

Clinical Application of Immunoreactivity of Dihydropyrimidine Dehydrogenase (DPD) in Gastric Scirrhus Carcinoma Treated with S-1, a New DPD Inhibitory Fluoropyrimidine

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Abstract. *Background:* A highly specific antibody against recombinant human dihydropyrimidine dehydrogenase (DPD) has been developed to immunohistochemically assess DPD expression in tumors. A new oral DPD inhibitory fluoropyrimidine (DIF), S-1, is reportedly effective against gastric scirrhus carcinoma. *Patients and Methods:* In this study, the relationship between immunoreactivity to DPD in biopsy specimens and the effects of chemotherapy were investigated in 61 patients treated with first-line fluoropyrimidine-based chemotherapy (S-1:DIF, 5-FU:non-DIF) for gastric scirrhus carcinoma. *Results:* The response rate was significantly higher in patients with DPD-positive tumors than in those with DPD-negative tumors in the S-1 group (45.5%, 10.0% : $p < 0.05$), as compared to the 5-FU group (0%, 5.6%: $p = 0.398$). According to the median survival time, there was no significant difference between patients with DPD-positive tumors (364 days) and those with DPD-negative tumors (406 days; $p = 0.626$) in either the S-1 group or the 5-FU group (181 days and 256 days, respectively; $p = 0.543$). *Conclusion:* This study indicates that S-1 may be effective even in gastric scirrhus carcinoma with a high level of DPD activity.

Borrmann-type-4 gastric cancer, clinically synonymous with gastric scirrhus carcinoma, is generally resistant to systemic chemotherapy. This type of gastric cancer is characterized by diffuse malignant lesions with indistinct borders, and is usually diagnosed at a very advanced stage. High rates of lymph node metastasis, invasion of neighboring structures

and peritoneal dissemination pose a great challenge for medical care. The outcome is usually poor, with 5-year survival rates ranging from 0% to 20% (1). Although most gastric scirrhus carcinomas are resistant to conventional 5-fluorouracil (5-FU)-based regimens, several recent case studies have reported a good response to S-1 (2, 3) Many studies have demonstrated that dihydropyrimidine dehydrogenase (DPD) is a biomarker for response in patients treated with 5-FU-based chemotherapy (4-7). DPD is a rate-limiting enzyme in the metabolism of 5-FU, and its expression by tumors is thought to attenuate the response to 5-FU (8-10). Since more than 80% of the administered dose of 5-FU is degraded in the liver by DPD to fluorinated β -alanine, the level of DPD activity is also a major determinant of 5-FU toxicity (11).

Recently, encouraging clinical results have led to the development of a new generation of oral fluoropyrimidines, commonly referred to as DPD inhibitory fluoropyrimidines (DIF) (12, 13). S-1 is a combined preparation consisting of 1 M tegafur, 0.4 M 5-chloro-2,4-dihydroxypyridine (CDHP), and 1 M potassium oxonate (Oxo). CDHP is a potent inhibitor of DPD, approximately 180 times more active than uracil in inhibiting DPD *in vitro*, and maintains prolonged 5-FU concentrations in plasma and tumors (14-16) Oxo protects against 5-FU-induced gastrointestinal toxicity. Two phase II studies of S-1 monotherapy in patients with metastatic gastric cancer yielded response rates of about 50%, with minimal toxicity (17-19). S-1 is now used to treat advanced gastric cancer as a single agent or in combination with other anticancer agents, including cisplatin, CPT-11, paclitaxel and docetaxel (20).

A technique using highly specific antibodies against recombinant human DPD (rhDPD) has been developed to immunohistochemically assess DPD expression in tumors (21-23) and thereby predict the clinical response to 5-FU-based chemotherapy. Several studies have examined the relationship between the DPD immunoreactivity of tumors

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and the response to oral fluoropyrimidines, but the clinical impact of DPD activity on response remains unclear for new drugs such as S-1, and there are no reports on the treatment of gastric scirrhous carcinoma. In this study, intra-tumoral levels of DPD were assessed immunohistochemically using anti-DPD polyclonal antibodies, and the relationship between the immunoreactivity of DPD and the antitumor effects of S-1 were investigated. We propose that S-1 might circumvent the resistance to 5-FU in gastric scirrhous carcinoma with a high level of DPD activity. Our aim was to clarify the differences between the antitumor activities and mechanisms of action of S-1 as a DIF and 5-FU as a non-DIF.

Patients and Methods

Patients. Sixty-one patients with Borrmann-type-4 gastric scirrhous carcinoma, who received S-1 or 5-FU as first-line chemotherapy at the National Cancer Center Hospital (Tokyo, Japan) between February 2000 and January 2003, were studied retrospectively. Thirty-one patients were given S-1 and 30 were given 5-FU. Tumor biopsy specimens were obtained from all patients before chemotherapy.

Treatment schedule and evaluation of response. S-1 was administered at a dose of 40 mg/m² of body surface area (BSA) twice daily in one of the following doses: 40 mg (BSA < 1.25 m²), 50 mg (1.25 m² ≤ BSA < 1.50 m²), or 60 mg (BSA ≥ 1.50 m²). S-1 was given for 28 consecutive days, followed by a 14-day rest period. This period was defined as one course of treatment. S-1 was purchased from Taiho Pharmaceutical Co., Ltd. (Tokyo, Japan) in the form of 20 and 25 mg capsules. 5-FU (800 mg/m²/day) was administered as a 5-day (120 h) intravenous continuous infusion, repeated every 28 days, comprising one course of treatment.

Both treatments were continued until tumor progression, unacceptable toxicity, or refusal by the patient to continue further therapy. The response of measurable target lesions to chemotherapy was objectively evaluated according to the WHO criteria after each course of treatment. The response of primary lesions was also evaluated according to the roentgenographic and endoscopic criteria proposed by the Japanese Research Society of Gastric Cancer for "c-lesions" (24). Complete response (CR) was defined as the disappearance of all invasive findings. Partial response (PR) was defined as a decrease of 50% or greater in the affected area on X-ray films after barium administration, obtained in the same position as that before treatment. Progressive disease (PD) was defined as a 25% or greater increase in lesions or the appearance of new lesions. Responses not falling into any of these categories were classified as stable disease (SD). The survival time was calculated from the start date of the first course of treatment to the date of death or to the final date of confirmed survival.

Immunohistochemical examination. DPD immunoreactivity in the tumor biopsy specimens was examined with the use of an anti-recombinant human DPD polyclonal antibody (diluted at 1:1000, The Second Cancer Laboratory, Taiho Pharmaceutical Co., Ltd., Saitama, Japan). The tissues were routinely fixed in 10% formalin and embedded in paraffin wax. Sections 3 μm thick were cut and mounted

Table I. Patient characteristics in both regimen (S-1 : DIF, 5-FU : non-DIF) groups.

| Characteristics | S-1 | 5-FU | p-value |
|---|--------------|--------------|---------|
| Total number of patients | 31 | 30 | |
| Age, years, median (range) | 53.7 (30-73) | 58.2 (39-70) | 0.387 |
| Gender (men/women) | 16/15 | 18/12 | 0.592 |
| ECOG performance status | | | |
| 0 | 10 | 4 | 0.214 |
| 1 | 20 | 22 | |
| 2 | 1 | 4 | |
| Histological type | | | |
| Intestinal type | 2 | 1 | 0.978 |
| Diffuse type | 29 | 29 | |
| Number of organs involved | | | |
| 1 | 8 | 12 | 0.151 |
| 2 | 17 | 11 | |
| 3 | 6 | 7 | |
| Site of metastatic disease | | | |
| Peritoneum | 29 | 16 | 0.117 |
| Distant lymph nodes | 17 | 20 | 0.672 |
| Liver | 9 | 12 | 0.867 |
| Lung | 2 | 2 | 0.978 |
| Others | 3 | 5 | 0.330 |
| Surgery (total gastrectomy) | | | |
| yes | 17 | 11 | 0.126 |
| no | 14 | 19 | |
| Treatment duration, days, median (range) | 217 (27-767) | 76 (25-258) | 0.006 |
| Number of chemotherapy cycles, mean (range) | 5.0 (1-16) | 2.4 (1-5) | 0.045 |

on aminopropyltriethoxysilane-coated slides, and were deparaffinized with xylene and rehydrated in graded ethanol. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxidase in methanol for 30 min at room temperature. Representative specimens were evaluated by the following antigen retrieval procedure. Three types of soaking solutions were employed: 10 mM citrate buffer, pH 6.0, 10 mM citrate buffer, pH 7.0 and 1mM EDTA solution, pH 8.0. After pressure cooking, the sections were left at room temperature for 30 min. The sections were incubated with polyclonal antibody against DPD overnight at room temperature. The specificity of this antibody has been reported previously (21). After rinsing in phosphate-buffered saline (PBS), pH 7.2, the sections were incubated with universal immunoperoxidase polymer, anti-mouse and rabbit (Histofine Simple Stain MAX PO, Nichirei, Tokyo, Japan), at room temperature for 30 min. The reaction products were visualized in 50 mM Tris-HCl buffer, pH 7.6, containing 50 mg/dl diaminobenzidine tetrahydrochloride and 0.006% hydrogen peroxidase. The nuclei were lightly counterstained with Mayer's hematoxylin, and the specificity of immunostaining with the polyclonal antibody was checked by preabsorption experiments using representative samples. Before immunostaining, the diluted antibody was combined with recombinant human DPD (Taiho Pharmaceutical Co., Ltd.) at final concentrations of 0.01, 0.1, 1.0 and 10 μg/ml, at 37°C for 1 h. As a positive control, we employed tumor tissue obtained from a xenograft of the human pancreatic cancer cell line MIA PaCa-2 in nude mice, established to have high DPD expression. Negative

controls were prepared by substituting PBS for the primary antibody (Rabbit Immunoglobulin Fraction: DAKO ENVISION). The slides were counterstained with hematoxylin.

Evaluation of immunostaining. Immunohistochemical staining intensity was semiquantitatively graded (- to 3+) on the basis of the proportion of positively-stained cancer cells in the lesions: -, negative; 1+, less than 1/3 of cancer cells positive; 2+, from 1/3 to less than 2/3 of cancer cells positive; 3+, 2/3 or more of cancer cells positive. A staining intensity of - to 1+ was considered negative, and that of 2+ to 3+ was considered positive. Immunohistochemical staining was evaluated independently by four investigators blinded to clinical outcomes. Any disagreement was resolved by consensus.

Statistical analysis. The statistical significance of the relationships of DPD immunoreactivity and TS immunoreactivity to the patients' responses to chemotherapy was evaluated with χ^2 -tests. Survival curves were calculated with the Kaplan-Meier method and analyzed with the use of log-rank tests.

Results

Patients' characteristics. The patients' characteristics are provided in Table I. Thirty-four men and 27 women, with a median age of 55 years (range, 30-73 years) were included. Fifty-six patients (91.8 %) had a performance status of 0 or 1 on the Eastern Cooperative Oncology Group scale, and all patients received S-1 or 5-FU chemotherapy as first-line treatment, including preoperative neoadjuvant chemotherapy.

DPD immunoreactivity. DPD immunoreactivity was diffusely distributed in the cytoplasm of tumor cells, with some differences in staining intensity within a given tumor. All grading patterns of DPD immunoreactivity using anti-recombinant human DPD polyclonal antibody are shown in Figure 1.

Immunoreactivity and response to chemotherapy. The overall response rate was 22.6% (7/31) in the S-1 group and 3.3% (1/30) in the 5-FU group. Positive rates for DPD were, respectively, 35.5% (11/31) in the S-1 group and 40.0% (12/30) in the 5-FU group. Response rates were 45.5% (5/11) in patients with DPD-positive tumors and 10% (2/20) in those with DPD-negative tumors ($p=0.044$) in the S-1 group, as compared with 0% (0/12) and 5.6 % (1/18) ($p=0.398$), respectively, in the 5-FU group.

Relationship between survival and DPD activity. The median survival time of all patients was 340 days (S-1: 393 days, 5-FU: 226 days). The median survival times were 364 days in patients with DPD-positive tumors and 406 days in those with DPD-negative tumors in the S-1 group ($p=0.626$), as compared with 181 days and 256 days, respectively, in the 5-FU group ($p=0.543$). The median survival time did not

differ significantly between patients with DPD-positive tumors and those with DPD-negative tumors in either treatment group.

Discussion

Our study indicates that S-1 may be effective in the treatment of gastric scirrhou carcinoma with higher DPD activity. Several studies focusing on human cancer cell lines have suggested that intratumoral DPD levels, assessed on the basis of either enzymatic activity or mRNA expression, are good predictors of the response to 5-FU-based chemotherapy (25-27). Previous studies have also shown that inhibition of intratumoral DPD increases sensitivity to 5-FU, and that thymidylate synthase (TS) overexpression plays a major role in the resistance. Here, we focused on the antitumor effect of S-1 as a newly-developed DIF, and examined the correlation with a DIF antitumor effect and a biomarker (DPD). Immunohistochemical analysis has several important advantages over measuring protein and mRNA levels, since it is labor-saving, low-cost and can be used for tissue specimens fixed in formalin. We believe that it would be valuable to establish a simple and reliable method to assess DPD expression in biopsy specimens, since this is the only available material capable of providing information on the biological properties of tumors before chemotherapy. Antibodies against DPD have recently become available for immunohistochemical analysis, and studies have shown that DPD immunoreactivity correlates with DPD activity and the level of mRNA expression in cancer tissue. Cancer cells that express higher levels of DPD are considered more resistant to 5-FU and may be unresponsive to chemotherapy. However, our findings suggest that S-1 may be effective against gastric scirrhou carcinoma with higher DPD activity. Although there was no significant difference in median survival time between DPD-positive patients and -negative patients in the S-1 group ($p=0.626$) as compared with those of the 5-FU group ($p=0.528$), S-1 showed a higher response rate in tumors with a high DPD activity ($p<0.05$). These results indicates that S-1 could be more effective in gastric scirrhou carcinoma patients resistant to 5-FU only and with high DPD activity. One remarkable point was that all patients who responded to S-1 in the DPD-positive group showed shrinkage of primary lesions. Although DPD has been documented as an important determinant of chemosensitivity to 5-FU, most previous studies have found that the levels of DPD mRNA, protein and activity in tumors are unrelated to outcome. Our results, which showed no correlation of the DPD score in biopsy specimens with survival or time to progression, are in agreement with these findings.

In tumors with low DPD activity, inhibition of DPD by CDHP did not enhance cytotoxicity, even if tumor DPD activity was further reduced. In contrast, maximum

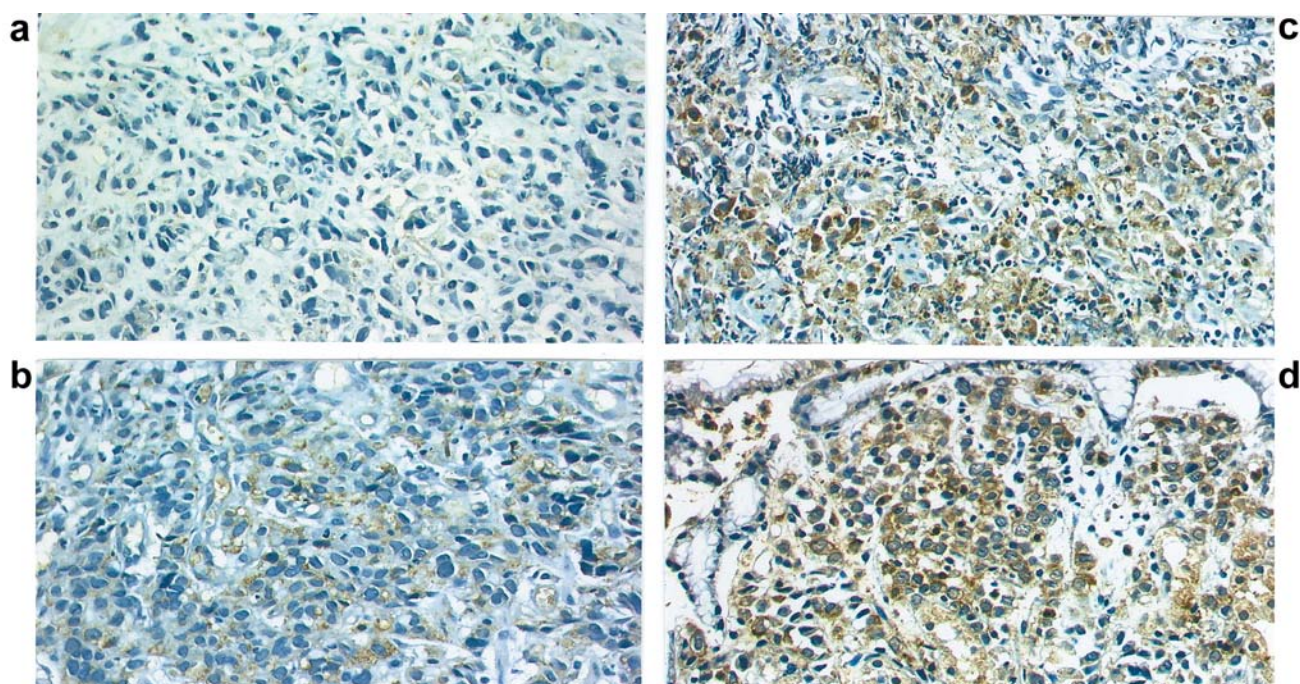


Figure 1. All grading pattern of immunohistochemical staining for DPD with polyclonal antibody (x400 magnification). Positive staining for DPD is observed in the cytoplasm of cancer cells, with some differences in staining intensity within a given tumor. (a. DPD -, b. DPD 1+, c. DPD 2+, d. DPD 3+). A staining intensity of - to 1+ was considered negative, and that of 2+ to 3+ was considered positive.

enhancement of the antitumor effect of S-1 would be expected in patients whose tumors have high DPD activity (28). Although the proportion of intratumoral DPD activity inhibited by CDHP is not clinically known, S-1 is expected to show antitumor effects, regardless of the status of intratumoral DPD. Similar to our results, several recent case studies have reported that S-1 is associated with shrinkage of primary lesions of Borrmann-type-4 gastric scirrhous carcinoma (2, 3). Although the mechanism of the response of primary lesions to S-1 remains unclear, strong inhibition of DPD, resulting in prolonged active concentrations of 5-FU in plasma and tumors, may be responsible for the shrinkage of these lesions.

In conclusion, our results suggest that S-1 may be effective against gastric scirrhous carcinoma, even in tumors with high levels of DPD activity. The relationship between DPD and the clinical response to other chemotherapeutic regimens should be investigated to determine whether intra-tumoral DPD activity is useful for selecting the best suited chemotherapeutic regimen. Further immunohistochemical studies on DPD with larger numbers of patients will hopefully contribute to the development of tailor-made DIF-based regimens designed to optimize response.

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