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Expression of Dihydropyrimidine Dehydrogenase in Cancer Cells but Not in Stromal Cells Predicts the Efficacy of Fluorouracil Treatment in Patients with Gastric Carcinoma

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Abstract. *Background: It is not known whether immunohistochemical quantification of dihydropyrimidine dehydrogenase (DPD) in cancer cells, stromal mononuclear cells and normal glands predicts the efficacy of fluorouracil (FU) derivatives in patients with T3 gastric adenocarcinoma. Materials and Methods: The levels of DPD in cancer cells, stromal cells and normal glands were measured immunohistochemically in 111 patients with T3 gastric carcinoma. Adjuvant chemotherapy with oral UFT (uracil/tegafur[4:1]) was administered to 95 patients for more than 1 year after surgery. Results: Forty-two (37.8%) patients demonstrated high DPD expression in the cytoplasm of their cancer cells. In patients with low DPD expression in cancer cells, the 5-year survival rates were 64.5% in patients given FU and 42.8% in those not given FU (p=0.014). Neither stromal cells nor normal glands affected the efficacy of FU treatment in relation to their DPD expression. Conclusion: DPD expression in cancer cells but not in stromal cells could be a predictor of the efficacy of FU chemotherapy in patients with T3 gastric carcinoma.*

Dihydropyrimidine dehydrogenase (DPD) is known as the first and rate-limiting enzyme in the catabolism of fluorouracil (FU) derivatives (1,2). 5-FU is one of the most useful drugs currently administered against gastrointestinal carcinomas, although the response rate as a single agent is only 10-20% (3). There are several reports concerning the relationship between DPD expression and prediction of FU chemosensitivity. Okabe *et al.* have suggested that immunohistochemical detection of tumoral DPD expression may be a means of predicting the clinical response to FU-based chemotherapy (4-6). DPD activity and the expression of DPD messenger RNA (mRNA) were shown to correlate with FU chemosensitivity (7-9). However, the optimal methodology for quantification of DPD expression remains controversial (10). Recently, Takechi *et al.* reported that DPD mRNA levels did not correlate with DPD protein expression (11).

DPD activity in human peripheral blood mononuclear cells (PBMC) or hepatic tissues is higher than that in cancerous tissues (12-14). Since 5-FU clearance is mainly correlated with DPD activity in PBMC and hepatic tissues, it is possible that DPD activity in the stromal mononuclear cells and normal glands may affect FU chemosensitivity. Guimbaud *et al.* demonstrated that DPD activity in colorectal carcinoma did not differ from that of normal colorectal tissue (15). On the contrary, Uetake *et al.* showed that the mRNA levels of DPD in normal colorectal mucosa was higher than that observed in tumor tissues (16). Previous reports have been documented based on the DPD activity or mRNA levels of tumor samples containing tumor cells, infiltrating mononuclear cells and sometimes cells from normal glands. However, there has been no precise examination of DPD expression in stromal mononuclear cells and normal glands.

Furthermore, the intratumoral levels of expression of the mRNA for the FU target enzyme TS are also predictors of the sensitivity of gastric carcinoma to FU-based chemotheapy (17). Salonga *et al.* reported that the colorectal tumors responding to 5-FU therapy had low expression values of DPD, TS and thymidine phosphorylase (6). However, little is known about the expression of DPD and TS in relation to the efficacy of FU for patients with gastric carcinoma. Immunohistochemistry seems to be the best method of identifying cancer cells, stromal cells and cells from normal glands in order to evaluate the prognostic significance of each factor. Therefore, we investigated the

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Key Words: 5-Fluorouracil, adjuvant chemotherapy, postoperative survival, dihydropyrimidine dehydrogenase, gastric carcinoma.

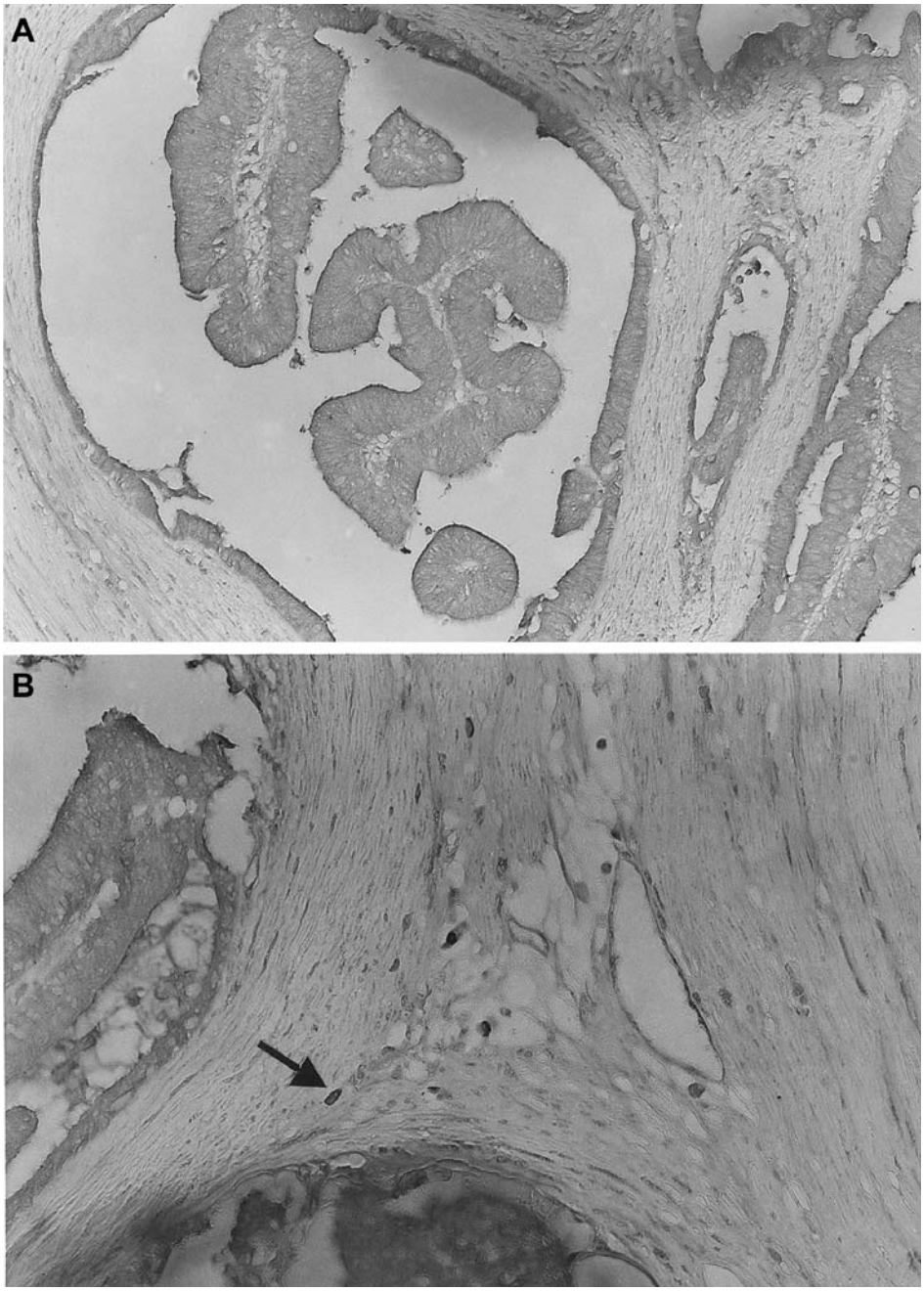

Figure 1. *DPD immunoreactivity in a paraffin-embedded section of gastric carcinoma:(A) DPD was well stained in the cytoplasm of gastric cancer cells, (B) DPD was well stained in the cytoplasm of mononuclear cells infiltrating into the stroma (arrow).*

expression of DPD in cancer cells, stromal mononuclear cells and cells from normal glands, using anti-recombinant human DPD polyclonal antibodies in patients with T3 gastric carcinomas, to clarify the effect on survival of patients in relation to postoperative FU chemotherapy. Then we performed a combined analysis of the effects of DPD and TS on FU chemosensitivity.

Materials and Methods

Clinical samples. One hundred and eleven patients with T3 gastric carcinoma, who underwent curative gastrectomy at our department from 1986 to 1992, were included in this study. All specimens obtained from these patients were histologically diagnosed as adenocarcinomas. Chemotherapy or radiotherapy was not carried out prior to surgery. Histology was classified into two subtypes;

Table I. *Relationship between the expression of DPD in cancer cells and clinicopathological factors.*

	Number of patients	DPD status High	DPD status Low	*p*-value
All patients	111	42	69	
Sex				
Male	69	26	43	
Female	42	16	26	0.999
Age				
<60	51	16	35	
>60	60	26	34	0.242
Tumor size (cm)				
<8.0	62	23	39	
>8.0	49	19	30	0.999
Histology				
Differentiated	31	16	15	
Undifferentiated	80	26	54	0.081
Lymph node metastases				
Positive	54	23	31	
Negative	57	19	38	0.334
Ly				
Positive	77	31	46	
Negative	34	11	23	0.525
V				
Positive	77	28	49	
Negative	34	14	20	0.674
TS				
Positive	29	16	13	
Negative	82	26	56	0.043

Table II. *Relationship between the expression of DPD in stromal mononuclear cells and clinicopathological factors.*

	Number of patients	DPD status Diffuse	DPD status Limitted	*p*-value
All patients	111	26	85	
Sex				
Male	69	17	52	
Female	42	9	33	0.818
Age				
<60	51	10	41	
>60	60	16	44	0.501
Tumor size (cm)				
<8.0	62	15	47	
>8.0	49	11	38	0.999
Histology				
Differentiated	31	9	22	
Undifferentiated	80	17	63	0.455
Lymph node metastases				
Positive	54	13	41	
Negative	57	13	44	0.999
Ly				
Positive	77	22	55	
Negative	34	4	30	0.080
V				
Positive	77	14	63	
Negative	34	12	22	0.056
TS				
Positive	29	10	19	
Negative	82	16	66	0.127

papillary and tubular adenocarcinomas were classified as differentiated, while poorly-differentiated and signet ring cell carcinomas were classified as undifferentiated. Ninety-five of the cases were treated by UFT (uracil/tegafur [4:1]) 300 mg/body/day for over 1 year after surgery. The other patients refused postoperative chemotherapy.

Immunohistochemistry of DPD and TS. Deparaffinized sections were antigen- retrieved by microwaving the sections for 15 minutes. Endogenous peroxidase activity was blocked by soaking the sections in 0.3% hydrogen peroxide in methanol for 20 minutes. After washing with Dulbecco's phophate-buffered saline (PBS), the sections were placed in 10% normal horse serum for 20 minutes to reduce non-specific staining. Then, the sections were incubated with anti-recombinant human DPD polyclonal antibodies (14) overnight at 4˚C in moist chambers. TS expression was studied immunohistochemically using a polyclonal antibody for human recombinant TS (Second Cancer Research Laboratory, Taiho Pharmaceutical Co. Ltd., Saitama, Japan) (18). Following antigen retrieval and blocking of endogeneous peroxidase activity, the sections were incubated with the aforementioned TS primary antibody (dilution, 1:200) at room temperature for 24 hours. In both stainings of DPD and TS, the sections were incubated with DAKO ENVISION+® (Dako Japan, Kyoto, Japan) at room temperature for 60 minutes. ENVISION+®-labeled polymer reagent is a peroxidase-labeled polymer conjugated to goat anti-rabbit and goat anti-mouse immunoglobulins in a Tris-HCl buffer containing carrier protein and an anti-microbial agent. The immunochemical reaction was revealed with a solution of 3.3'-diaminobenzidine tetrahydrochloride in a 50 mM Tris buffer (pH 7.6) containing 10 μL of 30% hydrogen peroxide. The reaction was stopped after 15 minutes by the addition of tap water. The sections were then briefly counterstained with methyl green and mounted.

Statistical methods. The immunoreactivity of DPD in cancer cells was evaluated by the percentage of stained cells as compared to the total number of tumor cells. Patients were classified into 2 groups: low expression (less than 5%) and high expression (above 5%). DPD expression in stromal mononuclear cells was assigned as either diffuse or limited (focal or no staining). The DPD staining in normal glands adjacent to the tumor was judged as positive or negative. TS immunoreactivity was evaluated by the ratio of stained cells to total tumor cells and reactivity was classified as either low expression (less than 10%) or high expression (above 10%). The intensity of immunoreactivity in cancer cells, stromal cells and normal glandular cells was not evaluated in this study.

The overall survival was estimated by the Kaplan-Meier method; death by all causes was included and statistical differences were analyzed by the Wilcoxon test. The relationship between the expression of DPD and the clinicopathological characteristics of patients with T3 gastric carcinoma was analyzed by the Chi-squared test. Associations were considered significant if the *p*-value was less than 0.05.

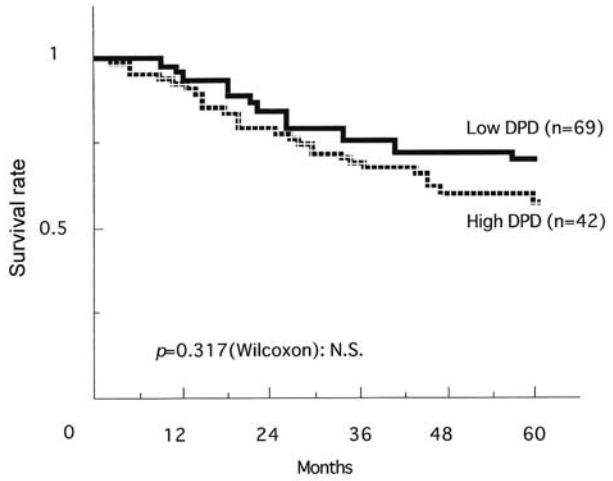

Figure 2. *Survival curves of patients with T3 gastric carcinoma in relation to DPD expression. There was no significant correlation between DPD expression levels and patient survival (p=0.317).*

Results

Immunohistochemistry of DPD. DPD immunoreactivity was detected in the cytoplasm of cancer cells (Figure 1A). High DPD expression was found in 42 (37.8%) cases. Table I shows the characteristics of patients as regards DPD expression in cancer cells. There was no relationship between DPD expression and gender, age, tumor size, lymph node metastasis, lymphatic involvement, or venous involvement. However, TS expression significantly correlated with DPD expression ($p<0.05$).

Numerous mononuclear cells infiltrating into the cancer stroma expressed DPD in some cases (Figure 1b). Diffuse DPD expression was found in 26 (23.4%) cases, while limited expression was observed in the remaining 85 cases (Table II). There was no significant correlation between DPD expression in stromal cells and clinicopathological factors. Neither TS nor DPD expression in the cancer cells correlated with DPD expression in stromal cells. However, carcinomas with diffuse DPD expression tended to accompany lymphatic or venous involvement.

Samples from normal gastric glands revealed DPD immunoreactivity in 33 cases (29.7%). Immunoreactivity was usually detected in the basal layer. There was no significant correlation between DPD expression in normal glandular tissue and clinicopathological factors (data not shown). However, there was a significant correlation between DPD levels in stromal cells and cells from normal glands. Diffuse DPD expression in stromal cells was found in 20 (60.6%) out of 33 cases, with positive DPD expression in the normal gland, whereas there were only 6 (7.7%) cases of diffuse expression in 78 cases with negative DPD expression in the normal gland ($p<0.01$).

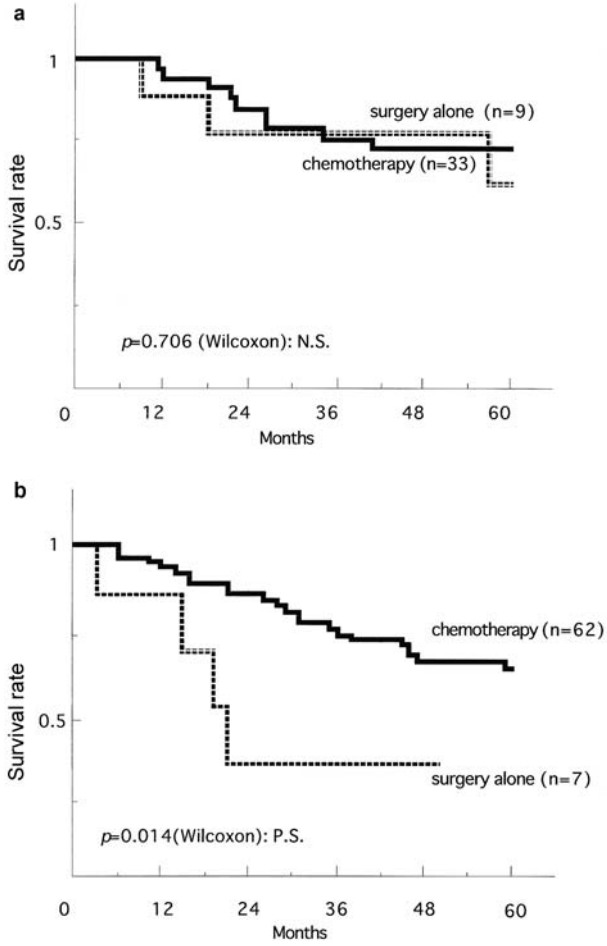

Figure 3. *Survival curves of patients with cancer cells displaying (a) high DPD expression and (b) low DPD expression. When tumors displayed low DPD expression, patients given FU survived significantly longer than those who were not given FU (p=0.014).*

DPD expression in cancer cells. The survival of patients with T3 gastric carcinoma is demonstrated in Figure 2 as it relates to DPD expression in cancer cells . There was no significant correlation between the DPD expression and the survival of patients ($p=0.317$). The efficacy of FU treatment on the survival of patients is shown in Figure 3. In patients with high DPD expression in cancer cells, no significant difference was observed as regards the survival of patients given FU and those not given FU (Figure 3a). However, patients with low DPD expression in cancer cells treated by FU survived longer than those not treated by FU ($p=0.014$). The 5-year survival rates were 64.5% and 42.8% in patients given FU and in those not given FU, respectively (Figure 3b). However, in patients given

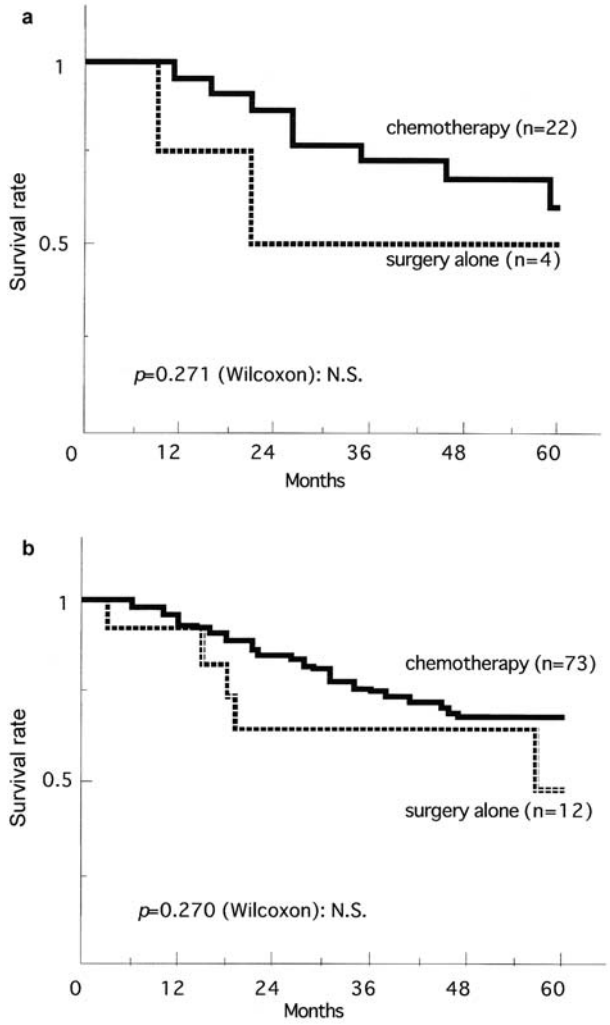

Figure 4. *Survival curves of patients with stromal mononuclear cells displaying (a) diffuse DPD expression and (b) limited DPD expression. FU treatment did not affect the survival of patients, irrespective of DPD expression of stromal cells.*

FU, no significant survival difference was noted between the groups with high DPD expression and low DPD expression.

DPD expression in stromal mononuclear cells. The relationship between DPD expression in mononuclear cells infiltrating into the cancer stroma and patient survival was analyzed. The 5-year survival rates were 63.6% in those with diffuse DPD expression and 68.5% in those with limited DPD expression. There was no significant difference between these two groups. Figure 4 shows the survival curves of patients with stromal mononuclear cells displaying (a) diffuse DPD expression and (b) limited DPD expression. Irrespective of DPD expression in stromal cells, patients treated with FU showed better survival rates than those not treated with FU. However, there was no significant difference.

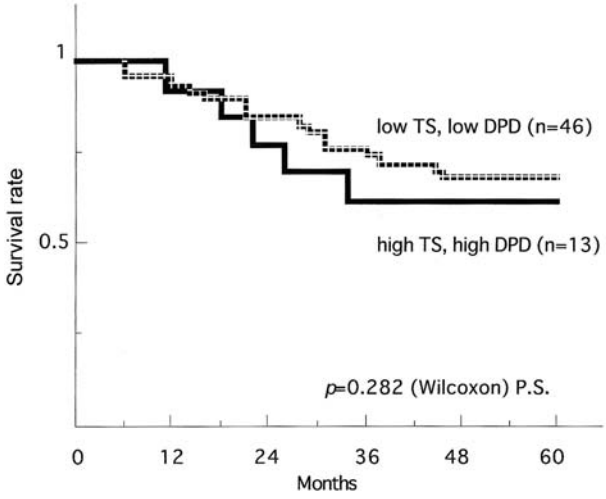

Figure 5. *Kaplan-Meier survival curves of FU- treated patients with cancer cells displaying low DPD / low TS immunoreactivity and high DPD / high TS immunoreactivity (p=282). There was no significant difference in survial between the groups.*

TS expression. In patients given FU treatment, survival curves were obtained by DPD expression and TS expression (Figure 5). Patients with low TS and low DPD tumors showed a better survival rate than those with high TS and high DPD tumors, although the difference was not significant. Survival curves of those with high TS and low DPD tumors and those with low TS and high DPD tumors were placed between the survival curves of those with high TS and high DPD tumors and those with low TS and low DPD tumors.

Discussion

There have been several reports that DPD activity or expression of DPD mRNA correlate with chemosensitivity to FU (7-9). Salonga *et al.* have demonstrated that low expression of all three genes for DPD, TS and thymidine phosphorylase is a predictive factor of longer survival in patients with colorectal carcinoma who are treated by FU (6). However, to date, little data has been collected that would indicate a significant correlation between DPD activity and FU chemosensitivity in patients with gastric carcinoma. Nevertheless, in the current study, patients given FU survived significantly longer than those not given FU when their tumors demonstrated low DPD expression. On the other hand, there was no significant difference in survival among patients with high- DPD cancer cells. These findings are consistent with previous reports demonstrating an inverse correlation between DPD activity and the efficacy of FU (8,19,20). Renal and hepatic clearance of 5-FU result in minimal amounts of an administered dose being available for

conversion to active metabolites; approximately 10%-20% is excreted unchanged in the urine and over 80% is catabolised by DPD, predominantly in the liver (1). Response rates to 5-FU therapy remain relatively low, at least in part due to the narrow therapeutic index and non-linear kinetics of the drug, which make it difficult to achieve a clinical response without producing significant systemic toxicity. As DPD clearly plays a key role in 5-FU pharmacokinetics, the prospective determination of DPD activity may help to identify patients likely to respond to FU therapy.

It should be noted that, in the current study, DPD expression did not affect the clinical efficacy of FU. The combined analysis of DPD and TS did not reveal these two factors as determinants of FU chemosensitivity. The recent development of a number of DPD inhibitors provides an alternative approach that may allow improved pharmacokinetic control and effective dosing of FU-based therapies (19,21-23). In the current study, UFT, containing uracil, a DPD inhibitor, was administered to patients with gastric carcinoma. UFT is one of the most common FU prodrugs in Japan. Uracil reduces inactivation of 5-FU not only in tumors but also in normal tissue. Therefore, UFT may be useful for patients irrespective of DPD expression. The important role of DPD and TS was reported in 5-FU chemosensitivity to colorectal carcinomas (6). However, DPD inhibitors may reduce the necessity to measure DPD levels prior to treatment.

We investigated DPD expression, not only in gastric cancer cells but also in stromal mononuclear cells and normal glandular cells. In previous studies, the enzyme activity and mRNA levels of DPD were measured in biopsy specimens that included cancer cells and stromal tissues (5,7). Thus, both DPD expression in cancer cells and stromal mononuclear cells, and occasionally in normal glandular cells, may affect the efficacy of FU treatment. To our knowledge, there is no report about the relationship between DPD expression in cancer cells, stromal cells and normal glands in patients with gastric carcinoma. Our data revealed no significant relationship between DPD expression in cancer cells and stromal cells or normal glands. However, the DPD expression in stromal cells correlated with that in normal glands. There was no significant correlation between the DPD expression in stromal cells or normal glands and the observed clinicopathological characteristics. Therefore, DPD levels in stromal cells or normal glands do not appear to be significant for the efficacy of FU as compared with DPD levels in cancer cells.

In colorectal cancer, the levels of DPD are higher in synchronous liver metastases than primary lesions (24). The effectiveness of FU treatment for patients with metachronous liver metastases may be difficult to predict based on the determination of primary lesions alone. On the contrary, the effectiveness of adjuvant FU treatment for patients with low DPD gastric carcinoma suggested that micrometastases in lymph nodes, liver, lung or peritoneum may have similar DPD expression as that observed in primary lesions (25,26). There are many reports of trials on adjuvant chemotherapy for curatively resected gastric carcinoma in the West and in Japan (27,28). However, no beneficial effects have been observed. Therefore, an approach for improving therapy with FU, by identifying the biochemical response determinants of this drug, seems to be important. Since the current series represents a retrospective investigation, it still remains important to determine the efficacy of adjuvant chemotherapy for curatively resected gastric cancers by carefully designed randomized controlled trials.

In conclusion, the DPD levels in cancer cells may predict the efficacy of FU therapy for patients with gastric carcinoma treated by curative surgery. However, the predictive potential may be limited when DPD inhibitors are used in combination with 5-FU. Further investigation is required to find useful indicators of chemosensitivity in treatments involving 5-FU and DPD inhibitors.

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