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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Running Title:

Biological markers in 5-FU and FP

Abstract

PURPOSE: In our previous phase II study of 5-fluorouracil and cisplatin (FP) for advanced gastric cancer, vascular endothelial growth factor (VEGF) was a predictor of chemoresponse, and patients having four or five favorable phenotypes out of p53 (-), bcl-2 (-), gluthathione S-transferase π (GST-π) (-), thymidylate synthase (TS) (-), and VEGF (+) survived longer than those having three or less. 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. **PATIENTS AND METHODS:** The above five markers were examined immunohistochemically in pre-treatment biopsy from 131 of 210 advanced gastric cancer patients (JCOG9205). **RESULTS:** Median survival times (MST) of 65 patients treated with 5-FUci and 66 patients with FP were 216 and 253 days (p=0.6953). Twenty patients with four or five of the above favorable phenotypes survived longer than the other 46 patients with three or less in FP (MST: 334, 243 days, p=0.0463). Survival times of 34 and 32 patients with VEGF (-) and (+) were similar in FP (MST: 269, 253 days, p=0.6317), whereas 30 patients with VEGF (+) survived shorter than 35 patients with VEGF (-) in 5-FUci (MST: 142 and 302 days, p=0.0043). **CONCLUSION:** The number of favorable phenotypes was confirmed to be a prognostic factor of gastric cancer patients treated with FP, and it is suggested that VEGF might be a selective marker between FP and 5-FUci.

Mini-abstract

Number of phenotypes of p53(-), bcl-2(-), GST-π (-), TS(-), VEGF(+) was a prognostic factor in treatment with FP for gastric cancer, and VEGF might be a predictor between FP and 5-FUci.

Key Words:

vascular endothelial growth factor, gastric cancer, 5-FU, CDDP

Introduction

Recently, many combination chemotherapy regimens including new agents have been developed, and show high response rates for advanced gastric cancer (1-8). However, in randomized phase III trials, no regimens have been reported indicating survival benefit to treatment with 5-fluorouracil (5-FU) alone (9-10), thus no standard chemotherapy has been established for advanced gastric cancer. In the phase III study of the Gastrointestinal Oncology Study Group in the Japan Clinical Oncology Group (GIOSG/JCOG), there was no significant difference in survival between the 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, and toxicities of 5-Fuci was lower than FP (11). Therefore, 5-FUci has still been recognized as one of the reference regimens for the present phase III study of advanced gastric cancer patients. It has also been reported that a good response to chemotherapy contributes to a long survival and cure of some patients (12) while severe toxicity associated with intensive chemotherapy causes deterioration in the patient's quality of life, especially in non-responders. Thus, it is very important to predict the effects of chemotherapy and to select an appropriate regimen for each patient before the commencement of chemotherapy.

Progress in basic research has revealed many factors and mechanisms implicated in sensitivity and resistance to chemotherapy, and some of these have been reported as having clinical impacts (13-16). However, there have been no reports of biological markers that are clearly useful for selecting chemotherapy regimens of advanced gastric cancer patients. In our previous study along the phase II study of FP for advanced gastric cancer, vascular

endothelial growth factor (VEGF) (+), p53 (-), bcl-2 (-), thymidylate synthase (TS) (-), and gluthathione S-transferase π (GST-π) (-) were shown to be favorable phenotypes for chemoresponses in 39 patients (17). Of these, patients with VEGF (+) showed a significantly higher response rate than those with VEGF (-). However, there was no difference in survival times between patients with (+) and (-) values in any of the above markers individually. There was a clear relationship between the number of favorable phenotypes and the response rates, and the 10 patients with four or five favorable phenotypes survived significantly longer than the 29 patients with three or fewer.

However, since our previous study was investigational, these results should be confirmed in another cohort. Moreover, the clinical utility of these markers for selecting chemotherapy regimens should be investigated and compared in a randomized phase III trial.

In this paper, we investigate the relationship between the expressions of the above five biological markers and the survival effects among the patients registered in the phase III study (JCOG9205 (11)) to confirm the results of our previous study, and to clarify the utility of these markers in selecting the chemotherapy regimens 5-FUci or FP. This study was approved by the chair of the Japan Clinical Oncology Group.

Patients and Methods

Patients

The source of the subjects were 280 patients enrolled into the phase III study (JCOG9205); 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. The 45 patients treated with UFTM were excluded from this study because UFTM treatment was stopped after the interim analysis of the phase III study had revealed no survival advantage and more severe toxic effects compared with 5-FUci (11). Three patients in the 5-FUci group, and one in the FP group, were also excluded because their biopsy samples were insufficient for immunostaining. Finally, the subjects of this study comprised 65 patients treated with 5-FUci and 66 with FP, from whom sufficient amounts of pre-treatment biopsy specimens were obtained endoscopically. These patients fulfilled the eligibility criteria of JCOG9205: (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 2 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/m^2 per day) on days 1 to 5. The FP schedule consisted of a drip infusion of CDDP (20 mg/m^2 per day) on days 1 to 5, together with the same dose of 5-FUci. These two treatments were repeated every four weeks until the appearance of disease progression, unacceptable toxicity, or the patient's voluntary withdrawal.

Immunohistochemistry

The biopsy samples obtained from 180 patients were immunostained as described in our previous study (17). All immunohistochemical examinations were performed on tissue sections from formalin-fixed and paraffin-embedded biopsy materials obtained endoscopically from primary tumors. Serial 3 □m thick slices were cut, deparaffinized in xylene, and dehydrated with graded ethanol, then immersed in methanol with 0.3% H_2O_2 for 20 minutes to inhibit endogenous peroxidase activity. The sections stained for p53 and TS were heated to 95 ^{o}C by microwave irradiation for 10 minutes in phosphate buffered saline (PBS) or 10 mM citrate buffer, respectively. The sections stained for VEGF were treated with 0.05% pepsin in 0.01 N HCl for 20 minutes at room temperature. After blocking with 10% normal swine serum in PBS (blocking buffer) for 60 minutes, 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), 1:20000; 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; and anti-VEGF antibody (Santa Cruz Biochemistry, CA, USA), 1:500. The sections were washed with PBS and then incubated for one hour with biotinylated secondary antibody diluted to 1:200. After washing with PBS, the sections were incubated with ABC reagent (Vector Laboratories, CA, USA), and the 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.

All immunostained specimens from the 180 patients were assessed by an investigator (N.B.) who was not informed of any clinical informations such as treatment schedules and clinical outcomes. The intensity of staining for p53 and GST-π was graded as (+) when strong, as ($\underline{+}$) 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, 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, and as (-) when weaker. TS expression was graded as (++), (+), (±), (-) based on the intensity of the staining. For all markers, patients were defined as positive when more than 20% of all cancer cells in each section showed (++) or (+).

Anti-tumor effects

The responses of measurable metastatic lesions and of primary lesions were evaluated according to the standard WHO criteria (18) and evaluation criteria proposed by the Japanese Gastric Cancer Association (19). All patients were followed for at least one year from their registration in 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 counted from the date of registration to the date of confirming disease progression, firstly by image diagnosis, secondly by clinical diagnosis, or to the date of death in patients without confirmation of disease progression. All clinical information was obtained from the JCOG data center.

Statistical analysis

Survival curves were calculated by the Kaplan-Meier method and compared with the log rank test. Patient characteristics and response rates were compared with a chi-squared test.

Results

Patient characteristics

Patient characteristics are given in Table I. The subjects constituted two thirds of all patients enrolled in JCOG9205. The numbers of patients treated with 5-FUci and FP were almost equal. The two groups were well balanced in age, sex, macroscopic type, histological type, and history of resection of the 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 metastasis (p=0.1196).

Overall survival and time to progression

Figure 1 shows the overall survival times of all subjects treated with 5-FUci or FP. There was no difference in survival between those treated with 5-FUci or with FP, with median survival times of 216 days in the 5-FUci group and 253 days in 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 pattern and positive rate for each biological marker was similar to our previous study (Table II). Table III shows the relationship between each biological marker and the chemoresponse. The overall response rates in the FP and 5-FUci groups were 44% (29/66) and 12% (8/65), respectively. Although the response rates of the patients with VEGF (-) were higher than those with VEGF (+) in the 5-FUci group (p=0.0599), there were no differences

in response rates between the other markers, (+) or (-), in either the 5-FUci or FP groups.

In the FP treatment group, 11 of the 20 patients (55%) with four or five favorable phenotypes, and 18 of the 46 patients (39%) with three or fewer, were responders (p=0.2326). However, there was no difference in the response rates between the 16 patients with four or five favorable phenotypes and the other 49 patients in the 5-FUci treatment group (favorable, 2/16 (13%); others, 6/49 (12%); p>0.9999).

Number of favorable phenotypes, survival, and time to progression

The 20 patients with four or five favorable phenotypes survived longer than the 46 patients with three or fewer in the FP treatment group (MST, 334 and 243 days, respectively; p=0.0463) (Fig. 2A), whereas there was no difference between the two types of patients in the 5-FUci group (MST, 203 and 216 days, respectively; p=0.315) (Fig. 2B). No significant differences were observed in TTP between the patients with four or five favorable phenotypes and the other patients either in the FP or the 5-FUci (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

Either in the 5-FUci or FP group, there was no significant difference in survival times between patients with (+) and (-) values in any of p53, bcl-2, TS, GST-π individually. As for VEGF, the survival times of the 32 (49%) patients with VEGF (+) and the 34 (51%) with VEGF (-) were identical in the FP treatment group (MST: 269 and 253 days, respectively; p=0.6317) (Fig. 3A), whereas the 30 patients with VEGF (+) showed 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 for patients with VEGF (-) was significantly longer than the TTP for 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 into the present study from the phase III study (JCOG9205) were equal in the three regimens. Patient characteristics and a positive rating for each biological marker were well balanced. These data indicate that biopsy samples were collected without any bias. The overall response rates, survival times, and TTP were very similar to the results of all enrolled patients to the phase III study (11). Although biopsy specimens were collected from only two thirds of the patients in JCOG9205, it is considered that the subjects of this study could represent the phase III study well.

Biopsy samples can be taken only from the superficial part of primary tumors and may not represent the biological behavior of the whole tumor exactly. Since many patients to be treated with chemotherapy are unresectable, only biopsy samples can be used to assess the biological markers. Takiuchi (20) and we (17) have reported 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 showed a higher response rate

than those with VEGF (-). These results suggest that assessment of biological markers in endocopical biopsy samples can yield useful information and that the expression of VEGF in the biopsy samples of gastric cancer patients might be a predictor of chemotherapeutic effects in regimens including CDDP.

The occurrence of positive VEGF was 47% (62/131) in the present study, which recapitulated the result of our previous study (51%, 20/39). The occurrence of other biological markers was also similar. These results implicated that the method for evaluating biological markers in this study was reproducible. In the present study there was no relationship between the expression of VEGF and chemoresponse to FP treatment while the response rates between patients with four or five favorable phenotypes was slightly higher than those with three or fewer. It is considered that these discrepancies are due to the following differences in the evaluation of responses between our previous study and the present study. Firstly, whereas patients in the previous study were recruited from a phase II study in which the primary endpoint was the response rate, the present study drew subjects from a phase III study in which survival time was the primary endpoint. Secondly, while all patients in the phase II study had primary tumors by which to evaluate the overall response to treatment, 33 of 131 (25%) patients had undergone gastrectomy before the initiation of chemotherapy in the phase III study. Thirdly, while a small number of institutions participated in the earlier phase II study, patients were enrolled from many institutions in the later phase III study.

In this study, the 20 patients with four or five favorable phenotypes survived longer than the other 46 patients with three or fewer in the FP group. This result recapitulates our previous findings on survival obtained from the phase II study. In the 5-FUci treatment group, there was no difference in survival between the two phenotype groups. However, either in the subset of

patients with four or five favorable phenotypes nor in those with three or less, the differences in survival between the FP and 5-FUci treatments were small. Moreover, there were no significant differences in TTP between favorable patients and others either in the FP or 5-FUci treatment groups. These results suggest that the number of favorable phenotypes may be a prognostic marker in patients treated with FP but not a selective marker between FP and 5-FUci.

VEGF promotes angiogenesis and the permeability of blood vessels and is associated with microvessel counts 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 (+) and (-) 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 (+) showed significantly a 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 alone. It is suggested that additional cisplatin to 5-FUci might overcome the malignant potential of VEGF although relationship between VEGF and the chemoresponse to FP (17, 20) was not so clear in the present study.

In the phase III study (JCOG9205) (11), FP treatment showed no survival benefit over treatment with 5-FUci, although the response rate and TTP after FP treatment was significantly better than after 5-FUci. This study showed that in the subset of patients with VEGF (-), 5-FUci treatment produced slightly longer survival times than FP, and the TTPs were identical. In contrast, in the subset of patients with VEGF (+), both survival and TTP of the patients treated with FP were longer than those with 5-FUci. These results suggest that VEGF

might be a selective marker between FP and 5-FUci.

In conclusion, the number of favorable phenotypes (>= 4 or <= 3) out of the markers VEGF (+), p53 (-), bcl-2 (-), TS (-), and GST- π (-) was a prognostic factor in advanced gastric cancer treated with FP. VEGF might be a selective marker to choose FP or 5-FUci. Although the methodology for evaluating biological markers in this paper might be immature compared to the present one such as microarray or proteomics, we could learn some lessens from the present study; 1) not a single markers cannot be prognostic and investigation of many markers is necessary especially for cytotoxic agents, 2) confirmation should be indispensable, 3) comparison in the phase III study is necessary to clarify the utility for selecting treatments. In near future, a clinical study investigating the usefulness of biological markers to select an appropriate chemotherapy is warranted.

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Table I　Patient Characteristics

		5-FUci (n=65)	FP (n=66)	p value
Age	Median (range)	63 (34-73)	63 (19-75)	p=0.6065
Gender	M/F	49/16	48/18	p=0.7257
Performance status (ECOG scale)	0/ 1/ 2/ unknown	34/18/11/2	24/35/5/2	p=0.0242
Macroscopic type	Expansive/ Infiltrative/ Others	15/48/2	11/53/2	p=0.6520
Histological type	Intestinal/ Diffuse/ Unknown	29/31/5	32/29/5	p=0.9019
Tumor extent	Locally advanced/ Ascites/ Metastatic	15/33/17	17/39/10	p=0.1196
Resection of the primary tumor	+/-	17/48	16/50	p=0.8011

The source of the subjects was a phase III study of Japan Clinical Oncology Group (JCOG9205), continuous infusion of 5-fluorouracil (5-FUci) v.s. a combination of 5-FU and cisplatin (CDDP). Pre-treatment biopsy were available in 65 patients treated with 5-FUci and 66 patients with FP.

Table II Expression of Biological Markers 5-FUci and FP

		5-FUci n=65	FP n=66
VEGF	(+)	30 (49)	32 (47)
	(-)	35 (51)	34 (53)
TS	(+)	37 (57)	21 (32)
	(-)	28 (43)	45 (68)
p53	(+)	28 (43)	28 (42)
	(-)	37 (57)	38 (58)
GST-π	(+)	38 (58)	41 (62)
	(-)	27 (42)	25 (38)
Bcl-2	(+)	7 (11)	11 (17)
	(-)	58 (89)	55 (83)

Expressions of vascular endothelial growth factor (VEGF), thynidylate synthase (TS), p53, Gluthathione S-transferase π (GST- π) and bcl-2 were examined immunohistochemistry.

Table III Expression of Biological Markers and Responses to 5-FUci and FP

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

Expressions of vascular endothelial growth factor (VEGF), thynidylate synthase (TS), p53, gluthathione S-transferase π (GST- π) and bcl-2 were examined immunohistochically. 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.

Figure 1: Survival curves of patients treated with 5-FUci and FP

Overall survival of 66 patients treated with FP (—) and 65 treated with 5-FUci (⋮⋮).

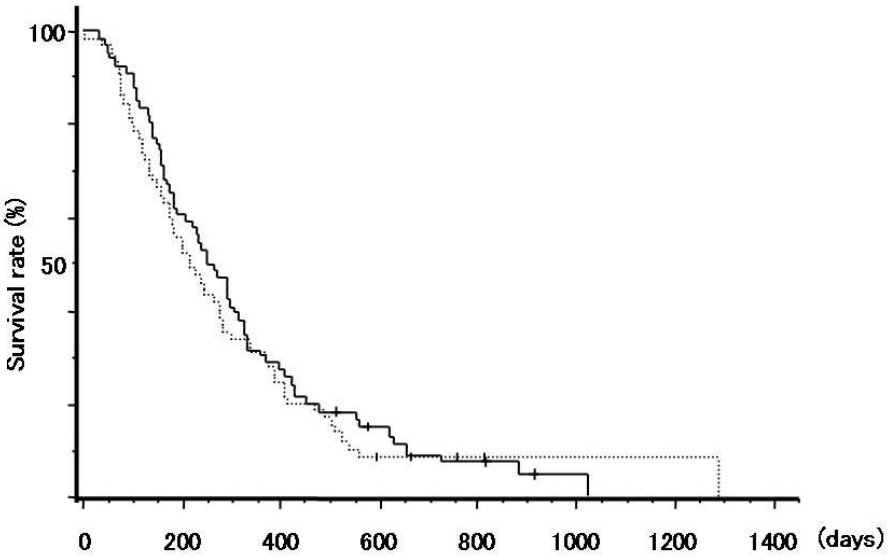

Figure 2: Number of favorable phenotypes and survival

Overall survival of patients (—) with 4 or 5 favorable phenotypes out of VEGF (+), TS (-), p53 (-), bcl-2 (-), GST- π (-), and those (·····) with 3 or fewer, after treatment with FP (A) or 5-FUci (B).

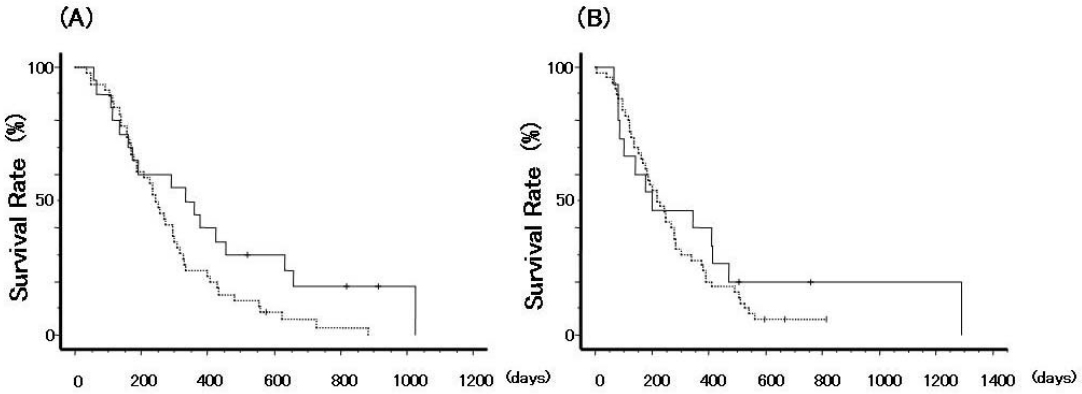

Figure 3: Expression of VEGF and survival

Overall survival of patients (—) with VEGF (+), and those (·····) with VEGF (-) after treatment with FP (A) or 5-FUci (B).