Dihydropyrimidine dehydrogenase and orotate phosphoribosyltransferase in esophageal cancer patients: Correlation with clinicopathological factors and prognosis

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Abstract. Thymidylate synthase (TS), dihydropyrimidine dehydrogenase (DPD) and orotate phosphoribosyltransferase (OPRT) are fluoropyrimidine metabolic enzymes which play important roles in the response of cancer patients to chemotherapy. In esophageal cancer, little is known about the relationship between the expression of these enzymes and corresponding clinicopathological features. In the present study, TS, DPD and OPRT expression levels were evaluated in 72 resected esophageal cancer specimens using immunohistochemistry. The relationship between enzyme expression and clinicopathological features was assessed using Fisher's exact test or the χ^2 test (categorical variables), or the Mann-Whitney rank-sum test (continuous variables). Survival curves were calculated using the Kaplan-Meier method, and differences evaluated using the log-rank test. The Cox proportional hazards model was also used. High DPD expression was associated with depth of invasion, nodal status, tumor stage, lymphatic invasion and venous invasion ($P<0.001$, $P=0.004$, $P<0.001$, $P=0.006$, $P=0.038$, respectively), as well as with decreased patient survival ($P=0.007$). In patients receiving adjuvant chemotherapy, low DPD expression did not significantly improve recurrence-free survival. OPRT was particularly expressed in esophageal cancer cells as compared to normal squamous cells. High OPRT expression was associated with depth of invasion and venous invasion ($P=0.006$, $P=0.003$, respectively). To conclude, in esophageal cancer DPD expression was associated with tumor progression and prognosis, and OPRT expression was correlated with carcinogenesis and tumor progression.

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Introduction

5-Fluorouracil (5-FU) is a widely used anticancer agent that is currently considered a key drug in clinical chemotherapy for gastrointestinal cancers, including esophageal, gastric and colorectal cancer (1-3). The response rate of the drug and its derivatives is dependent upon inter-individual differences in anabolic and catabolic enzyme activities. 5-FU is catabolized to 2-fluoro-ß-alanin by the first- and rate-limiting enzyme dihydropyrimidine dehydrogenase (DPD) in its metabolic pathway. The functional effects of 5-FU are believed to occur through its active metabolite, 5-fluorodeoxyuridine monophosphate (FdUMP), which together with the co-enzyme 5,10-methylenetetrahydrofolate (M-THF) forms a covalent ternary complex with the DNA *de novo* synthesizing enzyme thymidylate synthase (TS) (4,5). This complex blocks the conversion of deoxyuridine monophosphate (dUMP) to thymidine monophosphate (dTMP) and thus inhibits DNA synthesis. Hence, the pharmacogenetic variability of 5-FU-related enzymes such as DPD and TS may be a major determinant of outcome for gastrointestinal cancer patients treated with 5-FU. The expression patterns of TS and DPD have been studied numerous times in an attempt to predict the sensitivity of 5-FU and clinical outcome in various cancers (6-11).

Orotate phosphoribosyltransferase (OPRT) is the first-limiting enzyme in the conversion of 5-FU and leads, in the presence of 5-phosphoribosyl-l-pyrophosphate as a co-factor, to the formation of FdUMP (12,13). Previous studies have demonstrated that adenovirus-mediated transduction of the OPRT gene results in the marked sensitization of colon, gastric, liver and pancreatic cancer cells to 5-FU cytotoxicity (14,15). Thus, OPRT is an important enzyme in 5-FU activation. It is moreover the rate-limiting enzyme in the *de novo* process of DNA and RNA synthesis, which converts orotic acid to orotidine 5'-phosphate (16). Few studies have evaluated the relationship between OPRT and corresponding clinicopathological features, as a sensitive antibody for OPRT had yet to be established. However, a new highly sensitive antibody for OPRT is currently available (17).

To the best of our knowledge, there have been few reports describing the association of TS and DPD expression with carcinogenesis and prognosis in esophageal cancer, and there

have been no reports regarding OPRT expression in esophageal cancer patients. Hence, little is known about the relationship between these fluoropyrimidine metabolic enzymes and clinicopathological features in esophageal cancer. In the present study, we analyzed the expression levels of TS, DPD and OPRT in surgically resected specimens of esophageal cancer using immunohistochemistry, and assessed the correlation between the expression patterns of these enzymes and factors such as pathologic spread and prognosis.

Patients and methods

This study was approved by our institutional review board for retrospective review and analysis with a waiver of individual consent.

Patient characteristics. Formalin-fixed, paraffin-embedded tumor tissue was retrospectively collected from 72 patients who underwent esophageal resection for esophageal cancer between January 1996 and December 2000 in the Department of Surgical Oncology at the Research Institute for Radiation Biology and Medicine of Hiroshima University, Hiroshima, Japan. Transhiatal esophagectomy was performed on 12 patients with lower thoracic or abdominal esophageal carcinoma, while the remaining patients underwent transthoracic esophagectomy without cervical lymph node dissection. Any patient who had received preoperative therapy (neoadjuvant chemotherapy and/or radiotherapy) was excluded from the study. All patients were pathologically classified according to the 2002 TNM classification (6th edition).

Immunohistochemistry. The Dako LSAB Kit (Dako, Carpinteria, CA, USA) was used for immunohistochemical analysis. Briefly, sections were deparaffinized with xylene and gradually rehydrated with graded ethanol. Endogenous peroxidase was inactivated by 0.03% hydrogen peroxide in methanol for 15 min. To retrieve the antigenicities of TS and DPD, hydrated heating at 120˚C in 1 mM EDTA solution (pH 8.0) was performed in a pressure cooker for 12 min. After pressure cooking, the sections were left to cool at room temperature in soaking solution for 30 min. Immunostaining for OPRT was performed without an antigen retrieval step. Primary antibodies included a rabbit polyclonal anti-TS antibody (1:250 dilution), a rabbit polyclonal anti-DPD antibody (1:500 dilution) and a rabbit polyclonal anti-OPRT antibody (1:1000 dilution). All antibodies were provided by Taiho Pharmaceutical Co. (Tokushima, Japan). Sections were consecutively treated overnight with anti-TS and anti-DPD antibody, and with anti-OPRT antibody at room temperature for one hour followed by sequential 30-min incubation with biotinylated anti-rabbit IgG and peroxidase-labeled streptavidin. Staining was complete after a 30-sec incubation with substrate-chromogen solution. Sections were counterstained with 0.1% hematoxylin. The results of the antibody staining were graded according to the percentage of stained target cells. When 50% of the cells were stained, the result was considered high expression. The immunostained sections were independently reviewed by 2 investigators (Y.T. and Y.S.).

Statistical analysis. The association between the expression of each metabolic enzyme for 5-FU and individual clinical and pathological variables (age, gender, location, pathological stage, histological classification and vascular invasion) was assessed using Fisher's exact test or the χ^2 test (categorical variables), or the Mann-Whitney rank-sum test (continuous variables).

The association between individual, clinical and pathological variables (age, gender, pathological stage and grade, vascular invasion, TS, DPD and OPRT expression) and survival was assessed using the Cox proportional hazards regression model. Survival time was determined as the time from resection to death. For survivors, survival times were recorded as the last date that patients were known to be alive. Survival probabilities were estimated using the Kaplan-Meier method. Log-rank tests were implemented to compare survival curves among the various subgroups of patients. All statistical tests were two-tailed. All analyses were performed with SPSS software (version 10.5, SPSS Inc., Chicago, IL, USA). A P-value <0.05 was considered statistically significant.

Results

Patient characteristics and fluoropyrimidine metabolic enzyme immunohistochemistry. Immunostaining was used to evaluate 72 patient samples for each of the fluoropyrimidine metabolic enzymes. TS was expressed primarily in the cytoplasm and infrequently in the nuclei. DPD and OPRT staining was localized in the cytoplasm of tumor cells (Fig. 1A, B and D). The expression patterns of TS and DPD were similar in normal esophageal squamous cells and in cancer tissue. Normal esophageal squamous cells did not stain for OPRT (Fig. 1C). TS was highly expressed in all 72 tumors (100%). DPD was highly expressed in 26 of the 72 tumors (36%). OPRT was highly expressed in 62 of the 72 tumors (86%). Patient clinical and pathological characteristics are depicted in Table I. High DPD expression was associated with depth of invasion, nodal status, tumor stage, lymphatic invasion and venous invasion (P<0.001, P=0.004, P<0.001, P=0.006, P=0.038, respectively). High OPRT expression was associated with depth of invasion and venous invasion (P=0.006, P=0.003, respectively). None of the patients with low OPRT expression had received adjuvant chemotherapy.

Survival analysis. The median follow-up duration for all patients was 38 months, and for surviving patients 59 months. Actuarial 1-, 2-, 3- and 5-year survival rates for this population of patients with resected esophageal cancer were 74, 67, 55 and 47%, respectively. The role of DPD or OPRT and other clinical and pathological variables in predicting prognosis in esophageal cancer was evaluated using single-variable Cox proportional hazards regression analysis (Table II). High DPD expression was associated with decreased patient survival (P=0.007; Fig. 2). Median survival among patients with low DPD-expressing tumors was longer than among patients with high DPD-expressing esophageal cancer (not reached versus 25 months). Depth of invasion, nodal status, TNM stage and venous invasion were also predictive of overall patient survival. Age, gender, tumor grade, adjuvant chemotherapy and OPRT expression did not significantly influence survival in this group of patients; however, high OPRT expression acted as a poor prognostic factor (P=0.11; Fig. 3). Multivariate analysis showed that, as a prognostic factor, DPD expression

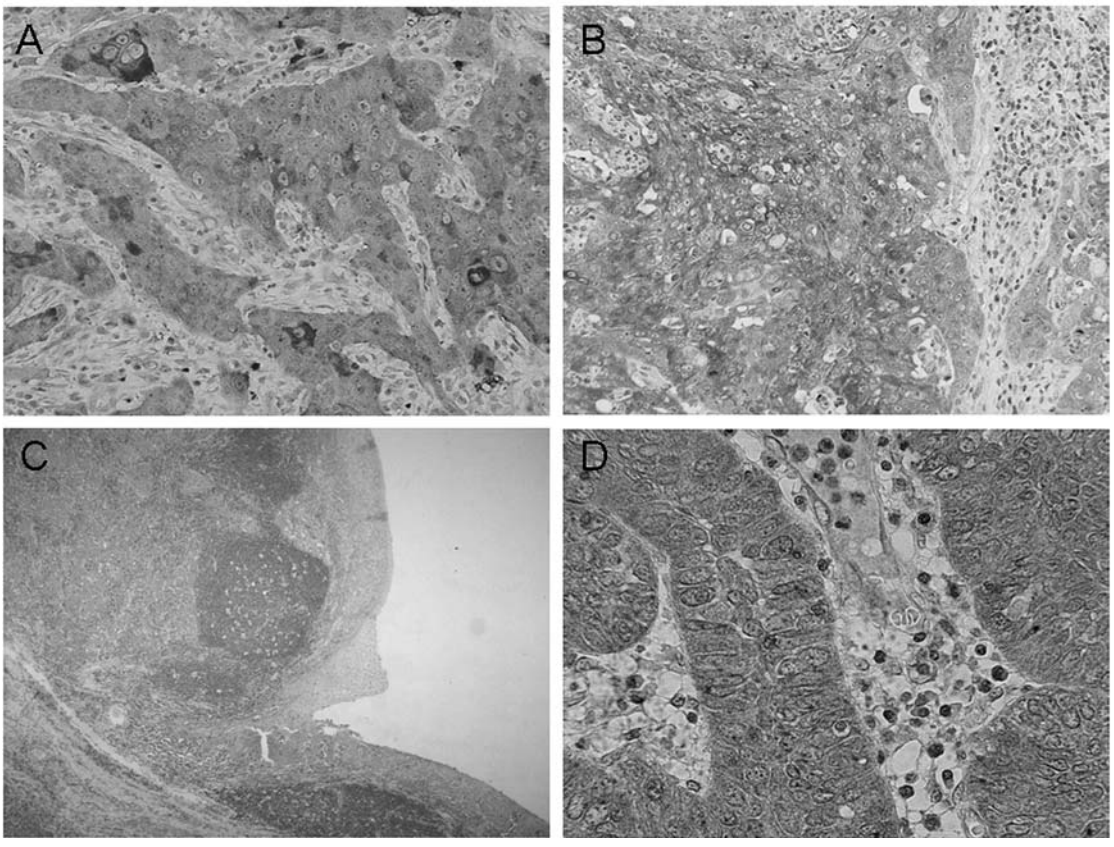

Figure 1. Immunohistochemistry of TS (A, x400), DPD (B, x400), and OPRT (C, x40 and D, x400) in esophageal cancer. High TS, DPD, and OPRT immunoreactivities were observed in the cytoplasm of most carcinoma cells (A, B and D). Normal esophageal squamous cells did not stain for OPRT (C). TS, thymidylate synthase; DPD, dihydropyrimidine dehydrogenase; OPRT, orotate phosphoribosyltransferase.

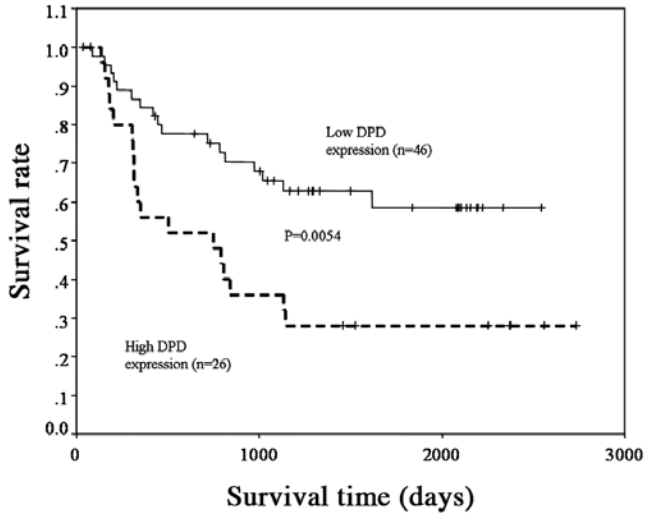

Figure 2. Actuarial survival of patients with resected esophageal cancer (n=72). Survival in patients with low DPD-expressing esophageal cancer (n=46) was significantly (P=0.0054) longer than in patients with high DPD-expressing tumors (n=26). DPD, dihydropyrimidine dehydrogenase.

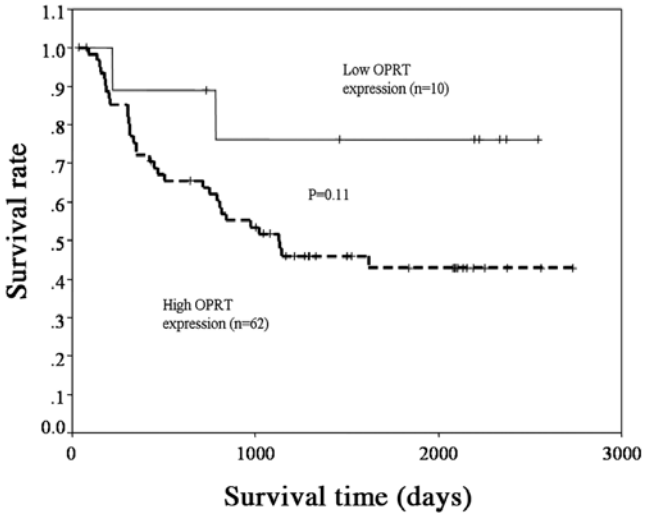

Figure 3. Actuarial survival of patients with resected esophageal cancer (n=72). Survival in patients with low OPRT-expressing esophageal cancer (n=10) was longer than in patients with high OPRT-expressing tumors (n=62, P=0.11). OPRT, orotate phosphoribosyltransferase.

tended to be less indicative than lymph node metastasis, depth of invasion and venous invasion (Table III).

DPD expression and response to adjuvant chemotherapy. The influence of DPD expression on response to adjuvant chemotherapy was further analyzed in resected esophageal cancer. In patients receiving adjuvant chemotherapy, low DPD expression did not significantly improve recurrence-free survival (P=0.67; Fig. 4). The expression of OPRT and response to adjuvant chemotherapy could not be analyzed as none of the patients with low OPRT expression had received adjuvant chemotherapy.

Table I. Clinicopathological characteristics of 72 patients with resected esophageal cancer evaluated for DPD and OPRT expression.[a]

		OPRT expression			DPD expression		
	Total (%)	High (%)	Low (%)	P-value	High (%)	Low (%)	P-value
No. of patients	72	62 (86)	10 (14)		26 (36)	46 (64)	
Age (years)	64.1±8.2	64.7±8.3	60.2±6.8	0.144	64±7.7	64.2±8.6	0.977
Gender				0.397			0.070
Male	58 (81)	51 (82)	7 (70)		24 (92)	34 (74)	
Female	14 (19)	11 (18)	3 (30)		2 (8)	12 (26)	
Location				0.522			0.584
Ut	6 (8)	6 (10)	0 (0)		3 (12)	3 (7)	
Mt	42 (58)	34 (55)	8 (80)		16 (62)	26 (60)	
Lt	16 (22)	14 (23)	2 (20)		6 (23)	10 (23)	
Ae	5 (7)	5 (8)	0 (0)		1 (3)	4 (10)	
Depth of invasion				0.006			<0.001
T1	42 (58)	32 (52)	10 (100)		8 (31)	34 (74)	
T2	7 (10)	7 (11)	0 (0)		5 (19)	2 (4)	
T3	16 (22)	16 (26)	0 (0)		9 (35)	7 (15)	
T4	7 (10)	7 (11)	0 (0)		4 (15)	3 (7)	
Nodal status				1.000			0.004
N0	33 (46)	28 (45)	5 (50)		6 (23)	27 (59)	
N1	39 (54)	34 (55)	5 (50)		20 (77)	19 (41)	
UICC stage				0.258			<0.001
I	30 (42)	25 (40)	5 (50)		4 (15)	26 (57)	
II	15 (21)	11 (18)	4 (40)		6 (23)	9 (20)	
III	15 (21)	15 (24)	0 (0)		9 (35)	6 (13)	
IV	12 (16)	11 (18)	1 (10)		7 (27)	5 (10)	
Histologic grade (SCC)				0.661			0.790
Well differentiated	7 (10)	6 (10)	1 (10)		2 (8)	5 (11)	
Moderately differentiated	32 (44)	29 (47)	3 (30)		13 (50)	19 (41)	
Poorly differentiated	29 (40)	24 (39)	5 (50)		10 (38)	19 (41)	
Other	4 (6)	3 (4)	1 (10)		1 (4)	3 (7)	
Lymphatic invasion				0.070			0.006
0	8 (11)	6 (10)	2 (20)		1 (4)	7 (16)	
1	38 (53)	31 (53)	7 (70)		10 (40)	28 (62)	
2	11 (15)	10 (17)	1 (10)		6 (25)	5 (11)	
3	12 (17)	12 (20)	0 (0)		7 (31)	5 (11)	
Venous invasion				0.003			0.038
0	26 (36)	18 (31)	8 (80)		5 (21)	21 (47)	
1	39 (54)	37 (63)	2 (20)		17 (71)	22 (49)	
2	4 (6)	4 (6)	0 (0)		2 (8)	2 (4)	
Adjuvant chemotherapy				0.054			0.100
Yes	19 (26)	19 (31)	0 (0)		10 (38)	9 (20)	
No	53 (74)	43 (69)	10 (100)		16 (62)	37 (80)	

[a]Data are presented as the number (percentage) or as the mean ± SD. DPD, dihydropyrimidine dehydrogenase; OPRT, orotate phosphoribosyltransferase; SCC, squamous cell carcinoma.

Table II. Univariate analyses of prognostic factors in patients with resected esophageal cancer evaluated for DPD and OPRT expression.

	No.	Median survival (months)	5-year survival (%)	RR (95% CI)	P-value
Age (years)					0.585
≤64	35	Not reached	51.4	1	
>64	37	34	42.9	1.2 (0.62-2.3)	
Gender					0.136
Female	14	Not reached	70.7	0.45 (0.16-1.3)	
Male	58	38	41.8	1	
Depth of invasion					<0.001
T1, T2	49	Not reached	65.1	1	
T3, T4	23	11	11.6	5.6 (2.8-11)	
Nodal status					<0.001
N0	33	Not reached	75.1	1	
N1	39	14	22.4	6.7 (2.9-15)	
UICC stage					<0.001
I, II	50	Not reached	65.7	1	
III, IV	22	10	6.8	6.6 (3.3-13)	
Histologic grade (SCC)					0.136
Well differentiated	12	Not reached	66.7		
Moderately differentiated	29	38	35.0		
Poorly differentiated	26	Not reached	55.1		
Lymphatic invasion					0.107
Negative	8	Not reached	100.0	1	
Positive	61	34	40.1	27 (0.49-1478)	
Venous invasion					0.002
Negative	26	Not reached	76.6	1	
Positive	43	28	30.2	4.02 (1.66-9.77)	
Adjuvant chemotherapy					0.122
Yes	19	17	36.8	1.74 (0.86-3.5)	
No	53	Not reached	51.0	1	
OPRT expression					0.131
High	62	38	42.8	3.0 (0.72-13)	
Low	10	Not reached	76.2	1	
DPD expression					0.007
High	26	25	28.0	2.49 (1.28-4.84)	
Low	46	Not reached	58.6	1	

DPD, dihydropyrimidine dehydrogenase; OPRT, orotate phosphoribosyltransferase; SCC, squamous cell carcinoma.

Discussion

The current study revealed that all 72 esophageal cancer specimens exhibited high expression of TS. Therefore, we were unable to assess the relationship between TS expression and the clinicopathological factors of esophageal cancer. Harpole *et al* reported a high expression level of TS (56%) associated with decreased survival in patients treated with trimodality therapy for esophageal cancer (18). They used a monoclonal antibody of TS, and the histology of esophageal cancer in the report was mainly adenocarcinoma (69.5%). Although adenocarcinoma is the most common form (>50%) of esophageal cancer in North America and many Western European countries, approximately 90% of esophageal cancer in Japan is squamous cell carcinoma (19). In this study, 67 of the 72 patients had squamous cell carcinoma. In Chinese patients with squamous cell carcinoma, high TS protein expression was observed in 41 of 51 cases (80%) by

Table III. Multiple regression analysis of prognostic factors in resected esophageal cancer.

	No.	Median survival (month)	5-year survival (%)	RR (95% CI)	P-value
Depth of invasion					0.027
T1, T2	49	Not reached	65.1	1	
T3, T4	23	11	11.6	2.541 (1.11-5.82)	
Nodal status					0.005
N0	33	Not reached	75.1	1	
N1	39	14	22.4	3.76 (1.48-9.57)	
Venous invasion					0.535
Negative	26	Not reached	76.6	1	
Positive	43	28	30.2	1.42 (0.47-4.24)	
DPD expression					0.729
High	26	25	28.0	1.14 (0.55-2.35)	
Low	46	Not reached	58.6	1	

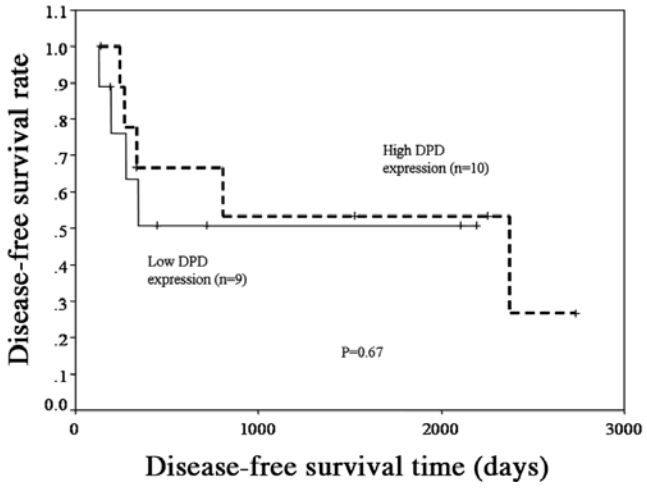

Figure 4. Recurrence-free survival of patients receiving adjuvant chemotherapy (n=19). DPD expression was not significantly associated with recurrence-free patient survival. DPD, dihydropyrimidine dehydrogenase.

immunohistochemistry (9). Our finding of a high TS expression rate similar to that observed in the Chinese study suggests that TS expression might be specific to esophageal squamous cell or squamous cell carcinomas.

There have been a few reports regarding the correlation between DPD and esophageal cancer. Noguchi *et al*, using an enzyme-linked immunosorbent assay, reported that no significant difference was detected between mean DPD concentrations in the esophageal tumor tissue and normal tissue of 33 patients with esophageal cancer (10). The current study demonstrated that high DPD expression was associated with depth of invasion, nodal status, tumor stage, lymphatic invasion and venous invasion. Moreover, high DPD expression was associated with poor prognosis in esophageal cancer. These results suggest that DPD expression might be associated with tumor progression and prognosis in esophageal cancer.

There have been no reports on OPRT expression in esophageal cancer. Kamoshida *et al* investigated the expression of TS, DPD, thymidine phosphorylase and OPRT in various types of cancer, including lung, stomach, colon, liver, gall bladder, pancreas, kidney, bladder and breast cancer, using the immunoperoxidase method. The expression patterns of enzymes in normal epithelial tissue were generally the same as those in the respective cancer tissue (20). In the present study, the OPRT expression rate was high (86%), and the expression of OPRT protein in esophageal cancer was remarkably higher than in normal esophageal squamous cells. Moreover, high OPRT expression was associated with depth of invasion and venous invasion. These results suggest that OPRT expression may be correlated with carcinogenesis and tumor progression in esophageal cancer. Mizutani *et al* reported that OPRT mRNA activity levels in specimens of bladder cancer and renal cell cancer were higher than in those from the normal bladder and kidney (16,21). OPRT expression may be specific for certain types of carcinoma, such as bladder cancer, renal cell cancer and esophageal cancer.

The current study did not examine the relationship between TS, OPRT expression and 5-FU sensitivity, as all patients had high TS expression and none of those with low OPRT expression had received adjuvant chemotherapy. DPD was not correlated with a response to fluorouracil-based adjuvant chemotherapy in this study, while in a previous study regarding colon cancer, lower DPD activity in cancer cells was associated with an improved response to 5-FU chemotherapy (11). In the present study, a small number of patients had received adjuvant chemotherapy. These patients showed a tendency towards having a poorer prognosis (Table II). As a result, it was difficult to assess the relationship between DPD expression and 5-FU sensitivity.

This is the first report on the expression of fluoropyrimidine metabolic enzymes, including OPRT, and associated clinicopathological features in esophageal cancer patients who did not receive preoperative therapy. Further studies are currently being conducted to assess the relationship between the expression of DPD or OPRT and chemosensitivity in esophageal cancer patients treated with 5-FU-containing neoadjuvant chemotherapy.

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