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ORIGINAL ARTICLE

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Tumor dihydropyrimidine dehydrogenase expression is a useful marker in adjuvant therapy with oral fluoropyrimidines after curative resection of colorectal cancer

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Abstract *Purpose*: Dihydropyrimidine dehydrogenase (DPD) is the rate-limiting enzyme of 5-fluoropyrimidine (5-FU) catabolism. We examined whether tumor DPD expression is an effective marker in adjuvant therapy with oral fluoropyrimidines after curative resection of colorectal cancer. *Methods*: We studied 89 patients with stage II–III colorectal cancers who had undergone curative resections and received oral 5-FU-based adjuvant chemotherapy. The levels of DPD expression in tumor and normal colonic mucosa were measured by an enzyme-linked immunosorbent assay. In 53 tumor samples, DPD enzymatic activity was also analyzed in order to evaluate the relationship between DPD expression and enzymatic activity. *Results*: DPD expression significantly correlated with DPD enzymatic activity in these 53 tumors ($r = 0.56$; $P < 0.001$). DPD expression in the tumors was significantly lower than in normal mucosa (47.1 ± 30.8 and 56.4 ± 18.5 U/mg protein, respectively; $P < 0.05$). We designated the cut-off value of tumor DPD as its median value (46.0 U/mg protein). Patients with low DPD expression had longer disease-free intervals than those with high DPD expression according to univariate analysis ($P = 0.026$). In a multivariate analysis, low DPD expression was significantly and independently associated with better survival. *Conclusions*: Tumor DPD expression is a useful marker for use with adjuvant chemotherapy with oral fluoropyrimidines after curative resection of colorectal cancer.

Keywords Dihydropyrimidine dehydrogenase · Oral fluoropyrimidines · Colorectal cancer

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Introduction

5-Fluorouracil (5-FU) is one of the most widely used chemotherapeutic agents for colorectal cancer. More than 80% of an administered dose of 5-FU is eliminated by catabolism via dihydropyrimidine dehydrogenase (DPD, EC 1.3.12), the rate-limiting enzyme [5]. A previous in vitro analysis conducted on 19 human cancer cell lines revealed that DPD activity in tumor cells is significantly related to 5-FU sensitivity: the lower the DPD activity, the greater the 5-FU cytotoxicity [1]. This experimental result was confirmed in a human cancer xenograft model, further demonstrating the significant relationship between 5-FU efficacy and tumor DPD activity or expression [13]. Clinical confirmation of the predictive value of DPD has been provided by Salonga et al. [25], who reported that DPD mRNA in colorectal tumors is inversely associated with 5-FU responsiveness, as responding patients had lower DPD expression than nonresponding patients. These data suggest that DPD expression in tumors may be a determining factor for 5-FU responsiveness [19]. However, the significance of tumor DPD expression has not been evaluated with respect to adjuvant chemotherapy for colorectal cancers. Therefore, the aim of this study was to evaluate whether tumor DPD expression is a useful marker for guiding adjuvant therapy with oral fluoropyrimidines after curative resection of colorectal cancer.

Materials and methods

Patients

We studied 89 patients with stage II–III colorectal cancers who underwent "curability A" resections from February 1996 to December 1998 at the Division of Surgical Oncology, Department of Translational Medical Sciences, Nagasaki University Graduate School of Biomedical Sciences. The study did not include patients with either synchronous or metachronous multiple colorectal cancers. The American Joint Committee on Cancer Classification and stage grouping was used to classify the tumors [9]. "Curability A" resections were postoperatively defined according to the Japanese Classification of Colorectal Carcinoma [14]. Each tumor was histopathologically classified in accordance with the World Health Organization criteria [10]. The 89 patients included 42 patients with stage II cancer and 47 with stage III cancer. Of the 89 tumors, 21 were classified as well-differentiated adenocarcinomas, 65 were classified as moderately differentiated adenocarcinomas, and 3 were classified as poorly differentiated adenocarcinomas. None of the patients had received preoperative chemotherapy or radiotherapy. All patients received oral adjuvant chemotherapy that started 3 weeks after curative surgery and continued for 12 months (UFT 400 mg twice daily). The mean age of the patients was 61 years (range 29–74 years). Of the 89 patients, 46 were male and 43 were female, and 45 tumors were located in the colon and 44 tumors were located in the rectum. All of the patients underwent standard follow-up examinations, including laboratory testing every 3 months. Chest roentgenograms, computed tomography, and abdominal ultrasonography were performed every 6 months, while colonoscopies were performed annually. The median follow-up period was 48 months (minimum 4.2 months). Written informed consent was obtained from all patients.

Sample preparation

Frozen material from the primary tumors and normal colonic mucosa stored at −80°C was homogenized in a tenfold volume of 10 m*M* Tris-HCl buffer (pH 7.4) containing 15 m*M* NaCl, 1.5 m*M* $MgCl_2$, and 50 μ*M* potassium phosphate. Next, these homogenates were centrifuged at 105,000 *g* for 1 h at 4°C. The supernatant was stored at −80°C until used. The total protein concentration was analyzed using a Bio-Rad protein assay kit (Bio-Rad, Tokyo, Japan). A portion of the supernatant was dialyzed overnight at 4°C in a buffer containing 20 m*M* potassium phosphate (pH 8.0), 1 m*M* EDTA, and 1 m*M* 2-mercaptoethanol, before being stored at −80°C until assayed for DPD enzymatic activity.

DPD enzymatic activity assay

To confirm the correlation between DPD enzymatic activity and DPD expression, we measured DPD enzymatic activities in 53 tumor samples based upon the method described by Takechi et al. [27]. Briefly, aliquots of the prepared sample (25 μl) were mixed with 25 μl of enzyme reaction mixture (2 m*M* dithiothreitol, 5 m*M* $MgCl_2$, 20 μ*M* [6-^{14}C]5-FU, 100 μ*M* NADPH), incubated at 37°C for 30 min, and applied to thin-layer chromatography plates (silica gel 60 F254; Merck, Darmstadt, Germany). DPD activity was determined by measuring the sum of the dihydrofluorouracil and 2-fluoro-β-alanine products formed from [6-^{14}C]5-FU. The plates were developed with a mixture of ethanol and 1 *M* ammonium acetate (5:1, v/v), according to the method of Ikenaka et al. [12]. 5-FU and the catabolized products formed from 5-FU were visualized and quantified using an image analyzer (BAS-2000, Fujix, Tokyo).

Enzyme-linked immunosorbent assay for DPD

The DPD expression levels in 89 colorectal tumors and paired normal colonic mucosa specimens were measured by enzyme-linked immunosorbent assay (ELISA). The amount of DPD sandwiched with the two anti-DPD monoclonal antibodies (clone 4B9 and 3A5) was estimated by measuring its absorbance at 450 nm [20]. The measured amount of DPD was calibrated with that measured in the standard solutions [20].

Statistical analysis

Statistical analyses were performed using the computer program STATISTICA (StatSoft, Tulsa, Okla.). The degree of correlation between DPD expression and DPD enzymatic activity was determined using the least squares method. Wilcoxon's matched-pairs signed-ranks test was used to compare DPD expression in the carcinomas and in the normal mucosa. Three variables with continuous data—age, maximum tumor diameter, and DPD expression—were classified into two groups based upon the their median values (63 years, 4.8 cm, 46.0 U/mg protein, respectively). Categorical data were analyzed by the Chi-squared or Fisher's exact test. The definition of the end-point for survival analysis was postoperative recurrence. Survival was analyzed using the Kaplan-Meier method and differences between the survival curves were tested for significance using the log-rank test [16, 23]. Multivariate analysis was performed using Cox's proportional hazard regression model in order to assess the effects of different variables upon patient survival [4]. All tests were two-tailed and a *P*-value of less than 0.05 was considered significant.

Results

DPD enzymatic activity and DPD protein expression

In 53 colorectal carcinomas, the levels of DPD expression correlated significantly with the levels of DPD enzymatic activity (Fig. 1). The correlation coefficient between them was 0.56 (95% confidence interval, 0.34–0.72; $P < 0.001$). Because the DPD ELISA was less expensive and labor intensive to perform than DPD enzymatic activity assays, we determined DPD expression in 89 colorectal carcinomas and paired specimens of normal mucosa using ELISA. The mean value and standard deviation (SD) of DPD in carcinomas and normal colonic mucosa specimens were 47.1 ± 30.8 U/mg protein (range 3.3–275.3, median 46.0 U/mg protein) and 56.4 ± 18.5 U/mg protein (range 25.5–107.3, median 54.6 U/mg protein), respectively ($P < 0.05$). We designated the cut-off value of tumor DPD at 46.0 U/mg protein (median value of tumor DPD).

Clinicopathologic features and DPD expression

There were no differences between the high and low DPD expression groups with respect to the following categories: age, gender, tumor location, maximum tumor diameter, lymphatic involvement, and stage (Table 1). Well-differentiated adenocarcinomas and venous involvement were more frequent in the low DPD expression group than in the high DPD expression group (Table 1, $P < 0.05$).

Prognosis

During the follow-up period, 15 patients relapsed; specifically, 8 patients had hematogenous recurrences (7 in the liver and 1 in the lung), 3 had peritoneal recurrences, 3 had lymph node recurrences, and 1 had a local recurrence. Based upon univariate analysis, the patients with low DPD expression had a longer disease-free interval than those with high DPD expression (Fig. 2, Table 1). In order to avoid the problem of colinearity, variables such as N classification and lymphatic involvement were excluded from the multivariate analysis. Low DPD expression was found to be significantly and independently associated with better survival (Table 2).

Discussion

Based upon prospective studies, Japanese clinical oncologists have often administered oral 5-FU-based adjuvant chemotherapy to patients with stage II–III colorectal cancers after curative resection [17, 24]. Recently, Carmichael et al. [3] have reported that there are no significant differences in efficacy between intravenous chemotherapy with 5-FU/leucovorin and oral chemotherapy with UFT/leucovorin in metastatic colorectal cancer. Moreover, in the interim report of the National Surgical Adjuvant Breast and Bowel Project C-06, there were no differences in toxicity grade or spectrum between intravenous and oral adjuvant chemotherapy for stage II–III colorectal cancers [26]. In addition, patients prefer oral chemotherapy to intrave-

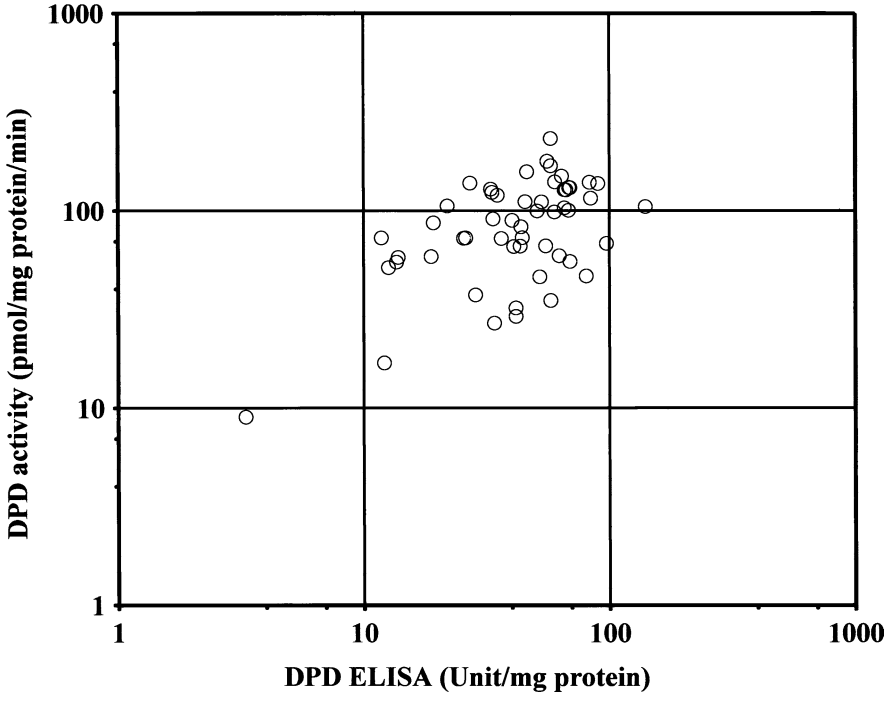

Fig. 1 Correlation between DPD expression and DPD enzymatic activity in 53 colorectal cancers. DPD expression shows a significant correlation with DPD enzymatic activity ($r = 0.56$; 95% confidence interval, 0.34–0.72; $P < 0.001$)

Table 1 Distribution of the clinicopathologic features and DPD expression groups, and prognostic variables for disease-free survival by univariate analysis

Variables	Number of patients		Univariate analysis	
	DPD low	DPD high	Hazard ratio (95% CI)	*P*-value
Age (years)				
<63	20	23	1	
≥63	24	22	0.39 (0.11–1.39)	0.15
Gender				
Female	24	19	1	
Male	20	26	1.29 (0.46–3.56)	0.63
Tumor location				
Colon	20	25	1	
Rectum	24	20	1.18 (0.43–3.26)	0.75
Maximum tumor diameter (cm)				
<4.8	18	26	1	
≥4.8	26	19	0.81 (0.29–2.24)	0.69
Histologic grade				
Well-differentiated adenocarcinoma	17	4	1	
Moderately/poorly differentiated adenocarcinoma	27	41	3.19 (0.72–14.21)	0.13
Lymphatic involvement				
Absent	1	4	1	
Present	43	41	1.32 (0.17–10.07)	0.79
Venous involvement				
Absent	3	13	1	
Present	41	32	2.30 (0.51–10.35)	0.28
Stage				
II (no lymph node metastasis)	22	20	1	
III (lymph node metastasis)	22	25	1.99 (0.68–5.82)	0.21
DPD expression				
Low			1	
High			7.69 (1.01–58.73)	0.05

nous chemotherapy [2]. Therefore, it is important to discover or develop an efficacious marker for tumor sensitivity to 5-FU-based oral adjuvant chemotherapy in colorectal cancer patients.

More than 80% of an administered dose of 5-FU is eliminated by catabolism through DPD, the rate-limiting enzyme [5]. The gold standard for estimating the tissue DPD level is measuring DPD enzymatic activity. However, DPD enzymatic activity assays using radioisotopes are expensive and labor-intensive, and thus difficult to use clinically. Therefore, in this study, we evaluated DPD protein expression and enzymatic activity simultaneously in 53 tumor samples. We found a significant correlation between these parameters, and

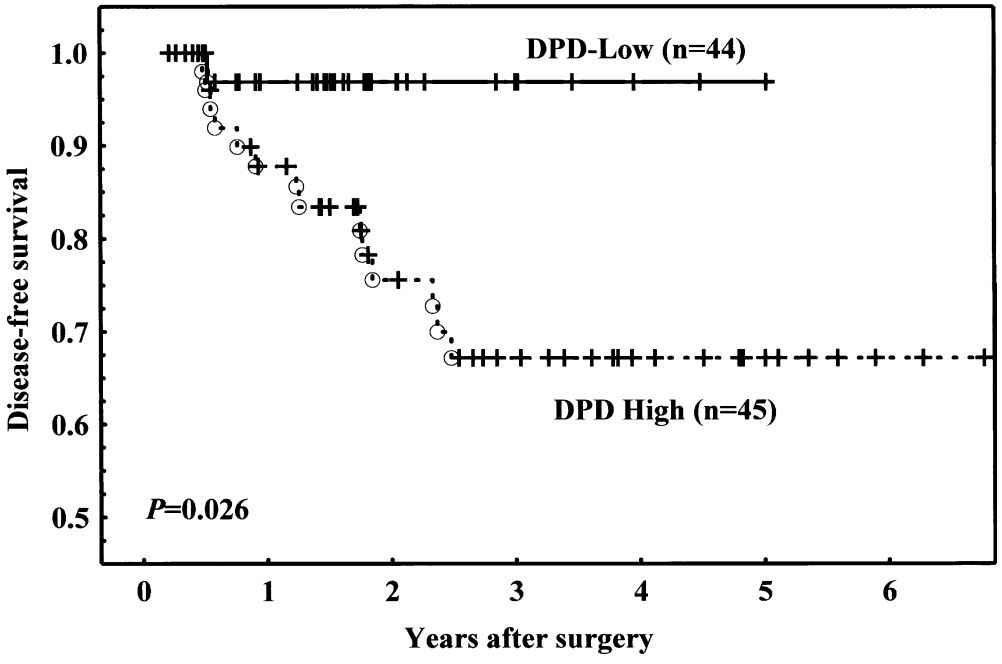

Fig. 2 Disease-free survival in 89 patients according to DPD expression

Table 2 Prognostic variables for disease-free survival by multivariate analysis

Variable	Multivariate analysis	
	Hazard ratio (95% CI)	*P*-value
Histologic grade		
Well-differentiated adenocarcinoma	1	
Moderately/poorly-differentiated adenocarcinoma	2.45 (0.54–11.10)	0.24
Venous involvement		
Absent	1	
Present	3.27 (0.72–14.85)	0.13
Stage		
II	1	
III	1.99 (0.68–5.86)	0.21
DPD expression		
Low	1	
High	8.05 (1.04–62.48)	0.05

further analyzed DPD levels using an ELISA. The findings are consistent with those of previous studies in which DPD levels were analyzed by enzymatic activity assays and ELISAs in 241 samples from various human tumors [20]. Moreover, an immunohistochemical study using DPD monoclonal antibodies revealed that DPD protein expression levels correlate with DPD enzymatic activities in human colorectal cancers [28].

In this study, DPD expression in colorectal carcinomas was significantly lower than its expression in normal colonic mucosa. McLeod et al. [18] have reported that DPD activity is higher in normal colonic mucosa than in colorectal carcinoma, and Johnston et al. [15] have reported that DPD mRNA levels are 37-fold lower in tumors than in adjacent normal colonic mucosa. The reason for this decreased tumor uracil catabolism remains unclear. The downregulation of DPD in tumors is in direct contrast to the overexpression of enzymes involved in the pyrimidine salvage pathway (thymidine kinase, uridine phosphorylase, and thymidine phosphorylase), which are observed in colorectal tumors but not in normal mucosa [22].

In some clinical studies, the usefulness of tumor DPD activity as a marker of response to 5-FU in cancer chemotherapy has been assessed. Etienne et al. [6] analyzed 52 head and neck cancer patients who received intravenous 5-FU-based chemotherapy, and found that complete responders exhibited lower tumor DPD activities than partial or nonresponding patients. Salonga et al. [25] measured tumor DPD mRNA expression in 33 pretreatment biopsies from advanced colorectal cancer patients who subsequently received treatment with intravenous 5-FU/leucovorin. The range of DPD mRNA expressions in those nonresponsive tumors were significantly broader than the range noted for responding tumors. These reports support our findings. Tumor DPD expression is a useful marker for guiding adjuvant chemotherapy with oral fluoropyrimidines after curative resection of colorectal cancer. On the other hand, in a prospective study of 103 colorectal cancer patients with liver metastases who received intravenous 5-FU-based chemotherapy, DPD activity in the liver metastases could not predict their clinical response (complete or partial response vs stabilization or progression of disease) to chemotherapy [8]. These differences may be due to differences in tissue origin of the samples used to estimate tumor DPD levels (primary tumor or liver metastasis). Etienne et al. also reported that DPD activity was significantly higher in liver metastases than in primary tumors, and DPD activities in liver metastases and primary tumors were not correlated [8].

Tanaka-Nozaki et al. measured 5-FU concentrations and DPD protein expressions in primary tumors from 40 patients with colorectal cancer who were preoperatively treated with doxifluridine (800–1200 mg/day) over a period of 14 days and during a preoperative period of 6–12 h [29]. They reported that the 5-FU concentrations in the tumors were inversely associated with tumor DPD protein levels. These results suggest that, after curative surgery, 5-FU concentrations in the latent tumor cells of micrometastases may be higher in patients with low DPD expression than in patients with high DPD expression.

Several factors are known to influence DPD activity. Harris et al. [11] demonstrated that DPD activity follows a circadian variability in rat livers (sevenfold) as well as in human blood lymphocytes (twofold). However, because of inherent collection difficulties, no such data exist for DPD activity in human tumor tissue. Yamashita et al. [30] reported that tumor DPD expression levels in 97 Dukes A–D stage colorectal cancers were significantly lower in female patients ($n = 34$) than in male patients ($n = 63$). In this study, we could not find a gender difference in tumor DPD expression levels (male 59.3 ± 47.7, female 46.7 ± 37.5 U/mg protein; $P = 0.13$, Mann-Whitney *U*-test). On the other hand, 5-FU clearance is not only influenced by DPD activity but also by liver function [7]. In this study, none of the patients had impaired liver function.

Although it is unknown whether there is a racial difference in tumor DPD expression, there is no racial difference in DPD activities in peripheral mononuclear cells [21]. Our results suggest that estimating DPD expression in tumors would provide useful information regarding oral adjuvant chemotherapy for colorectal cancer.

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