SPANDIDOS PUBLICATIONS

The expression profiles of orotate phosphoribosyltransferase and dihydropyrimidine dehydrogenase in gastric cancer and their clinical significance

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Abstract. Orotate phosphoribosyltransferase (OPRT) is an enzyme that causes the activation of 5-fluorouracil (5-FU). Dihydropyrimidine dehydrogenase (DPD) is known to catabolize 5-FU, which is widely used in chemotherapeutic treatments for patients with a variety of malignant tumors including gastric and colorectal cancer. The expression and activities of these two enzymes therefore play important roles in the response of cancer patients to chemotherapy. However, little is known about the expression of these enzymes in gastric cancer. In the present study, we further elucidate the expression patterns of ORPT and DPD and their clinicopathological significance by immunohistochemical analysis in 221 and RT-PCR in 36 gastric cancer samples. The expression of OPRT by immunohistochemical analysis was detected in 117 (52.9%) cases, whereas DPD was detected in 66 (29.9%) cases. Moreover, the level of expression of OPRT was found to correlate with the depth of tumor invasion and a poorer prognosis. Although the mRNA and protein expression of OPRT and DPD levels did not correlate, an inverse correlation in the expression of OPRT and DPD was observed by RT-PCR. The survival benefit of post-operative adjuvant chemotherapy could not be confirmed in our present analysis. However, among the patients who had received such treatment with 5-FU or its derivatives, the prognosis in cases with low DPD levels was better than that in cases with high DPD expression by immunohistochemical analysis. These results indicate that the expression of OPRT and DPD are important predictors of both survival and the response to adjuvant chemotherapy in gastric cancer patients.

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Introduction

Recent advances in chemotherapy, such as biochemical modulation and combinations of new therapeutic agents, have made a significant and positive impact upon both patient response and survival in gastric cancer (1-4). However, standard treatment regimens for gastric tumors have not yet been established (3,5). In addition, a prediction of patient response which might facilitate personalized therapy, is currently of great interest in the treatment of cancer patients (6,7).

5-Fluorouracil (5-FU) and its derivatives have been widely used and are considered to be key drugs in the treatment of solid tumors, including gastric cancer. 5-FU itself is inactive and needs to be metabolized to 5-fluorouridine 5'-monophosphate (FUMP) in cells, upon which it inhibits RNA and DNA synthetic processes. The main mode of function of 5-FU is thought to act through its active metabolite, 5-fluorodeoxyuridine monophosphate (FdUMP). Together with the coenzyme 5,10-methylene tetrahydrofolate (M-THF), FdUMP forms a covalent ternary complex with the DNA *de novo* synthesizing enzyme thymidylate synthase (TS), blocking the conversion of deoxyuridine monophosphate (dUMP) to thymidine monophosphate (dTMP) and thus inhibiting DNA synthesis (8-11). Orotate phosphoribosyltransferase (OPRT) is the first-limiting enzyme to metabolize 5-FU to FUMP (8) and dihydropyrimidine dehydrogenase (DPD) is a rate-limiting enzyme in the catabolism of 5-FU (12). Therefore, high OPRT and low DPD activities in cancer cells will, in principle, facilitate a more effective response to chemotherapy with 5-FU. Recent studies have demonstrated that high expression of DPD mRNA and strong activity of this enzyme is associated with resistance to 5-FU with colorectal cancer and gastric cancer (13-17). Other studies have demonstrated that high expression of OPRT mRNA and strong activity of this enzyme is associated with effect to 5-FU with colorectal cancer and gastric cancer (18,19). However, there have not been any previous reports on the significance of OPRT and DPD expression, detected by immunohistochemical staining, in gastric cancer.

In our present study, we further elucidated the significance of OPRT and DPD expression in gastric cancer patients by

Table I. Patient characteristics.[a]

Sex	
Male	139
Female	82
Depth of invasion (T)	
T1	34
T2	94
T3	63
T4	30
Lymph node metastasis (N)	
N0	74
N1	54
N2	54
N3	39
Histological type[b]	
Well	123
Poor	98
Stage	
IA	24
IB	45
II	38
IIIA	29
IIIB	21
IV	64

[a]Clinicopathological factors were determined according to Japanese classification of gastric carcinoma (32). [b]Poor histological type consisted of por1, por2 and sig.

Table II. Profile of 5-FU based adjuvant chemotherapy following surgery.

Stage B	
Chemo (-)	15
Chemo (+)	27
UFT[a]	15
MMC+5-FU[b]	6
MMC+5-FU, UFT	5
Others	1
Stage II	
Chemo (-)	5
Chemo (+)	31
UFT	17
MMC+5-FU	6
MMC+5-FU, UFT	5
Others	3
Stage IIIA	
Chemo (-)	7
Chemo (+)	21
UFT	13
MMC+5-FU	0
MMC+5-FU, UFT	4
Others	4
Stage IIIB	
Chemo (-)	4
Chemo (+)	15
UFT	9
MMC+5-FU	0
MMC+5-FU, UFT	1
Others	5

[a]UFT was taken orally at a dose of 300 mg/day for two years. [b]MMC+5-FU, MMC was injected 10 mg on operative day and 5-FU was taken orally at a dose of 150 mg/day for two years.

immunohistochemistry and RT-PCR. In addition, the association between post-operative adjuvant chemotherapy and the expression levels of these enzymes was analyzed to further investigate the possibilities for personalized therapies in the treatment of gastric cancer.

Patients and methods

Patients and tissue samples. We obtained 221 specimens of primary gastric cancer that were removed at Hiroshima University Hospital from 1990 to 2000. The patient group comprised 139 men and 82 women and their clinicopathological background is shown in Table I. Within this group, 94 individuals who were classified as either stage IB, II or III, received post-operative adjuvant chemotherapy including 5-FU and/or its derivatives for 12 to 24 months after surgery. The adjuvant chemotherapy regimens are summarized in Table II.

Immunohistochemistry. A Dako LSAB kit (Dako, Carpinteria, CA, USA) was used for immunohistochemical staining according to the manufacturer's instructions. Briefly, paraffin-embedded sections (4 μm) were deparaffinized in xylene and rehydrated through a graded ethanol series. For DPD antigen retrieval, the slides were heated in a pressure cooker in a 1-mM EDTA solution for 12 min (20). This step was not required for the detection of OPRT, as reported previously (21). Endogenous peroxidase was inactivated by treatment with 3% H_2O_2-methanol for 10 min and non-specific binding was blocked with 0.3% albumin/PBS for 10 min. Subsequently, the specimens were incubated overnight at room temperature with primary antibodies against OPRT and DPD, diluted at 1:1000 and 1:500, respectively, in TBS-T. Polyclonal antibodies against OPRT and DPD were generously donated by Taiho Pharmaceutical Co., Ltd., Japan. After rinsing in PBS-T, the slides were processed by sequential 30-min incubations with either biotinylated anti-rabbit or anti-mouse IgG and peroxidase-labeled streptavidin. Staining was then completed after a few minutes of incubation with a substrate-chromogen solution and the sections were counterstained with 0.1%

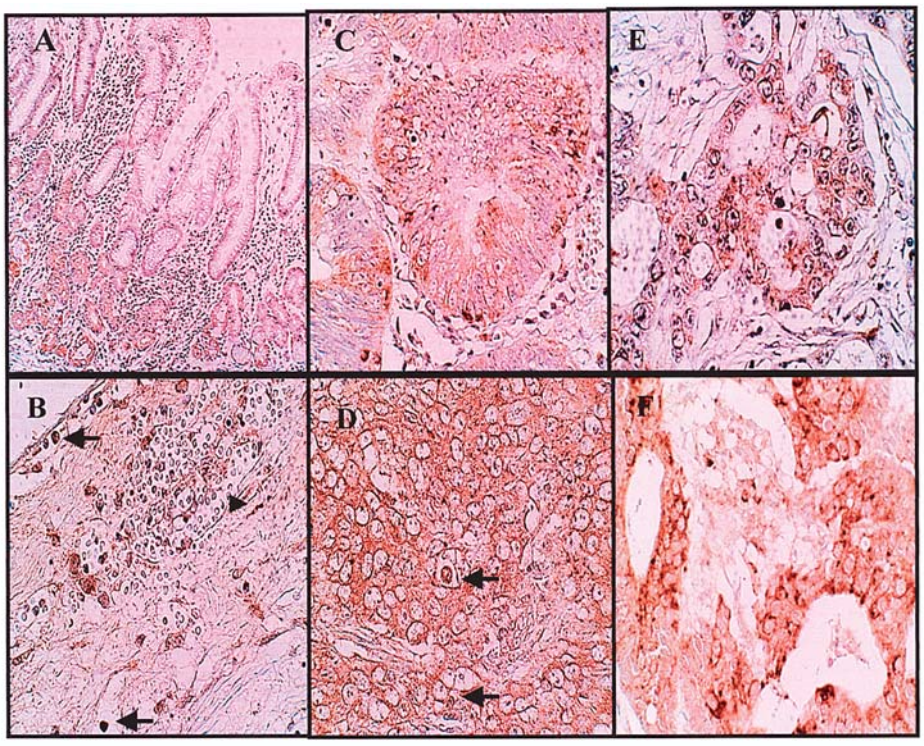

Figure 1. Expression profile of OPRT and DPD in gastric cancer tissue. OPRT expressed strongly in the basal layer in normal gastric mucosa (A) and was observed in lymphocytes (→) and in fibroblasts (➤) (B). OPRT showed a homogeneous distribution in the cytoplasm of gastric cancer cells. (D), OPRT expression was observed granular in the nucleus (→) in cases where the tumor cells had invaded more deeply into the stomach wall. (C), Focal staining pattern of OPRT in gastric cancer cells (tub1). (D), Diffuse staining pattern of OPRT in gastric cancer cells (por1). DPD showed diffuse staining in the cytoplasm of the cancer cells. (E), Focal staining pattern of DPD in gastric cancer cells (tub2). (F), Diffuse staining pattern of DPD in gastric cancer cells (tub2). (A), x100 magnification; (B-F) x400 magnification.

hematoxylin, dehydrated and mounted. The staining of these gastric cancers was examined by two investigators without prior knowledge of the clinical outcome. Stained sections for OPRT and DPD were classified into three groups, based on the proportion of positively stained cancer cells in the lesion. The classifications were: negative (no stained or positive in <10% of cancer cells), focal staining (positive staining in 10-50% of cancer cells) or diffuse staining (positive staining in >50% of cancer cells). We deemed both focal and diffusely stained cases as positive results.

RT-PCR. We obtained 36 frozen specimens of primary gastric cancer and normal tissue at the time of operation and isolated RNA in each case using the RNeasy mini kit (Qiagen Inc., Chatsworth, CA, USA), according to the manufacturer's instructions. One microgram of each RNA sample was then converted to cDNA using the GeneAmp RNA PCR Core Kit (Applied Biosystems, Tokyo, Japan), and PCR was performed with the QuantiTect SYBR Green PCR kit (Roche) in a 10-μl reaction mixture containing 5 μl of Sybr-Green, 0.2 μl of each primer, 3.6 μl RNase free water and 1 μl of each cDNA. The PCR conditions for OPRT and DPD amplification consisted of an initial 30 sec degradation at 95˚C, followed by 55 cycles of 95˚C for 5 sec, 55˚C for 5 sec and 72˚C for 30 sec. This was followed by a final 15 sec extension at 65˚C. To control for variations in the cDNA concentrations, the OPRT and DPD mRNA levels were normalized to ß-actin. We calculated the ratios of both OPRT and DPD to ß-actin in both normal gastric mucosa and gastric cancer tissue.

The OPRT primer sequences were 5'-TCCTGGGCAGAT CTAGTAAATGC-3' (OPRT-1107F) and 5'-TGCTCCTCAG CCATTCTAACC-3' (OPRT-1282R). The DPD primer sequences were 5'-AATGATTCGAAGAGCTTTTGAAGC-3' (DPD-F11) and 5'-GTTCCCCGGATGATTCTGG-3' (DPD-R11). ACTB primer sequences were 5'-CCAACTGGGACG ACATGGAG-3' (ACTB-L) and 5'-GCACAGCCTGGATAG CAACG-3' (ACTB-R).

Statistical analysis. Patient survival times were calculated using the Kaplan-Meier estimation method and differences between individual survival curves were evaluated by the log-rank test. Significant differences between gene expression levels and various clinicopathological factors were evaluated by the χ^2 test. A p-value <0.05 was regarded as statistically significant.

Results

Immunohistochemical detection of OPRT and DPD, and the correlation between this and clinicopathological factors. The expression of OPRT and DPD proteins in normal gastric mucosa was most often detected in a homogeneous pattern, and OPRT expression was strongly evident in the basal layer in particular (Fig. 1A). The expression of these proteins could also be observed in infiltrating lymphocytes and in surrounding fibroblasts (Fig. 1B). Representative staining of OPRT and DPD in gastric cancer tissue is shown in Fig. 1C-F. The expression of OPRT was detected in 117 of our gastric cancer cases (52.9%) and was found to be diffusely stained in

Table III. Correlation between clinicopathological factors and the expression of OPRT or DPD.[a]

Feature	OPRT-positive (%)	OPRT-positive diffuse stained (%)	DPD-positive (%)	DPD-positive diffuse stained (%)
T				
T1+T2	61 (47.7)	29 (22.7)	37 (28.9)	22 (17.2)
T3+T4	56 (60.2)	39 (41.9)	29 (31.2)	17 (18.3)
P-value	0.065	0.002	0.715	0.833
N				
N (-)	34 (45.9)	17 (23)	22 (29.7)	15 (20.3)
N (+)	83 (56.5)	51 (34.7)	44 (29.9)	24 (16.3)
P-value	0.139	0.075	0.975	0.468
Histological type				
Well	65 (52.8)	32 (26)	47 (38.2)	30 (24.4)
Poor	52 (53.1)	36 (36.7)	19 (19.4)	9 (9.2)
P-value	0.975	0.086	0.002	0.003
Stage				
IA	10 (41.7)	4 (16.7)	7 (29.2)	6 (25)
IB	21 (41.7)	10 (22.2)	14 (31.1)	9 (20)
II	16 (42.1)	7 (18.4)	9 (23.7)	3 (7.9)
IIIA	19 (65.5)	12 (41.7)	10 (34.5)	4 (13.8)
IIIB	11 (52.4)	7 (33.3)	7 (33.3)	4 (19)
IV	40 (62.5)	28 (43.8)	19 (31.2)	13 (20.3)

[a]The expression of the OPRT was detected mostly in advanced gastric cancer patients. In particular, diffusely stained OPRT correlated with the depth of invasion and tumor stage. The expression levels of DPD were significantly higher in more differentiated cancers.

68 of these positive samples (30.8%). OPRT expression was generally observed in a homogeneous distribution within the cytoplasm of gastric cancer cells. However, granular staining was detectable around the nuclei or in the nucleus in cases where the tumor cells had invaded more deeply into the stomach wall (Fig. 1D). In contrast, the expression of DPD was clearly observable in only 66 cases (29.9%), among which 39 samples (17.6%) showed diffuse staining in the cytoplasm of the cancer cells (Fig. 1E and F).

We next compared these immunostaining results with both the available clinicopathological data and prognosis of each corresponding patient. A summary of these findings is listed in Table III. The expression of the OPRT protein was mainly detectable in advanced gastric cancer cases. Of particular note was that the depth of tumor invasion and the stage of the tumor could be correlated with the occurrence of diffusely stained cases. However, there was no correlation found between the DPD protein expression levels and tumor staging, except for the fact that DPD expression was more often observed in differentiated gastric cancer types.

Interestingly, the prognosis of patients with a high level of expression of OPRT was significantly poorer than patients who were negative for this protein (Fig. 2A). The 5-year survival value for the former was determined to be 41.2%, and that of the latter was measured at 57.5% by Kaplan-Meier estimation (p=0.0052). With regard to the prognosis of patients with high DPD levels, there was no significant differences found when compared with patients showing low DPD expression (Fig. 2B). We then analyzed the prognosis of patients with or without a combination of both OPRT and DPD and found that a low expression of both of these enzymes was associated with a significantly better prognosis than other groups in our current subjects (Fig. 2C).

Prognosis in patients who had undergone chemotherapy after surgery. Although there was no significant survival benefit for patients following post-operative adjuvant chemotherapy with 5-FU-based regimens, in the small group of 94 cases in our present study (Fig. 3A), we analyzed the correlation between the expression of OPRT and DPD and the prognosis in these same patients. Although we hypothesized that the prognosis in patients with OPRT expression would be improved by adjuvant chemotherapy, as 5-FU would be more efficiently anabolized in occult tumor cells, the prognosis in these patients was in fact significantly poorer compared with individuals who were negative for this enzyme (p=0.0135, Fig. 3B). The 5-year survival rate of patients who were OPRT-positive was 52.3%, compared with 71.1% for OPRT-negative cases.

The prognosis of patients with or without DPD was also analyzed (Fig. 3C) and, as expected, the 5-year survival rate

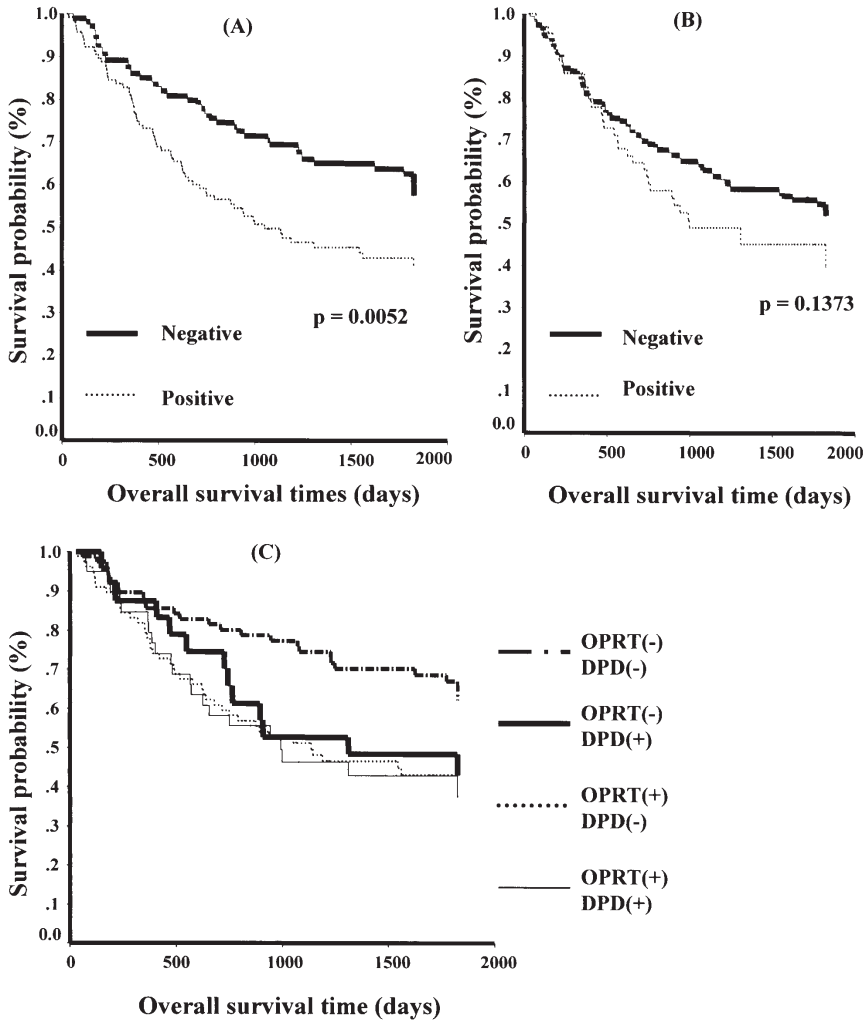

Figure 2. Kaplan-Meier survival curves of all gastric cancer patients in this study, according to OPRT (A) and DPD (B) immunohistochemistry. The survival curves were evaluated by the log-rank test. The patients with positive expression of OPRT protein were significantly poorer prognosis than the patients with negative expression of OPRT (p=0.0052). (C), Kaplan-Meier survival curves of all gastric cancer patients in this study, according to both OPRT and DPD immunohistochemistry. The 5-year survival rate in cases with negative expression for both proteins was 62.0%, and these patients had the best prognosis.

in DPD-positive cases was significantly poorer (45.9%) than patients who were negative for this enzyme (66.5%) (p=0.0274).

Measurement of OPRT and DPD mRNA levels by RT-PCR. We further examined the mRNA expression of OPRT and DPD by RT-PCR and compared these results with our immunohistochemical analysis of these genes. The relative expression levels of OPRT and DPD were determined in 36 patients. We established a cut-off value for the ratio of these expression levels, in normal gastric mucosa and gastric cancer cells, of 1.0 for OPRT and 1.7 for DPD. In 10/36 cases (27.8%) positive expression of OPRT mRNA was detected, whereas DPD mRNA expression was observed in 11 cases (30.6%) (Fig. 4). However, there was no correlation found between these mRNA expression profiles and the results of the immunohistochemistry. Moreover, the mRNA levels of OPRT and DPD did not correlate with the clinicopathological data for these patients (Table IV) and slight inverse correlation was observed between the expression of mRNA for OPRT and DPD (Fig. 4).

Discussion

We previously demonstrated the possibility of formulating personalized chemotherapies for gastric cancer patients using 5-FU-based regimens (22). Several molecular markers can be used to predict the response to chemotherapy, and 5-FU metabolism, in particular, has been extensively characterized in terms of the enzymes involved, including TS, DPD and OPRT. In general, inhibition of RNA and DNA function by 5-FU is thought to be controlled by three pathways. The primary pathway underlying this process is phosphorylation of 5-FU by OPRT to generate FUMP. The second pathway is the conversion of 5-FU by 5-fluorouridine phosphorylase (UP) into 5-fluorouridine (FUR) and the third is phosphorylation of FUR by 5-fluorouridine kinase (UK) also to generate FUMP. By the action of successive phosphorylations, 5-fluorouridine diphosphate (FUDP) is generated, and then converted into FUTP and subsequently uptaken by RNA. Although the precise mechanisms underlying these pathways are not yet well understood, OPRT has recently been

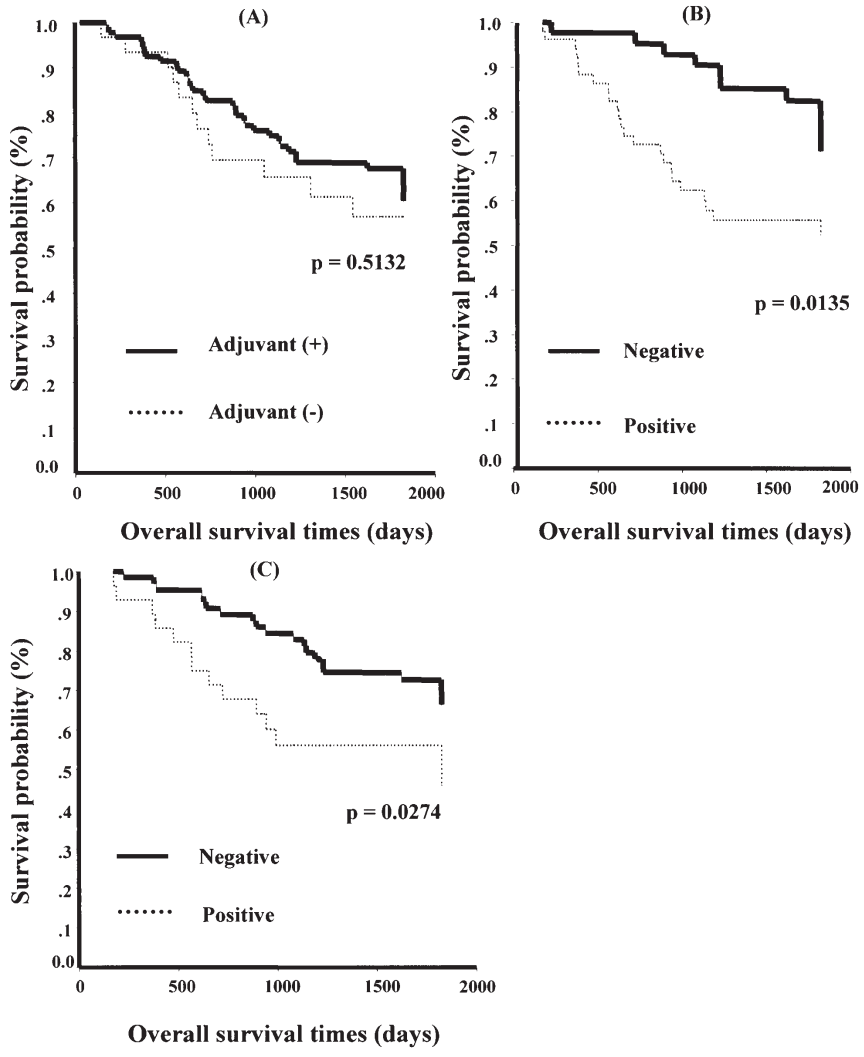

Figure 3. (A), Kaplan-Meier survival curves of gastric cancer patients, who were classified as stage IB-III, according to the occurrence of adjuvant chemotherapy after surgery. There was no statistically significant difference between these patient groups (p=0.5132). Kaplan-Meier survival curves of gastric cancer patients who had undergone 5-FU-based adjuvant chemotherapy after surgery, according to OPRT (B) and DPD (C) immunohistochemistry. The patients with positive expression of OPRT or DPD protein were significantly poorer prognosis than the patients with negative expression of OPRT or DPD.

characterized as one of the important factors that facilitates the functional effects of 5-FU.

Recent studies have demonstrated that the level of OPRT activity is low in tumors which have more invasive properties and are associated with lymph node metastases (23). Furthermore, it has been reported that the level of OPRT activity is higher in more differentiated tumors (24). In addition, patients who are positive for OPRT mRNA expression have a longer survival outcome than individuals with low OPRT expression (25). However, in our current study, the expression of OPRT protein was more frequently detected in cases where the tumor cells had invaded more deeply into the stomach wall and had metastasized to the regional lymph nodes. As expected therefore, the prognosis in patients with higher OPRT expression by immunohistochemistry was poorer. In other studies, it has also been reported that gastric and colon cancers with a higher level of expression of OPRT were more effectively treated by 5-FU-based chemotherapies (25-27). In our present study, it was therefore disappointing that we could not find any improvement in the prognosis of patients with high OPRT expression, even after 5-FU-based adjuvant chemotherapy. On the contrary, our findings indicate that cases with positive expression of OPRT had a considerably poorer prognosis than patients with negative expression. It may prove difficult to clarify the reason for these discrepancies because they have all been reported in retrospective studies, including our current report. However, it is worth clarifying that our present data show that the expression of OPRT can be detected even in normal gastric mucosa and interstitial cells, including fibroblasts and lymphocytes. Moreover, the activity and mRNA expression levels of the OPRT and DPD enzymes was measured following the homogenization of tumor tissues, which allows for the possibility of contamination by non-tumor cells. Hence, analysis of protein expression by immunohistochemistry allows for a more precise assessment of the cells that express the OPRT protein. These factors may partly explain the discrepancies between our current findings and previous reports.

DPD is a rate-limiting enzyme during the catabolism of 5-FU. In a previous study in colon cancer, high DPD activity was correlated with tumor progression (28). Furthermore, in additional reports, a lower activity of DPD in cancer cells was

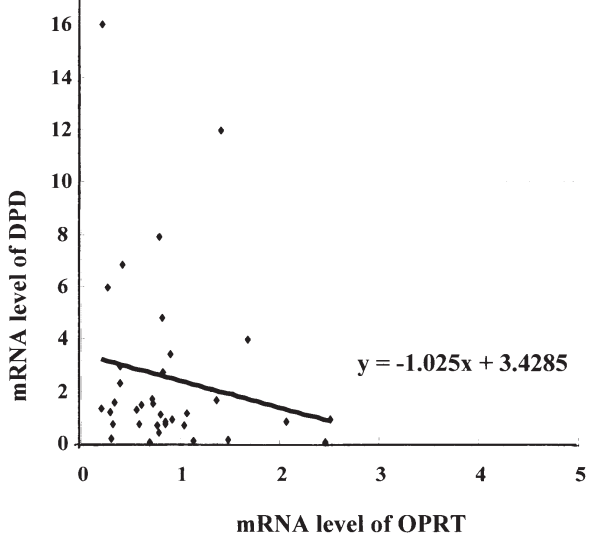

Figure 4. Correlation between the OPRT and DPD mRNA expression levels in 36 clinical samples. There was an inverse correlation between the transcript levels of these enzymes.

Table IV. Correlation with clinicopathological factors and expression of mRNA of OPRT or DPD.[a]

	Total	OPRT-positive (n=10) (%)	DPD-positive (n=11) (%)
T			
T1+T2	14	3 (21.4)	4 (28.6)
T3+T4	22	7 (31.8)	7 (31.8)
P-value		0.497	0.837
N			
N (-)	6	1 (16.7)	2 (33.3)
N (+)	30	9 (30)	9 (30)
P-value		0.318	0.871
Histological type			
Well	21	7 (33.3)	9 (42.9)
Poor	15	3 (20)	2 (13.3)
P-value		0.379	0.058
Stage			
IA	1	0	0
IB	3	1 (33.3)	1 (33.3)
II	8	2 (25)	2 (25)
IIIA	5	1 (20)	0
IIIB	9	2 (22.2)	3 (33.3)
IV	10	4 (40)	5 (50)

[a]Positive expression of OPRT mRNA was found in 10 cases (27.8%) and that of DPD was evident in 11 cases (30.6%). There was no correlation between clinicopathological factors and the expression levels of OPRT or the DPD transcripts.

associated with an improved response to 5-FU chemotherapy (15,29). In our present study in gastric cancer, although there were no apparent correlations between the DPD expression levels and clinicopathological results, patients with high expression of DPD showed a tendency for a poorer prognosis. Interestingly, in patients who had received adjuvant chemotherapy, the prognosis in cases of with high expression of DPD were considerably poorer, which is consistent with previously reported findings (30,31).

In our current experiments, we also analyzed the mRNA expression levels of OPRT and DPD by RT-PCR. As expected, there was no apparent correlation between the result of our immunohistochemical analysis and the mRNA data. This may possibly be due to contamination of the samples by non-tumor cells. Moreover, in patients with large amounts of connective tissue in the interstitial regions of their tumors, the mRNA levels may be reduced below their actual level.

In summary, although there are several issues to be resolved in the study of future gastric cancer treatments, including the expression pattern of OPRT, tumor progression and chemosensitivity, our present results indicate that OPRT and DPD are potentially very valuable prognostic markers in this disease. Furthermore, these enzymes may also play a pivotal role in predicting the sensitivity of gastric tumors to adjuvant chemotherapy with 5-FU and its derivatives.

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