Jpn. J. Cancer Res. **91**, 105–112, January 2000

Dihydropyrimidine Dehydrogenase and Messenger RNA Levels in Gastric Cancer: Possible Predictor for Sensitivity to 5-Fluorouracil

Yoichiro Ishikawa,[1] Tetsuro Kubota,[1,3] Yoshihide Otani,[1] Masahiko Watanabe,[1] Tatsuo Teramoto,[1] Koichiro Kumai,[1] Teiji Takechi,[2] Hiroyuki Okabe,[2] Masakazu Fukushima[2] and Masaki Kitajima[1]

[1]Department of Surgery, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582 and [2]Cancer Research Laboratory, Taiho Pharmaceutical Co., Ltd., 1-27 Misugidai, Hanno-city, Saitama 357-8527

We investigated the correlation between tumor sensitivity to 5-fluorouracil (5-FU) and enzymatic activities of thymidylate synthetase (TS) and dihydropyrimidine dehydrogenase (DPD) in human gastric cancer specimens. Forty-one patients with advanced gastric cancer gave informed consent and were enrolled in the study. Biopsy specimens of gastric cancer were obtained preoperatively through gastrofiberscopy and used to determine TS and DPD messenger RNA (mRNA) levels. TS and DPD enzyme activity and mRNA levels were also measured in resected tumor tissue samples obtained after surgical resection. TS and DPD activity were measured using the TS-binding assay and a radioenzymatic assay, respectively, while mRNA levels were measured by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR), with co-amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal standard. 5-FU sensitivity of resected tumor specimens was measured by the tetrazolium-based colorimetric assay (MTT assay). Both TS and DPD mRNA levels correlated well between biopsied and resected tumor specimens. A statistically significant correlation was also observed between mRNA levels in biopsied specimens and enzymatic activities in resected specimens. DPD levels significantly correlated with 5-FU sensitivity, such that high DPD activity and high DPD mRNA levels resulted in low sensitivity to 5-FU. In contrast, no correlation was observed between TS activity or TS mRNA levels and 5-FU sensitivity. We conclude that tumor DPD mRNA level, as assessed from biopsy specimens obtained by gastrofiberscopy, may be a useful indicator in predicting tumor sensitivity to 5-FU in patients with gastric cancer.

Key words: Thymidylate synthetase levels — Dihydropyrimidine dehydrogenase levels — Antitumor activity — 5-Fluorouracil — Human gastric cancer

5-FU is widely used in the treatment of gastrointestinal carcinomas and is considered to be one of the most effective drugs against gastric cancer. Since the efficacy rate of 5-FU treatment alone for gastric cancer is only 10–20%,[1)] one of the most frequently used protocols for gastric cancer is biomodulated 5-FU chemotherapy with folinic acid (leucovorin) and/or cisplatin.[2)] However, the clinical effects of these regimens are still unsatisfactory with regard to long-term survival.

5-FU is catabolized to 2-fluoro-β-alanine mainly in the liver by three enzymes; DPD (EC 1.3.1.2), the first and rate-limiting enzyme, followed by dihydropyrimidinase (EC 3.5.2.2) and β-ureidopropionase (EC 3.5.1.6). Several studies[3–7)] have demonstrated significant variation in tumoral DPD amongst different human tumor cell lines, human tumor xenografts and clinical samples, and elevated intratumoral DPD activity has been implicated in low antitumor activity of 5-FU due to increased 5-FU inactivation. Etienne *et al.*[6)] determined tumoral/non-tumoral 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 and non-responding patients. Moreover, some DPD inhibitors, such as 5-eniluracil,[8)] uracil[9)] and 5-chloro-2,4-dihydroxypyridine[10)] have been demonstrated to enhance 5-FU antitumor activity through inhibition of tumoral DPD activity in human tumor cell lines.

One of the main modes of action of 5-FU is thought to be through its active metabolite, FdUMP. FdUMP suppresses TS (EC 2.1.1.45) by forming covalent ternary complexes with 5,10-methylenetetrahydrofolate, which

[3] To whom correspondence should be addressed.
E-mail: tkubota@med.keio.ac.jp

Abbreviations: 5-FU, 5-fluorouracil; FdUMP, 5-fluoro-dUMP; TS, thymidylate synthetase; DPD, dihydropyrimidine dehydrogenase; β-ME, 2-mercaptoethanol; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H tetrazolium bromide; FCS, fetal calf serum; NADPH, nicotinamide adenine dinucleotide phosphate.

subsequently inhibits DNA synthesis.[11] Several reports have indicated that tumoral TS expression was related to the response to 5-FU-based chemotherapy and patient survival in gastric and colorectal cancers,[12–15] although increased expression of TS is not always recognized as a determining factor for 5-FU resistance.[6, 16, 17]

Therefore, we have focused our attention on the relationship between tumoral TS/DPD levels and tumor 5-FU sensitivity. With the advent of the polymerase chain reaction, alternative methods of TS and DPD determination have been applied to measure mRNA levels in small amounts of tissue, such as those obtained from biopsy specimens.[15, 18, 19] In our previous study using human tumor xenografts in nude mice, we reported that tumoral DPD mRNA levels were measurable by RT-PCR, and were a promising predictor of 5-FU sensitivity.[19]

The objective of the present study was to clarify the relationship between TS/DPD activity and mRNA levels, and 5-FU tumor sensitivity in human gastric cancer specimens.

PATIENTS AND METHODS

Patients Forty-one patients with advanced gastric cancer were enrolled in this study at Keio University Hospital between May 1997 and July 1998, after giving their informed consent. Scirrhous type gastric cancer was excluded from the study.

Chemicals 5-FU was purchased from Kyowa Hakko Kogyo Co., Ltd. (Tokyo). MTT was purchased from Sigma Chemical Co. (St. Louis, MO). [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 nicotinamide adenine dinucleotide phosphate (NADPH) was obtained from Sigma Chemical Co. All other chemicals used were the highest standard grade commercially available.

Biopsied specimens and fresh surgical specimens Biopsy specimens of gastric cancer for the assay of TS mRNA and DPD mRNA by the RT-PCR method were obtained preoperatively through gastrofiberscopy. Each biopsied specimen was confirmed histologically to contain cancer cells with reference to adjacent biopsied specimens. A total of five biopsy specimens of about 2 to 3 mm in size were collected and stored at −80°C using liquid nitrogen until use. After surgery, resected tumor tissues were divided into two pieces; one piece was stored in Hanks' balanced salt solution (GIBCO, Gaithersburg, MD) containing 100 IU/ml penicillin (GIBCO), 100 μg/ml streptomycin (GIBCO), and 0.25 μg/ml amphotericin B (GIBCO) and brought to the laboratory as soon as possible for the MTT assay. The other piece of tumor tissue, for which histological confirmation of malignancy was obtained, was immersed in liquid nitrogen and stored at −80°C until assayed for TS and DPD activities and mRNA levels.

Evaluation of 5-FU antitumor activity The MTT assay reported by Mosmann[20] with some modifications[21–23] was used to evaluate *in vitro* 5-FU sensitivity of the fresh surgical gastric cancer specimens.

Single-cell suspensions of the surgical specimens were prepared enzymatically by incubation for 30 min in 0.5 mg/ml pronase (Boehringer Mannheim GmbH, Mannheim, Germany), 0.2 mg/ml collagenase type I (Sigma) and 0.2 mg/ml DNase (Sigma). After centrifugation, tumor cells were suspended in RPMI 1640 (GIBCO) medium supplemented with 10% FCS (GIBCO), diluted to 2×10^5 cells/ml, and then plated into 96-well microplates (GIBCO) in volumes of 100 μl (10^4 cells per well). Equal volumes of 5-FU solution were added to give a final concentration of 50 μg/ml 5-FU as previously reported.[22] Control wells contained 100 μl of cell suspension and 100 μl of RPMI 1640 with 10% FCS. Plates were incubated for 48 h at 37°C in a humidified atmosphere of 95% air and 5% CO_2. At the end of incubation, 0.4% MTT and 0.1 *M* sodium succinate dissolved in 10 μl of phosphate-buffered saline were added to each well, and the plates were incubated for 3 h at 37°C. After the final incubation, 150 μl/well dimethyl sulfoxide was added, and the plates were mechanically shaken for 10 min to dissolve the formazan salt. Optical densities were read on a model EAR 340 easy reader (SLT-Labinstruments, Salzburg, Austria) at 540–630 nm. The sensitivity to 5-FU (T/C value) was calculated using the formula: $A/B\times100\%$, where A and B represent the mean absorbance of the treated and control wells, respectively.

Enzymatic assay For determination of TS binding activity to FdUMP, tumor tissues obtained from resected specimens were homogenized with 3 volumes of 200 m*M* Tris-HCl (pH 8.0) containing 20 m*M* β-ME, 100 m*M* NaF and 15 m*M* cytidine-5′-monophosphate, and centrifuged at 105 000*g* for 60 min. The resultant supernatants were used to determine the [6-^{3}H]FdUMP binding sites as a measure of TS activity according to the method of Spears *et al.*[24] with some modifications.[19]

The DPD enzymatic assay was based on a previously described method.[9] Briefly, tumor tissues were sonicated in 4 volumes of homogenization buffer (20 m*M* potassium phosphate (pH 8.0) containing 1 m*M* EDTA and 1 m*M* β-ME). Homogenates were centrifuged at 105 000*g* for 1 h at 4°C, and supernatants (cytosol fraction) were collected. Aliquots of the cytosol fractions (25 μl) were mixed with 25 μl of enzyme reaction mixture (2 m*M* dithiothreitol, 5 m*M* $MgCl_2$, 20 μM [6-^{14}C]5-FU, 100 μM NADPH), incubated at 37°C for 30 min, and applied to thin-layer chromatography plates (silica gel 60 F254, Merck, Darmstadt, Germany). Plates were developed with a mixture of ethanol and 1 *M* ammonium acetate (5:1, v/v), according to the

method of Ikenaka *et al*.[25] 5-FU and catabolized products formed from 5-FU were visualized and quantified using an image analyzer (BAS-2000, Fujix, Tokyo).

Semi-quantitative RT-PCR Semi-quantitative RT-PCR was performed using previously described methods.[19] Briefly, total RNA for each sample was isolated using the RNeasy mini kit (QIAGEN Inc., Chatsworth, CA) according to the manufacturer's instructions. Reverse transcription using 10 μg of total RNA was performed in a total volume of 100 μl containing 250 pmol of oligo$(dT)_{18}$, 80 U of rRNasin ribonuclease inhibitor (Promega, Madison, WI), 500 U of Moloney murine leukemia virus reverse transcriptase (GIBCO), 50 m*M* Tris-HCl (pH 8.3), 75 m*M* KCl, 3 m*M* $MgCl_2$, 10 m*M* dithiothreitol, and 0.5 m*M* dNTPs. Initially, RNA and oligo$(dT)_{18}$ were heated at 70°C for 10 min and immediately chilled on ice, and then the remaining reagents were added. The mixture was incubated for 15 min at 30°C, and then for 60 min at 42°C.

Three different concentrations of transcribed RNA were used as PCR templates. For accurate quantification, we confirmed that the PCR amplification was in the linear phase. PCR was carried out in a final volume of 50 μl containing cDNA template, TS (10 pmol each primer) and GAPDH (2 pmol each primer) or DPD (40 pmol each primer) and GAPDH (2 pmol each primer) primer combinations, 1.25 U of Ex Taq (Takara, Shiga) in 10× Ex Taq buffer (Takara) and 0.2 m*M* dNTPs, using a thermal cycler (PC-800, Astec, Tokyo). PCR profiles consisted of an initial 3-min denaturation at 94°C, followed by 30 cycles of 1 min denaturation at 94°C, 1 min annealing at 60°C and 2 min polymerization at 72°C, and a final 10-min extension at 72°C. PCR products were separated by 2% agarose gel electrophoresis, stained with ethidium bromide and visualized on a UV transilluminator. Gels were photographed on Type 667 film (Polaroid, Cambridge, MA), and images were scanned with an image scanner (JX-330, SHARP, Mahwah, NJ) and analyzed with Image Master 1D (Pharmacia Biotech, Tokyo). Relative amounts of TS mRNA and DPD mRNA were expressed as the ratios of TS to GAPDH RT-PCR products and DPD to GAPDH RT-PCR products, respectively.

Statistical analysis As TS and DPD levels exhibited asymmetrical distributions, non parametric tests were applied for statistical analysis. Linear regression analysis was performed with Instat for Macintosh version 2.01. The comparison of 5-FU sensitivity between groups of patients with high and low DPD levels was performed using the Mann-Whitney test. The criterion of statistical significance was $P \leq 0.05$.

RESULTS

Correlation between mRNA levels in preoperatively biopsied tumor specimens and resected tumor specimens As shown in Fig. 1, A and B, there was a statistically significant correlation between TS and DPD mRNA levels in biopsied tumor specimens and resected tumor specimens, with correlation coefficients of 0.93 for TS mRNA and 0.91 for DPD mRNA.

Correlation between mRNA levels in preoperatively biopsied tumor specimens and enzyme activities in resected tumor specimens TS and DPD mRNA levels in

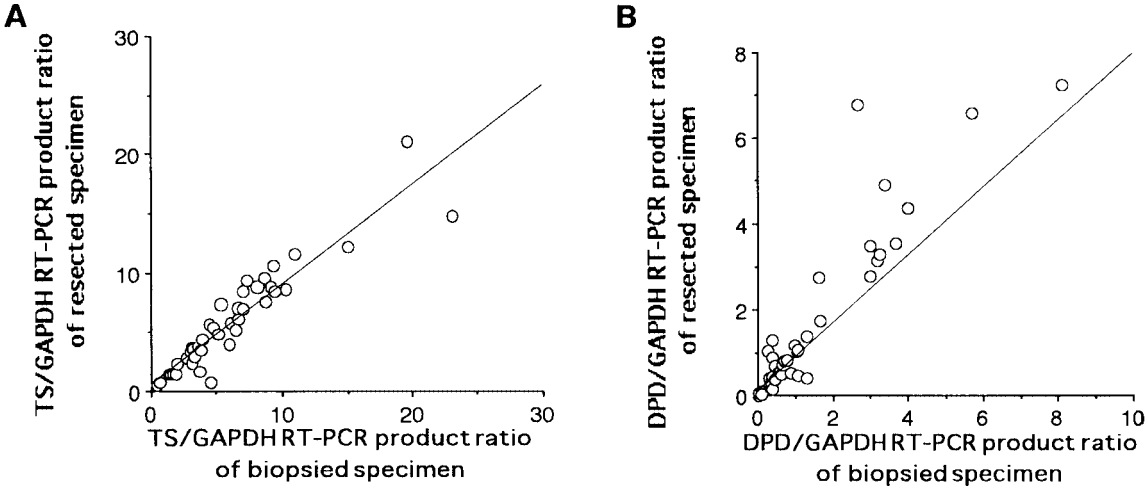

Fig. 1. Correlation between mRNA levels in biopsied tumor specimens and resected tumor specimens. Plots show the correlation between TS:GAPDH RT-PCR product ratios from biopsied and resected specimens (A), and DPD:GAPDH RT-PCR product ratios from biopsied and resected specimens (B) from gastric cancer patients. TS and DPD RT-PCR product levels are expressed as ratios to a GAPDH internal standard. Statistically significant correlations were observed for TS mRNA (r=0.93) and DPD mRNA (r=0.91). (A) y=0.84x+0.65, r=0.93, P<0.0001, (B) y=1.04x+0.12, r=0.91, P<0.0001.

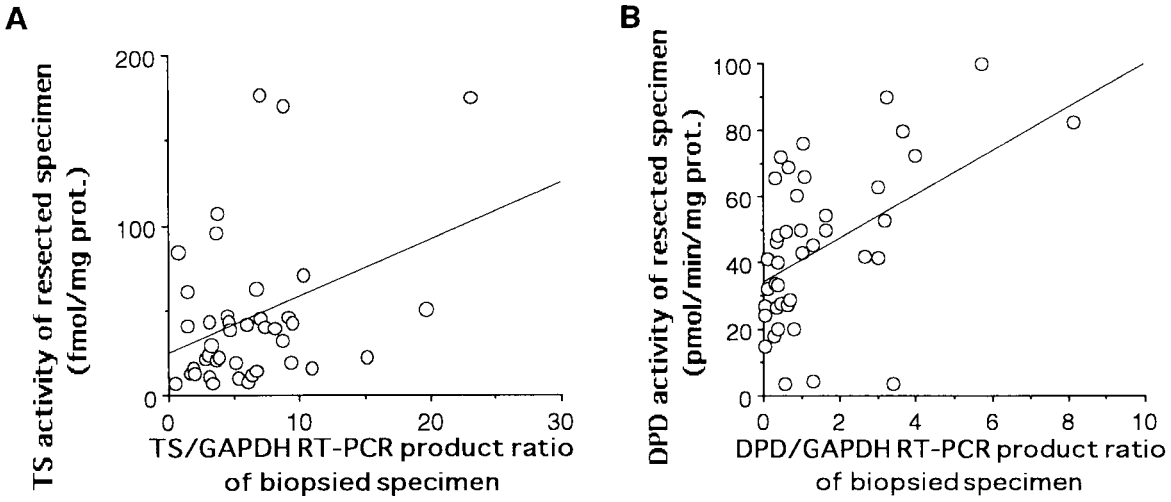

Fig. 2. Correlation between mRNA levels in biopsied tumor specimens and enzyme activity in resected tumor specimens. Plots show the correlation between TS:GAPDH RT-PCR product ratios from biopsied specimens and TS activity in resected specimens (A), and DPD:GAPDH RT-PCR product ratios from biopsied specimens and DPD activity in resected specimens (B) in gastric cancer patients. TS and DPD RT-PCR product levels are expressed as ratios to a GAPDH internal standard. Both TS mRNA and DPD mRNA in preoperatively biopsied specimens successfully predicted the enzyme activity in resected specimens. (A) $y=3.36x+24.58$, $r=0.36$, $P=0.021$, (B) $y=7.54x+34.06$, $r=0.54$, $P=0.0003$.

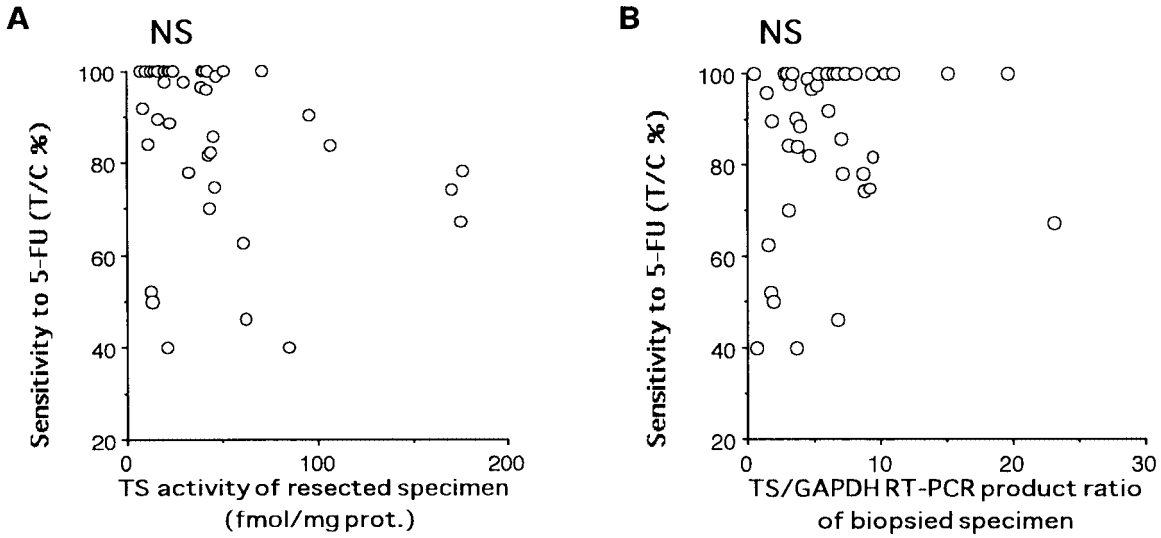

Fig. 3. Correlation between tumoral TS levels and the sensitivity to 5-FU. Plots show the correlation between TS activity in resected specimens and sensitivity to 5-FU (A), and TS:GAPDH RT-PCR product ratios of biopsied specimens and sensitivity to 5-FU (B) in gastric cancer patients. Sensitivity to 5-FU was measured by the MTT assay and T/C values were calculated using the formula: $A/B\times 100\%$, where A and B represent the mean absorbances of the treated and control wells, respectively.

biopsied specimens correlated with enzyme activities in resected specimens (Fig. 2). The coefficient of correlation was low for TS levels (Fig. 2A) and moderate for DPD levels (Fig. 2B).

Correlation between TS/DPD levels and sensitivity to 5-FU No significant correlation was observed between tumoral TS activity in resected specimens and tumor sensitivity to 5-FU (Fig. 3A), or between tumoral TS mRNA

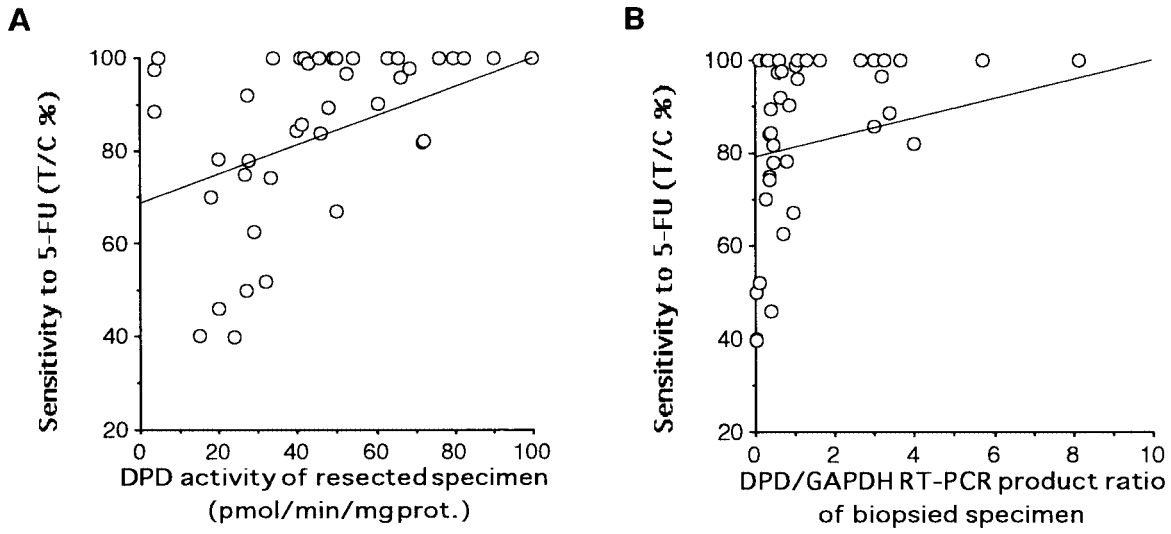

Fig. 4. Correlation between tumoral DPD levels and the sensitivity to 5-FU. Plots show the correlation between DPD activity in resected specimens and sensitivity to 5-FU (A), and DPD:GAPDH RT-PCR product ratios in biopsied specimens and sensitivity to 5-FU (B) in gastric cancer patients. Sensitivity to 5-FU was measured by the MTT assay and T/C values were calculated using the formula: $A/B \times 100\%$, where A and B represent the mean absorbances of the treated and control wells, respectively. (A) $y=0.37x+68.70$, $r=0.48$, $P=0.0015$, (B) $y=4.36x+79.00$, $r=0.41$, $P=0.0078$.

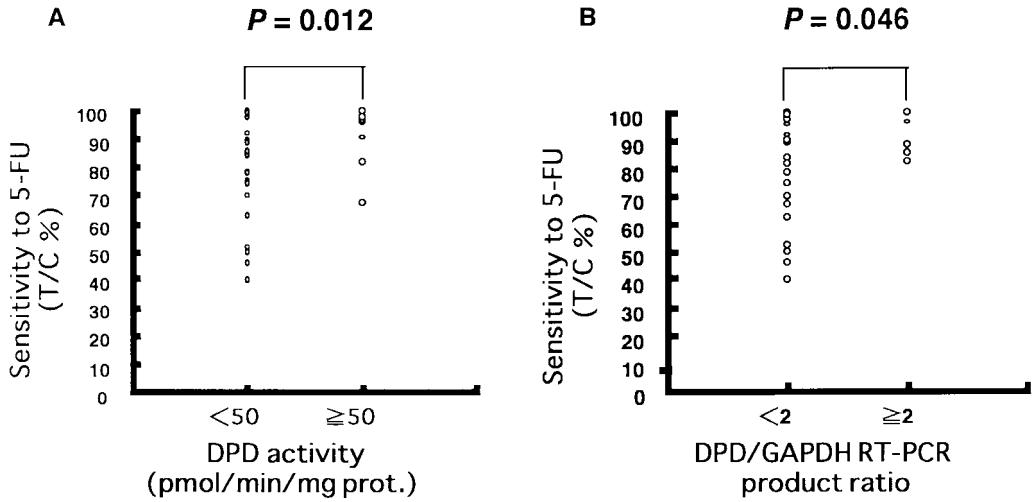

Fig. 5. Sensitivity to 5-FU according to DPD activity and DPD mRNA. Patients were categorized into high DPD activity group of resected specimens (≥50 pmol/min/mg protein) and low DPD activity group (<50 pmol/min/mg protein) (A), or high DPD mRNA group (≥2) and low DPD mRNA group (<2) in biopsied specimens (B). These cutoff levels successfully distinguished different sensitivities to 5-FU.

levels in biopsy specimens and 5-FU sensitivity (Fig. 3B). A correlation between tumoral DPD activity in resected specimens and sensitivity to 5-FU was observed, with a correlation coefficient of 0.48, which was statistically significant ($P=0.0015$) (Fig. 4A). High DPD activity resulted in low sensitivity to 5-FU, while low DPD activity was associated with high sensitivity to 5-FU. This tendency was also observed between tumoral DPD mRNA levels in biopsy specimens and sensitivity to 5-FU, with a correlation coefficient of 0.41 ($P=0.0078$) (Fig. 4B).

To estimate a possible cutoff point of DPD activity to determine resistance or sensitivity to 5-FU in gastric cancer patients, patients were categorized as having either high DPD activity (≥50 pmol/min/mg protein) or low DPD activity (<50 pmol/min/mg protein), or high DPD mRNA levels in biopsied specimens (≥2) or low DPD mRNA (<2). The high DPD activity group exhibited a significantly lower sensitivity to 5-FU than the low DPD activity group (Fig. 5A). Likewise, low sensitivity to 5-FU was associated with the high DPD mRNA group, and high sensitivity to 5-FU with the low DPD mRNA group (Fig. 5B).

DISCUSSION

The present study has shown a correlation of tumoral DPD activity and mRNA levels with 5-FU sensitivity in tumor specimens obtained from preoperative biopsy and surgical resection from 41 gastric cancer patients. High DPD activity and mRNA levels were associated with tumors with low 5-FU sensitivity, and low DPD activity was associated with high 5-FU sensitivity. This finding is consistent with our previous experimental study using seven human tumor xenografts in nude mice,[19] in which both tumoral DPD activity and mRNA levels significantly correlated with 5-FU sensitivity. These findings suggested that in patients with high basal DPD levels in tumors, 5-FU is quickly catabolized to 2-fluoro-β-alanine, resulting in the suppression of the anabolic pathway of 5-FU phosphorylation and the reduction of 5-FU sensitivity. Since the 5-FU-sensitive patients have a favorable outcome when treated with fluoropyrimidines as shown in our previous study,[23, 26] tumoral DPD levels might influence the survival of patients treated with fluoropyrimidines.

It was observed that DPD mRNA levels in preoperative biopsy specimens significantly correlated not only with mRNA levels in surgically resected specimens, but also with enzyme activity. We also examined tumoral DPD mRNA levels in biopsy specimens obtained from different portions of the same tumor in five gastric cancer patients and confirmed that the results for each tumor sample were substantially identical (data not shown). This suggested that there was little or no heterogeneity in DPD mRNA levels within the tumors. As a result, DPD mRNA level may be a better clinical parameter than DPD activity. Moreover, in an attempt to establish the critical level of DPD mRNA expression in gastric cancer, we found that there was a significant difference in 5-FU sensitivity between patients expressing high (≥2) and low (<2) DPD mRNA levels. This cutoff mRNA level may be a clinically useful parameter as even small sample amounts obtained from biopsy specimens before chemotherapy would be enough for the determination of mRNA levels, allowing the rational design of 5-FU-based chemotherapy for individual patients before surgery. However, some specimens were insensitive to 5-FU despite having DPD and DPD mRNA levels below the arbitrary cutoff point. This result was in contrast to our previous study using human tumor xenografts in nude mice.[19] Although the reasons for this are unclear at the present time, differences between human tumor xenografts and human surgical specimens may be due to differences in growth phase kinetics. For instance, it is possible that the xenografts were in the exponential growth phase while most of the surgical specimens were in the plateau phase.

No correlation between tumoral TS levels and 5-FU sensitivity was observed in this study. However, numerous experimental studies using colorectal, breast, or head and neck cancer cells have shown that either over-expression of the TS protein or high TS activity was associated with 5-FU resistance.[5, 27, 28] Johnston *et al.*[13, 14] also reported that tumoral TS mRNA expression had a statistically significant association with the response to protracted infusion of 5-FU-based chemotherapy and survival in patients with gastric and disseminated colorectal cancer. This discrepancy may be due to excess 5-FU substrate. We administered 60 mg per kg of 5-FU in nude mice in our previous study,[19] and used 50 μg per ml 5-FU in contact for 48 h in the present study. In our previous study, TS was completely inhibited and ribonucleotide reductase was saturated, resulting in increased incorporation of 5-FU into RNA in sensitive human tumor xenografts, which was not observed in less sensitive tumor strains.[29] It is possible, therefore, that excess 5-FU may have reduced the significance of TS levels in relation to the sensitivity of tumor cells to 5-FU in our present study.

Another possible factor may be the type of carcinoma used. Etienne *et al.*[6] 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 while DPD activity was significantly related to 5-FU responsiveness, no relationship could be demonstrated between TS activity and response to 5-FU therapy. In addition, different types of gastric carcinoma between Western countries (gastric type) and Japan (intestinal type) may be responsible for the different correlation between sensitivity to 5-FU and TS levels. Recently, Fata *et al.*[30] reported that low TS mRNA levels were an indicator for poor outcome for patients with gastric cancer in the U.S. This result was in contrast with previous reports[13, 14] and our own observations,[31] so the significance of TS and TS mRNA levels for sensitivity to 5-FU and prognosis remains controversial. Further investigation is required into the role of TS in 5-FU sensitivity and the prognosis of patients with advanced carcinoma.

(Received September 10, 1999/Revised October 26, 1999/Accepted October 29, 1999)

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) Shipper, D. L. and Wagener, D. J. Chemotherapy of gastric cancer. *Anticancer Drugs*, **7**, 137–149 (1996).
3) Naguib, F. N. M., el Kouni, M. H. and Cha, S. Enzymes of uracil catabolism in normal and neoplastic human tissues. *Cancer Res.*, **45**, 5405–5412 (1985).
4) 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 (1998).
5) 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 tumor sensitivity to fluorouracil. *Eur. J. Cancer*, **30A**, 1517–1522 (1994).
6) 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).
7) 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).
8) Baker, S. D., Khor, S. P., Adjei, A. A., Doucette, M., Spector, T., Donehower, R. C., Grochow, L. B., Sartorius, S. E., Noe, D. A., Hohnecker, J. A. and Rowinsky, E. K. Pharmacokinetic, oral bioavailability, and safety study of fluorouracil in patients treated with 776C85, an inactivator of dihydropyrimidine dehydrogenase. *J. Clin. Oncol.*, **14**, 3085–3096 (1996).
9) 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).
10) Shirasaka, T., Nakano, K., Takechi, T., Satake, H., Uchida, J., Fujioka, A., Saito, H., Okabe, H., Oyama, K., Takeda, S., Unemi, N. and Fukushima, M. Antitumor activity of 1 M tegafur-0.4 M 5-chloro-2,4-dihydroxypyrimidine-1 M potassium oxonate (S-1) against human colon carcinoma orthotopically implanted into nude rats. *Cancer Res.*, **56**, 2602–2606 (1996).
11) Langenbach, 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).
12) 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).
13) 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).
14) 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).
15) Leichman, C. G., Lenz, H. J., Leichman, L., Danenberg, K. D., 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).
16) Peters, G. J., Laurensse, E., Leyva, A., Lankelma, J. and Pinedo, H. M. Sensitivity of human, murine, and rat cells to 5-fluorouracil and 5′-deoxy-5-fluorouridine in relation to drug-metabolizing enzymes. *Cancer Res.*, **46**, 20–28 (1986).
17) Aschele, C., Sobrero, A., Faderan, M. A. and Bertino, J. R. Novel mechanism(s) of resistance to 5-fluorouracil in human colon cancer (HCT-8) sublines following exposure to two different clinically relevant dose schedule. *Cancer Res.*, **52**, 1855–1864 (1992).
18) Diasio, R. B., Danenberg, K. D., Johnson, M. and Danenberg, P. V. 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).
19) Ishikawa, Y., Kubota, T., Otani, Y., Watanabe, M., Teramoto, T., Kumai, K., Kitajima, M., Takechi, T., Okabe, H. and Fukushima, M. Dihydropyrimidine dehydrogenase activity and messenger RNA level may be related to the antitumor effect of 5-fluorouracil on human tumor xenografts in nude mice. *Clin. Cancer Res.*, **5**, 883–889 (1999).
20) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods*, **65**, 55–63 (1983).
21) Shimoyama, Y., Kubota, T., Watanabe, M., Ishibiki, K. and Abe, O. Predictability of *in vivo* chemosensitivity by *in vitro* MTT assay with reference to the clonogenic assay. *J. Surg. Oncol.*, **41**, 12–18 (1989).
22) 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).
23) 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).
24) 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).
25) Ikenaka, K., Shirasaka, T., Kitano, S. and Fujii, S. Effect of uracil on metabolism of 5-fluorouracil *in vitro*. *Gann*, **70**, 353–359 (1979).
26) 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 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).
27) Johnston, P. G., Drake, J. C., Trepel, J. and Allegra, C. J. Immunological quantitation of thymidylate synthase using the monoclonal antibody TS 106 in 5-fluorouracil-sensitive and -resistant human cancer cell lines. *Cancer Res.*, **52**, 4306–4312 (1992).
28) Van der Wilt, C. L., Pinedo, H. M., Smid, K. and Peters, G. J. Elevation of thymidylate synthase following 5-fluorouracil treatment is prevented by the addition of leucovorin in murine colon tumors. *Cancer Res.*, **52**, 4922–4928 (1992).
29) Inada, T., Ichikawa, A., Kubota, T., Ogata, Y., Moosa, 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).
30) Fata, F., Baylor, L., Lipton, R., Huang, Y., Karper, M., Park, J. M., Danenberg, K. D., Danenberg, P. V. and Kelsen, D. P. Low thymidylate synthase (TS) is not an independent predictor of outcome in patients with operable gastric cancer. Abstract #926. *Proc. ASCO*, **18**, 241 (1999).
31) Ishikawa, Y., Kubota, T., Otani, Y., Watanabe, M., Teramoto, T., Kumai, K., Takechi, T., Okabe, H., Fukushima, M. and Kitajima, M. Thymidylate synthetase and dihydropyrimidine dehydrogenase levels in gastric cancer. *Anticancer Res.*, **19** (1999), in press.