose, the mean (SD) C_{max} was 113 (36.1) μg/ml and the mean (SD) C_{min} was 15.4 (8.5) μg/ml; during the first dosing interval, the mean AUC (SD) was approximately 640 (174) μg day/ml and the mean (SD) $t_{1/2}$ was 5.6 (2.0) days. After multiple panitumumab administrations, the mean (SD) steady-state concentrations based on data from the third dose and beyond were 31.7 (21.3) mg/ml before infusion and 146 (34.6) mg/ml immediately after infusion; these values were 2.0- and 1.3-fold higher, respectively, than the corresponding value after the first administration.

Safety

All patients had at least one adverse event and most patients (98%) had at least one adverse event that was considered by the investigator to be possibly related to treatment with panitumumab. No patient discontinued the study because of an adverse event. Most adverse events were grade 1 or 2. Thirteen (25%) patients had an adverse event with a worst grade of 3, and three (6%) patients had events with a worst grade of 4. The most common grade 3 adverse events were anorexia ($n = 4$) and hypophosphatemia ($n = 3$). Five grade 4 adverse events occurred in three patients, including anemia, fatigue, abnormal hepatic function, hepatic failure and hyperuricemia. One patient had a grade 5 event that was attributed to disease progression within 30 days of the last dose of panitumumab.

Most treatment-related events were grade 1 or 2, and skin-related events were most common (Table 3). One patient had a grade 3 serious adverse event of deep vein thrombosis. No grade 4 or higher treatment-related adverse event was reported. Fifty-one (98%) patients had treatment-related skin toxicities. The most common skin toxicities were acne (81%), dry skin (62%), rash (46%), paronychia (33%), pruritus (33%), nail disorder (15%) and erythema (13%). Three (4%) patients had grade 3 treatment-related skin toxicities (acne, rash and paronychia). In a Kaplan–Meier analysis of skin toxicities, the median time to first toxicity was 6 days (95% CI: 5, 7) and the median time to most severe toxicity was 9 days (95% CI: 7, 13).

Table 3. Treatment-related adverse events[a] occurring in ≥10% of patients

Adverse event,[b] *n* (%)	All patients ($N = 52$)	
	All grades	Grade 3
Patients with any treatment-related adverse event	51 (98)	6 (12)[c]
Skin toxicities	51 (98)	3 (6)
Acne	42 (81)	1 (2)
Dry skin	32 (62)	0
Rash	24 (46)	1 (2)
Pruritis	17 (33)	0
Paronychia	17 (33)	1 (2)
Nail disorder	8 (15)	0
Erythema	7 (13)	0
Hypertrichosis	5 (10)	0
Hypomagnesemia	17 (33)	0
Fatigue	13 (25)	0
Stomatitis	12 (23)	0
Anorexia	11 (21)	1 (2)
Diarrhea	8 (15)	0
Vomiting	7 (13)	0
Constipation	5 (10)	0
Decreased weight	5 (10)	0

[a]The investigator considered there to be a reasonable possibility that the event may have been caused by panitumumab.
[b]Adverse events were coded using the MedDRA dictionary version 9.0. Grades based on the National Cancer Institute Common Toxicity Criteria version 2.0 with the exception of skin or nail toxicities, which were graded using the modified Common Terminology Criteria for Adverse Events (CTCAE) version 3.0.
[c]The remaining treatment-related grade 3 events not included in the table were anemia ($n = 1$) and hypophosphatemia ($n = 1$).

Grade 3 or 4 laboratory toxicities were seen in 12 (23%) patients; 11 (21%) patients had grade 3 and 1 (2%) patient had a grade 4 laboratory toxicity. Nineteen patients had grade 1 and two patients had grade 2 hypomagnesemia. Non-transient anti-panitumumab antibodies were seen in two (4%) patients; these patients did not have any severe or serious adverse events. Serum antibodies from these two patients did not exhibit neutralizing activity.

There were no investigator-reported adverse events of infusion reactions in this study. In a conservative *post hoc* analysis of adverse event terms (categories of acute infusion reaction/cytokine release syndrome and allergic reaction/hypersensitivity occurring on the day of infusion and resolving the same day or the day after the infusion), potential infusion reactions were reported in 6 (12%) patients, yielding a per-infusion incidence of 7/367 (2%). All potential infusion reactions were grade 1 and included pyrexia ($n = 3$), vomiting ($n = 1$), hypertension ($n = 1$) and fatigue ($n = 1$).

DISCUSSION

The ORR (13.5%) in this study of panitumumab in Japanese patients with mCRC was similar to rates reported in prior studies in non-Japanese patients (11,14,15). Consistent with results from prior studies that examined the relationship between level of EGFR expression and efficacy of anti-EGFR monoclonal antibodies (15–19), no apparent correlation was observed between panitumumab efficacy and percentage of tumor cell membrane EGFR staining. These observations suggest that EGFR staining may not identify patients who are more likely to respond to anti-EGFR antibody therapy, and patients should not be denied this treatment based on EGFR testing. Patients with grade 2 or 3 skin-related adverse events had higher response rates and longer PFS than patients with grade 1 events. These findings are consistent with studies associating skin toxicity with response to anti-EGFR antibodies (15–17,19).

The presence of mutated *KRAS* in tumors has been seen to be a negative predictor of response to anti-EGFR monoclonal antibody therapies (12,20). In a pooled analysis (21) of patients with available *KRAS* status in this study and a prior Phase 1 study in Japanese patients with mCRC (9), all patients who had a response to panitumumab had tumors that expressed wild-type *KRAS*. Although a comparison of efficacy between Japanese patients with tumors expressing wild-type *KRAS* and those with mutated *KRAS* is not conclusive because of the small sample size, our findings are

consistent with other panitumumab (12) and cetuximab studies (22).

The pharmacokinetic profile of patients who were tested in this study was similar to those from prior studies in Japanese patients (23) and non-Japanese patients (24). At the 6 mg/kg Q2W dose and schedule, steady-state concentrations are attained by the third infusion.

Similar to observations in prior clinical trials of the anti-EGFR monoclonal antibodies (15–17,19), the most common adverse events reported in this study were skin-related. The skin reactions were primarily mild to moderate in severity. Only 6% of skin-related adverse events were severe (grade $\geq$3) compared with $\sim$16% in prior studies (7).

In conclusion, this Phase 2 study examined the effects of panitumumab in Japanese patients with mCRC who developed disease progression or relapsed while on or after prior fluouropyrimidine, irinotecan and oxaliplatin chemotherapy. Results from this study indicate that panitumumab at a dose of 6 mg/kg Q2W was well tolerated and exhibited clinically meaningful antitumor activity in this patient population. The pharmacokinetic and safety profiles were similar to those observed in previous non-Japanese panitumumab clinical studies.

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Conflict of interest statement

None declared.

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Impact of vascular endothelial growth factor receptor 1, 2, and 3 expression on the outcome of patients with gastric cancer

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Tumor angiogenesis is a multistep interactive process in which vascular endothelial growth factor (VEGF) and its receptors have a major role. However, the clinical significance of these molecules in gastric cancer (GC) remains unclear. Our study group comprised 86 patients who underwent gastrectomy and subsequently received chemotherapy for recurrent or residual tumor. Using immunohistochemical techniques, we analyzed the expression of VEGF receptors (VEGF-R) 1, 2, and 3. VEGF-R1 expression (defined as >5% staining) was found in the tumor cells of 65 tumors (76%) and in the stromal vessels of 36 tumors (42%). VEGF-R2 expression was found in tumor cells and stromal vessels of 0 and 46 tumors (0 and 53%), respectively, and VEGF-R3 expression was found in tumor cells and stromal vessels of 0 and 75 tumors (0 and 87%), respectively. Univariate analysis revealed that VEGF-R expression correlated with shorter survival (VEGF-R1 in stromal vessels, $P = 0.001$; VEGF-R2 in stromal vessels, $P = 0.009$; VEGF-R3 in stromal vessels, $P = 0.005$) and lower response to S-1 (VEGF-R1 in stromal vessels, $P = 0.039$). Multivariate analysis of potential prognostic factors showed that VEGF-R1 and VEGF-R2 in stromal vessels were independent predictors of poor outcome. Our data suggest that VEGF-R expression can be a predictor of unfavorable clinical outcome in GC. VEGF-R are promising candidates as therapeutic targets. (*Cancer Sci* 2009; 100: 310–315)

Gastric cancer (GC) is the second leading cause of cancer-related death worldwide, accounting for over 20 deaths per 100 000 population annually in East Asia (China, Japan), Eastern Europe, and parts of Central and South America.[1] Recently, many chemotherapy regimens using new agents have been developed that show high response rates for advanced GC, and progress in basic research has revealed many factors and mechanisms implicated in sensitivity and resistance to chemotherapy.

Angiogenesis reportedly plays an important role in cancer invasion and metastasis. Vascular endothelial growth factor (VEGF) and VEGF receptor (VEGF-R) represent important regulators of angiogenesis, and increased expression of this family of molecules has been documented in various cancer cell lines[2] and tissues.[3,4] Previous clinical studies have demonstrated that increased expression of VEGF or its family is associated with the grade of angiogenesis and the prognosis for various human cancers.[5–9]

In GC, several studies have found that expression of VEGF ligands and subtypes correlates with prognosis,[10–12] and expression of soluble VEGF-R1 is also a predictor of prognosis.[13] However, the distribution, frequency, and prognostic value of VEGF-R expression in GC have not been clarified. The present study investigated relationships between VEGF-R expression and prognosis in patients with advanced GC.

Materials and Methods

Patients. Subjects were 86 patients who underwent surgery for primary GC and received chemotherapy for the treatment of recurrent or residual tumors at the National Cancer Center Hospital (NCCH). Inclusion criteria were as follows: histologically proven advanced GC; unresectable, locally advanced, or metastatic disease; no prior chemotherapy and no prior adjuvant or neoadjuvant chemotherapy; specimens of primary GC were obtained before the start of chemotherapy by surgical resection or biopsy at NCCH; radiographically measurable disease; first-line chemotherapy was received from January 1995 to December 2004; tumor response and survival times were confirmed; adequate bone marrow, liver, and renal function; and written informed consent. The tissue samples were collected retrospectively from patients who met these criteria. Measurable disease was assessed by computed tomography. Response was evaluated according to the standard International Union against Cancer (UICC) guidelines as complete response (CR), partial response (PR), no change (NC), or progressive disease (PD). The response rate was calculated as the ratio of CR + PR to CR + PR + NC + PD.[14] Written informed consent was obtained before treatment and evaluation of tumor samples.

Immunohistochemical staining. Serial 4-μm sections were made from formalin-fixed paraffin-embedded tissue. Sections were dewaxed in xylene and rehydrated through a graded alcohol series. Antigen retrieval was carried out by incubating sections in target-retrieval solution (Dako Japan, Tokyo, Japan) for 40 min in a 95°C water bath and cooling for at least 20 min.

After quenching endogenous peroxidase with peroxidase-blocking reagent (Dako Japan) for 5 min and washing with Tris-buffered saline containing Tween 20, sections were incubated with the primary antibody (Table 1).

Immunoreaction was detected using the following secondary antibody systems: CSA-II (Dako Japan) for VEGF-R1, VEGF-R2, and VEGF-R3; and the Envison + kit (Dako Japan) for CD34, D2-40, CD31, and factor VIII, according to the instructions of the manufacturer. Sections were counterstained using Mayer's hematoxylin.

Evaluation of immunostaining. The entire specimen was examined at low magnification (×40), and positive cells were counted in areas with strong immunoreactivities at high magnification (×200). The number of immunoreactive cells was counted in three fields of view that exhibited the most positive staining, and the average ratio of immunoreactive cells to the

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Table 1. Antibodies used for immunohistochemistry

Antigen	Antibody	Manufacturer	Dilution	Incubation time (min)
CD34	M 7165	Dako Japan	1:100	30
D2-40	M 3619	Dako Japan	1:50	30
CD31	M 0823	Dako Japan	1:50	Overnight
Factor XIII	N 1505	Dako Japan	1:2	30
VEGF-R1	AF 321	R&D	1:150	15
VEGF-R2	AF 357	R&D	1:50	15
VEGF-R3	AF 349	R&D	1:50	15

total number of cancer cells per field was calculated. The number of immunoreactive vessels was counted in three fields of view that demonstrated the most positive staining, and the average ratio of immunoreactive vessels to the total number of CD34-positive and D2-40-positive vessels per field was calculated. Staining results for VEGF-R1, VEGF-R2, and VEGF-R3 were classified by estimating the percentage of epithelial cells and vessels showing specific immunoreactivity: negative (defined as <5% staining) or positive (defined as >5% staining).[7] Two researchers evaluated the immunostaining results without being informed of the clinical data.

Statistical analysis. We examined objective tumor response to chemotherapy overall survival. Overall survival were calculated as the period from the start of first-line chemotherapy until disease progression or death from any cause, respectively. If patients were lost to follow up, data were censored at the date of the last evaluation. Statistical analysis was carried out using Stat View version 5 software (SAS Institute, Cary, NC, USA). Pearson's correlations were used to assess VEGF and VEGF-R expression, and a χ^2-test was used to assess relationships between VEGF and VEGF-R expression and therapeutic effect. Each factor and overall survival were determined by Kaplan–Meier methods and analyzed using a log-rank test. Multivariate analysis was carried out using a Cox proportional hazard model.

Results

Clinicopathological characteristics. The clinicopathological characteristics of the patients are shown in Table 2. Patients comprised 69 (80%) men and 17 (20%) women, with a median age of 61 years. Tumor stage (assessed according to TNM classification at the time of surgery) was I, II, or III in 35 patients, and distant metastasis was confirmed at the time of surgery (stage IV) in 51 patients. Histopathologically, 39 patients had intestinal-type adenocarcinoma and 47 displayed diffuse-type adenocarcinoma. All patients received chemotherapy; first-line chemotherapy comprised S-1 in 29 patients, 5-fluorouracil (5-FU) in 24 patients, cisplatin (CDDP) and irinotecan (CPT-11) in 28 patients, and other agents in the remaining five patients. The median follow-up time was 13.3 months (range 1.0–71.7 months).

Expression of VEGF-R1, VEGF-R2, and VEGF-R3. VEGF-R1 was immunoreactive in tumor cells (not only in the membrane, but also in the cytoplasm) and tumor stromal vessels (Fig. 1a). VEGF-R1 expression was found in tumor cells of 65 tumors (76%) and in stromal vessels of 36 tumors (42%) (Table 3).

VEGF-R2 and VEGF-R3 were immunoreactive mainly in tumor stromal vessels (Fig. 1b–d). VEGF-R2 expression was found in tumor cells and stromal vessels of 0 and 46 tumors (0 and 53%), respectively, and VEGF-R3 expression was found in tumor cells and stromal vessels of 0 and 75 tumors (0 and 87%), respectively. The three types of VEGF-R were not markedly correlated with each other in terms of expression.

Table 2. Patient characteristics (n = 86)

Characteristic	n
Sex	
Male	69
Female	17
Median age (years)	61 (range 39–84)
Tissue type	
Intestinal	39
Diffuse	47
pStage†	
I	2
II	11
III	22
IV	51
ECOG performance status	
0	42
1	41
2	3
Metastases	
Liver	25
Abdominal lymph node	43
Peritoneum	23
Lung	4
Other	4
First-line chemotherapy	
S-1	29
5-Fluorouracil	24
Cisplatin + irinotecan	28
Other	5

†Japanese classification. ECOG, Eastern Cooperative Oncology Group.

Table 3. Distribution of vascular endothelial growth factor receptor (VEGF-R) 1, VEGF-R2, and VEGF-R3 expression

Status	VEGF-R1				VEGF-R2		VEGF-R3	
	Cytoplasm		Vessel		Vessel		Vessel	
	n	%	n	%	n	%	n	%
Negative (<5%)	21	24	50	58	40	47	11	13
Positive (>5%)	65	76	36	42	46	53	75	87

Relationship of VEGF-R expression with response to chemotherapy and survival. The response rate was 38% (11/29) in the S-1 group, 4% (1/24) in the 5-FU group, and 43% (12/28) in the CDDP and CPT-11 group (Table 4). In the S-1 group, the response rate was lower in the 15 patients in whom stromal vessels stained positive for VEGF-R1 than in the 14 patients in whom stromal vessels did not (20 vs 57%, χ^2-test $P = 0.039$). In the other groups, the response rates were not markedly affected by expression of VEGF-R.

To clarify the relevance of marker positivity in prediction of disease outcome, staining results for VEGF-R1, VEGF-R2, and VEGF-R3 were correlated with patient survival according to the log-rank test. A univariate analysis revealed that VEGF-R expression correlated with shorter survival (VEGF-R1 in stromal vessels, 11.2 vs 15.9 months, $P = 0.001$, Fig. 2a; VEGF-R2 in stromal vessels, 11.0 vs 15.6 months, $P = 0.009$, Fig. 2b; VEGF-R3 in stromal vessels, 12.8 vs 24.3 months, $P = 0.005$, Fig. 2c). Moreover, multivariate analysis of potential prognostic factors showed that VEGF-R1 and VEGF-R2 expression by stromal vessels were independent predictors of poor outcome in advanced GC (Table 5).

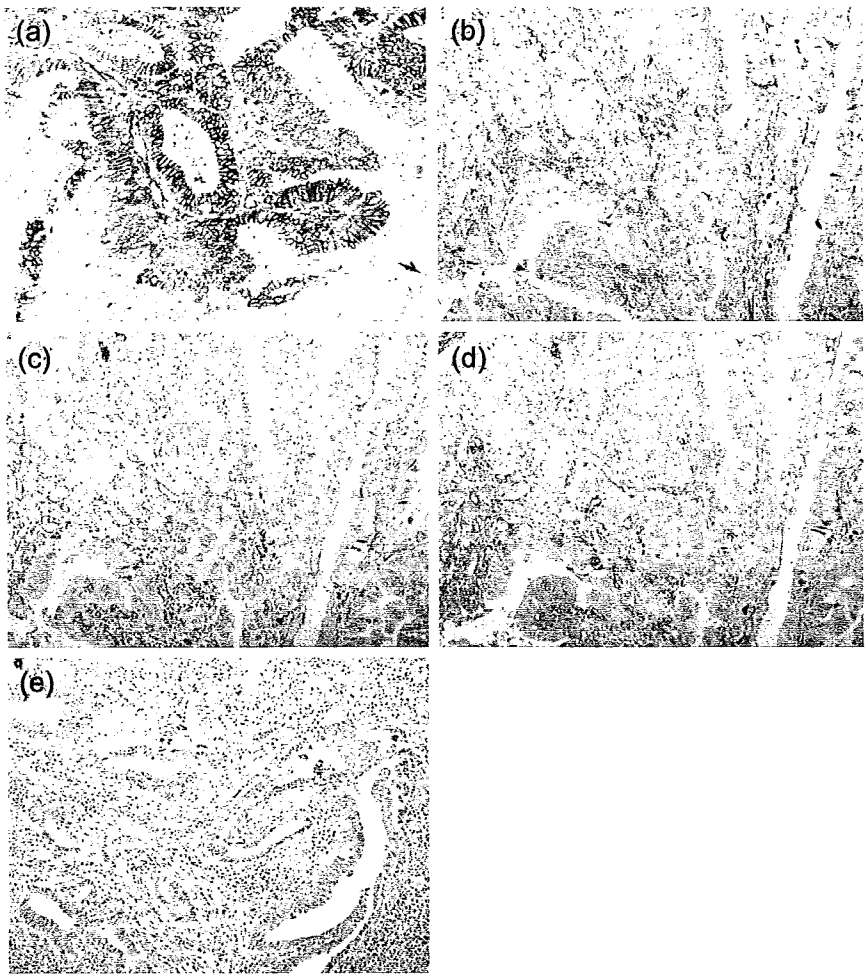

Fig. 1. Typical examples of (a) CD34 staining, (b) D2-40 staining, (c) CD31 staining, (d) factor VIII staining, and (e) negative controls. (a) Vascular endothelial growth factor receptor (VEGF-R) 1 is mainly expressed in tumor cells, secondarily on stromal vessels. (b–d) VEGF-R2 and VEGF-R3 are mainly expressed on stromal vessels. Original magnification, ×200.

Table 4. Relationship between vascular endothelial growth factor receptor (VEGF-R) expression and response to chemotherapy

First-line regimen	*n*	Total response (%)	VEGF-R1				VEGF-R2		VEGF-R3	
			Cytoplasm		Stromal vessels		Stromal vessels		Stromal vessels	
			Positive (%)	Negative (%)	Positive (%)	Negative (%)	Positive (%)	Negative (%)	Positive (%)	Negative (%)
S-1	29	38	32	57	20	57	31	44	37	50
			$P = 0.234$		$P = 0.039$		$P = 0.474$		$P = 0.715$	
Cisplatin and irinotecan	28	43	33	47	45	41	47	38	46	25
			$P = 0.255$		$P = 0.570$		$P = 0.445$		$P = 0.887$	
5-Flurouracil	24	4	0	4	0	4	4	0	4	0
			–		–		–		–	

Discussion

In the present study, we analyzed VEGF-R expression levels in primary tumors from 86 patients with advanced GC. Our goal was to determine whether such expression levels are related to treatment outcomes such as survival and response. We found that expression of VEGF-R1 and VEGF-R2 in stromal vessels in GC specimens were significant predictors of poor survival in advanced GC. Recently, several studies have reported that the genetic profile of patients is related to the outcome of cancer therapy. In colorectal cancer, VEGF-R2 expression for metastatic tumors was higher when compared to non-metastatic tumors,[5] and in head and neck cancer[15] and breast cancer,[16] some studies have documented that VEGF-R3 expression correlates with lymph node metastasis and malignancy,[7,9,14,17] whereas others have not observed this relationship.[18–20] Further investigations are needed to clarify interactions among VEGF-R subtypes and the effects of VEGF expression in stroma on angiogenesis and lymphangiogenesis. In GC, several studies have reported correlations between the expression of VEGF and poor prognosis, or lymphatic metastasis. However, most studies examined survival from the date of surgery to the time of event. In the present study, we examined the expression of VEGF-R, objective tumor response to chemotherapy, and overall survival; the latter two being calculated as the period from the start of first-line chemotherapy until disease progression or death from any cause, respectively.

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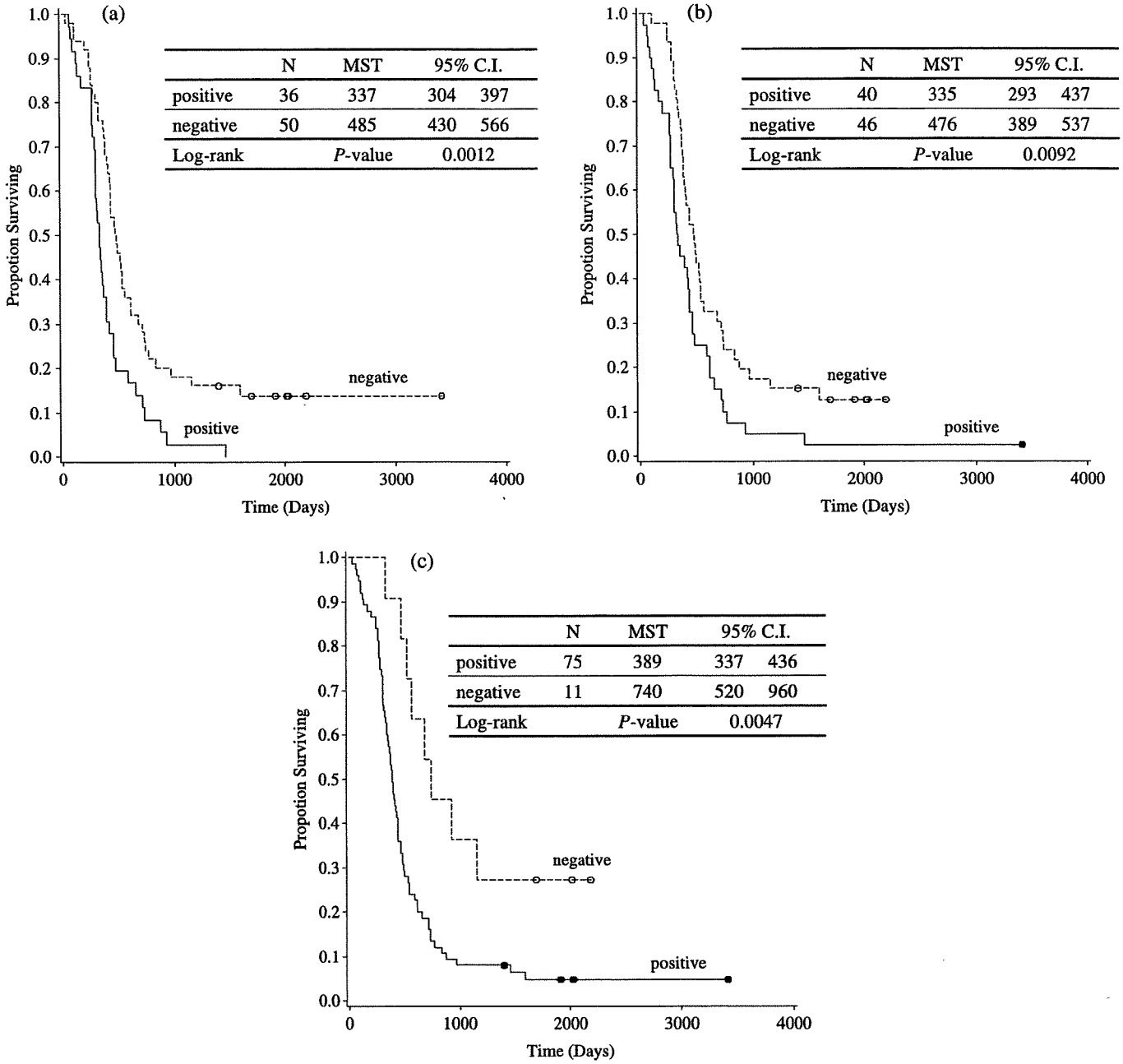

Fig. 2. Impact of (a) vascular endothelial growth factor receptor (VEGF-R) 1, (b) VEGF-R2, and (c) VEGF-R3 expression in stromal vessels on patient survival.

Table 5. Impact of vascular endothelial growth factor receptor (VEGF-R) expression on patient survival from first-line chemotherapy (multivariate analysis)

	Parameter	Hazard ratio	95% confidence interval.		*P*-value
VEGF-R1 (vessel)		**1.75**	**1.09**	**2.80**	**0.020**
PS	1, 2 versus 0	1.45	0.62	2.27	0.109
Tissue type	Diffuse vs intestinal	0.64	0.64	1.00	0.052
Metastasis site	2≥ versus 1	1.5	0.89	2.55	0.132
VEGF-R2 (vessel)		**1.76**	**1.12**	**2.75**	**0.014**
PS	1, 2 versus 0	1.56	1.00	2.46	0.052
Tissue type	Diffuse versus intestinal	0.64	0.41	1.01	0.055
Metastasis site	2≥ versus 1	1.69	1.01	2.81	0.045

PS, Performance Status.

After treatment with S-1, patients with positive staining for VEGF-R1 in stromal vessels showed a lower response rate (20 vs 57%, $P = 0.039$) and shorter survival (10.2 vs 20.2 months, hazard ratio = 3.62: data not shown) than those with negative staining, whereas there was no difference with CDDP and CPT-11. The number of patients treated with S-1 was small, but Boku *et al.* have reported the relationship between VEGF status and the effects of S-1 and 5-FU; patients expressing VEGF showed a slightly lower response rate and relatively shorter survival than those who did not.[21,22] The mechanisms behind this relationship are unclear,[23] but expression of VEGF-R may become a prognostic marker relevant in deciding on a treatment strategy of 5-FU-based drugs.

Our analysis revealed that VEGF-R expression was correlated with shorter survival (VEGF-R1 in stromal vessels, $P = 0.001$; VEGF-R2 in stromal vessels, $P = 0.009$; and VEGF-R3 in stromal vessels, $P = 0.005$), and multivariate analysis of potential prognostic factors showed that VEGF-R1 and VEGF-R2 in stromal vessels were independent predictors of poor outcome. VEGF-R2 is a potent regulator of vascular endothelial cells and has been directly linked to tumor angiogenesis and blood vessel-dependent metastasis. VEGF-R1 may contribute to pathological vascularization directly by stimulating endothelial cell function and indirectly by mediating recruitment of bone marrow progenitor cells.[24] Furthermore, Carmeliet and coworkers demonstrated synergy between the VEGF-R1- and VEGF-R2-specific ligands, indicative of cross-talk between the receptors, allowing modulation of a variety of VEGF-R-dependent signals.[25] In GC, the expression of VEGF or VEGF-C, which are intimately involved in regulation of the lymphangiogenic process, has been reported to be correlated with a poor prognosis.[10,11,26] Juttner *et al.* found that the presence of VEGF-D and its receptor VEGF-R3 was associated with lymphatic metastasis.[12] Given these results, expression of the VEGF family appears to affect the prognosis of GC.

Our immunostaining evaluation revealed that VEGF-R is expressed in tumor cells and tumor stromal vessels.VEGF-R2, which is expressed primarily in vascular endothelial cells, is believed to be the major mediator of angiogenesis in human malignancy, as it regulates activation of downstream effector molecules such as the phosphoinositide 3-kinase plus AKT and mitogen-activated protein kinase pathways. It also potentiates endothelial differentiation, DNA synthesis, and proliferation.[27,28] On the other hand, VEGF-R3 is expressed primarily in lymphatic endothelial cells and regulates lymphangiogenesis.[29] Recently, some studies have documented that the expression of VEGF-R has been observed in tumor cells in several cancers,[30–35] and in the autocrine VEGF–VEGFR loop in cancer cells. Fan *et al.* demonstrated that incubation with VEGF-A or VEGF-B significantly increased colorectal cancer cell migration; however, treatment with a VEGF-R1 antibody blocked this effect.[30] Giatromanolaki *et al.* demonstrated that phosphorylated VEGF-R2 plus KDR receptors are largely expressed in colon cancer cells and intratumoral vasculature, and their expression is associated with tumor diameter and poor histological differentiation.[31] In GC, Tian *et al.* demonstrated that VEGF-R2-positive tumor cells could be stimulated by exogenously added VEGF.[32] In our study, patients with strong positive staining (defined as >50% staining) for VEGF-R1 in the cytoplasm of tumor cells showed shorter survival (12.6 vs 14.2 m, $P = 0.044$; data not shown) than others. Thus, these results suggest that the autocrine VEGF–VEGF-R loop function may contribute to cancer cell proliferation.

In conclusion, our study provides evidence that VEGF-R expression in GC specimens is a risk factor for poor survival in patients with advanced GC. The results of our analysis can help to identify patient subgroups at higher risk for poor disease outcome in GC.

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