Dihydropyrimidine Dehydrogenase Activity and Messenger RNA Level May Be Related to the Antitumor Effect of 5-Fluorouracil on Human Tumor Xenografts in Nude Mice

Yoichiro Ishikawa, Tetsuro Kubota,[1] Yoshihide Otani, Masahiko Watanabe, Tatsuo Teramoto, Koichiro Kumai, Masaki Kitajima, Teiji Takechi, Hiroyuki Okabe, and Masakazu Fukushima

Department of Surgery, School of Medicine, Keio University, Tokyo 160-8582 [Y. I., T. K., Y. O., M. W., T. Te., K. K., M. K.]; and Cancer Research Laboratory, Taiho Pharmaceutical Co. Ltd., Saitama 357-8527 [T. Ta., H. O., M. F.], Japan

ABSTRACT

We investigated the correlation between tumor sensitivity to 5-fluorouracil (5-FU) and the enzymatic activity and mRNA levels of thymidylate synthetase (TS) and dihydropyrimidine dehydrogenase (DPD) using human tumor xenografts in nude mice. Three gastric carcinoma xenografts (SC-1-NU, St-4, and H-111), two colon carcinoma xenografts (Co-4 and Col-3-JCK), one pancreatic carcinoma xenograft (PAN-3-JCK), and one breast carcinoma xenograft (MX-1) were inoculated into nude mice. When the resultant tumors reached 100–300 mg, 5-FU was administered i.p. at a dose of 60 mg/kg in a schedule of three times every 4 days, and the antitumor activity of 5-FU was evaluated as the relative mean tumor weight in treated mice compared to control mice. Xenografts were also inoculated into untreated nude mice. When tumors weighed 200–300 mg, tumor tissues were resected for measurement of tumoral TS and DPD. TS and DPD activities were detected by the TS-binding assay and a radioenzymatic assay, respectively. mRNA levels were measured by semiquantitative reverse transcription-PCR, with glyceraldehyde-3-phosphate dehydrogenase coamplified as an internal standard. TS and DPD activities ranged from 84.7 to 775.5 fmol/mg protein and from not detectable to 79.7 pmol/min/mg protein, respectively. TS and DPD mRNA levels ranged from 0.51 to 9.90 and from not detectable to 0.93, respectively. The enzymatic activities of TS and DPD were correlated with observed mRNA levels. DPD levels were significantly correlated with 5-FU sensitivity, with high DPD activity and high DPD mRNA level resulting in low sensitivity to 5-FU. In contrast, no correlation between TS level and 5-FU sensitivity was observed. Tumoral DPD activity and DPD mRNA level may be useful indicators in predicting the antitumor activity of 5-FU.

INTRODUCTION

Although 5-FU[2] is considered to be one of the most effective agents against gastrointestinal carcinomas, its efficacy rate is only 10–15% for gastric and colon cancer (1, 2). Two main modes of action have been proposed for 5-FU through its active metabolites, FdUMP and 5-fluoro-UTP. FdUMP suppresses TS (EC 2.1.1.45) by forming a covalent ternary complex with 5,10-methylenetetrahydrofolate, which subsequently inhibits DNA synthesis (3). 5-Fluoro-UTP is incorporated into cellular RNA, resulting in RNA dysfunction (4). Several reports (5–9) have indicated that TS expression in clinical tumor samples was significantly related to the response to 5-FU in gastric and colorectal cancer patients. Johnston *et al.* (7) have reported that TS expression level was an important independent prognosticator of survival in primary rectal cancer patients.

5-FU is catabolized to 2-fluoro-β-alanine mainly in the liver via three enzymes: DPD (EC 1.3.1.2), the first and rate-limiting enzyme, followed by dihydropyrimidinase and β-ureidopropionase. Several recent studies (10–13) concerned with 5-FU antitumor effects have demonstrated that tumoral DPD activity varied between different human tumor cell lines and clinical samples of patients with head and neck, liver, and colorectal cancers. Etienne *et al.* (11) determined tumoral:nontumoral DPD activity ratios (normalized DPD) in tumor biopsy specimens from head and neck cancer patients before administration of 5-FU-based chemotherapy and reported that complete responders exhibited significantly lower normalized DPD values than partial or nonresponding patients. Moreover, some DPD inhibitors, such as 5-etynyluracil (14), uracil (15), and 5-chloro-2,4-dihydroxypyridine (16), have been demonstrated to enhance the antitumor activity of 5-FU in human tumor cell lines. Beck *et al.* (10) measured TS and DPD activities in a panel of 19 human tumor cell lines in parallel with 5-FU responsiveness and showed that both TS and DPD activities

Received 8/28/98; revised 11/19/98; accepted 12/9/98.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom requests for reprints should be addressed, at Department of Surgery, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. Phone: 81-3-3353-1211 ext. 2334; Fax: 81-3-3355-4707; E-mail: tkubota@med.keio.ac.jp.

[2] The abbreviations used are: 5-FU, 5-fluorouracil; FdUMP, 5-fluoro-dUMP; TS, thymidylate synthetase; DPD, dihydropyrimidine dehydrogenase; q4d×3, three times every 4 days; RW, relative mean tumor weight; β-ME, β-mercaptoethanol; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

were significantly correlated with 5-FU effectiveness; *i.e.*, the greater the enzymatic activity, the higher the 5-FU IC_{50}.

To evaluate factors determining appropriate antitumor agents, we have investigated several chemosensitivity tests, including human tumor clonogenic assay (17), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H tetrazolium bromide assay (18, 19), and histoculture drug response assay (20, 21), which predict the sensitivity of tumor cells and/or tumor tissues to different agents with high rates of predictability. Although these assays are suitable for the evaluation of the efficacy of agents with different modes of action, significant amounts of tumor tissue are required to perform these methods.

The objective of this study was to clarify the correlation between TS/DPD activity and mRNA levels with the antitumor activity of 5-FU on human tumor xenografts serially transplanted in nude mice.

MATERIALS AND METHODS

Human Tumor Xenograft and Nude Mice. Three gastric carcinoma xenografts (SC-1-NU, St-4, and H-111), two colon carcinoma xenografts (Co-4 and Col-3-JCK), one pancreatic carcinoma xenograft (PAN-3-JCK), and one breast carcinoma xenograft (MX-1) were used in this study. SC-1-NU (22) and H-111 (23) were kindly provided by Dr. M. Yamauchi (Nagoya University, Nagoya, Japan) and Dr. M. Fujita (Osaka University, Osaka, Japan) respectively. Col-3-JCK (24) and PAN-3-JCK (25) were established at the Central Institute for Experimental Animals (Kawasaki, Japan) and provided by Dr. T. Nomura. MX-1 was established by Giovanella *et al.* (26) and was kindly provided by Dr. K. Inoue, Cancer Chemotherapy Center (Tokyo, Japan). The other lines were established in the Pathology Division of the National Cancer Center Research Institute (Tokyo, Japan; Ref. 27). These xenografts were established 15–20 years ago and have been serially passaged in our laboratory without any changes in histological findings and chemosensitivity. Male nude mice with a BALB/cA genetic background were purchased from CLEA Japan, Co., Ltd. (Tokyo, Japan). Six- to 8-week-old mice weighing 20–22 g were used for the experiments.

Chemicals. 5-FU was purchased from Kyowa Hakko Kogyo, Co., Ltd. (Tokyo, Japan). [6-^{3}H]FdUMP (16.9 Ci/mmol) was obtained from Moravek Biochemicals, Inc. (Brea, CA). [6-^{14}C]5-FU (56 mCi/mmol) was from American Radiolabeled Chemicals Co. (St. Louis, MO), and NADPH was from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were the highest standard grade commercially available.

Antitumor Activity of 5-FU. Tumor tissue fragments, each measuring $\sim 3 \times 3 \times 3$ mm, were inoculated into s.c. tissues of the bilateral dorsum of ether-anesthetized nude mice, using a trocar needle. Implanted tumors were measured (length and width) with a sliding caliper three times weekly by the same observer.

Tumor weight was calculated according to the method of Geran *et al.* (28) from linear measurements using the formula: tumor weight (mg) = length (mm) $\times$ [width (mm)]^2/2. When tumors reached 100–300 mg, tumor-bearing mice were allocated randomly to test groups, each consisting of five mice, and treatment was initiated. 5-FU was administered i.p. at a dose of 60 mg/kg in a schedule of q4d$\times$3, determined to be the maximum tolerated dose in nude mice in our previous report (27).

RW was calculated as $RW = W_i/W_0$, where W_i is the mean tumor weight at any given time and W_0 is the mean tumor weight at the time of initial treatment. The antitumor activity of 5-FU was evaluated as the lowest *T*/*C* value (%) during the experiment, where *T* is the RW of the treated group and *C* is the RW of the control group at any given time. The antitumor activity was evaluated as sensitive when the lowest *T*/*C* value was $\leq$42%, which was calculated from $(0.75)^3$, corresponding to a 25% reduction of each dimension (29).

Collection of Tumor Tissues. When the s.c. inoculated tumor xenografts reached exponential growth in untreated nude mice, the mice were sacrificed, and tumors weighing 200–300 mg were resected for measurement of TS and DPD activity and mRNA levels. Three tumors of each xenograft were collected and immersed in liquid nitrogen immediately after resection and stored at -80°C until analysis.

FdUMP Binding Assay. Tumors were homogenized with three volumes of 200 mM Tris-HCl (pH 8.0) containing 20 mM β-ME, 100 mM NaF, and 15 mM CMP and centrifuged at 105,000 $\times$ *g* for 60 min. The resultant supernatant was used for the determination of TS activity according to the method of Spears *et al.* (30) using [6-^{3}H]FdUMP as substrate.

DPD Enzymatic Assay. The DPD enzymatic assay was based on the method described by Takechi *et al.* (15). Briefly, tumor tissues were sonicated in four volumes of homogenization buffer [20 mM potassium phosphate (pH 8.0) containing 1 mM EDTA and 1 mM β-ME]. Each homogenate was centrifuged at 105,000 $\times$ *g* for 1 h at 4°C, and supernatant (cytosol fraction) was collected. The enzyme reaction mixture contained 10 mM potassium phosphate (pH 8.0), 0.5 mM EDTA, 0.5 mM β-ME, 2 mM DTT, 5 mM $MgCl_2$, 20 μM [6-^{14}C]5-FU, 100 μM NADPH, and 25 μl of the cytosol fraction in a final volume of 50 μl and incubated at 37°C for 30 min. DPD activity was determined by measuring the sum of the dihydrofluorouracil and 2-fluoro-β-alanine products formed from [6-^{14}C]5-FU. Supernatant aliquots (5 μl) were applied to TLC plates (silica gel 60 F254; Merck, Germany) and developed with a mixture of ethanol and 1 M ammonium acetate (5:1, v/v), according to the method of Ikenaka *et al.* (31). Each product was visualized and quantified using an image analyzer (BAS-2000; Fujix, Tokyo, Japan).

Semiquantitative RT-PCR. Semiquantitative RT-PCR was performed using method of Takechi *et al.* (32). Total RNA for each homogenate was isolated using the RNeasy mini kit (Qiagen Inc., Chatsworth, CA) according to manufacturer instructions. Total RNA yields and purity were determined spectrophotometrically by measuring the absorbance of aliquots at 260 and 280 nm. RNA integrity was checked by visualizing rRNA bands by agarose gel electrophoresis in the presence of formaldehyde.

Reverse transcription with 10 μg of total RNA was carried out in a total volume of 100 μl containing 250 pmol of oligo(dT)$_{18}$, 80 units of rRNasin RNase inhibitor (Promega, Madison, WI), and 500 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD) in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, and 0.5 mM dNTPs. Initially, RNA and oligo(dT)$_{18}$ were heated at 70°C for 10 min and immediately chilled on ice,

and then the other reagents were added and incubated for 15 min at 30°C and 60 min at 42°C.

For PCR, cDNA aliquots were diluted in sterile water, depending on transcript abundance. Three cDNA concentrations for TS/GAPDH primer or DPD/GAPDH primer combinations were used. For accurate quantification using this method, measurements must be taken in the linear phase of the reaction, in which cDNA concentration is directly proportional to signal intensity. A range of cDNA concentrations was used to determine the linear phase of the PCR. PCR primers, summarized in the Table 1, were designed according to human TS (33), DPD (34), and GAPDH (35) cDNA sequences. Comparison to mouse sequences showed these primers to be specific for human TS/DPD/GAPDH sequences. Mouse TS and GAPDH cDNA sequences were obtained from published data (36, 37), and the mouse DPD cDNA sequence was quoted from Takechi *et al.* (32).

PCR was carried out in a final volume of 50 μl containing cDNA template, TS and GAPDH or DPD and GAPDH primers, 1.25 units of Ex Taq (Takara, Shiga, Japan) in 10× Ex Taq buffer (Takara), and 0.2 mM dNTPs, using a thermal cycler (PC-800; Astec, Tokyo, Japan). Ten pmol of each TS primer and 2 pmol of each GAPDH primer were used for the TS PCR, and 40 pmol of each DPD primer and 2 pmol of each GAPDH primer were used for the DPD PCR. The PCR profile consisted of an initial 3-min denaturation at 94°C, followed by 30 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 60°C, and 2 min of polymerization at 72°C and a final 10-min extension at 72°C.

PCR products were separated by 2% agarose gel electrophoresis. Gels were stained with ethidium bromide, visualized on a UV transilluminator and photographed on Type 667 films (Polaroid, Cambridge, MA). Images were scanned with an image scanner (JX-330; Sharp, Mahwah, NJ) and analyzed with Image Master 1D (Pharmacia Biotech). Relative amounts of TS mRNA and DPD mRNA were expressed as ratios of TS to GAPDH RT-PCR products and DPD to GAPDH RT-PCR products, respectively.

Statistical Analysis. Linear regression analysis was performed on Instat for Macintosh Version 2.01.

RESULTS

Chemosensitivity of Human Tumor Xenografts to 5-FU. *In vivo* chemosensitivity of the human tumor xenografts is shown in Table 2. SC-1-NU, H-111, and Co-4 were sensitive to 5-FU, with lowest *T*/*C* ratios of 8.4, 31.3, and 13.4%, respectively, whereas St-4, Col-3-JCK, PAN-3-JCK, and MX-1 were less sensitive to 5-FU, with lowest *T*/*C* ratios of 87.6, 98.0, 88.6, and 54.1%, respectively.

TS and DPD Levels. TS and DPD levels in the seven human tumor xenografts are shown in Table 3. TS activity was measurable in all human tumor xenografts and varied within a 10-fold range (84.7 ± 10.7–775.5 ± 131.5 fmol/mg protein in seven xenografts). DPD activity was measurable in all but one human tumor xenograft (Co-4) and varied over a 113-fold range (0.7 ± 0.4–79.7 ± 25.1 pmol/min/mg protein in six xenografts). TS mRNA was measurable in all human tumor xenografts and varied within a 20-fold range (0.51 ± 0.07–9.90 ± 1.38 TS:GAPDH ratio in seven xenografts). DPD mRNA was measurable in all but two human tumor xenografts (Co-4 and H-111) and varied over a 13-fold range (0.07 ± 0.02–0.93 ± 0.17 DPD:GAPDH ratio in five xenografts). Values represented the mean and SDs of results from three tumors, each weighing ~200–300 mg.

Table 1 Primers for TS, DPD, and GAPDH amplification

mRNA	Bases	Sequences (5′ → 3′)	Product size (bp)
TS	989–1010	GAATCACATCGAGCCACTGAAA	579
	1567–1547	GTGTTACTCAGCTCCCTCAGA	
DPD	2516–2536	TCCTCCAGGTATGCAGTGCCA	514
	3029–3009	GTTATGGTGGGCAGGTGGGTT	
GAPDH	483–494	CAACAGCCTCAAGATCATCAGC	328
	810–790	TTCTAGACGGCAGGTCAGGTC	

Correlation between DPD/TS Activity and Their mRNA Levels. As shown in Fig. 1*A*, there was a statistically significant correlation between DPD activity and DPD mRNA level, with a coefficient of correlation of 0.81. A positive correlation was also noted between TS activity and TS mRNA level (Fig. 1*B*) with a coefficient of correlation of 0.62.

Correlation between DPD Levels and the Sensitivity to 5-FU. A correlation between DPD activity and xenograft sensitivity to 5-FU is shown in Fig. 2*A*. Tumor DPD activity significantly correlated with tumor sensitivity to 5-FU with a coefficient of correlation of 0.94, which was statistically significant at $P = 0.0017$. High DPD activity resulted in low sensitivity to 5-FU, whereas low DPD activity was associated with high sensitivity to 5-FU. This tendency was also observed between tumor DPD mRNA level and sensitivity to 5-FU, with a coefficient of correlation of 0.79 (Fig. 2*B*), which was also statistically significant ($P = 0.03$). High DPD mRNA level was correlated with low sensitivity to 5-FU in the human tumor xenografts.

Correlation between TS Levels and the Sensitivity to 5-FU. Tumor TS activity and sensitivity to 5-FU are shown in Fig. 3*A*. A trend of high TS levels occurring in tumors with low sensitivity to 5-FU was observed; however, this trend was not statistically significant. Tumor TS mRNA level also did not correlate with 5-FU sensitivity, as shown in Fig. 3*B*.

DISCUSSION

Here, we demonstrated a correlation between DPD activity and DPD mRNA level with sensitivity to 5-FU in seven human tumor xenografts, with high DPD activity and high DPD mRNA level occurring in tumors with low sensitivity to 5-FU. Several studies using human tumor cell lines (10) and clinical samples (11) have also reported that 5-FU catabolism in tumor cells is a probable determining factor in 5-FU responsiveness. Diasio *et al.* (38) measured DPD expression by quantitative PCR assay in tumor specimens from 32 colorectal cancer patients treated with 5-FU and reported that low levels of DPD expression were seen in all of the 5-FU responders compared with much higher levels in the 5-FU nonresponders. These findings suggest that 5-FU is quickly catabolized to 2-fluoro-β-alanine in tumors with high basal DPD activity and/or high basal DPD mRNA level, result-

Table 2 *In vivo* chemosensitivity of human tumor xenografts

	RW[a]						
Drug	SC-1-NU	St-4	H-111	Co-4	Col-3-JCK	PAN-3-JCK	MX-1
5-FU[b]	8.4	87.6	31.3	13.4	98.0	88.6	54.1

[a] The sensitivity to 5-FU was expressed as the RW of drug-treated mice compared to control mice.
[b] 5-FU, 60 mg/kg i.p. q4d×3.

Table 3 TS and DPD levels in human tumor xenografts[a]

Xenograft	TS[b]	TS mRNA[c]	DPD[d]	DPD mRNA[e]
SC-1-NU	597.8 ± 87.9	9.90 ± 1.38	4.5 ± 0.06	0.07 ± 0.02
St-4	537.9 ± 222.1	6.17 ± 0.74	79.7 ± 25.1	0.32 ± 0.05
H-111	313.0 ± 43.2	4.97 ± 1.42	0.7 ± 0.4	ND[f]
PAN-3-JCK	140.0 ± 20.2	0.51 ± 0.07	62.3 ± 21.3	0.31 ± 0.06
MX-1	231.2 ± 42.9	6.44 ± 0.50	13.2 ± 3.95	0.17 ± 0.04
Co-4	84.7 ± 10.7	5.60 ± 1.62	ND	ND
Col-3-JCK	775.5 ± 131.5	6.94 ± 2.09	79.4 ± 11.7	0.93 ± 0.17

[a] Values represent means and SDs of results from three tumors, each weighing ~200–300 mg.
[b] TS activity, in fmol/mg protein.
[c] TS mRNA level, expressed as TS:GAPDH RT-PCR product ratio.
[d] DPD activity, in pmol/min/mg protein.
[e] DPD mRNA level, expressed as DPD:GAPDH RT-PCR product ratio.
[f] ND, not detected.

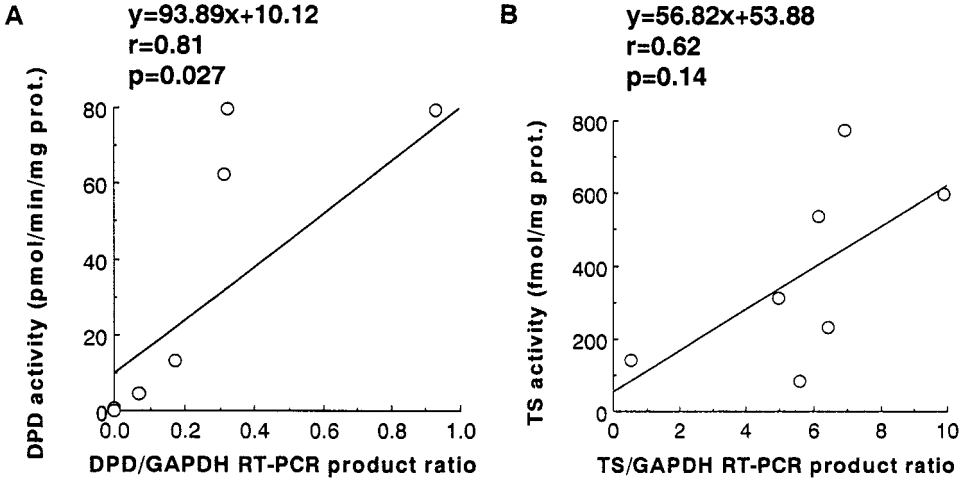

Fig. 1 Correlation between DPD and TS activity and mRNA levels. Plots show the correlations between DPD activity and DPD:GAPDH RT-PCR product ratio (*A*) and TS activity and TS:GAPDH RT-PCR product ratio (*B*) in seven human tumor xenografts. DPD and TS RT-PCR product levels are expressed as ratios to GAPDH internal standard.

ing in the suppression of the anabolic pathway of 5-FU phosphorylation.

Etienne *et al*. (11) measured both TS and DPD activity in tumor biopsy specimens from 62 head and neck cancer patients before administration of 5-FU based chemotherapy and reported that DPD activity was a factor significantly related to 5-FU responsiveness, but no relationship was demonstrated between TS activity and response to 5-FU therapy. In contrast, Danenberg and colleagues (8, 9) reported that tumoral expression of TS mRNA had a statistically significant association with response to protracted infusion of 5-FU based chemotherapy and survival in patients with gastric and disseminated colorectal cancer.

However, correlation between tumoral TS levels and 5-FU antitumor activity was not evident in this study. We have previously reported that, in xenografts with high TS and low DPD levels, like SC-1-NU, excessive FdUMP suppressed TS completely, and the concentration of 5-FU in RNA increased gradually, resulting in both TS inhibition and RNA dysfunction (39). The excessive RNA dysfunction in SC-1-NU may be related to the low correlation between TS levels and sensitivity to 5-FU observed in this study. However, it should be noted that the 5-FU administered in this study was by bolus injection of the maximum tolerated dose, which is different to the usual clinical dose and the protracted infusion protocol in clinical therapy.

Lenz *et al.* (40) reported that p53 status and response to

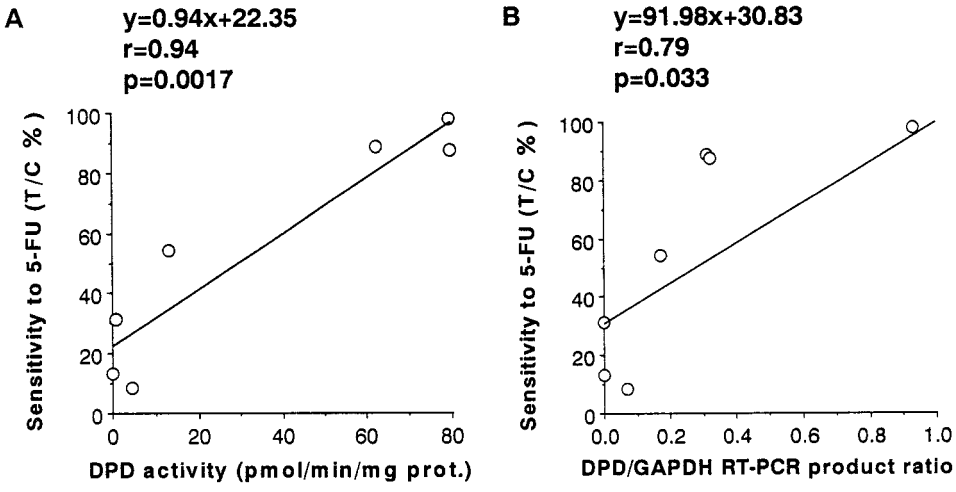

Fig. 2 Correlation between DPD levels and the sensitivity to 5-FU. Plots show the correlations between DPD activity and sensitivity to 5-FU (*A*) and DPD:GAPDH RT-PCR product ratio and sensitivity to 5-FU (*B*) in seven human tumor xenografts. 5-FU (60 mg/kg) was administered i.p. in a schedule of q4d×3, and the sensitivity to 5-FU expressed as the RW of the treated mice compared to that of control mice (*T/C* value). DPD activity and mRNA level significantly correlated with the sensitivity to 5-FU.

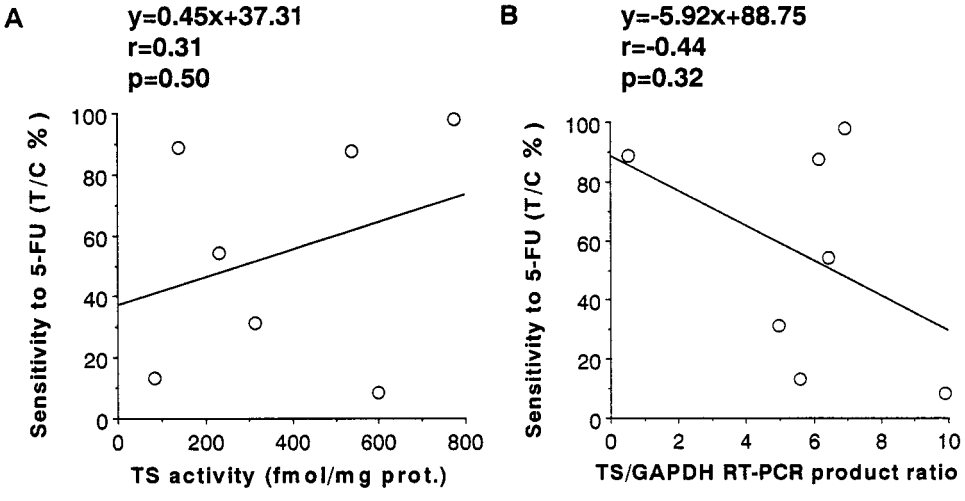

Fig. 3 Correlation between TS levels and the sensitivity to 5-FU. Plots show the relationships between TS activity and sensitivity to 5-FU (*A*) and TS:GAPDH RT-PCR product ratio and sensitivity to 5-FU (*B*) in seven human tumor xenografts. 5-FU (60 mg/kg) was administered i.p. in a schedule of q4d×3 and the sensitivity to 5-FU expressed as the RW of the treated mice compared to that of control mice (*T/C* value). Neither TS activity nor mRNA level correlated with sensitivity to 5-FU.

5-FU chemotherapy were associated in the patients with disseminated colorectal cancer and that the patients with wild-type *p53* had significantly lower TS levels compared to the patients with mutated *p53*. They also reported that p53 and TS expression in primary stage II colon cancer are associated and appear to influence recurrence and survival (41). However, no reports were published concerning the correlation between p53 and DPD. Because only DPD was correlated with the sensitivity to 5-FU in this study, p53 would hardly be significant correlated with the sensitivity to 5-FU in the xenografts used in this study. However, the correlation of p53 status and TS/DPD activity with their mRNA levels should be investigated further in another study.

To discuss the correlation between TS/DPD levels and the sensitivity to 5-FU, we assumed that the levels of TS and DPD remain constant throughout the growth of the tumor. This is particularly troublesome, considering the circadian rhythm reported with regard to DPD activity (42). To avoid this change in DPD activity due to circadian rhythm, all of the experiments were conducted at the same time period (2:00–3:00 p.m.). In our preliminary experiment, when TS and DPD activity and their mRNA levels were determined in different growth phases, DPD activity and mRNA levels were constant through each growth phase, but TS activity changed in three of seven xenografts (data not shown). To prevent this variance, we assessed TS and DPD activity and their mRNA levels in the similarly sized tumors (200–300 mg).

This study has shown that there was a statistically significant correlation between DPD activity and DPD mRNA level in the human tumor xenografts. The semiquantitative RT-PCR method for determination of DPD mRNA is more convenient than radioenzymatic assay for DPD activity because RT-PCR dose not require radioisotopes or relatively large amounts of tumor tissue. Because even small amounts of samples obtained from biopsy specimens before chemotherapy are enough for the assay of DPD mRNA, RT-PCR detection of DPD mRNA may be useful in designing 5-FU-based chemotherapy for individual patients. This study suggests that determination of DPD mRNA level obtained from biopsy specimens before chemotherapy is a promising predictor for sensitivity to 5-FU. In addition, in

tumors with high DPD mRNA level, DPD inhibitors, such as 5-etynyluracil (14), uracil (15), and 5-chloro-2,4-dihydroxypyridine (16), may be potential modulators in promoting the antitumor activity of 5-FU.

REFERENCES

1. Macdonald, J. S., and Gohmann, J. J. Chemotherapy of advanced gastric Cancer: present status, future prospects. Semin. Oncol., *15* (Suppl. 4)*:* 42–49, 1988.

2. Hafstrom, L., Rudenstam, C-M., Domellof, L., and the Swedish Gastro-Intestinal Tumour Adjuvant Therapy Group. A randomized trial of oral 5-fluorouracil versus placebo as adjuvant therapy in colorectal cancer Dukes' B and C: results after 5 years observation time. Br. J. Surg., *72:* 138–141, 1985.

3. Langenback, R. J., Danenberg, P. V., and Heidelberger, C. Thymidylate synthetase: mechanism of inhibition by 5-fluoro-2′-deoxyuridylate. Biochem. Biophys. Res Commun., *48:* 1565–1571, 1972.

4. Matsuoka, H., Ueo, H., Sugimachi, K., and Akiyoshi, T. Preliminary evidence that incorporation of 5-fluorouracil into RNA correlates with antitumor response. Cancer Invest., *10:* 265–269, 1992.

5. Peters, G. J., van der Wilt, C. L., van Groeningen, C. J., Smid, K., Meijer, S., and Pinedo, H. M. Thymidylate synthase inhibition after administration of fluorouracil with or without leucovorin in colon cancer patients: implications for treatment with fluorouracil. J. Clin. Oncol., *12:* 2035–2042, 1994.

6. Johnston, P. G., Fisher, E. R., Rockette, H. E., Fisher, B., Wolmark, N., Drake, J. C., Chabner, B. A., and Allegra, C. J. The role of thymidylate synthase expression in prognosis and outcome of adjuvant chemotherapy in patients with rectal cancer. J. Clin. Oncol., *12:* 2640–2647, 1994.

7. Johnston, P. G., Lenz, H-J., Leichman, C. G., Danenberg, K. D., Allegra, C. J., Danenberg, P. V., and Leichman, L. Thymidylate synthase gene and protein expression correlate and are associated with response to 5-fluorouracil in human colorectal and gastric tumors. Cancer Res., *55:* 1407–1412, 1995.

8. Lenz, H-J., Leichman, C. G., Danenberg, K. D., Danenberg, P. V., Groshen, S., Cohen, H., Laine, L., Crookes, P., Silberman, H., Baranda, J., Garcia, Y., Li, J., and Leichman, L. Thymidylate synthase mRNA level in adenocarcinoma of the stomach. J. Clin. Oncol., *14:* 176–182, 1995.

9. Leichman, C. G., Lenz, H-J., Leichman, L., Danenberg, K., Baranda, J., Groshen, S., Boswell, W., Metzger, R., Tan, M., and Danenberg, P. V. Quantitation of intratumoral thymidylate synthase expression predicts for disseminated colorectal cancer response and resistance to protracted-infusion fluorouracil and weekly leucovorin. J. Clin. Oncol., *15:* 3223–3229, 1997.

10. Beck, A., Etienne, M. C., Cheradame, S., Fischel, J. L., Formento, P., Renee, N., and Milano, G. A role for dihydropyrimidine dehydrogenase and thymidylate synthase in tumour sensitivity to fluorouracil. Eur. J. Cancer, *30A:* 1517–1522, 1994.

11. Etienne, M. C., Cheradame, S., Fischel, J. L., Formento, P., Dassonville, O., Renee, N., Schneider, M., Thyss, A., Demard, F., and Milano, G. Response to fluorouracil therapy in cancer patients: the role of tumoral dihydropyrimidine dehydrogenase activity. J. Clin. Oncol., *13:* 1663–1670, 1995.

12. Jiang, W., Lu, Z., He, Y., and Diasio, R. B. Dihydropyrimidine dehydrogenase activity in hepatocellular carcinoma: implication in 5-fluorouracil-based chemotherapy. Clin. Cancer Res., *3:* 395–399, 1997.

13. McLeod, H. L., Sludden, J., Murray, G. I., Keenan, R. A., Davidson, A. I., Park, K., Koruth, M, and Cassidy, J. Characterization of dihydropyrimidine dehydrogenase in human colorectal tumours. Br. J. Cancer, *77:* 461–465, 1988.

14. Milano, G., Fischel, J. L., Spector, T., Etienne, M. C., and Formento, P. Enhancement of fluorouracil cytotoxicity by inhibiting dihydropyrimidine dehydrogenase activity with 5-etynyluracil cytotoxicity by inhibiting dihydropyrimidine dehydrogenase activity with 5-ethynyluracil. Proc. Am. Assoc. Cancer Res., *36:* 292, 1995.

15. Takechi, T., Uchida, J., Fujioka, A., and Fukushima, M. Enhancing 5-fluorouracil cytotoxicity by inhibiting dihydropyrimidine dehydrogenase activity with uracil in human tumor cells. Int. J. Oncol., *11:* 1041–1044, 1997.

16. Takechi, T., Uchida, J., Takeda, S., and Fukushima, M. Enhancement of 5-fluorouracil cytotoxicity by dihydropyrimidine dehydrogenase inhibitor, 5-chloro-2,4-dihydroxypyrimidine. Proc. Jpn. Cancer Assoc., *87* (Suppl.)*:* 639, 1996.

17. Kubota, T., Kawamura, E., Kurihara, H., Ishibiki, K., and Abe, O. Clinical study of clonogenic assay-with reference to adjuvant cancer chemotherapy after operation. J. Jpn. Surg. Soc., *87:* 154–161, 1986.

18. Suto, A., Kubota, T., Shimoyama, Y., Ishibiki, K., and Abe, O. MTT assay with reference to the clinical effect of chemotherapy. J. Surg. Oncol., *42:* 28–32, 1989.

19. Saikawa, Y., Kubota, T., Furukawa, T., Suto, A., Watanabe, M., Kumai, K., Ishibiki, K., and Kitajima, M. Single-cell suspension assay with an MTT end point is useful for evaluating the optimal adjuvant chemotherapy for advanced gastric cancer. Jpn. J. Cancer Res., *85:* 762–765, 1994.

20. Furukawa, T., Kubota, T., and Hoffman, R. M. Clinical application of the histoculture drug response assay. Clin. Cancer Res., *1:* 305–311, 1995.

21. Kubota, T., Sasano, N., Abe, O., Nakao, I., Kawamura, E., Saito, T., Endo, M., Kimura, K., Demura, H., Sasano, H., Nagura, H., Ogawa, N., Hoffman, R. M., and the Chemosensitivity Study Group for the Histoculture Drug-Response Assay. Potential of the histoculture drug-response assay to contribute to cancer patient survival. Clin. Cancer Res., *1:* 1537–1543, 1995.

22. Nagai, S., Yamauchi, M., Andoh, T., Nishizawa, M., Satta, T., Kodera, Y., Kondou, K., Akiyama, S., Ito, K., and Takagi, H. Establishment and characterization of human gastric and colonic xenograft lines resistant to CPT-11 (a new derivative of camptothecin). J. Surg. Oncol., *59:* 116–124, 1995.

23. Fujita, F., Fujita, M., Taguchi, T., Shimozuma, K., Sakamoto, Y., Kimoto, Y., and Hirai, T. Multifactorial analysis of parameters influencing chemosensitivity of human cancer xenografts in nude mice. Int. J. Cancer., *43:* 637–644, 1989.

24. Yamada, Y., Nakatani, K., Watanabe, A., Okumura, T., Yano, T., Yamada, Y., and Nakano, H. Expression of malignant potential of human colon cancer in nude mice: preliminary report. J. Jpn. Surg. Soc., *91:* 787, 1990.

25. Nomura, T., Sakurai, Y., and Inada, M. The Nude Mouse and Anticancer Drug Evaluation, pp. 84–87. Tokyo: Japanese Journal of Cancer and Chemotherapy Publishers Inc., 1996.

26. Giovanella B. C., Stehlin J. S., and Williams, L. J., Jr. Heterotransplantation of human malignant tumors in "nude" thymusless mice. II. Malignant tumors induced by injection of cell cultures derived from human solid tumors. J. Natl. Cancer Inst. (Bethesda), *52:* 921–930, 1974.

27. Kubota, T., Nakada, M., Tsuyuki, K., Inada, T., Asanuma, F., Ishibiki, K., and Abe, O. Cell kinetics and chemosensitivity of human carcinomas serially transplanted into nude mice. Jpn. J. Cancer Res., *77:* 502–507, 1986.

28. Geran, R. I., Greenberg, N. H., Macdonald, M. M., Schumacher, A. H., and Abbott, B. J. Protocols for screening chemical agents and natural products against animal tumors and other biological systems. Cancer Chemother. Rep., *3:* 51–61, 1972.

29. Kubota, T., Ishibiki, K., and Abe, O. The clinical usefulness of human xenografts in nude mice. Prog. Clin. Biol. Res., *276:* 213–225, 1988.

30. Spears, C. P., Shahinian, A. H., Moran, R. G., Heidelberger, C., and Corbett, T. H. *In vivo* kinetics of thymidylate synthetase inhibition in 5-fluorouracil-sensitive and -resistant murine colon adenocarcinomas. Cancer Res., *42:* 450–456, 1982.

31. Ikenaka, K., Shirasaka, T., Kitano, S., and Fujii, S. Effect of uracil on metabolism of 5-fluorouracil *in vitro*. Gann, *70:* 353–359, 1979.

32. Takechi, T., Okabe, H., Fujioka, A., and Fukushima, M. Relationship between protein levels and gene expression of dihydropyrimidine dehydrogenase in human tumor cells during growth in culture and in nude mice. Jpn. J. Cancer Res., *89:* 1144–1153, 1999.

33. Takeishi, K., Kaneda, S., Ayusawa, D., Shimizu, K., Gotoh, O., and Seno, T. Nucleotide sequence of a functional cDNA for human thymidylate synthase. Nucleic Acid Res., *13:* 2035–2045, 1985.

34. Yokota, H., Fernandez-Salguero, P., Furuya, H., Lin, K., McBride, O. W., Podschun, B., Schnackerz, K. D., and Gonzalez, F. J. cDNA cloning and chromosome mapping of human dihydropyrimidine dehydrogenase, an enzyme associated with 5-fluorouracil toxicity and congenital thymine uraciluria. J. Biol. Chem., *269:* 23192–23196, 1994.

35. Tokunaga, K., Nakamura, Y., Sakata, K., Fujimori, K., Ohkubo, M., Sawada, K., and Sakiyama, S. Enhanced expression of a glyceraldehyde-3-phosphate dehydrogenase gene in human lung cancers. Cancer Res., *47:* 5616–5619, 1987.

36. Perryman, S. M., Rossana, C., Deng, T., Vanin, E. F., and Johnson, L. F. Sequence of a cDNA for mouse thymidylate synthase reveals striking similarity with the prokaryotic enzyme. Mol. Biol. Evol., *3:* 313–321, 1986.

37. Sabath, D. E., Broome, H. E., and Prystowsky, M. B. Glyceraldehyde-3-phosphate dehydrogenase mRNA is a major interleukin 2-induced transcript in a cloned T-helper lymphocyte. Gene, *91:* 185–191, 1990.

38. Diasio, R. B., Danenberg, K., Johnson, M., and Danenberg, P. Quantitation of dihydropyrimidine dehydrogenase expression in tumor specimens of patients treated with 5-fluorouracil using a quantitative polymerase chain reaction assay. Proc. Am. Assoc. Cancer Res., *39:* 188,1998.

39. Inada, T., Ichikawa, A., Kubota, T., Ogata, Y., Moossa, A. R., and Hoffman, R. M. 5-FU-induced apoptosis correlates with efficacy against human gastric and colon cancer xenografts in nude mice. Anticancer Res., *17:* 1965–1972, 1997.

40. Lenz, H-J., Hayashi, K., Salonga, D., Danenberg, K. D., Danenberg, P. V., Metzger, R., Banerjee, D., Bertino, J. R., Groshen, S., Leichman, L. P., and Leichman, C. G. p53 point mutation and thymidylate synthase messenger RNA levels in disseminated colorectal cancer: an analysis of response and survival. Clin. Cancer Res., *4:* 1243–1250, 1998.

41. Lenz, H-J., Danenberg, K. D., Leichman, C. G., Florentine, B., Johnston, P. G., Groshen, S., Zhou, L., Xiong, Y. P., Danenberg, P. V., and Leichman, L. P. p53 and thymidylate synthase expression in untreated stage II colon cancer: association with recurrence, survival, and site. Clin. Cancer Res., *4:* 1227–1234, 1998.

42. Harris, B. E., Song, R., Soong S-J., and Diasio, R. B. Relationship between dihydropyrimidine dehydrogenase activity and plasma 5-fluorouracil levels with evidence for circadian variation of enzyme activity and plasma drug levels in cancer patients receiving 5-fluorouracil by protracted continuous infusion. Cancer Res., *50:* 197–201, 1990.-