Immunohistochemical Dihydropyrimidine Dehydrogenase Expression is a Good Prognostic Indicator for Patients with Dukes' C Colorectal Cancer

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Abstract. *Background: Dihydropyrimidine dehydrogenase (DPD) is an enzyme that catabolizes 5-fluorouracil (5-FU), which is widely used for chemotherapy in patients with advanced colorectal cancer (CRC). However, the clinical importance of tumor dihydropyrimidine dehydrogenase (DPD) expression in patients with CRC treated with 5-FU remains unclear. Materials and Methods: We investigated DPD activities in normal mucosa (N) and tumors (T) by enzyme-linked immunosorbent assay (ELISA) in 64 surgically resected patients with Dukes' C CRC who were treated orally with postoperative adjuvant FU-based chemotherapy. We also immunohistochemically investigated DPD expression in these specimens. The clinicopathological importance of DPD activity and expression was evaluated in the patients. Results: Positive DPD expression was detected in 28 tumors (43.8%) and tumor DPD activity significantly correlated with tumor DPD immunoreactivity (p=0.0121). Further, tumor DPD activity and immunoreactivity also correlated with lymph node metastatic status (p=0.0409). The disease-free survival rate of patients with positive- tumor DPD expression was significantly worse than that of patients with negative-tumor DPD expression (39.3% vs. 72.2%, p=0.0127). However, DPD activity in tumors or normal mucosa did not correlate with patient prognosis. Tumor DPD expression appeared to be an important poor prognostic factor in patients with Dukes' C CRC by multivariate analysis (p=0.013). Conclusion: Immunohistochemical DPD expression in tumors is a useful prognostic parameter in patients with Dukes' C CRC treated with postoperative adjuvant FU-based chemotherapy.*

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5-Fluorouracil (5-FU) is one of the most frequently used chemotherapeutic agents in treatments for advanced colorectal cancer (CRC). However, the therapeutic effects of 5-FU differ among patients and response rates to 5-FU are reported to be only about 20% (1-4). Differences in the effectiveness of 5-FU chemotherapy might be based on differences in the sensitivities of carcinoma cells to 5-FU (5). 5-FU is converted to fluorodeoxyuridine-5'-monophosphate (FdUMP) by thymidine phosphorylase (TP) in tumors. FdUMP forms a stable, tight-binding ternary complex with thymidylate synthase (TS) and 5,10-methylene-tetra-hydrofolate. As a result, FdUMP prevents the methylation of deoxyuridine-5'-monophosphate (dUMP) to deoxy-thymidine-5'-monophosphate (dTMP) by TS and inhibits DNA synthesis (6). On the other hand, the majority of an administered dose of 5-FU is degraded to inactive metabolites (2-fluoro-ß-alanine), mainly in the liver or in the tumor by dihydropyrimidine dehydrogenase (DPD). High DPD activity and high messenger RNA (mRNA) expression level of DPD have been reported to correlate with the 5-FU chemoresistance of cancer cells *in vitro* and *in vivo* (7-15). However, the immunohistochemical DPD expression of CRC has been evaluated in just one paper (16). Moreover, we could not find any reports that examined the correlation between immunohistochemical DPD expression and disease-free survival in CRC patients treated with postoperative adjuvant FU-chemotherapy.

In this study, we analyzed DPD activities in tumors and in normal mucosa of colon and rectum by enzyme-linked immunosorbent assay (ELISA). As well, we analyzed DPD expression in tumors by immunohistochemistry. We also
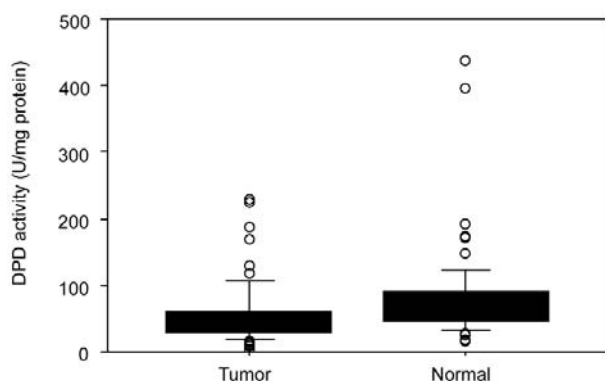
Figure 1. *DPD activities of 64 colorectal carcinomas (55.1±46.2 U/mg protein) are significantly lower than those of noncancerous colorectal mucosa (79.9±71.3 U/mg protein) (p=0.0005).*
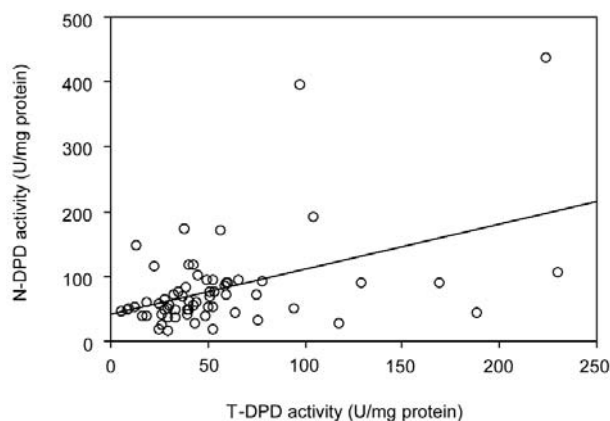

Figure 2. *Significant correlation of DPD activity detected by ELISA between tumor and normal mucosa (r=0.698, p=0.0002).*

investigated the clinical importance of DPD activity and expression in patients with Dukes' C CRC treated with 5-FU.

Materials and Methods

Tissue preparations. We obtained tumors and normal mucosas from 64 patients who underwent colorectal resection with curative intent between January 1992 and December 2001, and who gave informed consent for the use of their tissues. Samples, approximately 1 g of tissue, were collected immediately after resection of the specimen. Non-tumorous tissues were obtained from an area sufficiently distant from the tumor. Half of the tissue was fixed in 10% buffered-formalin and embedded in paraffin. Sections (4 μm thick) were prepared and stained for histopathological diagnosis and immunohistochemical analysis. The other half of the tissue was stored at -80˚C until use.

Assay of DPD activity. Each tumor and normal tissue sample was homogenized in a 10-fold volume of 10 mM Tris-HCl buffer (pH7.4) containing 15 mM NaCl, 1.5 mM $MgCl_2$ and 50 μM potassium phosphate. The homogenate was centrifuged at 10,000 x g for 15 minutes at 4˚C. Supernatants were then dialyzed overnight at 4˚C in a 20 mM potassium phosphate buffer (pH7.4) containing 1 mM 2-mercaptoethanol. The protein concentration was determined by the methods of Lowry *et al.* (17). DPD activity was determined by a sandwich ELISA with two monoclonal antibodies specific to human DPD, according to the methods described by Mori *et al.* (18). DPD levels in tissues were expressed as Unit (U)/mg protein, with one unit equivalent to the amount of DPD protein that catabolizes 1 pmole of 5-FU per minute.

Immunohistochemical staining and evaluation of DPD expression. Paraffin-embedded sections were dewaxed in xylene, rehydrated gradually with graded alcohols and antigen retrieval was performed by microwaving for 15 minutes. Endogenous peroxidase was blocked by incubation of samples in 3% hydrogen peroxide in methanol. After being washed 3 times in phosphate buffer saline (PBS), they were placed in 10% normal equine serum (Cosmo Bio Co. Ltd.,

Tokyo Japan) in PBS for 30 minutes to reduce nonspecific staining. The sections were subsequently incubated with polyclonal antibody against DPD (dilution, 6:1000, Second Cancer Research Laboratory, Taiho Pharmaceutical Co. Ltd., Saitama Japan) at 4˚C overnight. After being washed 3 times in PBS, slides were incubated with HISTOFINE SIMPLESTAIN MAX-PO (R)® (Nichirei Corporation Co. Ltd., Tokyo Japan) at room temperature for 1 hour. Immunohistochemical reactions were revealed with a solution of 3,3'-diaminobenzidine tetrahydrochloride. The sections were then counterstained with methyl green. DPD-positive cancer cells were counted randomly at 200 x magnification among 1×10^3 carcinoma cells, and the population of DPD-positive cells recorded. Staining grades were evaluated independently by two investigators (K.O. and M.M.) without prior knowledge of the clinicopathological details. Immunoreactivity was graded as follows: positive, more than 10% of carcinoma cells were stained; negative, no detectable expression or fewer than 10% of carcinoma cells were stained. Normal colorectal mucosa including macrophages and plasma cells was used as a positive control; it is known that these cells are well stained by DPD antibodies (16). The negative control was made by omission of the primary antibody during the process of immunohistochemical staining.

Patients. Clinicopathological findings of CRC were defined according to Dukes' classification (19) and the Japanese classifications of colorectal carcinomas (20). All 64 patients had been diagnosed as Dukes' C and curative colorectal resection performed between January 1992 and December 2001. None of the patients received preoperative chemotherapy and all were followed until December 2002. Postoperative adjuvant 5-FU-based chemotherapy was instituted for all 64 patients and oral intake of 5-FU derivative, such as 300 mg/day of Carmofur (Nihon Schering Co., Ltd., Osaka, Japan), 600 mg/day of Doxifluridine (Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) or 300 mg/day of Uracil plus Tegafur (Taiho Pharmaceutical Co., Ltd., Tokyo, Japan), was continued for 6 to 12 months. The types of cancer recurrence were established by diagnostic imaging (computed tomography and ultrasonography) done at least twice a year.

Table I. *Correlation between DPD activities and clinicopathological variables.*

Clinicopathological variables	Number(%)	T-DPD (U/mg prot)	p value	N-DPD (U/mg prot)	p value	T-DPD/N-DPD	p value
Age							
< 69y	33(51.6)	43.0±20.8	0.3681	71.3±40.0	0.9038	0.828±0.778	0.6820
69y ≤	31(48.4)	68.0±60.7		89.1±93.7		0.978±0.897	
Gender							
Male	39(60.9)	55.7±47.1	0.7516	77.0±62.9	0.8097	0.929±0.952	0.8419
Female	25(39.1)	54.2±45.6		84.5±83.8		0.856±0.640	
Location of tumor							
Colon	32(50.0)	50.8±46.2	0.1379	82.5±91.7	0.2622	0.820±0.809	0.3404
Rectum	32(50.0)	59.4±46.5		77.3±43.6		0.981±0.874	
Histological type							
Well-diff.	20(31.3)	50.6±29.0	0.2329	82.1±81.0	0.9617	0.799±0.478	0.1252
Moderately-diff.	35(54.7)	47.6±39.2		82.8±74.3		0.776±0.773	
Others	9(14.1)	94.1±79.1		63.8±25.4		1.609±1.343	
Depth of invasion							
mp	4(6.3)	64.3±56.3	0.8203	77.9±31.3	0.6445	0.924±0.845	0.4332
ss/a1	6(9.4)	42.0±17.4		74.2±31.3		0.629±0.280	
se/a2	48(75.0)	55.0±49.6		83.8±80.5		0.869±0.790	
si/ai	6(9.4)	62.6±33.9		56.2±24.3		1.407±1.446	
Lymph node metastasis							
n1	49(76.6)	44.4±28.8	0.0100	68.8±35.7	0.5313	0.788±0.588	0.2776
n2/n3	15(23.4)	90.1±71.0		116.3±129.0		1.267±1.341	
Lymphatic invasion							
absent	15(23.4)	61.5±40.7	0.1729	93.3±92.7	0.5955	1.018±1.030	0.5955
present	49(76.6)	53.1±47.9		75.8±63.9		0.865±0.781	
Venous invasion							
absent	14(21.9)	53.8±52.0	0.4901	118.4±133.3	0.6261	0.675±0.423	0.3894
present	50(78.1)	55.5±45.0		69.1±35.7		0.964±0.916	
Tumor size							
large(50mm≤)	36(56.3)	56.1±39.7	0.2974	77.4±64.3	0.9838	1.006±0.983	0.3037
small(<50mm)	28(43.7)	53.9±54.1		83.2±80.4		0.766±0.597	

Well/Moderately-diff.: Well/Moderately-differentiated adenocarcinoma. Others: Signet-ring cell carcinoma and Mucinous adenocarcinoma
mp: Tumor invasion of muscularis propria. ss/a1: Tumor invasion of subserosa/through muscularis propria into non-peritonealized part. se/a2: Tumor invasion of serosa/ non-peritonealized part, pericolic, or perirectal tissues. si/ai: Direct tumor invasion of other organs and structures
n1: Lymph node metastasis to Group 1. n2/n3: Lymph node metastasis to Group 2 and/or 3.

Statistical analysis. Differences between the two groups were evaluated by the Mann-Whitney's *U*-test. Differences among more than three groups were evaluated by the Kruskal-Wallis rank test. Relationships between various parameters were evaluated statistically using the Chi-squared or Fisher's exact probability test. Survival rates were calculated by the Kaplan-Meier method. Disease-specific survival rates were used; that is, only recurrences caused by colorectal cancer were taken as outcome events and all other deaths were considered as censored ones. The log rank test was used for comparisons of the two survival curves. Multivariate analysis was by the Cox's proportional hazard regression model. Differences were considered significant when the *p*-value was less than 0.05.

Results

The DPD activities and their correlation with clinicopathological findings. The average tumor (T) DPD activity (55.1 U/mg protein, range 5.2-230.2, median 42.3) was lower than that in normal mucosa (N) DPD activity (79.9 U/mg protein, range 15.8-437.4, median 61.2, $p=0.0005$, Figure 1). T-DPD activity showed a significantly positive correlation with that of N-DPD ($r=0.698$, $p=0.0002$, Figure 2). We examined the correlation between DPD activities (T-DPD, N-DPD, T-DPD/N-DPD ratio) and major clinicopathological findings
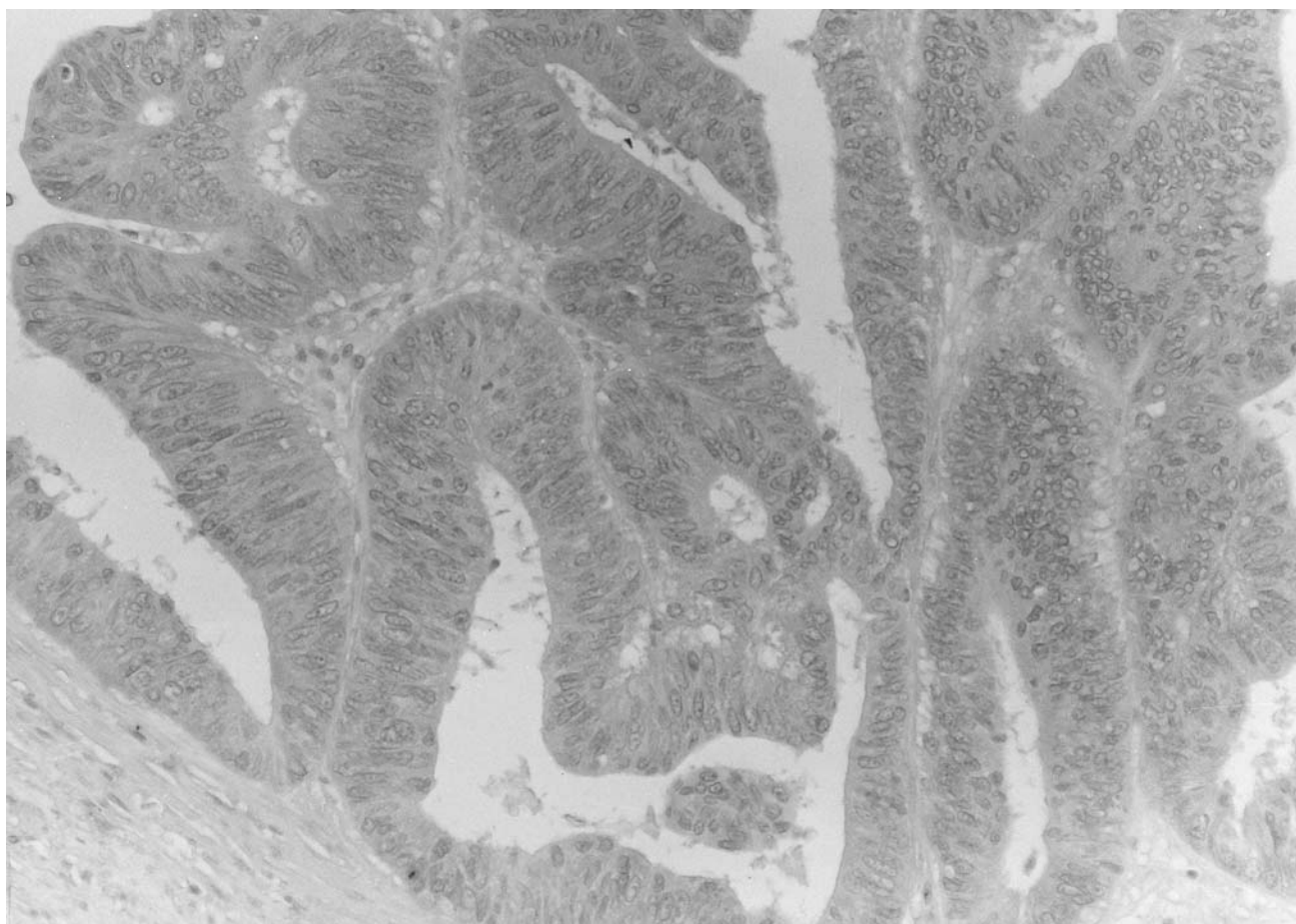
Figure 3. *Immunohistochemical DPD expression in advanced colon adenocarcinoma cells (x 200).*

(Table I); no significant correlation was demonstrated, except for the extent of lymph node metastasis and T-DPD activity ($p=0.01$).

Immunohistochemical DPD expression and correlation with clinicopathological findings. Figure 3 shows a representative case with well stained DPD in the cytoplasm of cancer cells, but the intensity was not uniform among the patients. DPD was also strongly stained in the cytoplasm of mononuclear cells, especially in macrophages and plasma cells. The staining intensity of these cells was used as an internal control. Twenty-eight tumors (43.8%) were DPD-positive according to our criteria for immunoreactivity. There was significant correlation between immunohistochemical DPD expression and the extent of lymph node metastasis ($p=0.0409$, Table II), but there were no significant differences between immunohistochemical DPD expression and other clinicopathological variables. The DPD activities of tumors determined by ELISA and immunoreactivity were significantly correlated ($p=0.0121$, Figure 4).

Univariate analysis of disease-free survival. The 5-year disease-free survival rate of Dukes' C colorectal cancer in the present study was 57.8%. The patients were divided into two sub-groups according to the median of T-DPD activity (median 42.3 U/mg protein) and of T-DPD/N-DPD ratio (median 0.717). There were no significant differences in disease-free survival rates between the two groups with low T-DPD activity (60.6%, n=33) and high T-DPD activity (54.8%, n=31; $p=0.8752$), and between the two groups with low T-DPD/N-DPD ratio (57.9%, n=38) and high T-DPD/N-DPD ratio (57.7%, n=26; $p=0.7908$). However, the survival curve of the 36 patients with DPD-negative tumors was significantly better than that of the 28 patients with DPD-positive tumors (5-year disease-free survival rates, 72.2% vs. 39.3%; $p=0.0127$, Figure 5).

Multivariate analysis. Twelve variables (age, gender, tumor location, histological type, depth of invasion, lymph node metastasis, lymphatic invasion, venous invasion, tumor size, T-DPD activity, T-DPD/N-DPD ratio and DPD

Table II. *Immunohistochemical DPD expression and clinicopathological variables.*

Clinicopathological variables	Number(%)	Expression of DPD positive	negative	*p* value
Age				
<69y	33(51.6)	15	18	0.9749
69y≤	31(48.4)	13	18	
Gender				
Male	39(60.9)	18	21	0.8212
Female	25(39.1)	10	15	
Location of tumor				
Colon	32(50.0)	15	17	0.8011
Rectum	32(50.0)	13	19	
Histological type				
Well-diff.	20(31.3)	6	14	0.1727
Moderately-diff.	35(54.7)	16	19	
Others	9(14.1)	6	3	
Depth of invasion				
mp	4(6.3)	0	4	0.2828
ss/a1	6(9.4)	2	4	
se/a2	48(75.0)	23	25	
si/ai	6(9.4)	3	3	
Lymph node metastasis				
n1	49(76.6)	18	31	0.0409
n2/n3	15(23.4)	10	5	
Lymphatic invasion				
Absent	15(23.4)	5	10	0.5274
Present	49(76.6)	23	26	
Venous invasion				
Absent	14(21.9)	4	10	0.3219
Present	50(78.1)	24	26	
Tumor size				
Large(≥50mm)	36(56.3)	18	18	0.3741
Small(<50mm)	28(43.7)	10	18	

Well/Moderately-diff.: Well/Moderately-differentiated adenocarcinoma. Others: Signet-ring cell carcinoma and Mucinous adenocarcinoma. mp: Tumor invasion of muscularis propria. ss/a1: Tumor invasion of subserosa/through muscularis propria into non-peritonealized part. se/a2: Tumor invasion of serosa /non-peritonealized part, pericolic, or perirectal tissues. si/ai: Direct tumor invasion of other organs or structures. n1: Lymph node metastasis to Group 1. n2/n3: Lymph node metastasis to Group 2 and/or 3.

Figure 4. *Significant correlation of DPD activity of tumor and DPD immunoreactivity (p=0.0121).*

Figure 5. *Disease-free survival curves of patients with positive and negative immunohistochemical expression of DPD.*

immunoreactivity) were analyzed using the Cox's proportional hazards regression model to determine the prognostic factors of Dukes' C CRC patients. Analyses revealed that tumor location, lymphatic invasion and DPD immunoreactivity independently contributed to disease-free survival (Table III).

Discussion

We found that the level of T-DPD activity was significantly lower than that of N-DPD, but showed significant positive correlation with 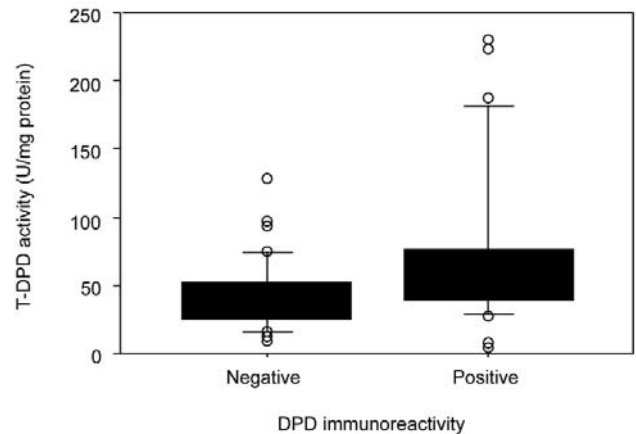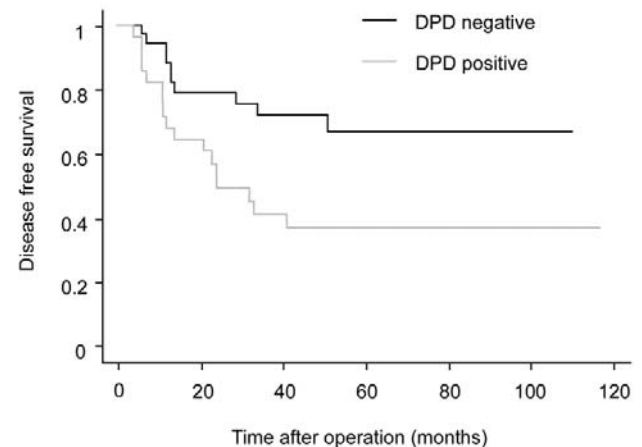N-DPD activities. The results concurred with those in other reports (14, 18, 21). The reason for the decreasing DPD activity in tumor cells remains unclear. However, the down-regulation of DPD expression in tumors is in direct contrast with the overexpression of enzymes of the pyrimidine salvage pathway (thymidine kinase, uridine phosphorylase and thymidine phosphorylase), which is observed in colorectal tumors but not in normal mucosa (22). Decreased catabolism of uracil and thymine possibly leads to an increase in pyrimidine nucleotide pools for use in DNA and RNA biosynthesis in tumor cells (23). Thus, the down-regulation of DPD expression may create a favorable environment for tumor growth. On the other

Table III. *Multivariate analysis of disease-free survival in Dukes' C CRC.*

Variables	Hazard ratio	95% Confidence interval	*p* value
Age (≥69 *vs.* 69<)	2.057	0.773-5.473	0.1487
Gender (Female *vs.* Male)	0.388	0.141-1.070	0.0673
Location of tumor (Colon *vs.* Rectum)	0.219	0.073-0.659	0.0069
Histological type (Well-diff. *vs.* Others)	0.575	0.189-1.752	0.3302
Depth of invasion (se/a2+si/ai *vs.* mp + ss/a1)	0.487	0.116-2.047	0.3258
Lymph node metastasis (n1 *vs.* n2/n3)	0.535	0.163-1.758	0.3024
Lymphatic invasion (Absent *vs.* Present)	0.223	0.060-0.826	0.0247
Venous invasion (Absent *vs.* Present)	1.128	0.336-3.793	0.8451
Tumor size (50mm≤ *vs.* <50mm)	1.651	0.696-3.916	0.2553
T-DPD activity (High *vs.* Low)	0.495	0.176-1.390	0.1818
T-DPD/N-DPD ratio (High *vs.* Low)	1.236	0.493-3.100	0.6508
DPD immunoreactivity (Negative *vs.* Positive)	0.318	0.118-0.861	0.0241

Well-diff.: Well-differentiated adenocarcinoma. Others: Moderately-differentiated adenocarcinoma, Signet-ring cell carcinoma and Mucinous adenocarcinoma.
mp: Tumor invasion of musclaris propria. ss/a1: Tumor invasion of subserosa/through muscularis propria into non-peritonealized part. se/a2: Tumor invasion of serosa/non-peritonealized part, pericolic, or perirectal tissues. si/ai: Direct tumor invasion of other organs or structures.
n1: Lymph node metastasis to Group 1.
n2/n3: Lymph node metastasis to Group 2 and/or 3.

hand, high DPD activity in normal colorectal mucosa may reduce the toxic side-effects of 5-FU on normal mucosa, such as diarrhea or bacterial translocation in patients treated with 5-FU.

DPD immunoreactivity of tumor cells and T-DPD activity were significantly correlated; a result that is consistent with the findings of Takenoue *et al.* (16). We also found that both DPD activity and immunoreactivity in tumors were significantly high in patients with group 2 or 3 lymph node metastasis compared to those with group 1. Our results support that DPD activity determined by ELISA and immunohistochemical DPD expression reflects the metastatic potential of tumors. Shirota *et al.* (15) also

reported that intratumoral DPD mRNA levels were significantly associated with tumor progression and metastasis. However, other reports (16, 21, 24) could not demonstrate any significant correlation between tumor DPD activity or expression and clinicopathological factors in CRCs. The reason for this discrepancy might be due to the differences of patient backgrounds. Our patients were all Dukes' C, while others were of all stages. The restricted stage and the background of our patients might explain the significance of our results. We also calculated the tumor/normal tissue ratio of DPD activity in order to correct the individual differences. It has been reported that the tumor/normal tissue ratio of DPD activity was higher in tumors at an advanced stage and with positive vessel invasion (24). However, we could not find any significant relationship between the tumor/normal tissue ratio of DPD activity and clinicopathological features in patients with Dukes' C CRC.

Rather than T-DPD activity determined by ELISA, DPD expression was demonstrated immunohistochemically to be a useful prognostic predictor for patients with Dukes' C CRC. T-DPD activity determined by ELISA contained DPD protein originating from human peripheral blood mononuclear cells. On the other hand, the immunohistochemical method could evaluate DPD expression of cancerous tissues limited to the actual tumor cells. Therefore, immunohistochemical analysis of DPD may reflect more exact DPD levels of tumor cells than ELISA. Thus, DPD immunoreactivity of tumors was recognized as an independent prognostic factor in patients with Dukes' C CRC treated with 5-FU.

DPD immunoreactivity is more informative than DPD activity determined by ELISA for predicting the effectiveness of postoperative adjuvant FU-based chemotherapy in advanced CRC patients. If DPD immunoreactivity in CRC is positive, we believe there is a need to consider other anti-cancer drugs such as anti-DPD drugs [1M Tegafur-0.4M 5-chloro-2,4-dihydroxypyridine-1M potassium oxonate (S-1)].

Acknowledgements

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