Prognostic value of 5-fluorouracil metabolic enzyme genes in Dukes' stage B and C colorectal cancer patients treated with oral 5-fluorouracil-based adjuvant chemotherapy

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Abstract. 5-Fluorouracil (5-FU) is the most commonly used anticancer drug for colorectal cancer (CRC). In this study, we aimed to clarify the prognostic value of the expression of the 5-FU metabolic enzyme genes, including orptate phosphoribosyl transferase (OPRT), dihydropyrimidine dehydrogenase (DPD), thymidylate synthetase (TS) and thymidylate phosphorylate (TP) genes in CRC patients treated with oral 5-FU-based adjuvant chemotherapy. We examined 103 CRC patients with Dukes' stage B and C who underwent oral 5-FU-based adjuvant chemotherapy. Formalin-fixed, paraffin-embedded tumor specimens from primary CRC tissues were dissected by laser-captured microdissection and quantification of mRNA levels of OPRT, DPD, TS and TP were measured by real-time reverse transcription (RT) PCR. The relationship between these 5-FU metabolic enzyme gene levels and disease-free and overall survival rates were examined. The disease-free and overall survival curves of the OPRT mRNA high-expression group were significantly longer than that of the OPRT mRNA low-expression group. The disease-free and overall survival curves of the DPD mRNA high-expression group were significantly shorter than that of the DPD mRNA low-expression group. In contrast, there were no significant differences between the TS or TP mRNA high-expression and low-expression groups in the disease-free and overall survival curves. In a multivariate Cox regression analysis, it was demonstrated that the OPRT mRNA level is an independent prognostic variable for disease-free and overall survival. These results suggest that the OPRT mRNA is a useful indicator in the prediction of disease-free and overall survival in Dukes' B and C stage CRC patients treated with oral 5-FU-based adjuvant chemotherapy.

Introduction

Colorectal cancer (CRC) is one of the most common malignancies and 5-fluorouracil (5-FU) is widely used as a first-line of adjuvant chemotherapy in patients with CRC (1,2). In western countries, adjuvant chemotherapy such as 5-FU plus leucovorin (LV) (5-FU/LV), infusional 5-FU, LV and oxaliplatin (FOLFOX) has been established as a generalized regimen for CRC patients undergoing curative resection (3,4). In Japan, an oral form of the 5-FU-related drug such as Tegaful plus Uracil, doxifluridine (5'-DFUR) was developed for convenient administration and has been widely used in adjuvant chemotherapy for CRC (5,6). However, the clinical effectiveness of oral 5-FU-based chemotherapy differs among patients (7). In designing optimal individualized adjuvant treatment, it is essential to predict the response to 5-FU and prognosis in each patient.

It is known that several 5-FU-related metabolic pathway enzymes are correlated with sensitivity to 5-FU (8-12). The antitumor effect of 5-FU is found in the inhibitor element of thymidylate synthase (TS), an essential DNA synthetic enzyme, by 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP) and the incorporation of 5-FU metabolites into RNA and DNA (8). The initial metabolism of 5-FU into nucleotides is essential for its action by one or more of the following pathways: directly to 5-fluorouridine-5'-mono-phosphate (FUMP) by orotate phosphoribosyl transferase (OPRT); indirectly to FUMP in a sequence of reactions with conversion of 5-FU to 5-fluorouridine phosphorylated by uridine phsophorylase (UP); indirectly to FdUMP by 2'-deoxy-5-fluorouridine (FUdR) catalyzed by thymidine phosphorylase (TP) (9,10). Furthermore, it is known that 5-FU is categorized to 2-fluoro-ß-alanine in the liver and other tissues by dihydropyrimidine dehydrogenase (DPD) (8,11,12). However, the prognostic value of these 5-FU-related metabolic enzymes in patients treated with 5-FU-based adjuvant chemotherapy is still controversial. It has been reported that a high-expression level of TS and low-expression levels of DPD were correlated with poor prognosis (13,14). In contrast, it has been reported that OPRT, though not TS and DPD have a prognostic value in

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patients treated with 5-FU-based adjuvant chemo-therapy (15). It was thought that one of the reasons for these confused results is the purity of tumor samples. To resolve this problem, the present study used a laser microdissection system in order to enhance the purification of the colorectal tumor specimens.

In this study, using the real-time RT-PCR followed by sampling used by a laser microdissection, we evaluated the prognostic value of these 5-FU-related metabolic pathway genes in CRC patients with Dukes' stage B and C who were treated with oral 5-FU-based adjuvant therapy.

Patients and methods

Patients and treatment. One hundred and three CRC patients with Dukes' stage B and C who underwent complete resection of CRC without preoperative chemotherapy between 1995 and 2005 were enrolled. The average age of the patients was 63±15 years (range, 31 to 80; median 66). The average follow-up time was 34±18 months (range 4-60; median 30). This study was approved by the Ethics Committee of Teikyo University and each patient provided written informed consent. These patients were treated with oral 5-FU-based adjuvant chemotherapy. The patients received oral Tegaful plus Uracil (300 mg/m²/day) (UFT: Taiho Pharmaceutical Co. Ltd., Tokyo, Japan) or Tegaful, Uracil plus LV (75 mg/day) for a minimum of 1 year. The development of local recurrence, distant metastases, and death were re-evaluated in each patient. The evaluation consisted of the pertinent medical history, physical examination and repetition of imaging studies, including a CT scan of the abdomen.

Microdissection of tumor specimen. A representative formalin-fixed, paraffin-embedded tumor specimen was selected after examination of the hematoxylin and eosin stained slides. Ten micron thick sections were stained with neutral fast red to enable visualization of histology for laser capture microdissection (P.A.L.M. Microlaser Technologies AG, Munich, Germany), which was performed in order to ensure that only tumor cells were studied.

RNA extraction and cDNA synthesis. RNA was isolated from formalin-fixed, paraffin-embedded specimens using the MasterPure Purification kit (Epicenter, Madison, WI). After RNA isolation, cDNA was derived from each sample according to a previously described procedure (16).

Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR). As target genes, OPRT, DPD, TS and TP mRNA were examined and ß-actin mRNA was used as an internal control. Sequences of primers and probes are described in Table I. Target cDNA sequences were amplified by quantitative PCR using a fluorescence-based real-time detection method (ABI 7000, Perkin Ermer Co., USA) as described previously (17). In brief, the 25 µl PCR reaction mixture contained 600 nmol/l dUTP, 5.5 mmol/l MgCle and Taq Man buffer A containing a reference dye. The PCR conditions were 50°C for 10 sec and 95°C for 10 min, followed by 42 cycles at 95°C for 15 sec and 60°C for 1 min. The levels of OPRT, DPD, TS and TP mRNA were normalized by ß-

Table I. Sequence of primers and probes.

Primer / Probe		Sequence
OPRT	F	TAGTGTTTTGGAACTGTTGAGGTT
	R	CTTGCCTCCCTGCTCTCTGT
	Probe	6FAM-TGGCATCAGTGACCTTCAAGCCCTCCT
DPD	F	AGGACGCAAGGAGGGTTTG
	R	GTCCGCCGAGTCCTTTACTGA
	Probe	6FAM-CAGTGCCTACAGTCTCGAGTCTGCCAGTG
TS	F	GCCTCGGTGTGCCTTTCA
	R	CCCGTGATGTGCGCAAT
	Probe	6FAM-TCGCCAGCTACGCCCTGCTCA
TP	F	CCTGCGGACGGAATCCT
	R	GCTGTGATGAGTGGCAGGCT
	Probe	6FAM-CAGCCAGAGATGTGACAGCCACCGT
ß-actin	F	TGAGCGCGGCTACAGCTT
	R	TCCTTAATGTCACGCACGATTT
	Probe	6FAM-ACCACCACGGCCGAGCGG

actin mRNA. All evaluations of data were performed without knowledge of the clinical status of the patients and controls.

Statistical analysis. The correlation between the mRNA levels and clinicopathological parameters were evaluated using the Fisher's exact test and the Chi-squared test. The Student's t-test was used to compare the dose of Tegaful between the 5-FU-related mRNA high-expression and low-expression groups. The disease-free survival time and overall survival time were examined according to the Kaplan-Meier survival curve and the statistical significance was calculated using the log-rank test. The Cox proportional-hazards regression analysis was used to estimate univariate and multivariate hazard ratios for disease-free survival and overall survival. A $p < 0.05$ was regarded as statistically significant.

Results

Comparison of administered dose of 5-FU. The levels of OPRT, DPD, TS and TP mRNA were normalized by ß-actin mRNA. The patients were divided into a 5-FU metabolic enzyme mRNA high-expression group and a low-expression group with a cut-off value. The cut-off values obtained by median levels were 1.33 for OPRT (range, 0.60-3.53), 0.67 for DPD (range, 0.17-2.70), 1.58 for TS (range, 0.60-3.80) and 2.71 for TP (range, 0.41-7.17) mRNA.

Table II shows the comparison of the dose of Tegaful in the mRNA high-expression and low-expression groups of OPRT, DPD, TS or TP genes. It was shown that a total dose of Tegaful did not result in any significant differences between the high-expression and low-expression groups.

Clinicopathological features versus TS, DPD, OPRT and TP mRNA levels. The relationship between OPRT, DPD, TS and TP gene expressions and the clinicopathological factors were examined (Table III). No statistically significant differences in

Comparison of a total dose of Tegaful (g) in the high-expression and low-expression groups of 5-FU metabolic enzyme

mRNA	OPRT mRNA	DPD mRNA	TS mRNA	TP mRNA
High-expression group	196±112	220±276	213±190	207±176
Low-expression group	224±254	200±289	235±245	214±156

Table III. The relationship of clinicopathological factors and OPRT, DPD, TS, TP mRNA levels.

Variables	OPRT		DPD		TS		TP	
	High (n=66)	Low (n=37)	High (n=46)	Low (n=57)	High (n=54)	Low (n=49)	High (n=51)	Low (n=52)
Tumor size								
<5 cm	35 (53.0)	15 (40.5)	23 (50.0)	27 (47.4)	26 (48.2)	24 (49.0)	25 (49.2)	25 (48.1)
≥5 cm	31 (47.0)	22 (59.5)	23 (50.0)	30 (52.6)	28 (51.9)	25 (51.0)	26 (51.0)	27 (51.9)
		p=0.30		p=0.79		p=1.00		p=1.00
Histological type								
(Well)	49 (74.2)	24 (64.9)	31 (67.4)	42 (73.7)	37 (68.5)	36 (73.5)	38 (74.5)	35 (67.3)
(Moderate, Poor, Muc)	17 (25.8)	13 (35.1)	15 (32.6)	15 (26.3)	17 (31.5)	13 (26.5)	13 (25.5)	17 (32.7)
		p=0.37		p=0.52		p=0.67		p=0.52
Depth of invasion								
(-)	63 (95.5)	31 (83.8)	39 (84.8)	55 (96.5)	50 (92.6)	44 (89.8)	49 (96.1)	45 (85.6)
(+)	3 (4.6)	6 (16.2)	7 (15.2)	2 (3.5)	4 (7.4)	5 (10.2)	2 (3.9)	7 (13.5)
		p=0.07		p=0.07		p=0.73		p=0.16
Lymphatic invasion								
(-)	41 (62.1)	24 (64.9)	27 (58.7)	38 (66.7)	37 (68.5)	28 (57.1)	29 (58.9)	36 (69.2)
(+)	25 (37.9)	13 (35.1)	19 (41.3)	19 (33.3)	17 (31.5)	21 (42.9)	22 (43.13)	16 (30.8)
		p=0.83		p=0.42		p=0.31		p=0.22
Venous invasion								
(-)	32 (48.5)	18 (48.7)	22 (47.8)	28 (49.1)	27 (50.0)	23 (46.9)	27 (53.9)	23 (44.2)
(+)	34 (51.5)	19 (51.4)	24 (52.2)	29 (50.9)	27 (50.0)	26 (53.1)	24 (47.1)	29 (55.8)
		p=1.00		p=1.00		p=0.84		p=0.43
Lymph node metastasis								
(-)	37 (56.1)	15 (40.5)	20 (43.5)	32 (56.1)	28 (51.9)	24 (48.9)	25 (49.0)	27 (51.9)
(+)	29 (43.9)	22 (59.5)	26 (56.5)	25 (43.7)	26 (48.2)	25 (51.0)	26 (51.0)	25 (48.1)
		p=0.15		p=0.24		p=0.84		p=0.43

clinicopathological factors were observed between these metabolic genes in the high-expression and low-expression groups.

Survival versus OPRT, DPD, TS and TP mRNA levels. Kaplan-Meier disease-free and survival curves of CRC patients were examined between the levels of 5-FU metabolic enzyme mRNA high-expression and low-expression groups. Disease-free survival was analyzed for patients with a curative resection (Fig. 1). In this analysis, the high-OPRT mRNA groups showed a significantly longer survival as compared with that of the low-OPRT mRNA group. Furthermore, patients who were in the high-DPD mRNA expression group showed a significantly shorter survival time than those in the low-DPD mRNA group. In contrast, the disease-free survival in the high-expression groups of TS or TP mRNA did not show any significant differences compared with that of the low-expression group. Overall survival curves were analyzed for all patients as shown in Fig. 2. The OPRT high-expression groups showed a significantly longer survival as compared with that of the OPRT low-expression group. The DPD high-expression group showed a significantly shorter survival rate than that of the DPD low-expression group. In contrast, TS or TP mRNA did not show any significant differences between the high-expression and the low-expression groups.

Univariate and multivariate analysis of OPRT, DPD, TS and TP mRNA levels as predictors of survival. We conducted a univariate and multivariate Cox proportional hazard regression
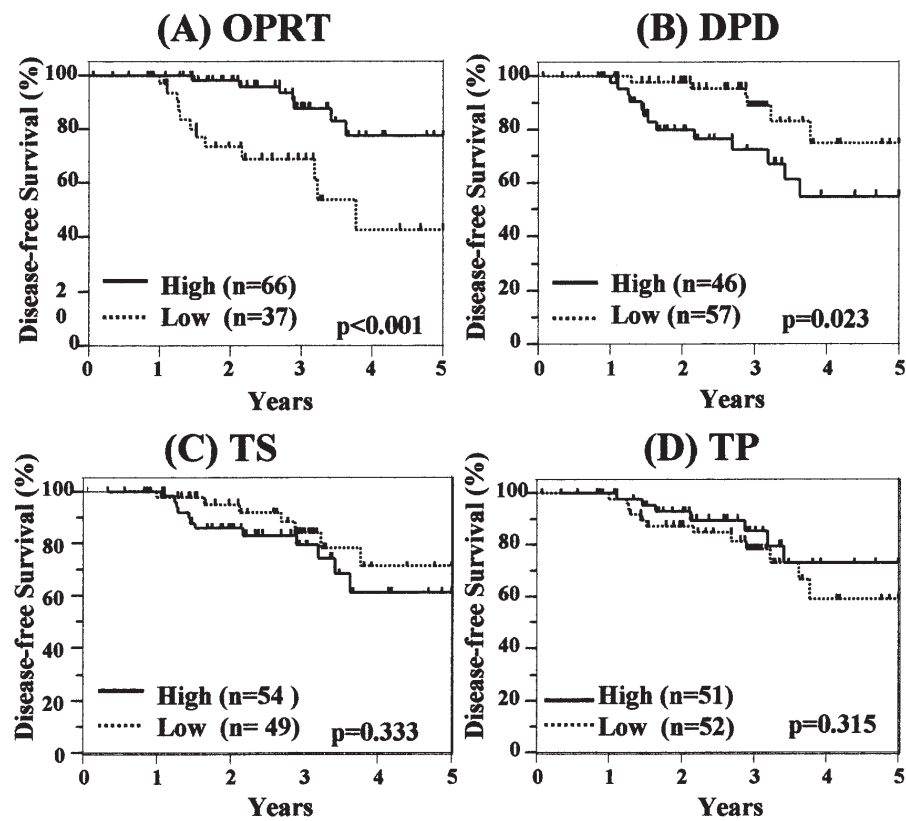
Figure 1. Disease-free survival analysis based on the expression of 5-FU metabolic enzyme genes. Disease-free survivals of OPRT mRNA (A), DPD mRNA (B), TS mRNA (C) and TP mRNA (D) were analyzed by the Kaplan-Meier method. Significant differences were shown between the high-expression and low-expression groups in OPRT mRNA and DPD mRNA.
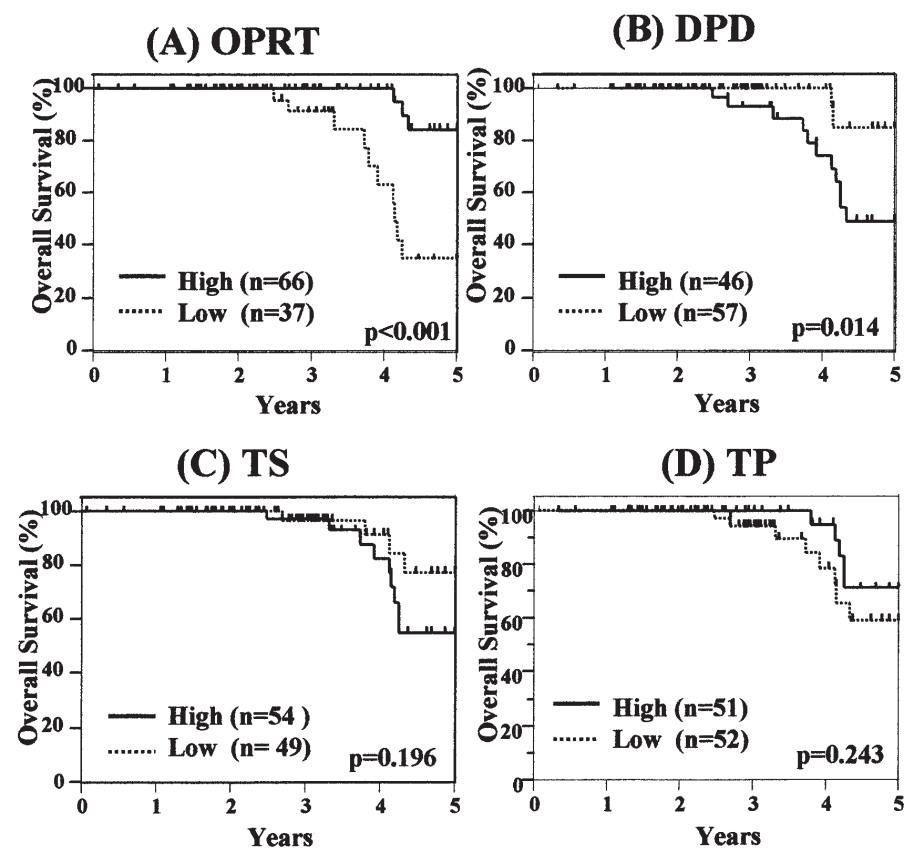

Figure 2. Overall survival analysis based on the expression of 5-FU metabolic enzyme genes. Overall survival of OPRT mRNA (A), DPD mRNA (B), TS mRNA (C) and TP mRNA (D) were analyzed by the Kaplan-Meier method. Significant differences were shown between the high-expression and low-expression groups in OPRT mRNA and DPD mRNA.

SPANDIDOS PUBLICATIONS Cox regression analysis for disease-free survival.

Variables	Univariate Analysis		Multivariate Analysis	
	Hazard Ratio (95% CI)	P-value	Hazard Ratio (95% CI)	P-value
Tumor size	1.37 (0.87-2.25)	0.18	-	
Histological type	1.46 (0.92-2.30)	0.11	-	
Depth of invasion	2.72 (1.52-4.52)	0.02	1.43 (0.76-2.55)	0.25
Lymphatic invasion	1.40 (0.89-2.23)	0.14		
Venous invasion	2.29 (1.32-4.75)	0.002	1.85 (1.04-3.90)	0.03
Lymph node metastasis	2.64 (1.52-5.48)	0.001	2.17 (1.22-4.56)	0.007
OPRT	0.48 (0.29-0.76)	0.002	0.60 (0.35-0.99)	0.04
DPD	1.71 (1.07-2.89)	0.02	1.29 (0.77-2.29)	0.33
TS	1.26 (0.79-2.06)	033	-	
TP	0.79 (0.48-1.24)	0.31	-	

Table V. Cox regression analysis for overall survival.

Variables	Univariate Analysis		Multivariate Analysis	
	Hazard Ratio (95% CI)	P-value	Hazard Ratio (95% CI)	P-value
Tumor size	1.62 (0.92-3.10)	0.09	-	
Histological type	1.34 (0.76-2.33)	0.29	-	
Depth of invasion	2.05 (1.12-3.58)	0.02	1.18 (0.62-2.12)	0.58
Lymphatic invasion	1.19 (0.689-2.10)	0.52	-	
Venous invasion	1.82 (1.00-3.85)	0.04	1.36 (0.71-3.07)	0.36
Lymph node metastasis	3.43 (1.52-14.63)	0.001	2.38 (0.89-10.78)	0.08
OPRT	0.36 (0.17-0.64)	0.001	0.48 (0.21-0.93)	0.03
DPD	2.31 (1.19-5.89)	0.01	1.43 (0.67-3.80)	0.37
TS	1.45 (0.84-2.80)	0.19	-	
TP	0.72 (0.39-1.25)	0.25	-	

analysis for disease-free survival (Table IV). In a univariate analysis, depth of invasion, venous invasion, lymph node metastasis, OPRT mRNA and DPD mRNA showed a significant association for disease-free and overall survival. A Cox multivariate analysis was performed for factors that showed a significance in a univariate analysis and venous invasion, lymph node metastasis and OPRT mRNA showed a significant correlation for disease-free survival.

We then examined univariate and multivariate Cox proportional hazard regression analysis for overall survival (Table V). In a univariate analysis, depth of invasion, venous invasion, lymph node metastasis, OPRT mRNA and DPD mRNA showed a significant association for overall survival. A Cox multivariate analysis was performed for factors that showed a significance in a univariate analysis and OPRT mRNA showed a significant correlation for overall survival.

These results suggest that the OPRT mRNA expression levels could be a useful prognostic indicator for disease-free and overall survival of CRC patients with Dukes' stage B and C who are treated with adjuvant chemotherapy based on 5-FU oral administration.

Discussion

In this study, we showed that the OPRT mRNA level makes it possible to predict a prognosis in Dukes' B and C stage CRC patients treated with oral 5-FU-based adjuvant chemotherapy.

As to the methodology of 5-FU metabolic pathway enzymes, protein activity, immunohistochemistry, enzyme-linked immunosorbent assay (ELISA) and mRNA levels have been reported (13-15). Concerning the routine measurement of these enzymes, protein activity measurements are not technically feasible because of the complexity in the use of radioisotopes. In contrast, the real-time RT-PCR method is reproducible, simple, rapid and suitable for use even with very small amounts of tumor tissues such as a biopsy. Furthermore, it has been thought that inaccurate results occur when the surrounding normal tissues are contaminated in tumor samples. To resolve this problem, we introduced a laser micro-dissection system in combination with real-time RT-PCR.

Several molecular markers associated with 5-FU metabolic pathway enzymes such as OPRT, DPD, TS and TP have been examined for prediction of the prognosis in CRC patients

treated with 5-FU-based chemotherapy (13-15,18-22). In our study, all patients were treated with Tegafur-Uracil combination therapy. It is known that Tegaful is converted to 5-FU, which kills cancer cells and Uracil inhibits degradation of the relased 5-FU.

OPRT is anticipated to play a key role in the first step, leading to DNA synthesis inhibition and RNA dysfunction. Recently, using the enzyme activity method though not mRNA, Ochiai *et al* reported that patients with high levels of OPRT activity have a longer survival outcome than individuals with low levels (15). Ichikawa *et al* reported that the survival time of patients with a high OPRT/DPD ratio is significantly longer than that of patients with a low OPRT/DPD ratio in metastatic CRC-treated with 5-FU-based chemotherapy (23). In this study, using the real-time RT-PCR followed by sampling used by laser microdissection, we demonstrated that the disease-free and overall survival times of the OPRT mRNA high-expression group were significantly longer than that of the low-OPRT mRNA group. Furthermore, in the multivariate Cox regression analysis, we demonstrated that the OPRT mRNA level is an independent prognostic value for disease-free survival and overall survival. To the best of our knowledge, our paper is the first study that demonstrates the prognostic value of OPRT mRNA of tumor cells in Dukes' stage B and C patients treated with 5-FU-based oral adjuvant chemotherapy.

DPD is a rate-limiting enzyme released during the catabolism of 5-FU. In a previous study, it was reported that high DPD mRNA levels affect the acquisition of resistance to 5-FU (24). Many reports show that patients with low-DPD levels have a better prognosis to 5-FU than those with high-DPD levels (14,25). In our Kaplan-Meier survival curve analysis, the low-DPD mRNA group showed a significantly longer survival time than those in the high-DPD mRNA group. In the Cox univariate analysis, our study demonstrated that the DPD mRNA expression in tumors was related to survival. In a multivariate analysis, however, DPD mRNA level did not show an independent prognostic value. Ichikawa *et al* reported that the DPD gene expression had no predictive value for survival supporting our results (24).

The prognostic value of TS in colorectal cancer patients also appears to be controversial. Several studies have reported that TS levels predict an overall outcome for patients in the early stages of CRC (26,27). Elder *et al* reported that patients whose tumors expressed the highest expression of TS had a significantly longer disease-free survival if they were treated with adjuvant therapy compared with surgery (28). In contrast, in the present study, it was difficult to predict the efficacy of TS mRNA levels for prognosis. This discrepancy might be explained by the differences in the treatment schedule and drugs based on 5-FU such as Tegaful, UFT, 5'-DFUR and TS-1. In our study, we treated the Dukes' B or C patients with oral administration of Tegaful and Uraci or UFT, Uracil plus LV.

It is known that TP has activity as an angiogenesis-inducing factor and as a metabolic enzyme for the fluoropyrimidines. The prognostic significance of TP mRNA expression in CRC is still unclear. Nishimura *et al* reported that the prognosis of high TP groups is better than that of low TP groups (29). Salonga *et al* reported that TP together with

high levels of TS are reported to be associated with a poor response to 5-FU (30). In the present study, however, TP mRNA did not show a prognostic value for disease-free survival and overall survival.

In this study, we have shown that the OPRT mRNA level is useful for the prediction of a prognosis in Dukes' B and C stage CRC patients treated with oral 5-FU-based adjuvant chemotherapy. The information of the OPRT gene appears to be a promising approach for optimal individualized adjuvant treatment.

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