Significance of Biological Markers for Predicting Prognosis and Selecting Chemotherapy Regimens of Advanced Gastric Cancer Patients between Continuous Infusion of 5-FU and a Combination of 5-FU and Cisplatin

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Background: Our previous phase II study of 5-fluorouracil (5-FU) and cisplatin (FP) for treatment of advanced gastric cancer showed that strong immunoreactivity for vascular endothelial growth factor (VEGF) is associated with chemoresponse. Patients with four or five of the favorable phenotypes, p53 (-), bcl-2 (-), glutathione S-transferase π (-), thymidylate synthase (-), and VEGF (+), survived longer than those with three or less of these phenotypes. The purpose of this study is to confirm our previous results and to compare the significance of those markers between continuous infusion of 5-FU (5-FUci) and FP.

Methods: Pretreatment biopsies from 131 of 210 advanced gastric cancer patients enrolled to JCOG9205 were analyzed immunohistochemically for the presence of the five markers.

Results: Median survival times of patients treated with 5-FUci (n = 65) or FP (n = 66) were 216 and 253 days, respectively (P = 0.6953). After FP treatment, patients with four or five favorable phenotypes (n = 20) survived longer than those with three or less favorable phenotypes (n = 46) (334 days and 243 days, respectively; P = 0.0463), and the survival times of 34 and 32 patients with VEGF (-) and (+) were similar (269 days and 253 days, respectively; P = 0.6317). After 5-FUci, 30 patients with VEGF (+) survived for a shorter time than 35 patients with VEGF (-) (142 days and 302 days, respectively; P = 0.0043).

Conclusion: The number of favorable phenotypes is prognostic for gastric cancer patients treated with FP, and VEGF has a different impact on survival between treatment with 5-FUci and FP.

Key words: vascular endothelial growth factor – gastric cancer – 5-fluorouracil – cisplatin

INTRODUCTION

Many combination chemotherapy regimens for treatment of advanced gastric cancer have recently been developed using new agents and shown high response rates (1–8). However, a standard chemotherapy has not been established for treatment of advanced gastric cancer because there are no reports from randomized phase III trials showing a survival benefit to the treatment with 5-fluorouracil (5-FU) alone (9,10). In the phase III study of the Gastrointestinal Oncology Study Group (GIOSG) of the Japan Clinical Oncology Group (JCOG), there was no significant difference in survival between continuous infusion of 5-FU (5-FUci) and a combination of 5-FU and cisplatin (FP) despite a higher response
rate and longer time to progression (TTP) of FP compared with 5-FUci, associated with lower toxicity of 5-FUci than of FP (11). Therefore, 5-FUci was considered a reference regimen for the present phase III study of advanced gastric cancer patients. It has also been reported that a better response to chemotherapy contributes to longer survival and cures in some patients (12). However, severe toxicity associated with intensive chemotherapy causes deterioration of patients’ quality of life, especially that of nonresponders. Thus, the ability to predict the effects of chemotherapy and to select an appropriate regimen for each patient before commencement of chemotherapy is important.

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

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

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

PATIENTS AND METHODS

PATIENTS

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

TREATMENT SCHEDULE

The treatment schedule for the 5-FUci group comprised a continuous infusion of 5-FU (800 mg/m² per day) on days 1–5. The FP schedule consisted of a drip infusion of CDDP (20 mg/m² per day) on days 1–5, together with the same dose of 5-FUci as the 5-FUci group. These two treatments were repeated every four weeks until the appearance of disease progression, unacceptable toxicity, or the patient’s voluntary withdrawal from the study.

IMMUNOHISTOCHEMISTRY

Biopsy samples from 180 patients were immunostained as described in our previous report (17). All immunohistochemical analyses were performed using tissue sections from formalin-fixed, paraffin-embedded biopsy material obtained endoscopically from primary tumors. Serial 3 μm thick slices were cut, deparaffinized in xylene, dehydrated with graded ethanol and then immersed in methanol containing 0.3% H₂O₂ for 20 min to inhibit endogenous peroxidase activity. Sections stained for p53 and TS were heated to 95°C by microwave irradiation for 10 min in phosphate buffered saline (PBS) or 10 mM citrate buffer, respectively. Sections stained for VEGF were treated with 0.05% pepsin in 0.01 N HCl for 20 min at room temperature. After blocking with 10% normal swine serum in PBS (blocking buffer) for 60 min, all sections were incubated overnight at room temperature with the primary antibodies diluted in blocking buffer to the following concentrations: anti-p53 antibody (Nichirei, Tokyo, Japan), 1:2000; anti-bcl-2 antibody (DAKO, Glostrup, Denmark), 1:40; anti-GST-π antibody (MBL, Nagoya, Japan), 1:24000; anti-TS antibody (TS106 (16)), 1:200; anti-VEGF antibody (Santa Cruz Biochemistry, CA, USA), 1:500. The sections were washed with PBS and then incubated for 1 h with biotinylated secondary antibody diluted 1:200. After washing with PBS, the sections were incubated with ABC reagent (Vector Laboratories, CA, USA) and the color reaction was.
developed in Tris buffer containing 2% 3,3'-diaminobenzidine and 0.3% hydrogen peroxide. The sections were then counterstained with hematoxylin or methyl green.

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

**ANTI-TUMOR EFFECTS**

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

**STATISTICAL ANALYSIS**

Survival curves were constructed using the Kaplan–Meier method and compared using the Log-rank test. Patient characteristics and response rates were compared using a χ² test.

**RESULTS**

**PATIENT CHARACTERISTICS**

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

**OVERALL SURVIVAL AND TIME TO PROGRESSION**

Figure 1 shows the overall survival times of subjects treated with 5-FUci or FP. There was no significant difference in survival between patients treated with 5-FUci or with FP;
Expressions of vascular endothelial growth factor (VEGF), thymidylate synthase (TS), p53, glutathione S-transferase π (GST-π) and bcl-2 were examined immunohistochemically. Values in parentheses are percentages.

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

<table>
<thead>
<tr>
<th>Biological Marker</th>
<th>5-FUci (n = 65)</th>
<th>FP (n = 66)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF (+)</td>
<td>30 (49)</td>
<td>32 (47)</td>
</tr>
<tr>
<td>VEGF (−)</td>
<td>35 (51)</td>
<td>34 (53)</td>
</tr>
<tr>
<td>TS (+)</td>
<td>37 (57)</td>
<td>21 (32)</td>
</tr>
<tr>
<td>TS (−)</td>
<td>28 (43)</td>
<td>45 (68)</td>
</tr>
<tr>
<td>p53 (+)</td>
<td>28 (43)</td>
<td>28 (42)</td>
</tr>
<tr>
<td>p53 (−)</td>
<td>37 (57)</td>
<td>38 (58)</td>
</tr>
<tr>
<td>GST-π (+)</td>
<td>38 (58)</td>
<td>41 (62)</td>
</tr>
<tr>
<td>GST-π (−)</td>
<td>27 (42)</td>
<td>25 (38)</td>
</tr>
<tr>
<td>Bcl-2 (+)</td>
<td>7 (11)</td>
<td>11 (17)</td>
</tr>
<tr>
<td>Bcl-2 (−)</td>
<td>58 (89)</td>
<td>55 (83)</td>
</tr>
</tbody>
</table>

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

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

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

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

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

Expression of Biological Markers and Response

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

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

Number of Favorable Phenotypes, Survival and Time to Progression

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

VEGF, Survival and Time to Progression

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

Discussion

The recruitment rates of patients from the phase III study (JCOG9205 (11)) into the present study were equal among the three regimens. Patient characteristics and rates of positive reactions for biological markers were well balanced. These data indicate that biopsy samples were collected without bias. The overall response rates, survival times and
TTPs were similar to those of patients enrolled in the phase III study (11). Although biopsy specimens were collected only from two-thirds of the patients enrolled in JCOG9205 (11), the subjects of this study were considered representative of those of the phase III study.

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

These results suggest that assessment of biological markers using endoscopic biopsy samples can yield useful information and that the expression of VEGF in the biopsy samples of gastric cancer patients may be a predictor of chemotherapeutic effects in CDDP containing regimens.

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

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

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

**Figure 3.** Overall survival of patients (solid line) with VEGF (+) and those (dotted line) with VEGF (−) after treatment with FP (A) or 5-FUci (B).
higher than that of patients with three or less favorable phenotypes. It could be argued that these discrepancies indicate that expression of VEGF and the number of favorable phenotypes are not good predictive markers of chemotherapy response to FP. However, Takiuchi et al. (20) reported that immunohistochemical expression of VEGF can predict the response to FP in patients with gastric cancer. Nagashima et al. (21) reported that VEGF and the number of favorable phenotypes of similar biological markers were predictive markers of chemotherapy to irinotecan plus CDDP and of survival. Several reports have described differences in chemoresponse between phase II and III studies of the same chemotherapy regimens. It is possible that the discrepancies between our previous and present studies were caused by the difference in the method of evaluating responses between the phase II and III studies of FP.

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

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

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

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

Acknowledgments

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Conflict of interest statement
None declared.

References


