Biological Markers as a Predictor for Response and Prognosis of Unresectable Gastric Cancer Patients Treated with 5-Fluorouracil and cis-Platinum


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ABSTRACT
We investigated the utility of examining biological markers to predict chemoresponse and survival. The subjects consisted of 39 unresectable gastric cancer patients treated with a combination of 5-fluorouracil and cis-platinum. The expression of p53, bcl-2, thymidylate synthase (TS), glutathione S-transferase \( \pi \) (GST-\( \pi \)), and vascular endothelial growth factor (VEGF) in the formalin-fixed biopsy samples of primary tumors before chemotherapy was examined immunohistochemically. The positive rate for VEGF, bcl-2, TS, p53, and GST-\( \pi \) was 51, 10, 46, 38, and 69%, respectively. VEGF-positive cases showed a higher response rate than did negative cases (11 of 20 versus 2 of 19 cases; \( P = 0.0057 \)). The cases that were negative for p53, TS, bcl-2, and GST-\( \pi \) were more likely to respond to chemotherapy than the cases that were positive for these markers. The 10 cases having 4 or 5 favorable phenotypes (VEGF positive, p53 negative, bcl-2 negative, TS negative, and GST-\( \pi \) negative) survived longer than the remaining 29 cases (\( P = 0.0069 \)). Multivariate analysis revealed that the number of favorable phenotypes (\( \geq 4 \) versus \( \leq 3 \)) had a greater impact on survival than performance status (0 versus 1 or 2), age (>60 years versus \( \leq 60 \) years), macroscopic type (scirrhous versus nonscirrhous), histological type (intestinal versus diffuse), or tumor extent (locally advanced versus metastatic). Immunohistochemical examination of biological markers in biopsy samples may be useful in predicting the clinical outcome of unresectable gastric cancer patients treated with 5-fluorouracil and cis-platinum.

INTRODUCTION
The combination of 5-FU\(^3\) and CDDP (FP), is one of the most common chemotherapy regimens for gastric cancer patients (1–3). Rougier et al. (3) have reported that the patient’s PS, tumor location, macroscopic type, and histological type influence the response of advanced gastric cancer patients treated with FP, and that patients with good PS and nonscirrhous type tumors showed better prognosis. In recent years, due to numerous advances in basic research, many biological markers related to antineoplastic sensitivity have been identified. Mutant p53 and bcl-2 proteins protect cancer cells from apoptosis induced by many antineoplastic agents (e.g., topoisomerase inhibitors, DNA-reactive drugs, and antimetabolites) and confer cytotoxic drug resistance (4–6). GST-\( \pi \) is an enzyme that plays an important role in cellular detoxification, and increases in this enzyme have been associated with resistance to antineoplastic agents such as CDDP (7–9). TS, which is a target of 5-FU, influences the chemosensitivity of 5-FU (10). Clinically, these biological markers have been reported to have some impact on chemoresponse and survival (11–17). Because drug delivery is important for the sensitivity of tumors to antineoplastic agents, VEGF may contribute to chemoresistance through the promotion of angiogenesis and/or vascular permeability (18, 19). In this study, we investigated whether immunohistochemical examination of p53, bcl-2, TS, GST-\( \pi \), and VEGF in biopsy samples of primary tumors was a useful method for predicting the response and prognosis of unresectable gastric cancer patients treated with FP.

SUBJECTS AND METHODS
Study Population. Subjects were 39 patients with unresectable gastric cancer, consisting of 31 of 57 patients enrolled in the Japanese Clinical Oncology Group Phase II trial of FP (1) who were treated in our hospitals and 8 of 15 consecutive patients treated with the same regimen at the National Cancer Center Hospital East between July 1992 and December 1994.

The abbreviations used are: 5-FU\(^3\), 5-fluorouracil; CDDP, cis-platinum; FP, combination of 5-FU and CDDP; VEGF, vascular endothelial growth factor; GST-\( \pi \), glutathione S-transferase \( \pi \); PS, performance status; TS, thymidylate synthase.

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\(^3\) The abbreviations used are: 5-FU, 5-fluorouracil; CDDP, cis-platinum; FP, combination of 5-FU and CDDP; VEGF, vascular endothelial growth factor; GST-\( \pi \), glutathione S-transferase \( \pi \); PS, performance status; TS, thymidylate synthase.
These 39 cases fulfilled the following recruitment criteria: (a) age, 70 years or less; (b) PS, 2 or less on the European Oncology Group scale; (c) no prior treatment; (d) adequate bone marrow, hepatic, and renal functions; and (e) primary tumors from which it was possible to obtain a sufficient amount of cancerous tissue for examining five biological markers by biopsy before chemotherapy.

**Treatment Schedule and Evaluation of Response.** The treatment consisted of a protracted infusion of 5-FU (800 mg/m²/day) and a 30-min infusion of CDDP (20 mg/m²/day) for 5 consecutive days and was repeated every 4 weeks until progression of disease. The response to chemotherapy in measurable lesions was assessed 4 weeks after the start of each course and evaluated by the standard WHO criteria (20). In addition, the response in the primary lesions was assessed according to the roentgenographic and endoscopic criteria proposed by the Japanese Research Society for Gastric Cancer (21). Survival time was estimated from the start of the first course to the date of death or to the final date of survival confirmation.

**Immunohistochemical Examination.** All immunohistochemical examinations were performed on tissue sections of formalin-fixed, paraffin-embedded biopsy materials from primary tumors. Serial 3-μm-thick slices were cut, deparaffinized in xylene, dehydrated with graded ethanol, and then immersed in methanol with 0.3% H₂O₂ for 20 min to inhibit endogenous peroxidase activity. The sections stained for p53 and TS were heated to 95°C by microwave irradiation for 10 min in PBS or 10 mm citrate buffer, respectively. The sections stained for VEGF were treated with 0.05% pepsin in 0.01 HCl for 20 min at room temperature. After blocking with 10% normal swine serum in PBS (blocking buffer) for 60 min at room temperature, 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), 1:20,000; anti-bcl-2 antibody (DAKO, Glostrup, Denmark), 1:40; anti-GST-π antibody (MBL, Nagoya, Japan), 1:24,000; anti-TS antibody [TS106 (15)], 1:200; and anti-VEGF antibody (Santa Cruz Biochemistry, Santa Cruz, CA); 1:500. The sections were washed with PBS and then incubated with biotinylated secondary antibody diluted 1:200. After washing with PBS, the sections were incubated with ABC reagent (Vector Laboratories, Burlingame, CA), and color reaction was developed in 2% 3,3'-diaminobenzidine and 0.3% hydrogen peroxide in Tris buffer. The sections were then counterstained with hematoxylin or methyl green. Immunohistochemical staining was assessed independently by each of two investigators who were blinded to the clinical outcome. The intensity of p53 and GST-π staining was graded as follows: ++, strong; +, faint; and −, no visible staining. For bcl-2, the intensity of staining was graded as follows: ++, stronger than that in lymphocytes; +, equal to that in lymphocytes; and −, weaker than that in lymphocytes. For all three markers, cases were defined as positive when more than 20% of all cancer cells in each section showed ++ or + staining according to the criteria of Okuyama et al. (9) and Ohsaki et al. (17). The staining of VEGF was graded as follows: +, the staining intensity in cancer cells was stronger than that in the stromal cells; ±, the staining intensity was equal to that in the stromal cells; and −, the staining intensity was weaker than that in the stromal cells. These cases graded as + were defined as positive. TS expression was scored from 0–3 based on the intensity of the staining (22), with scores of 1–3 defined as positive. Diffuse or focal staining was not assessed.

**Statistical Analysis.** Subjects were categorized as positive or negative according to the immunohistochemical results. We investigated the relationship between the expression of each biological marker and chemoresponse using the χ² test or Fisher’s exact test and the survival using the Kaplan-Meier method (log-rank test). The Cox proportional regression analysis was performed with a SAS program to examine the significance of these biological markers for survival using clinicopathological features as covariates. The clinicopathological features were categorized as follows: PS, 0 versus 1 or 2; age, >60 years versus ≤60 years; macroscopic type, scirrhous versus nonscirrhous; histological type, intestinal versus diffuse; and tumor extent, locally advanced versus metastatic (Table 1).

### RESULTS

#### Biological Marker Expression

Positive staining for p53 was observed in the nuclei of cancer cells, whereas positive staining for bcl-2 was observed in the cytoplasm (Fig. 1, A and B). GST-π was observed in the nucleus and/or cytoplasm (Fig. 1C). TS expression was observed in a perinuclear site. Positive staining for VEGF in cancer cells is shown in Fig. 1E. For all biological markers, the staining pattern was heterogeneous, and not all cancer cells were stained. The percentage of cases positive for VEGF, bcl-2, TS, p53, and GST-π was 51, 10, 46, 38, and 69%, respectively.

#### Biological Marker Expression and Response

The overall chemotherapy response rate was 33% (13 of 39 cases). Table 2 shows the relationship between the expression of each
Fig. 1 Immunohistochemistry of biological markers p53, bcl-2, GST-π, VEGF, and TS. Positive staining for p53 (A) is observed in the nuclei, and positive staining for bcl-2 (B) is observed in the cytoplasm of cancer cells. GST-π expression (C) is observed in the nucleus and/or cytoplasm, and TS expression (D) with a perinuclear location is observed. The staining of VEGF (E) in cancer cells is more intensive than that in stromal cells. Cases were defined as positive when more than 20% of all cancer cells in each section showed positive staining for p53, bcl-2, or GST-π. VEGF staining was defined as positive when the staining intensity in cancer cells was stronger than that in stromal cells. TS expression was scored as positive or negative based on the staining intensity.

biological marker and the chemoresponse. VEGF-positive cases showed a significantly higher response rate than did VEGF-negative cases. As for the other biological markers, those cases negative for p53, bcl-2, GST-π, and TS showed higher response rates than those positive for these markers. Thus, we designated VEGF positive, TS negative, p53 negative, bcl-2 negative, and GST-π negative as favorable phenotypes for chemoresponse.

**Biological Marker Expression and Survival.** The median survival time among all cases was 243 days. The survival curves of VEGF-positive and -negative cases were very similar (Fig. 2). Among the 27 nonresponders, the 10 VEGF-positive cases did not survive as long as the 17 VEGF-negative cases (median survival time, 158 versus 198 days). As a single factor, the cases with phenotypes favorable for chemoresponse of each
of the biological markers survived slightly longer than the cases without such favorable phenotypes (p53, P = 0.1527; bcl-2, P = 0.4756; TS, P = 0.6987; and GST-π, P = 0.2102).

Combination of Biological Markers. The immunohistochemical results for each of these five markers were independent from each other. The relationship between the number of favorable phenotypes and chemoresponse is shown in Table 3. Ten cases with 4 or 5 favorable phenotypes showed a high response rate (70%, 7 of 10 cases) and survived longer than the remaining 29 cases (Fig. 3; median survival time 317 versus 198 days; P = 0.0069).

Multivariate Analysis. The Cox proportional regression analysis revealed that none of these five biological markers had a significant impact on survival as a single factor. The number of favorable phenotypes (≥4 versus ≤3) had a greater impact on survival than PS, age, tumor extent, macroscopic type, or histological type (Table 4).

DISCUSSION

Many biological mechanisms have been implicated in playing a role in the sensitivity of tumors to antineoplastic agents. In the present study, VEGF was shown to be a good marker for predicting chemoresponse. To our knowledge, there have been no reports describing a relationship between VEGF and chemoresponse. Because VEGF contributes to tumor angiogenesis and vascular permeability, good drug delivery through VEGF may explain the relationship between chemoresponse and VEGF.

It has been reported that TS protein levels and mRNA levels of nonresponders were higher than those of responders in 5-FU-based regimens (15, 16, 22). Nabeya et al. (23) reported that gastric/esophageal adenocarcinoma cells expressing mutant p53 protein were resistant to 5-FU and CDDP. Okuyama et al. (9) reported a correlation between the expression of GST-π and CDDP sensitivity as evaluated by an in vitro succinate dehydrogenase inhibition test in 62 gastric cancer patients. bcl-2 is also known to inhibit apoptosis and is related to resistance to antineoplastic agents (5, 6). Gilbert et al. (24) and Ali-Osman et al. (25) graded the immunostaining of GST-π and reported the relationship between the expression of GST-π and patients’ survival. It is possible that the grading of GST-π expression may correlate to the patient’s survival better than negative/positive categorization. However, these reports did not refer to chemoresponse. Nishimura et al. (14) categorized the expression of GST-π as high or low and reported that it correlated well with chemoresponse to platinum-based chemotherapy and survival in patients with head and neck cancer. Thus, we categorized the expression of GST-π as negative or positive and investigated the relationship between GST-π expression and chemoresponse. In the present study, p53-negative, TS-negative, GST-π-negative, and bcl-2-negative cases showed relatively higher response rates than did the corresponding positive cases.
cases. Thus, we designated VEGF positive, p53 negative, TS negative, GST-π negative, and bcl-2 negative as favorable phenotypes for chemoresponse.

Although all patients who survived longer than 500 days were VEGF-positive cases, the survival curves of VEGF-positive and -negative cases were very similar in spite of the significant difference in response rates. VEGF promotes progression and metastasis and is associated with poor prognosis in various tumors (26, 27). As for the other four markers, there have been more than a few reports correlating them with the survival of the patients treated with chemotherapy (11–17). In the present study, none of these four biological markers had a significant impact on survival as a single factor. The small number of the subjects in this study may be a primary reason for these results. However, the cases with each of the five favorable phenotypes survived slightly longer than did the cases without them, and it is possible that favorable phenotypes for chemoresponse may be favorable phenotypes for survival. Rougier et al. (3) reported that PS and macroscopic type were prognostic factors in advanced gastric cancer patients treated with FP. In the present study, the number of favorable phenotypes had a greater impact on survival than these clinicopathological features. Because the staining pattern of each marker was heterogeneous, and the therapeutic regimen of this study was a combination chemotherapy, it seems reasonable to suppose that combined mechanisms of these biological markers were related with chemoresponse and survival. Combinations of clinical features can be used as a prognostic index (28). In a similar manner, it may be useful to use combinations of biological markers to predict clinical outcome. Furthermore, it is expected that combinations of clinicopathological features and biological phenotypes may also be useful. In conclusion, VEGF was a good marker for predicting the chemoresponse of unresectable gastric cancer patients treated with FP, and the number of favorable phenotypes might be a good predictor of chemoresponse and survival.

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REFERENCES


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Table 4  Cox proportional regression analysis for survival

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<th>Variable</th>
<th>Categories</th>
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<td>No. of favorable phenotypes</td>
<td>≥4 vs. ≤3</td>
<td>0.0074</td>
<td>4.097 (1.458–11.512)</td>
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<td>Histological type</td>
<td>intestinal vs. diffuse</td>
<td>0.1196</td>
<td>1.852 (0.852–4.024)</td>
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<td>Tumor extension</td>
<td>locally advanced vs. metastatic</td>
<td>0.1652</td>
<td>2.114 (0.734–6.085)</td>
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<td>PS</td>
<td>0 vs. 1 and 2</td>
<td>0.2032</td>
<td>1.580 (0.781–3.197)</td>
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<td>Macromorphic type</td>
<td>nonscirrhous vs. scirrhous</td>
<td>0.7149</td>
<td>1.079 (0.718–1.623)</td>
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<td>Age (yr)</td>
<td>≤60 vs. &gt;60</td>
<td>0.9010</td>
<td>1.048 (0.498–2.205)</td>
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* CI, confidence interval.
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