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Role of thymidine phosphorylase and dihydropyrimidine dehydrogenase in tumour progression and sensitivity to doxifluridine in gastric cancer patients

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Abstract

This study was designed to investigate the role of thymidine phosphorylase (TP) and dihydropyrimidine dehydrogenase (DPD) on tumour progression and sensitivity to 5′-deoxy-5-fluorouridine (5′-DFUR). Tumour tissue was obtained from surgically resected samples from 93 patients with primary gastric cancer. Tumour TP and DPD expression levels were determined by the enzyme-linked immunosorbent assay (ELISA) system and compared with several clinicopathological factors and *in vitro* sensitivity to 5′-DFUR. DPD showed no correlation with any clinicopathological factors. However, the TP level was significantly correlated with the depth of tumour, lymphatic invasion and venous invasion. In comparison with 5′-DFUR sensitivity, there was a weak inverse correlation between the DPD level and the sensitivity to 5′-DFUR ($r_s = -0.361$). Furthermore, the TP/DPD ratio showed a significant correlation with 5′-DFUR sensitivity ($r_s = 0.634$). In a subgroup of patients with postoperative 5′-DFUR administration, the survival rate was significantly better in patients with a high TP/DPD ratio ($n = 8$) than in those with low TP/DPD ratio ($n = 14$) ($P = 0.0140$). These results suggest that sensitivity to 5′-DFUR is predictable by measurement of both TP and DPD levels.

Keywords: Thymidine phosphorylase; Dihydropyrimidine dehydrogenase; Gastric cancer; Drug sensitivity testing; Doxyfluridine

1. Introduction

Thymidine phosphorylase (TP: EC2.4.2.4) is known to: have a high homology with platelet-derived endothelial growth factor (PD-ECGF); have activity as an angiogenesis-inducing factor [1]; be related to tumour growth/progress in gastric [2–4], breast [5] and colorectal cancers [6]. Furthermore, TP is reportedly an enzyme that converts capecitabine and its intermediate metabolite, 5′-deoxy-5-fluorouridine (doxifluridine; 5′-DFUR), to 5-fluorouracil (5-FU) [7]. It is also reported that TP activity in tumour tissue correlates with 5-FU concentration in tumour tissue following capecitabine administration [8], and with tumour sensitivity to capecitabine and 5′-DFUR [9,10].

Recently, it was found that dihydropyrimidine dehydrogenase (DPD: EC1.3.1.2), an initial and rate-limiting catabolic enzyme, is involved in the pharmacokinetics and toxicity of 5-FU [11,12], and it has been reported that patients with DPD deficiency show severe toxicity to 5-FU administration [13]. It has also been reported that in tumours with high DPD activity, 5-FU decomposition is accelerated resulting in resistance to 5-FU [14–16].

Thus, in the present study, in order to search for a parameter to predict sensitivity to 5′-DFUR, we employed patients with resected gastric cancer, and assayed TP as well as DPD in the tumour tissue to investigate relationships with clinicopathological factors, postoperative survival period and *in vitro* tumour sensitivity to 5′-DFUR.

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2. Patients and methods

2.1. Patients

A total of 93 gastric cancer patients who underwent gastrectomy at our Department from May 1998 to December 2001 were enrolled in this study. Tumour tissue and adjacent normal tissue were obtained from surgically-resected samples and stored at −80 °C until use. Patient characteristics, determined according to the Japanese Classification of Gastric Carcinoma [17], are shown in Table 1. There were 67 patients with lymph node metastasis, 19 patients with peritoneal metastasis and 6 patients with hepatic metastasis. Of these 93 patients, 37 patients did not receive any chemotherapy after surgery mainly due to the patient's refusal, 56 patients received adjuvant chemotherapy (22 received 5′-DFUR, 14 uracil plus tegafur, 11 TS-1 (IM tegafur 0.4M 5-chloro-2,4-dihydroxypyridine and IM potassium oxonate), 8 cisplatin plus 5-FU and 1 5-FU) The treatment regimen was selected by physician in charge. All patients gave written informed consent to enter this study that was started after being approved by the local ethics committee.

Table 1
Patient characteristics[a] ($n=93$)

Age (years)	
Range	(30–85)
Median	64
Gender	
Male	60
Female	33
Depth of tumour invasion	
T1	10
T2	31
T3	38
T4	14
Lymph node metastasis	
N0	26
N1	25
N2	33
N3	9
Peritoneal metastasis	
P0	74
P1	19
Hepatic metastasis	
H0	87
H1	6
Histological type[b]	
Diff	34
Undiff	59
Lymphatic invasion	
−	7
+	86
Venous invasion	
−	10
+	83
Stage	
Ia	9
Ib	11
II	18
IIIa	14
IIIb	9
IV	32

[a] Clinicopathological factors were determined according to Japanese Classification of Gastric Carcinoma [17].
[b] Diff, differential type; Undiff, undifferentiated type.

2.2. TP and DPD levels

The enzyme levels in the specimens were assayed by enzyme-linked immunosorbent assay (ELISA) system as previously described in Refs. [18,19]. These enzyme levels were expressed as U/mg protein, where one U in TP is an amount equivalent to one μg of 5-FU produced in an hour, and one U in DPD is an amount equivalent to catabolise one pmol of 5-FU per minute.

2.3. In vitro *tumour sensitivity test to 5′-DFUR*

Sensitivity of freshly resected tumours to 5′-DFUR was determined by an adenosine triphosphate (ATP) assay with serum-free culture developed in our department [20], if a relatively large amount of tumour was obtained. Briefly, each tumour sample was sliced into small fragments and digested enzymatically to obtain a suspension of single cells. Cells at a concentration of 2×10^4 cells/180 μl were dispensed onto a 96-well microtitre plate for a total of 3–6 wells in each drug untreated control group and drug-treated group. Twenty microlitres of physiological saline was added to each well in the control group. In the drug treated group, 20 μl of 5′-DFUR at a final concentration of 5.0 μg/ml, which corresponds to a peak plasma concentration following an oral administration of 5′-DFUR with a general dose, was added to each well and the plate was incubated for 72 h in 5% CO_2 at 37 °C. Cell viability was determined by measuring intracellular ATP content by a bioluminescence method. The assay was regarded as evaluable when, after incubation, the ATP level in the control group was more than 2.0 nM. Relative tumour growth inhibition rate (IR%) was calculated as follows: (1−average ATP level in the drug-treated group/average ATP level in the control group)×100.

2.4. Statistical analysis

Statistical analysis was performed on a personal computer with Stat View™ ver. 5.0 software (SAS Institute Inc., Cary, NC, USA). Statistical differences were evaluated between two groups using the Mann–Whitney test and Wilcoxon signed rank test, and for three or more groups using the Kruskal–Wallis test. To evaluate correlations between two variables, linear regression

analysis was performed to calculate Spearman's rank correlation coefficients. The survival rate was calculated by the Kaplan–Meier method and a statistical analysis was performed using the log rank test. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. TP and DPD expression levels in gastric cancer

TP levels in tumour and normal tissues were measured in all 93 samples resulting in respective values (mean ± S.D.) of 146 ± 127 U/mg protein and 49 ± 27 U/mg protein (Fig. 1). The TP level was significantly higher in tumour than in normal tissues ($P < 0.0001$). There was a weak correlation of TP levels between tumour and normal tissues ($R = 0.232$, $P = 0.0261$) (Fig. 2). DPD levels in tumour and normal tissues were also measured in all samples resulting in values (mean + S.D.) of 71 ± 64 and 60 ± 49 U/mg protein (Fig. 1). There was no significant correlation of the DPD levels between tumour and normal tissues (Fig. 2). There were also no correlations between the TP level and DPD level in tumour and normal tissues. In comparisons of the levels of the enzymes and clinicopathological factors, DPD showed no correlation with any clinicopathological factors. Whereas, TP levels significantly correlated with the depth of tumour invasion ($P = 0.0293$), lymphatic invasion ($P = 0.0008$) and venous invasion ($P = 0.0002$) (Table 2).

3.2. Correlation between tumour sensitivity to 5′-DFUR and TP as well as DPD levels

In 52 of 93 samples, relatively large amounts of tumour were obtained and *in vitro* drug sensitivity testing performed. In 45 out of these 52 samples (87%), tumour sensitivity to 5′-DFUR was evaluated and relationships with the TP and DPD levels were investigated. There was no correlation between the TP level and the sensitivity to 5′-DFUR (Fig. 3a). On the contrary, there was a weak inverse correlation between the DPD level and the sensitivity to 5′-DFUR ($r_s = -0.361$, $P = 0.0142$) (Fig. 3b). Furthermore, calculation of the TP/DPD ratio resulted in a significant correlation with *in vitro* tumour sensitivity to 5′-DFUR ($r_s = 0.634$, $P < 0.0001$) (Fig. 3c).

3.3. Association between TP and DPD level and overall survival

Overall survival was calculated according to the TP and DPD levels and the TP/DPD ratio. The median

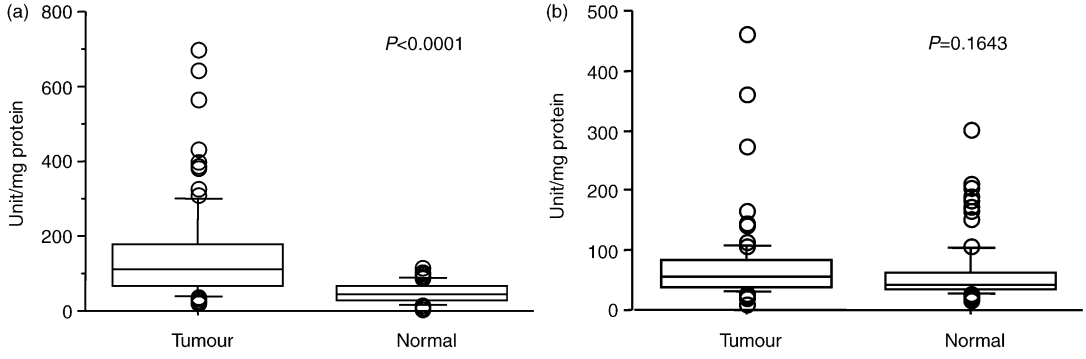

Fig. 1. Comparisons of TP (a) and DPD (b) levels between tumour and normal tissues. TP levels were significantly higher in tumour tissues than in normal tissues. No difference was observed in DPD levels between tumour and normal tissues.

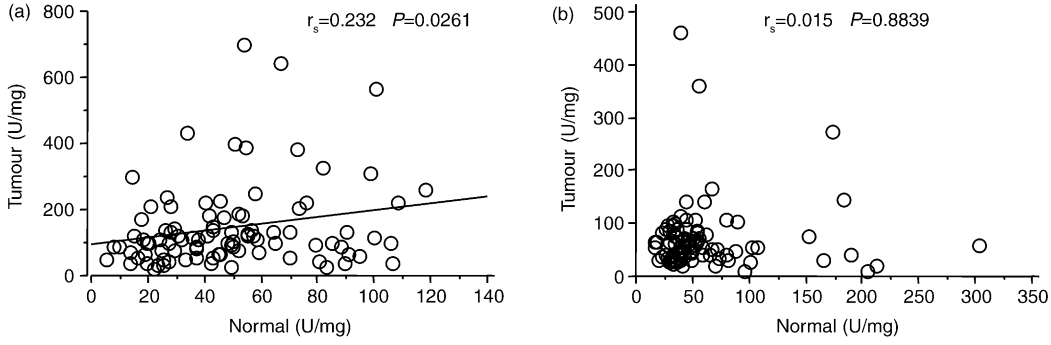

Fig. 2. Correlation between TP (a) and DPD (b) levels in tumour and normal tissues. There was a weak correlation of the TP level between tumour and normal tissues. No correlation was observed in the DPD levels between tumour and normal tissues.

Table 2
Comparison of clinicopathological factor and TP DPD levels

Clinicopathological factors[a]	TP[b]	Statistics[c]	DPD[b]	Statistics
Depth of tumour invasion				
T1	87±114	$P=0.0293$	45±29	$P=0.0961$
T2	145±104		75±78	
T3	152±126		70±57	
T4	171±172		85±63	
Lymph node metastasis				
N0	131±107	$P=0.5089$	73±85	$P=0.9377$
N1	161±127		66±37	
N2	135±128		69±46	
N3	181±180		90±104	
Peritoneal metastasis				
P0	157±138	$P=0.2017$	69±69	$P=0.0678$
P1	102±46		78±33	
Hepatic metastasis				
H0	147±127	$P=0.2346$	73±65	$P=0.1997$
H1	121±129		45±21	
Lymphatic invasion				
−	48±32	$P=0.0008$	47±33	$P=0.1318$
+	154±128		73±65	
Vessel invasion				
−	53±30	$P=0.0002$	46±29	$P=0.0569$
+	157±129		74±66	

S.D., standard deviation; TP, thymidine phosphorylase; DPD, dihydropyrimidine dehydrogenase.

[a] Clinicopathological factors are determined according to Japanese Classification of Gastric Carcinoma [17].

[b] Values represent mean±S.D. TP and DPD levels are expressed as U/mg protein.

[c] Mann–Whitney test between the two groups; Kruskal–Wallis test among more than three groups.

follow-up period was 548 days (range: 29–1374 days). There were 31 deaths during this period. Cut-off levels of TP, DPD and the TP/DPD ratio were set at the median values. There were no differences in the patient's background between each subgroup. TP level had no impact on overall survival after surgery in an analysis of all patients enrolled in this study (Fig. 4a). There were also no differences in survival according to the DPD level (data not shown). Whereas, in the subgroup of patients with postoperative 5′-DFUR administration (22 patients), the survival rate was significantly better in patients with a high TP/DPD ratio than in those with a low TP/DPD ratio (Fig. 4b).

4. Discussion

Although HPLC is a well-known method to measure TP activity, results obtained by either HPLC or ELISA have been reported to show a high coincidence [18]. DPD has also been measured by a radio-enzymatic assay, and again a high coincidence is reported between DPD activity obtained by a radio-enzymatic assay and the protein expression level assayed by ELISA [19]. Moreover, the benefits of ELISA include: no need for special equipment, no need for radioisotopes, results obtained rapidly with a high sensitivity, and only a small test sample amount is needed.

In the present study, a significantly higher TP expression was observed in tumour than in normal tissues. It has also been reported that, in cases of breast [21], gastric [2–4] and colorectal [6] cancers, immunostaining or results from the ELISA method also showed higher TP expressions in tumour tissues than in the surrounding normal tissues. In the present study, there was a weak relationship between TP levels in the tumour and normal tissues. Although the regulating mechanism for TP expression in tumours remains unclear, TP expression is reportedly accelerated by hypoxia [22], cytokines such as tumour necrosis factor-alpha (TNF-alpha), INF, interleukin-1 (IL-1) [23], or anticancer drugs such as cyclophosphamide, paclitaxel, and docetaxel [24].

It is also reported that TP has a high homology with platelet derived-endothelial cell growth factor (PD-ECGF) and shows angiogenesis-inducing activity and that TP expression is closely related to tumour progress/growth [1]. Furthermore, studies on gastric cancer report that TP expression closely correlates with tumour invasion, haematogenous metastasis, lymph node metastasis, venous invasion, lymphatic invasion, and microvascular density [2–4,25]. Results of the present study showed a significantly higher TP expression in tumours with a deeper tumour invasion, lymphatic and venous invasion suggesting an angiogenesis-inducing role for TP in gastric cancer.

As for DPD, the present study showed a slightly higher expression in tumour tissues than in the surrounding normal tissues, but the difference was not significant. There are many reports indicating no difference in DPD activities in gastric [26] and colorectal [27] cancers. Etienne and colleagues [14], similar to our present study, reported that DPD levels do not differ between tumour and normal tissues. The regulating mechanism of DPD in tumour cells is not clear. Johnston and colleagues [27] reported that *DPD* mRNA levels were significantly lower in colorectal tumours than in normal mucosa, whereas the catalytic activity and protein level did not differ significantly. They speculated that DPD can be regulated at both transcriptional and translational levels. In our present study, no relationship was seen between DPD activity in tumour tissues and clinicopathological factors. We have previously reported that no correlation was obtained between DPD activity, determined by a radio-enzymatic assay, and clincopathological factors [16]. Ishikawa and colleagues [26]

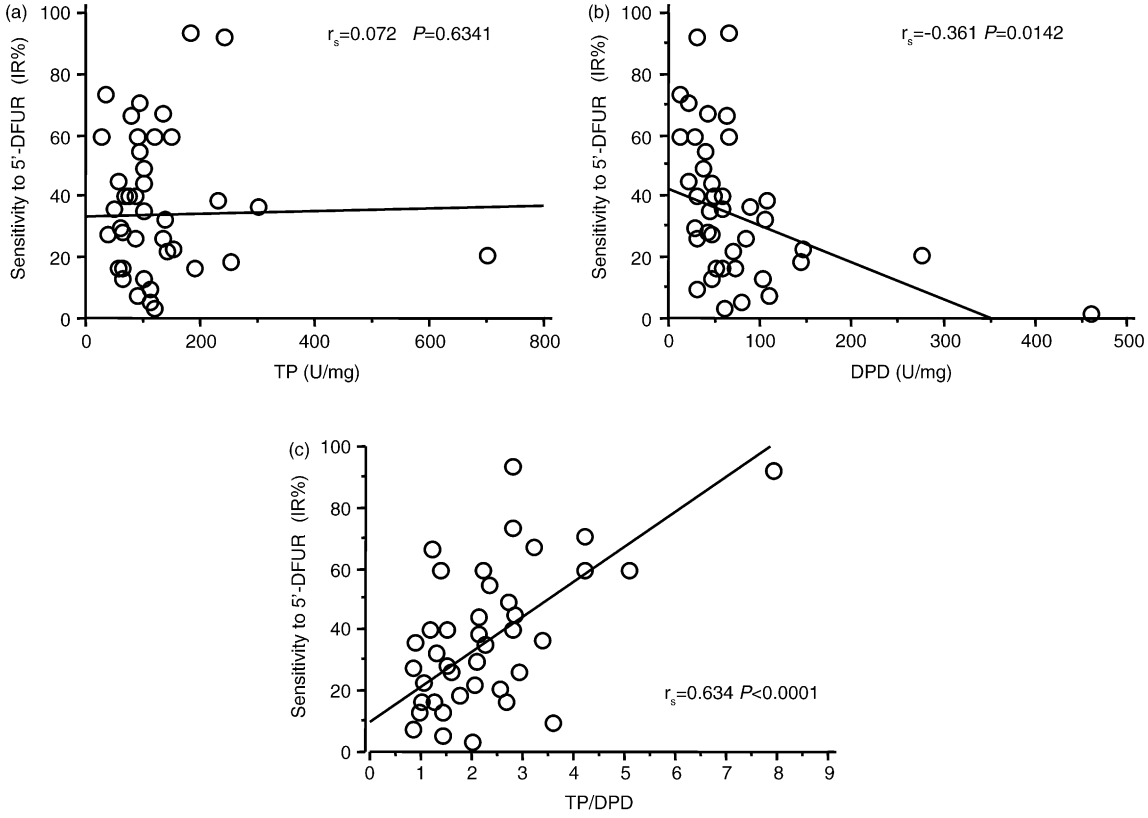

Fig. 3. Relationship of *in vitro* tumour sensitivity to 5′-DFUR with TP (a), DPD (b) and TP/DPD ratio (c). Tumours with a high DPD level showed relative resistance to 5′-DFUR. The TP/DPD ratio showed a significant correlation with the tumour sensitivity to 5′-DFUR.

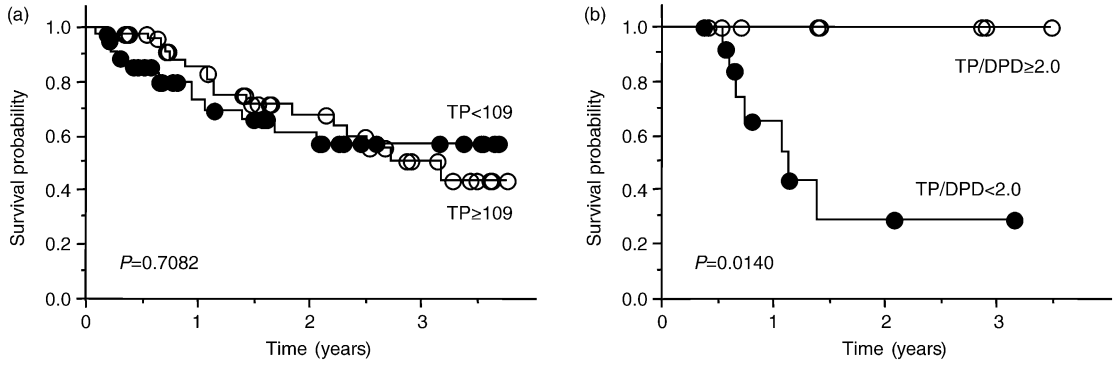

Fig. 4. Comparison of overall survival according to the tumour TP and DPD levels. There was no significant difference of survival according to TP level (a). In patients with postoperative 5′-DFUR administration ($n=22$), the survival rate was significantly better in patients with a high TP/DPD ratio (b).

also reported similar results. Thus, the role of DPD in gastric cancer growth/progression remains unclear.

It is known that TP has activity as an angiogenesis-inducing factor and as a metabolic enzyme for the fluoropyrimidines. For example, capecitabine, an analogue of 5-FU, and 5′-DFUR are finally metabolised to 5-FU by TP [7]. It is also reported that TP shows a significant affinity to 5′-DFUR, but that cells transfected with the *TP* gene show no change in sensitivity to 5-FU [28,29]. Correlation between TP expression and 5′-DFUR efficacy has also been proven clinically. For example, Koizumi and colleagues [10] reported that, among gastric cancer patients treated by drug regimens including 5′-DFUR, those with a high TP expression in their tumour tissues showed a significantly higher response rate to the regimens. The higher TP expression in tumour tissues than in normal tissues is the basis behind the rationale of tumour-selective activity of these anticancer drugs. Shulter and colleagues [8] investigated the relationship of TP and DPD activities in tumour and normal tissues from 19 colorectal cancer patients given capecitabine with 5-FU concentrations in both

tissues, and found that the TP activity was 3.7 times higher in the tumour tissues than in the normal tissues and that the 5-FU concentration in the tumour tissues was 3.2 times higher than in the normal tissues.

DPD is mainly found in the liver and catabolises over 80% of the 5-FU administered to patients [11]. For example, DPD activity in peripheral blood mononuclear cells (PBMC), which is assayed as a surrogate marker for the liver, reportedly showed a close relationship with the pharmacokinetics of 5-FU given continuously [12]. It is also known that patients with a DPD deficiency given 5-FU develop severe adverse drug reactions [13]. Basic and clinical studies confirm that DPD activity in tumour tissues correlates with tumour sensitivity to 5-FU. This indicates that 5-FU decomposition is accelerated in tumours with high DPD expression levels resulting in a reduction of the antitumour efficacy of 5-FU [14–16].

In the present study, we investigated the correlation between *in vitro* tumour sensitivity to either 5′-DFUR and TP/DPD expressions. Results showed that tumours with a high DPD expression were resistant to 5′-DFUR and the TP/DPD ratio demonstrated a strong correlation with the tumour sensitivity to 5′-DFUR. Collie-Duguid and colleagues [30] reported that the TP/DPD ratio was significantly higher in colorectal cancer tissues than in adjacent normal tissues and suggested that there is a preferential activation of fluoropyrimidines in tumour cells. Ishikawa and colleagues [9] employed nude mice with transplantable tumours, and examined the relationships of tumour sensitivity to capecitabine, 5′-DFUR, UFT and 5-FU with TP/DPD activities. They reported that there is a significant correlation between the TP/DPD ratio and tumour sensitivities to capecitabine as well as to 5′-DFUR. Results of our present study support these findings suggesting that tumour sensitivity to 5′-DFUR is regulated by activities of both the metabolic and catabolic enzymes.

In the present study, analysis of all of the patients showed no differences in survival with respect to TP or DPD expression levels. There are many other studies on the prognostic significance of TP in gastric cancer patients. For example, Maeda and colleagues [3] reported that TP is a prognostic factor in gastric cancer patients. Tanigawa and colleagues [4] reported that TP expression showed a relationship to microvascular density and haematogenous metastasis, but no difference was seen in prognosis according to the TP expression level. The findings of these studies may result from the different analytical methods employed and differences in patient selection. Thus, the prognostic significance of TP expression in gastric cancer patients is still unclear. Our present study showed that, in gastric cancer patients with a postoperative 5′-DFUR administration, a high TP/DPD ratio resulted in significantly better survival rates. This supposedly indicates that patients with a high TP/DPD ratio have an increased effect of 5′-DFUR to prolong survival periods. However, as this result was obtained from a subgroup analysis involving small numbers, the data should be interpreted with caution. Therefore, we intend to conduct a further prospective study on the clinical significance of TP and DPD.

Owing to recent progress in molecular biology, *TP* and *DPD* mRNA expression can be assayed using the reverse transcriptase-polymerase chain reaction (RT-PCR) method [30], where predicting tumour sensitivity to 5′-DFUR can be done using a minute amount of biopsy specimen. Furthermore, in the future, it is possible that gene expression data could be utilised to select susceptible patients prior to treatment and employed to establish a tailor-made treatment for cancer patients.

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