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ORIGINAL ARTICLE

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Dihydropyrimidine dehydrogenase expression in preoperative biopsy and surgically resected specimens of gastric carcinoma

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Abstract *Purpose:* 5-Fluorouracil (5-FU) is most commonly used to treat patients with gastrointestinal neoplasms, including gastric carcinoma. Dihydroxypyrimidine dehydrogenase (DPD), an enzyme involved in metabolism of 5-FU, is a key factor determining the sensitivity of the tumors to 5-FU. Particularly when preoperative chemotherapy based on 5-FU is attempted, it is critical to know in advance how much DPD the tumor expresses. We investigated preoperative biopsy and surgically resected specimens of gastric carcinomas immunohistochemically for the expression of DPD. *Methods:* The study group comprised 55 advanced gastric carcinoma patients who had undergone surgery. Sections of the biopsy and resected specimens were immunostained with anti-DPD polyclonal antibody. DPD immunoreactivity was classified into four groups based on staining intensity expressed as DPD score. We compared the DPD scores of the biopsy specimens with those of the resected specimens. The possible associations between DPD score and survival rate or clinicopathological parameters, including prognostic factors, were also analyzed. *Results:* The DPD scores of the biopsy specimens correlated with those of the resected specimens ($\kappa = 0.456$). In agreement with previous reports, the DPD scores of the gastric carcinomas were not associated with prognosis or with any clinicopathological factor. *Conclusions:* It is considered that immunohistochemical analysis of DPD expression in gastric carcinoma using biopsied tissue is a technically feasible method to assess the expression of DPD in the tumor prior to surgical resection.

Keywords Gastric carcinoma · 5-Fluorouracil · Dihydropyrimidine dehydrogenase · Biopsy · Immunohistochemical analysis

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Introduction

Gastric cancer is one of the most frequently occurring neoplasms worldwide. The overall 5-year survival rate of gastric carcinoma after surgery has gradually increased to approximately 20% in the Unites States and 71% in Japan, where a higher proportion of gastric cancers are diagnosed during the early stages [4, 9, 10, 14]. On the other hand, the prognosis for advanced gastric carcinoma is still dismal: only 7–10% of patients with stage IV disease survive 5 years in both countries [4, 9, 10]. For these patients, neoadjuvant chemotherapy is frequently chosen prior to surgical treatment [7, 14]. 5-Fluorouracil (5-FU) is one of the chemotherapeutic agents most widely used against gastric carcinoma [7, 14, 19]. Since chemotherapy does not always succeed in causing the tumor to regress in all patients, it is very important to predict the response of the tumor to 5-FU preoperatively. However, there is no established method to estimate the sensitivity of the tumor to 5-FU prior to surgery.

Over 80% of administered 5-FU is metabolically degraded by dihydroxypyrimidine dehydrogenase (DPD), a primary rate-limiting enzyme in the 5-FU metabolic pathway [1, 2, 3]. Several authors have reported that DPD activity and *DPD* mRNA expression are inversely correlated with chemosensitivity to 5-FU in vitro and in cancer patients [1, 2, 3, 5, 8, 12]. Therefore, DPD is not only a key modulator of 5-FU pharmacokinetics, but also a good predictor of responsiveness to 5-FU. On this basis, we considered it valuable to establish a simple

and reliable method to assess DPD expression in biopsied tissue since this is the only available material that will provide information on the biology of the tumor before surgery.

Antibodies against DPD have recently become available for immunohistochemical analysis [11, 13, 18], and it has been reported that immunoreactivity to DPD is also correlated with DPD activity and the level of mRNA expression in cancer tissue [13, 18]. To this end, applying immunohistochemical analysis of DPD to biopsied material seemed to be an ideal method for predicting 5-FU chemosensitivity. In this regard, we considered it a first priority to confirm that immunoreactivity to DPD in biopsied material reflected tumor expression of DPD.

We examined the immunoreactivity of DPD in preoperative biopsy specimens of gastric carcinoma and in surgically resected material. We discuss the feasibility of using immunohistochemical assessment of DPD with anti-DPD antibody in biopsy specimens for predicting chemosensitivity to 5-FU.

Materials and methods

Patients and specimens

The study population consisted of 55 patients (45 men and 10 women; mean age 60.0 years) who had undergone gastrectomy for primary gastric cancer between 1994 and 1996 at the Department of Surgery, Tokyo Postal Services Agency Hospital, Tokyo. All the patients were preoperatively diagnosed by up to six biopsy specimens (approximately 2 mm in size) taken with capped forceps through a gastroscope. The preoperative biopsies and surgically resected specimens were fixed in 10% formalin, and the specimens were dehydrated and embedded in paraffin for histopathological diagnosis. Advanced gastric cancer, i.e. a tumor invading the muscularis propria or more deeply, was required to have been confirmed in all the patients selected for this study.

All the clinicopathological data were obtained by retrospective review of the clinical records and pathological reports. Lymph node and distant metastases and stage of gastric carcinoma were determined according to the UICC TNM classification, fifth edition [15]. Other pathological parameters were described according to the Japanese Gastric Cancer Classification [6]. The details are summarized in Table 1.

Immunohistochemistry and scoring of DPD expression

Together with a paraffin block of the biopsy, a representative block containing the invasive portion of the primary tumor of each patient was selected for immunohistochemical staining. Sections were cut into 3.5-μm slices, dewaxed in xylene and rehydrated through a series of ethanol solutions. They were then incubated in 10 m*M* citrate buffer (pH 6.0), and boiled in a microwave oven for 20 min at 170 W to enhance the immunoreactivity. To eliminate endogenous peroxidase activity, the sections were incubated in 0.3% hydrogen peroxide for 20 min.

The sections were incubated with polyclonal rabbit antibody against human DPD (1:2000 dilution) [11, 13] for 10 h at 4°C. The primary antibody was visualized using a Histofine SAB-Po Kit (Nichirei, Tokyo), according to the instructions provided by the manufacturer. These processes were conducted automatically using the IHS-20 system (SAKURA Finetechnical Company, Tokyo) to avoid variations in immunostaining. Tumor tissue obtained from a xenograft of a human pancreatic cancer cell line MIAPaCa-2 in nude mice served as the positive control [13, 17]. Negative controls were established by substituting phosphate-buffered saline for the primary antibody. The slides were counterstained with hematoxylin.

DPD immunoreactivity was measured in the biopsied specimens and at the surface and invasive front of the surgically resected gastric cancer specimens. Immunoreactivity was assessed semiquantitatively by grading the staining intensity of cancer cells as negative (0), light (1), moderate (2), or intense (3). If the tissue was stained heterogeneously, it was described as, for example, 1–2 which indicates weak to moderate intensity. All immunohistochemical studies were done without knowledge of the clinical data. For analysis of the association between the immunoreactivity of a specimen and that in other parts of the tumor or clinicopathological parameters, the DPD score was defined as the maximum intensity of DPD staining in the biopsy or the resected cancer specimen.

Statistical analysis

The degree of correlation between DPD scores in the biopsy and resected tumor specimens was analyzed using weighted kappa statistics. The Chi-squared test was used to determine the statistical significance of the correlations between the DPD score and other clinicopathological factors such as gender, age, tumor location, macroscopic type, histological subtype, depth, lymph node metastases, distant metastases, stage of disease and curativity of the surgical operation. Differences in mean age or maximum tumor size in relation to DPD score were evaluated by a one-way analysis of variance. The survival rate was calculated starting from the time of surgical resection of the tumor. Survival curves were drawn according to the Kaplan-Meier method and differences in survival distributions were evaluated by the log-rank test. Cox proportional hazards modeling of factors potentially related to survival was performed to identify the factors that might have a significant influence on survival. Categories consisting of less than 5% (i.e. fewer than three cases) of the total number were gathered into other appropriate categories (pN3 and v3), or excluded (negative staining for DPD in the resected specimen, and "sig" and "muc" in histology) from these analyses except for the kappa statistics. *P*-values < 0.05 were considered statistically significant.

Table 1 Clinicopathological features of patients

No. of patients (male/female)	55 (45/10)
Age (years)	
Mean	60.0
Range	41–80
Maximum tumor dimension (mm)[a]	
Mean	64.8
Range	10–170
Tumor location in stomach	
Upper	14
Middle	16
Lower	21
Whole	4
Macroscopic type (1/2/3/4)[b]	6/26/19/4
Histological type (pap/tub1/tub2/por/sig/muc)[b]	3/6/15/28/2/1
Depth of invasion (mp/ss/se/si)[b]	11/25/17/2
Lymph node metastases (pN0/pN1/pN2/pN3)[c]	16/20/10/9
Distant metastasis (pM0/pM1)[c]	11/44
Lymphatic invasion (ly0/ly1/ly2/ly3)[b]	11/24/13/7
Venous invasion (v0/v1/v2/v3)[b]	24/20/10/1
UICC stage (IB/II/IIIA/IIIB/IV)[c]	15/15/8/4/13
Curativity (A/B/C)[b]	27/16/12

[a]Excluding one tumor of unknown size
[b]According to the Japanese gastric cancer classification, 13th edn
[c]According to the UICC TNM classification, 5th edn

Results

DPD expression in resected gastric carcinoma

Positive staining for DPD was seen in 53 (96%) of the 55 surgically resected gastric carcinomas (Table 2). Immunoreactivity for DPD was observed in the cytoplasm at various intensities (Fig. 1). In 35 carcinomas (63%), cancer cells on the surface of carcinoma stained with almost homogeneous intensity, while the others showed heterogeneous staining intensity. At the invasive front, 40 carcinomas (73%) showed homogeneous immunoreactivity for DPD. Eight carcinomas showed heterogeneous staining for DPD both on the surface and in the invasive portion of the tumor. The staining intensity differed between the surface and the invasive portion in 22 carcinomas (40%): 10 showed more intense immunoreactivity in the invasive portion, whereas the opposite was observed in the other 12. Therefore, 29 resected gastric carcinomas (53%) were found to exhibit heterogeneous expression of DPD (Table 2).

Table 2 Distributions of DPD immunoreactivity in the resected and biopsied gastric carcinoma

Patient no.	DPD immunoreactivity		
	Surface	Invasive front	Biopsy
1	2	2	1
2	2	3	1–2
3	1	1	1
4	3	3	2
5	2	1	1
6	3	2	1
7	2–3	2–3	2–3
8	0	2	0
9	1	1	0–1
10	1	1	1
11	1	1	1
12	1	2	2
13	2–3	2	2–3
14	2–3	2–3	2–3
15	1	1	1–2
16	2–3	3	2
17	2–3	2–3	2–3
18	1	1	1
19	2–3	1–2	2
20	2–3	2	2
21	1	1	1
22	2	2	2–3
23	2–3	2–3	2–3
24	2–3	2	2–3
25	1–2	2	2
26	1–2	1	1
27	1–2	1	1
28	2–3	2	2
29	1	2	1
30	1	1	2
31	1	1	1
32	1	1	2
33	2–3	2–3	2
34	2	2	1
35	2	2–3	2–3
36	1–2	1–2	2
37	2	1	2
38	0	0	0
39	1–3	1–3	0–1
40	2	2	1
41	1	1	1
42	1	0	1
43	1	1	1
44	1	2–3	2
45	0	0	1
46	1	1	1
47	0–1	0	0
48	1	1	1
49	1	1	1
50	1	1	1
51	2	2	2
52	1	1	2
53	1	2–3	2
54	1	1	1
55	1	1	1

DPD expression in biopsy specimens of gastric carcinoma

Positive immunoreactivity with anti-DPD antibody was seen in 52 (95%) of the 55 biopsy specimens. The immunostaining pattern was homogeneous in 40 biopsies (73%) and heterogeneous in 12 (22%; Table 2).

To determine to what extent the expression of DPD in the biopsy specimens reflected that in the resected carcinoma specimens, the maximum DPD immunoreactivity (DPD score) was compared. As shown in Table 3, a perfect concordance between these scores was observed in 29 carcinomas (53%). The DPD score in the biopsy was lower than that in the resected tumor in 20 carcinomas (36%), whereas the opposite was observed in the other 6 carcinomas (11%). The weighted kappa coefficient was 0.456 (95% confidence interval 0.292 to 0.620).

Relationship between DPD score and survival and clinicopathological factors

The overall relative survival rate at 5 years in this study was 42.8%, and the mean survival time was 3.7 years. To determine whether the expression of DPD was associated with prognosis, the survival curve was analyzed with regard to DPD score using the Kaplan-Meier method. As shown in Fig. 2, the survival rate was not related to the DPD score, in either the biopsies or the resected tumors ($P = 0.785$ and $P = 0.529$, respectively, log-rank test). To confirm that the patients constituted an ordinary cohort of gastric carcinoma patients, we also analyzed the survival curve in relation to other clinicopathological parameters. In a univariate analysis, maximum tumor size, depth of invasion, lymph node metastases, distant metastases, lymphatic infiltration, venous invasion, and curativity of the operation affected the survival rate of the patients (Table 4). These factors were further subjected to multivariate analysis using the Cox proportional hazards model (Table 5). UICC stage was excluded from this analysis because it was

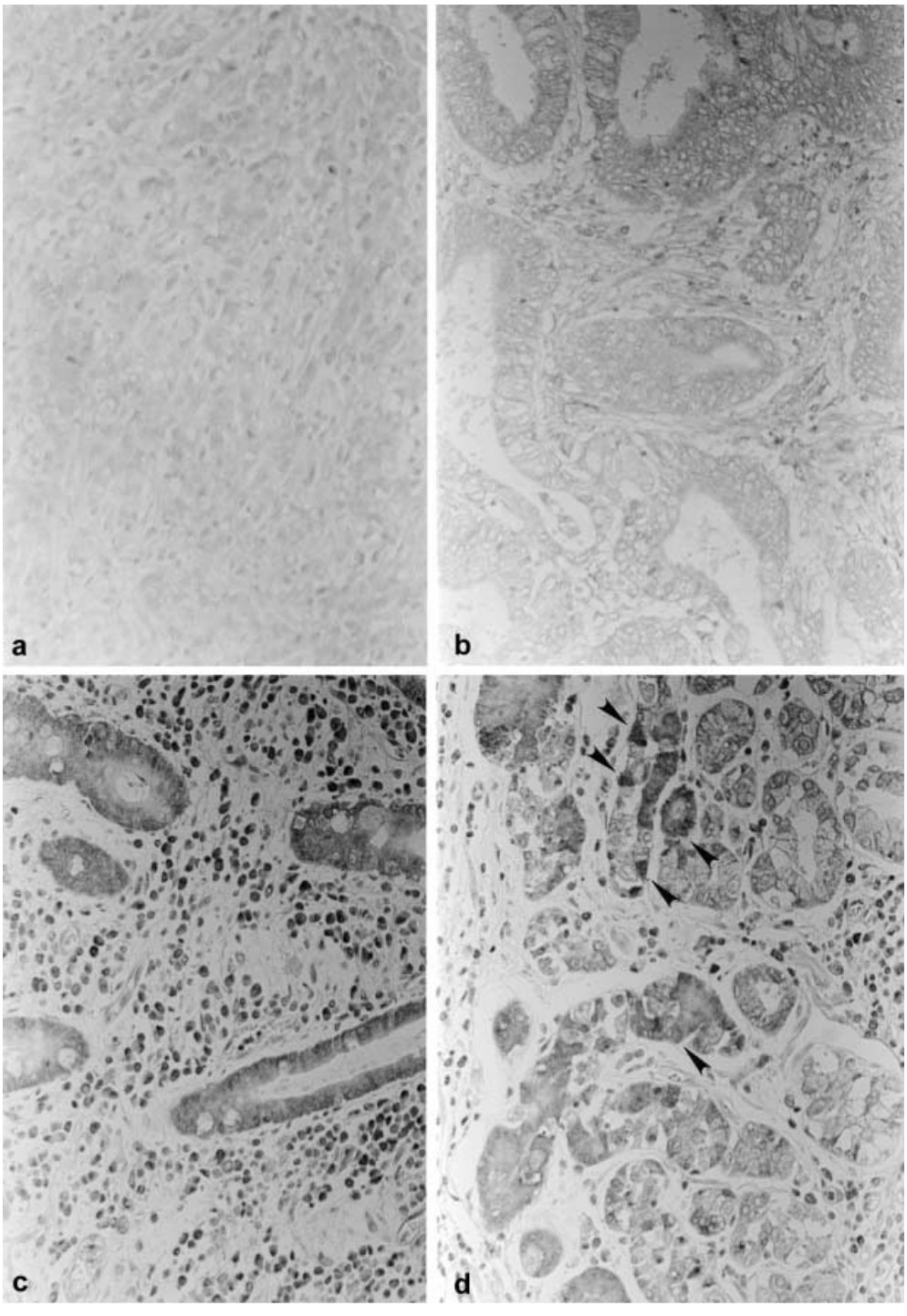

Fig. 1a–d Representative immunoreaction to DPD in gastric carcinoma (original magnification ×400): **a** negative (score 0), **b** light (score 1), **c** moderate (score 2), **d** intense (*arrowheads*; score 3)

determined automatically by the depth of invasion and metastases to lymph nodes and distant organs [15]. The analysis revealed that lymph node metastases and curativity were independent prognostic factors (Table 5, and data not shown).

Finally, we examined the association between DPD score and clinicopathological factors, including the potential prognostic factors. As seen in Table 6, no significant correlation was observed between DPD score in the resected tumors or biopsies and these parameters.

Discussion

We found that almost all gastric carcinomas immunoreacted with anti-DPD antibody. DPD was distributed in the cytoplasm of cancer cells and staining intensity varied among the carcinomas, in a similar manner to previous results with regard to gastric and colon carcinoma tissues [11, 18]. Approximately half the tumors showed focal heterogeneity of DPD expression,

Table 3 Correlation of DPD score between biopsied and resected specimens

Biopsy	Resected specimen			
	0	1	2	3
0	1	1	1	0
1	1	16	7	2
2	0	4	5	9
3	0	0	1	7

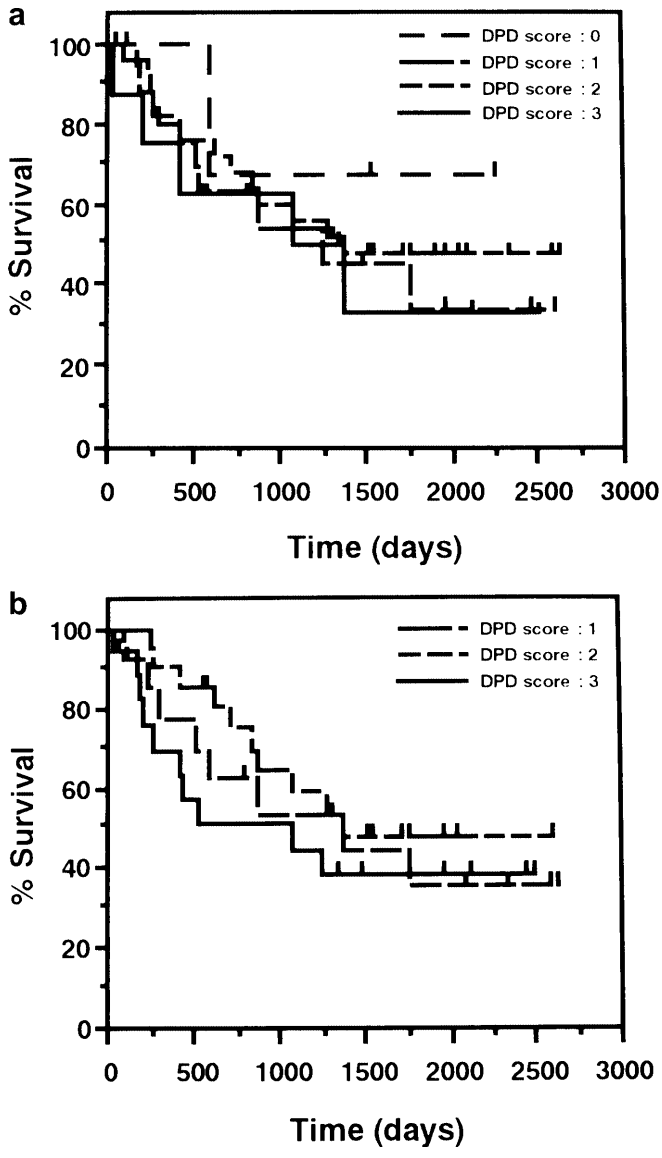

Fig. 2a, b Kaplan-Meier survival curves of patients with advanced gastric carcinoma. Survival curves are stratified according to DPD score in the biopsy (**a**) and in the resected tumor (**b**)

independently of the site within the tumor, since a similar number of tumors showed heterogeneous staining in the biopsy specimen, and on the surface and in the invasive portion of the tumor (Table 2). Moreover, a difference in the expression level of DPD was observed

Table 4 Univariate analysis of prognostic factors in gastric cancer patients

Parameter	*P*-value
DPD score in the biopsy (0/1/2/3)	0.7924
DPD score in the resected tumor (1/2/3)	0.5346
Sex (male/female)	0.2252
Age (years) (< 50/50–59/60–69/70–79/≥80)	0.2726
Tumor size (mm) (< 40/40–79/80–119/≥120)	0.0284
Tumor location (upper/middle/lower/whole)	0.4148
Macroscopic type (1/2/3/4)	0.129
Histological type (pap/tub1/tub2/por)	0.8761
Depth of invasion (mp/ss/se,si)	0.0003
Lymph node metastasis (pN0/pN1/pN2/pN3)	< 0.0001
Distant metastasis (pM0/pM1)	0.0001
Lymphatic invasion (ly0/ly1/ly2/ly3)	0.014
Venous invasion (v0/v1/v2,v3)	< 0.0001
Curativity (A/B/C)	< 0.0001

in 40% of the tumors between the surface and the invasive front. However, there was no tendency for the DPD expression level to be related to depth in these tumors (Table 2). Taken together, the expression of DPD varied randomly in gastric carcinoma tissue.

The main issue of this study was to investigate whether the expression level of DPD estimated by immunostaining in the biopsy specimens agreed with that in the surgically resected tumor. Quantitatively, DPD activity is an appropriate parameter for this comparison. In this study, however, we chose immunohistochemical staining because this simple method enabled us to confirm the histology of the specimens. Cancer cells that express DPD at higher levels are considered more resistant to 5-FU and may be expected to survive chemotherapy. Thus, in patients showing heterogeneous expression of DPD, it is reasonable to choose the maximum intensity (DPD score) for further analyses. We found a moderate agreement in the DPD scores between the biopsy and the surgically resected specimens (weighted kappa value 0.456; Table 3), suggesting that DPD expression measured immunohistochemically in preoperative biopsy specimens may be a substitute for that in resected tumor. The DPD score in the biopsies tended to be smaller than in the resected tumors (Table 3). Discrepancies in the DPD scores occurred probably because of the focal heterogeneity of DPD expression in most cases. However, incomplete staining due to the small amount of biopsied tissue would result in an underestimation of the immunoreactivity. Thus, increasing the volume and number of biopsies would lead to an improvement in the reliability of the preoperative estimation of DPD expression in the tumor.

Although it has been well documented that DPD determines the chemosensitivity to 5-FU, it has been shown in most previous studies that the expression level of *DPD* mRNA and protein and DPD activity in the tumor are not related to prognosis nor to prognostic factors [11, 16]. In agreement with this, DPD scores in the biopsy and the resected specimens did not affect the survival rate after surgery in our study pop-

Table 5 Cox proportional hazards model multivariate analysis of prognostic factors in gastric cancer patients

Parameter	Relative risk of death	95% CI	*P*-value
Lymph node metastasis			
pN0	0.034	0.002–0.583	0.0191
pN1	0.092	0.018–0.523	0.0067
pN2	0.272	0.055–1.405	0.1217
pN3	1		
Curativity			
A	0.025	0.002–0.243	0.0017
B	0.076	0.012–0.436	0.0042
C	1		

Table 6 Analysis of clinicopathological parameters and DPD score in biopsied and resected specimens

Parameter	*P*-value	
	Biopsies	Resected tumor
Gender	0.877	0.326
Age	0.160[a]	0.362[a]
Tumor size	0.917[a]	0.996[a]
Tumor location	0.292	0.294
Macroscopic type	0.795	0.527
Histological type	0.093	0.083
Depth of invasion	0.357	0.538
Lymph node metastasis	0.953	0.803
Distant metastasis	0.248	0.188
Lymphatic invasion	0.641	0.764
Venous invasion	0.538	0.389
Curativity	0.317	0.764

[a] *P*-value estimated by ANOVA

ulation (Fig. 2, Table 4). In addition, the expression level of DPD was found to be completely independent of the potential prognostic factors such as tumor size, depth of invasion, lymph node metastases, lymphatic infiltration and venous invasion in the current study (Table 6).

In conclusion, we first demonstrated that the expression level of DPD, as estimated by immunohistochemical analysis in the preoperative biopsy was comparable to that in the resected gastric carcinoma, suggesting its usefulness in the management of advanced gastric cancer. Some of the patients were treated with 5-FU-based chemotherapy after surgery, but it was neither standardized nor randomized in the current study. Hence, to confirm that immunohistochemical staining of DPD in the biopsy is indeed valuable in predicting chemosensitivity, a large-scale prospective study analyzing the relationship between DPD expression in biopsy tissue and response rate to 5-FU is necessary.

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