Expression of Dihydropyrimidine Dehydrogenase, Orotate Phosphoribosyl Transferase and Thymidylate Synthase in Patients with Primary Colorectal Cancer, and Associations with Site of First Metastasis

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Abstract. *Aim: The activity of the widely used anticancer drug 5-fluorouracil (5-FU) is determined by the presence of several enzymes, including dihydropyrimidine dehydrogenase (DPD), orotate phosphoribosyl transferase (OPRT), and thymidylate synthase (TS). This study compared the expression levels of these enzymes between primary colorectal cancer with and without distant metastases, and examined whether these expression patterns are associated with hematogenous metastasis. Materials and Methods: Among 40 patients with colorectal cancer, 20 had no metastasis and 20 had distant metastasis. Strong expression on immunohistochemistry was classified as positive, while weak, moderate or no expression was classified as negative. Results: Positive expressions of DPD, OPRT and TS in primary colorectal cancer tissue were seen in 47.5%, 75% and 20%, respectively. However, no relationships were observed among the expressions of DPD, OPRT and TS. Expressions of OPRT (p=0.029) and TS (p=0.017) in primary tissues were significantly associated with hematogenous metastasis. Patterns of the expression of the three enzymes varied, and were classified in six ways. A tendency was seen for primary colorectal cancer with DPD-high expression to have liver metastasis and for that with DPD-low expression, to have lung metastasis. Conclusion: High expression levels of OPRT and TS in colorectal cancer appear to be significantly involved in metastasis after curative surgery. The organs in which metastases arise may be controlled by the expression of DPD.*

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As a typical fluoropyrimidine anticancer agent, 5-fluorouracil (5-FU) has been a key chemotherapy drug for gastrointestinal cancer, for more than half a century (1). Fluoropyrimidines are the most widely used anticancer agents in colorectal cancer, and sensitivity to these agents affects prognosis (2). With advances in molecular biology, the mechanisms of action for 5-FU have come to be better understood, and studies have indicated that the expression of enzymes involved in 5-FU-related metabolic pathways are associated with both the therapeutic effects and the appearance of adverse effects.

Three main metabolic pathways are important for 5-FU effects, and the activity and amounts of the enzymes dihydropyrimidine dehydrogenase (DPD), orotate phosphoribosyl transferase (OPRT), and thymidylate synthase (TS) in each of these pathways are known to be related to the antitumor effects of 5-FU (3-5). Therefore, response to 5-FU can be predicted from the expressions of DPD, OPRT, and TS in tumor tissue, allowing for consideration of individualized approaches to chemotherapy (6, 7).

Relationships between these metabolic enzymes, cancer sensitivity, and antitumor effects were actively researched in the 1990s. With the rise of novel anticancer agents such as oxaliplatin and irinotecan in recent years, however, the results of past research have probably not been effectively utilized. While these new anticancer agents have strong antitumor effects, they are used in combination with 5-FU. These drugs have come to be used as adjuvant chemotherapies following curative resection of colon cancer. Performing adjuvant chemotherapy may not be useful in all cases, thus establishing targets to identify the specific need for such therapy is becoming more important.

Investigations of risk factors for postoperative recurrence have revealed various biomarkers. However, whether risk factors for recurrence are factors that require adjuvant therapy remains unclear. Tailor-made treatments are thus

Table I. *Patients' characteristics of primary lesion.*

		Total (n=40)	No recurrence (n=20)	Hematogenous metastasis (n=20)
Age, years	(mean±SD)	64.4±1.6	64.9±2.3	65.4±3.3
Gender	(Male/female)	20/20	10/10	10/10
Location	(Colon/rectum)	19/21	9/11	10/10
Median follow-up	(Months)	35.2	44.7	33.8
Depth of invasion	T2	2	1	1
	T3, T4	38	19	19
Lymph node metastasis	Negative	19	11	8
	Positive	21	9	12
Histological type	Well	22	11	11
	Mod	18	9	9

well: Well-differentiated adenocarcinoma, mod: moderately differentiated adenocarcinoma.

likely to see increasing demand in the future. Investigations of regimens that consider 5-FU-related metabolic enzymes will also be essential. The present study retrospectively examined the status of 5-FU-related metabolic enzymes and the postoperative recurrence in the primary tumor of advanced colorectal cancer, for which curative resection and postoperative adjuvant chemotherapy were performed.

Patients and Methods

Clinical samples. Surgical specimens were obtained from 40 patients (20 men, 20 women) with primary colorectal cancer who underwent radical resection in the Department of Surgery of Kurume University between 1999 and 2004. Written informed consent was obtained from each patient prior to treatment. Patients' characteristics are summarized in Table I. Twenty patients were recurrence-free more than five years after surgery. The remaining 20 patients had distant metastases postoperatively, with lung metastases in 10 patients and liver metastases in 10 patients. No patients with both lung and liver metastases were actively included in this study. The median age of patients at the time of operation was 64.4 years (range=17-83 years). The underlying pathology was rectal cancer in 21 patients and colon cancer in 19 patients. All patients received 5-FU postoperatively. The mean duration of follow-up was 68 months (range= 36-84 months). Histological differentiation was well-differentiated in 22 cases and moderate in 18. Depth of invasion was T2 in two cases and T3 or T4 in 38. Lymph node metastasis was negative in 19 patients and positive in 21.

Freshly resected specimens were immersed in 10% formalin for fixation, after lymph nodes in the mesentery had been promptly retrieved by the surgeon. Specimens were removed after 3-4 days, and all tumors were sliced and embedded in paraffin.

Immunohistochemistry. Immunohistochemical analysis of 5-FU-related enzymes, such as DPD, OPRT, and TS, was performed on each tumor tissue sample. The deepest-invading tissue samples of primary colorectal cancer were selected for immunohistochemistry. Expression of DPD, OPRT, and TS was determined using antibodies supplied by Taiho Pharmaceutical (Saitama, Japan) using a 4-μm section of paraffin-embedded tissue. Endogenous peroxidase activity was inhibited by incubating the slides in 3% H_2O_2 for 5 min. Each slide was incubated overnight with the appropriate antibody at 4˚C. For detection through staining, the ChemMate ENVISION method (DakoCytomation, Glostrup, Denmark) was used, with diaminobenzidine (DAB) as the chromogen.

Table II. *Expression of each enzyme in the primary site and in the site of recurrence.*

		No recurrence (n=20)	Hematogeous metastases (n=20)	*p*-Value
DPD	Neg.	11	10	0.759
	Pos.	9	10	
TS	Neg.	19	13	0.017
	Pos.	1	7	
OPRT	Neg.	8	2	0.029
	Pos.	12	18	

neg.: Negative, pos.: positive.

Evaluation of immunohistochemistry. Expressions of DPD, OPRT, and TS were each classified as either negative or positive. Negative expression was accepted when no expression, or only weak to moderate expression in the entire cytoplasm in >10% of cancer cells was observed. Positive expression was defined when strong expression of the entire cytoplasm was present in >10% of cancer cells.

Statistical analysis. Fisher's exact test or the Wilcoxon rank-sum test was used to compare each expression of DPD, OPRT, or TS in the tumor tissue and for metastasis status. Values of $p<0.05$ were regarded as significant. The relapse-free survival curve in this series was depicted using the Kaplan–Meier method, and levels of significance were tested using the log-rank test. Differences were considered statistically significant at the $p<0.05$ level. Associations between expressions of each DPD, OPRT, TS in primary tumors and metastasis after surgery were summarized in a descriptive manner of examination using 2×2 tables.

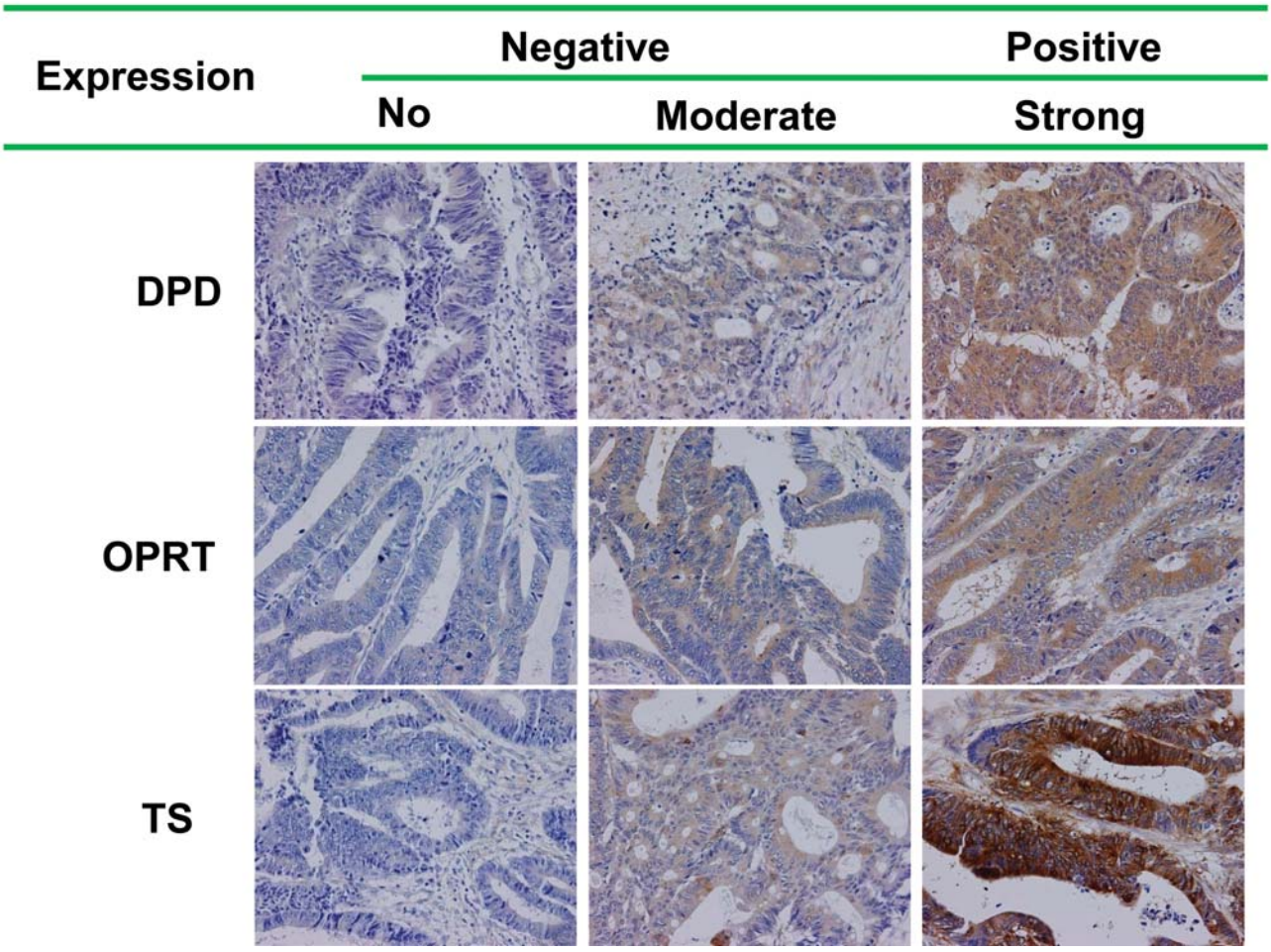

Figure 1. *Expression of DPD (Dihydropyrimidine dehydrogenase), OPRT (Orotate phosphoribosyl transferase) and TS (Thymidylate synthase) in primary colorectal cancer.*

Results

Expression of DPD, OPRT, and TS in primary colorectal cancer tissues. Expressions of these three enzymes in normal mucosa of the colon and rectum were as follows. DPD and TS were expressed on the surface and on the crypt epithelium. OPRT was expressed at various levels in the mucosa and was often highly expressed in normal mucosa adjacent to the tumor.

Expressions of each of the three enzymes were classified as positive or negative in expression intensity, as assessed by immunohistochemistry (Figure 1). Strong expression of DPD, OPRT, and TS was seen in 47.5% (19/40), 75% (30/40) and 20% (8/40) of cases, respectively. No associations between expressions of each enzyme were apparent. Patients with strong expression of OPRT and TS in primary tumors had significantly greater frequencies of metastasis to the liver, and to the lung, with values of $p=0.029$ and $p=0.017$, respectively (Table II). In particular, recurrence was found in 90% (18/20) of OPRT-positive cases. The level of DPD expression did not correlate with the presence of metastases. The relapse-free survival of the group with strong TS expression was significantly lower than those of the groups with moderate or no expression (Figure 2; log-rank $p<0.0001$). However, no significant differences in relapse-free survival rates were seen according to the expression of OPRT between the groups (Figure 3).

Relationship between expressions of DPD, OPRT and TS in primary tumor and hematogenous metastasis (Figure 4). Patterns of expression varied for the three enzymes, but were classifiable into six groups according to the combination of expression levels. The respective categories and their recurrences were compared for each site, and recurrence was not found to be related to DPD expression. Characteristically, however, lung metastasis was more frequent among patients

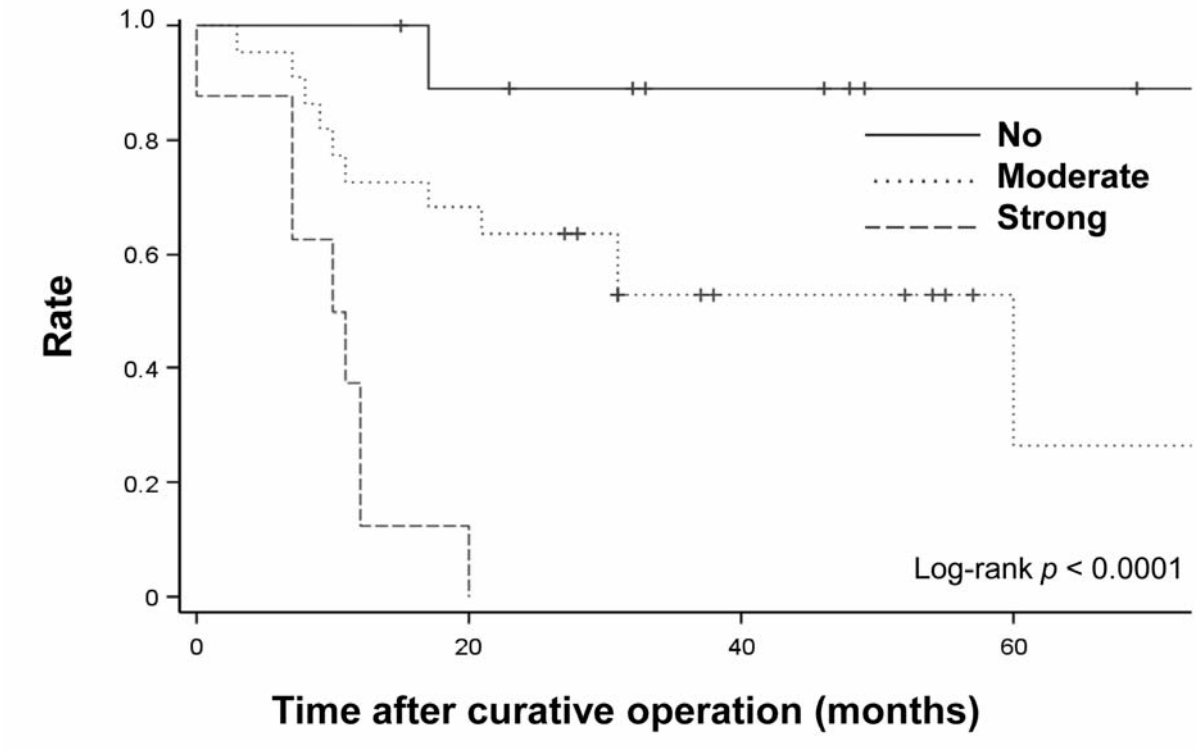

Figure 2. *Time-to-recurrence classified by Thymidylate Synthase expression levels*.

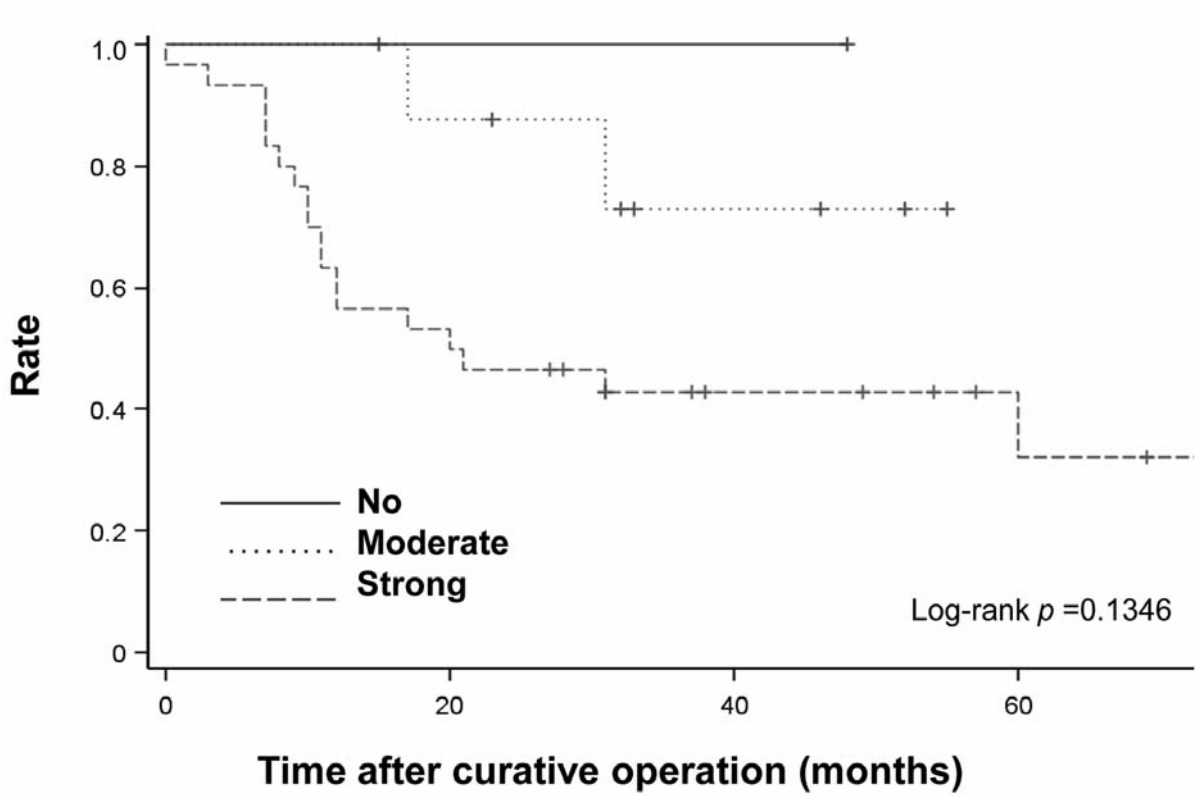

Figure 3. *Time-to-recurrence classified by Orotate phosphoribosyl transferase expression levels*.

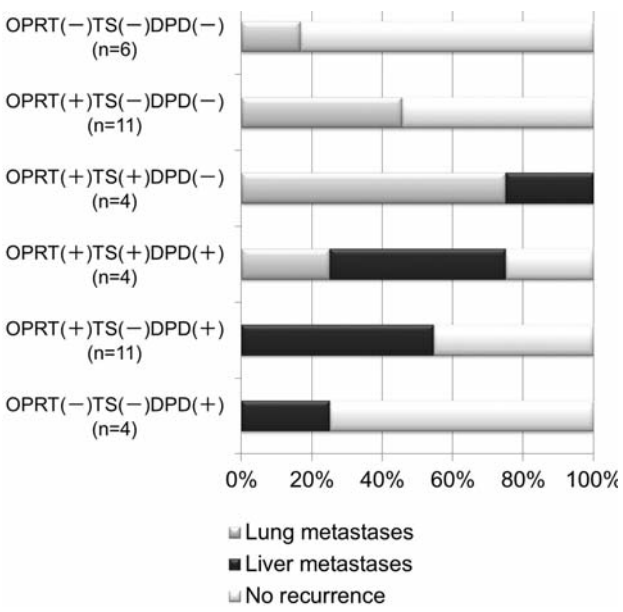

Figure 4. *Relationship between combination of expressions and site of metastasis.*

with DPD-negative tumors and liver metastasis was more frequent among DPD-positive cases. Almost no cases of recurrence were seen among patients with positive findings for either OPRT or TS.

Discussion

We investigated the expression of DPD, TS, and OPRT in primary tumor tissues from patients who had undergone postoperative adjuvant chemotherapy using fluoropyrimidine anticancer agents. To confirm the expression levels of 5-FU-related enzymes, we measured i) enzyme activity; ii) protein; iii) RNA; and iv) DNA. Each has measurement advantages and disadvantages, thus, investigations making use of the specific characteristics are necessary.

We performed immunohistological staining using resected specimens. Although qualitative protein testing with immunohistological stains lacks objectivity in some areas, this approach is inexpensive and offers a high level of versatility. In addition, localization of expression sites within the tumor and confirmation of heterogeneity can be achieved with relative ease. Conditions within the specimen, such as histological type and vascular invasion, can be evaluated at the same time. In this study, three researchers (Y.A., A.K., and T.M.) examined the specimens separately, but no significant differences were identified in the findings.

Expression levels of DPD, OPRT, and TS varied, and no results suggested correlations between each of these enzymes. The mRNA levels of *DPD* and *OPRT* have been reported to be inversely correlated (5), and OPRT and DPD enzyme activity can reportedly be predicted from 5-FU sensitivity (8).

Although some selection bias was present for the patients used in this study, to investigate relationships between enzyme expression in the primary tumor and in postoperative recurrence, the frequency of strong expression was highest with OPRT, and metastatic recurrence occurred in 90% of those cases. OPRT is thought to be very important in the anabolic pathway of 5-FU in humans (3). With the enzyme activity method, 5-FU sensitivity is correlated with high OPRT and low DPD levels (8), and the antitumor effects of 5-FU are considered (9).

In this study, a high frequency of metastatic recurrence was seen in patients with high expression of OPRT, even though they had undergone adjuvant chemotherapy, and 5-FU was not thought to exert substantial antitumor effects. This difference in results may have been due to the different measurement methods, suggesting the possibility that sensitivity and antitumor effects do not necessarily correspond. The possibility is also suggested that other related enzymes inhibit antitumor effects. However, we did not investigate these differences in the results, while further investigations are needed.

A huge amount of information about the mechanisms underlying cancer metastasis has been elucidated at the molecular, biological, and genetic levels. Risk factors for recurrence of various types of cancer have been investigated as metastasis biomarkers. Kawahara *et al*. (10) suggested that dUTPase, a 5-FU-related enzyme, may offer promise as a predictive biomarker of the metastatic potential in colorectal cancer, and positive expressions of OPRT and TS are also thought to represent potential predictive biomarkers.

Looking at the relationship between enzyme expression within tumors and recurrence sites, very interesting results were obtained, showing that lung metastasis occurred more often in cases with negative results for DPD, while liver metastasis occurred more frequently in cases with positive results for DPD. DPD may thus be related to the growth of cancer cells in the microenvironment of each organ. Expression of 5-FU-related enzymes in various types of cancer and normal sections of the same tissues has been investigated (11). The results showed that the protein expression of DPD in colorectal cancer tissues was significantly lower than in the matched non-cancerous tissues. However, no comparisons between normal tissues were made. Although data were not obtained for the liver and precise data were not shown, expression of DPD in normal lung appeared to be lower than that in colorectal cancer tissue. In addition, levels of *DPD* mRNA are reportedly higher in liver metastases than in primary tumors (12). Moreover, the 5-FU-related enzymes of the primary colorectal cancer can be used in predicting the therapeutic efficacy of 5-FU against liver metastases (13).

These findings also suggest that compatibility between organs with metastases and primary tumors contributes to the formation of metastasis. If the organs for which cancer cells have acquired metastatic traits could be predicted, prioritized follow-up and preventive treatment could probably be planned. Biomarkers are helpful in follow-up and individualized treatment, and DPD, OPRT and TS may all be considered as being such markers.

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