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Quantitative Measurement of Thymidylate Synthase and Dihydropyrimidine Dehydrogenase mRNA Level in Gastric Cancer by Real-time RT-PCR

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We used real-time reverse-transcription polymerase chain reaction (RT-PCR) to assay expression of the mRNA of thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD) in gastric cancer tissue with the objective of establishing a system to measure TS and DPD in ultra-low-volume samples. Nude mouse xenografts of 5 human gastric cancer cell lines and 85 clinical samples were used as the specimens in this study. Sensitivity to 5-fluorouracil (5-FU) was determined on the basis of the relative tumor proliferation rate in mice and the results of ATP assay using serum-free cultures of the clinical samples. mRNA expression was measured in tumor tissue by real-time RT-PCR using the ABI PRISM 7700 system. The values for expression of the mRNA for TS and DPD were corrected according to the level of glyceraldehyde-3-phosphate dehydrogenase mRNA expression. The xenografts yielded correlations between TS and DPD mRNA expression and the activity of the enzymes (TS: r_s=0.700, DPD: r_s=0.900), and an inverse correlation was noted between the mRNA levels and sensitivity to 5-FU (TS: r_s=−0.900, DPD: r_s=−0.800). The clinical samples showed an inverse correlation between 5-FU sensitivity and mRNA expression (TS: r_s=−0.518, DPD: r_s=−0.564). Sensitivity to 5-FU was noted only in cases in which TS mRNA expression and DPD mRNA expression were both low. Real-time RT-PCR can provide a highly sensitive assessment of TS and DPD mRNA expression in gastric cancer, and it was useful for predicting 5-FU sensitivity.

Key words: Real-time RT-PCR — Gastric cancer — Chemosensitivity testing — Thymidylate synthase — Dihydropyrimidine dehydrogenase

Although numerous multidrug regimens have been developed for the treatment of gastric cancer, their efficacy rates have not exceeded approximately 50%.[1,2] Thus, it is important to select patients in whom treatment is likely to be effective in order to improve the results of chemotherapy. *In vitro* anticancer drug sensitivity tests, which we developed with this goal in mind, have already yielded favorable results.[3] However, tests that involve cell culturing procedures are complicated by the need for special facilities, and there can be problems with failures attributable to low cell growth.

Standard regimens for gastric cancer involve some form of concomitant treatment that includes biomodulated 5-fluorouracil (5-FU) chemotherapy.[4,5] The mechanism of action of 5-FU has been explained in terms of incorporation of fluorouridine 5′-triphosphate (FUTP) into RNA, resulting in altered gene expression, or in terms of inhibition of thymidylate synthase (TS) by the active metabolite 5-fluorouridine monophosphate (FdUMP).[6–8] Several reports have indicated that TS expression in the tumors was related to the sensitivity to 5-FU-based chemotherapy both in the laboratory and clinically.[9–12] On the other hand, dihydropyrimidine dehydrogenase (DPD) which is both an initial and a rate-limiting catabolic enzyme of 5-FU has been reported to play an important role in the pharmacokinetics of 5-FU and was correlated with the antitumor effectiveness of 5-FU in cancer cell lines and tumors.[9,13–18]

Recently, both basic and clinical researches have shown a correlation between the antitumor effectiveness of 5-FU and TS or DPD mRNA levels in tumors by using semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR).[11,12,16,19–21] One of the major advantages of these methods is that they can be performed with ultra-low-volume samples as compared with measurement of the enzyme activities and *in vitro* chemosensitivity tests.

A real-time RT-PCR method based on *Taq*Man fluorescence methodology requires fewer steps after PCR than conventional PCR methods, simplifying the procedures

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and making it possible to maintain quantification subsequent to PCR, while allowing a broad dynamic range of measurements.[22] Quantitative measurement of genes may aid in the interpretation of results obtained by conventional techniques. In the treatment of gastrointestinal carcinomas, a correlation has been reported between the quantitative measurement of TS or DPD mRNA and survival after chemotherapy including 5-FU,[23–25] but there have been no reports on correlations between the results of quantitative assay of TS and DPD mRNA expressions and 5-FU based chemotherapy in gastric cancer. The objective of the present study was to clarify the significance of TS and DPD mRNA levels in predicting sensitivity to 5-FU based on ultra-small samples and to investigate the possible utility of this system by examining its relation to *in vitro* anticancer drug sensitivity tests.

MATERIALS AND METHODS

Animals Male BALB/cA nude mice (age 4 weeks; weight 18–20 g) were purchased from CLEA Japan, Co., Ltd. (Tokyo). The animals were housed under specific-pathogen-free conditions with laminar flow racks.

Drugs 5-FU was obtained from Kyowa Hakko Kogyo Co., Ltd. (Tokyo). [6-^{3}H]FdUMP (16.9 Ci/mmol) was obtained from Moravek Biochemicals, Inc. (Bera, CA). [6-^{14}C]5-FU (56 mCi/mmol) was purchased from American Radiolabeled Chemicals Co. (St. Louis, MO), and nicotinamide adenine dinucleotide phosphate (NADPH) was obtained from Sigma Chemical Co. (St. Louis, MO).

Tumors Five human gastric cancer cell lines (GCIY, GT3TKB, MKN-1, MKN-28, MKN-74) were obtained from Riken Cell Bank (The Institute of Physical and Chemical Research, Saitama). These lines (1×10^6 cells) were subcutaneously transplanted into the dorsal flank of each mouse, and the mice were sacrificed when the tumor volume reached 100 mm^3. The tumors were harvested for further experiments. Tumor pieces were stored at −80°C pending measurement of TS and DPD levels.

A total of 85 fresh human gastric cancer tissue samples were obtained from tissue surgically resected from patients undergoing gastrectomy in our department between June 1998 and January 2002. Patient characteristics, determined according to the Japanese Classification of Gastric Carcinomas, are shown in Table I.[26] Specimens of approximately 10 mm^3 were immediately stored at −80°C pending real-time RT-PCR. When a relatively large volume of tissue samples had been obtained, they were processed for *in vitro* chemosensitivity testing.

All patients gave written informed consent to participate in the study. Experiments were carried out after approval by the local ethics committee.

Antitumor activity of 5-FU in human gastric cancer xenografts Tumor tissue fragments were inoculated with a trocar needle into the dorsal flank of each nude mouse. When tumor volume [(major axis)×(minor axis)^2×1/2] reached approximately 100 mm^3, the tumor-bearing mice were randomly allocated to groups of five animals each, and 5-FU was administered intraperitoneally at a dose of 50 mg/kg, once every 4 days, repeated three times. The control group was injected with 0.9% NaCl solution. Tumor volume was measured every 2 days after drug administration. Relative tumor volume (RTV) was calculated as follows: (mean tumor volume during treatment)/(mean tumor volume at the start of treatment). Antitumor effect (inhibition rate; IR%) was calculated as follows: IR%=(1−mean RTV of treatment group/mean RTV of untreated group)×100.

All animal experiments were carried out in accordance with the Guidelines for the Welfare of Animals in Experimental Neoplasia.[27]

***In vitro* drug sensitivity testing of clinical samples of gastric cancer** The sensitivity to 5-FU of clinical samples was determined by ATP assay using a serum-free culture developed in our department.[3] Briefly, tumor samples were enzymatically digested to obtain single-cell suspensions. A 20 μl volume of 5-FU at a final concentration of 50 μg/ml was added to cells, at a concentration of 2×10^4/180 μl, and the cultures were incubated in 5% CO_2 at 37°C for 72 h. Cell viability was determined by measuring intracellular ATP content by the bioluminescence method. Sensitivity to 5-FU (IR%) was calculated as follows: IR%=(1−average ATP level in treatment group/average

Table I. Patients' Characteristics[a]

Gender	male	56	Histological type[b]	diff.	50
	female	29		undiff.	45
Depth of invasion	T1	27	Stage	Ia	14
	T2	24		Ib	8
	T3	31		II	15
	T4	13		IIIa	11
				IIIb	9
Lymph node metastasis	N0	27		IV	28
	N1	22			
	N2	28			
	N3	8			
Peritoneal metastasis	P (–)	69			
	P (+)	16			
Hepatic metastasis	H (–)	77			
	H (+)	8			

a) Clinicopathologic factors were determined according to Japanese Classification of Gastric Carcinima.[26]
b) dif., differentiated type; undif., undifferentiated type.

ATP level in untreated control group)×100. When IR% was more than 50%, the sample was judged to be sensitive to 5-FU according to our criteria.[3)]

TS binding assay Measurement of TS binding activity to FdUMP was based on the method of Ishikawa *et al.*[21, 28)] with minor modifications.[18)] Enzyme solution was obtained by homogenizing tumor tissues in 3 volumes of 0.2 *M* Tris-HCl (pH 8.0) containing 20 m*M* 2-mercaptoethanol and centrifuging at 10 500*g* for 60 min at 4°C. The supernatant obtained was incubated at 25°C for 3 h with 0.6 *M* NH_4HCO_3 (pH 8.0), 0.1 *M* 2-mercaptoethanol, 0.1 *M* NaF, and 15 m*M* 5-CMP. The solution was incubated at 30°C for 20 min with [6-^{3}H]FdUMP, 2 m*M* tetrahydrofolate, 16 m*M* ascorbate, 9 m*M* formaldehyde, 15 m*M* 5-CMP, 20 m*M* 2-mercaptoethanol, and 100 m*M* NaF and centrifuged at 2000*g* for 5 min. The supernatant was removed, and trichloroacetic acid was added to the pellet. The tritiated water formed during incubation was then assayed with a liquid scintillation counter. The samples to measure total TS content were prepared by fully dissociating the ternary complex present in the cytosol to unbound TS at pH 8.0 in the preincubation period. To determine the [6-^{3}H]FdUMP binding sites as a measure of TS content, protein concentration was determined using a protein assay kit (Bio-Rad, Richmond, CA). TS content was expressed as pmol/mg of protein.

DPD activity Measurement of DPD activity was based on the method of Takechi *et al.*[29)] with minor modifications.[18)] Briefly, enzyme solution was obtained from tumor tissue by homogenization in 3 volumes of 0.2 *M* potassium phosphate (pH 8.0) containing 1 m*M* 2-mercaptoethanol and 1 m*M* EDTA. The solution was then centrifuged at 10 500*g* for 60 min at 4°C, and the supernatant was collected. Enzyme solution (25 μl) was mixed with an equal volume of reaction mixture (2 m*M* dithiothreitol (DTT), 5 m*M* $MgCl_2$, 20 μM [6-^{14}C]5-FU (56 Ci), 100 μM NADPH) and incubated at 37°C for 10 min or 30 min. After centrifugation, a 5-μl aliquot of the supernatant was applied to a thin-layer chromatography plate (silica gel 60 F254; Merck, Darmstadt, Germany). Plates were developed with a mixture of 99% ethanol and 1 *M* ammonium acetate (5:1, v/v). The densities of 5-FU and degradation products were calculated using an image analyzer (Bio-Rad). DPD activity is expressed as pmol/mg of protein/min.

Real-time RT-PCR Total RNA was isolated by disruption of frozen sections in 1 ml of "TRIZOL" (GIBCO/BRL, Cleveland, OH) according to a guanidinium isothiocyanate-phenol-chloroform-based method[30)] and was purified according to the RNA clean-up protocol. The amount of total RNA was measured spectrophotometrically in terms of absorbance of aliquots at 260 nm.

A 1-μg sample of total RNA was incubated with 1 μl of "random hexamer" (Perkin Elmer Cetus, Branchburg, NJ) in 9 μl of solution for 10 min at 70°C and chilled at 4°C. Next, reverse transcription of total RNA was performed in a total volume of 20 μl containing 4 μl of "5× RT buffer" (GIBCO/BRL), 2 μl of 100 m*M* DTT, 4 μl of 2.5 m*M* dNTP, and 1 μl of "Superscript II RNase H reverse transcriptase" (GIBCO/BRL). The samples were incubated at 37°C for 10 min and 90°C for 60 min, and the cDNA synthesized was stored at −20°C until used.

All PCRs were performed by quantitative real-time methods using the "ABI PRISM 7700 Sequence Detection System" (Applied Biosystems, Foster City, CA). PCR was performed in a 50-μl reaction mixture containing 25 μl of "*Taq*Man Universal PCR Master Mix" (Perkin Elmer Biosystems, Foster City, CA), 200 n*M* of each primer, 5 n*M* probe, 5 μl of each cDNA sample or appropriately diluted cDNA plasmid sample, and ddH_2O in 0.2 ml MicroAmp optical tubes (Perkin Elmer Cetus). Thermal cycling con-

Table II. Sequences of Oligonucleotide Primers and Sequence-specific Probe Sequences for TS, DPD, and GAPDH

Gene and oligonucleotide	Sequence	Corresponding cDNA sequence
TS		
Forward primer	5′-GAA TCA CAT CGA GCC ACT GAA A-3′	882–903
Reverse primer	5′-CAG CCC AAC CCC TAA AGA CTG-3′	1099–1078
Probe	5′-(FAM) TTC AGC TTC AGC CAG AAC CCA GA (TAMRA)-3′	905–927
DPD		
Forward primer	5′-AAT GAT TCG AAG AGC TTT TGA AGC-3′	1755–1778
Reverse primer	5′-GTT CCC CGG ATG ATT CTG-3′	1862–1844
Probe	5′-(FAM) TGC CCT CAC CAA AAC TTT CTC TCT TGA TAA GGA (TAMRA)-3′	1791–1823
GAPDH		
Forward primer	5′-GAA GGT GAA GGT CGG AGT C-3′	66–84
Reverse primer	5′-GAA GAT GGT GAT GGG ATT TC-3′	291–272
Probe	5′-(TET) CAA GCT TCC CGT TCT CAG CC (TAMRA)-3′	262–243

ditions comprised an initial denaturation step at 95°C for 10 min and 50 cycles at 95°C for 15 s and 60°C for 1 min. To control for variation in the number of cells, mRNA was quantified in relation to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Primers and probes for TS, DPD, and GAPDH were designed using "Primer Express" (Perkin Elmer Cetus) (Table II). A standard curve was constructed with eight points representing 10-fold serial dilutions of each cDNA plasmid. The value of each point was equivalent to the value of each cDNA plasmid PCR product from 50 copies to 5×10^8 copies. The TS, DPD, and GAPDH cDNA plasmids were provided by Taiho Pharmaceutical Co., Ltd. (Saitama). The standard curves of TS, DPD and GAPDH are shown in Fig. 1. Relationships between TS mRNA and between DPD mRNA levels are expressed as ratios of TS to GAPDH RT-PCR products and of DPD to GAPDH RT-PCR products, respectively.

Statistical analysis Statistical analysis was performed using "StatView V. 5.0 software" (SAS Institute, Inc., Cary, NC). The Spearman rank correlation coefficient was calculated to evaluate the correlation between the two variables. Statistical differences were evaluated between 2 groups using the Mann-Whitney test, and for 3 or more groups using the Kruskal-Wallis test. A *P* value less than 0.05 was considered to be statistically significant.

RESULTS

TS and DPD levels, sensitivity to 5-FU in human gastric cancer xenografts Table III shows the sensitivity to 5-FU, and the TS and DPD levels of five human gastric cancer xenografts. The maximum IR% value was adopted as the antitumor effect value. MKN-74 showed the highest sensitivity to 5-FU, and GT-3TKB showed the lowest sensitivity.

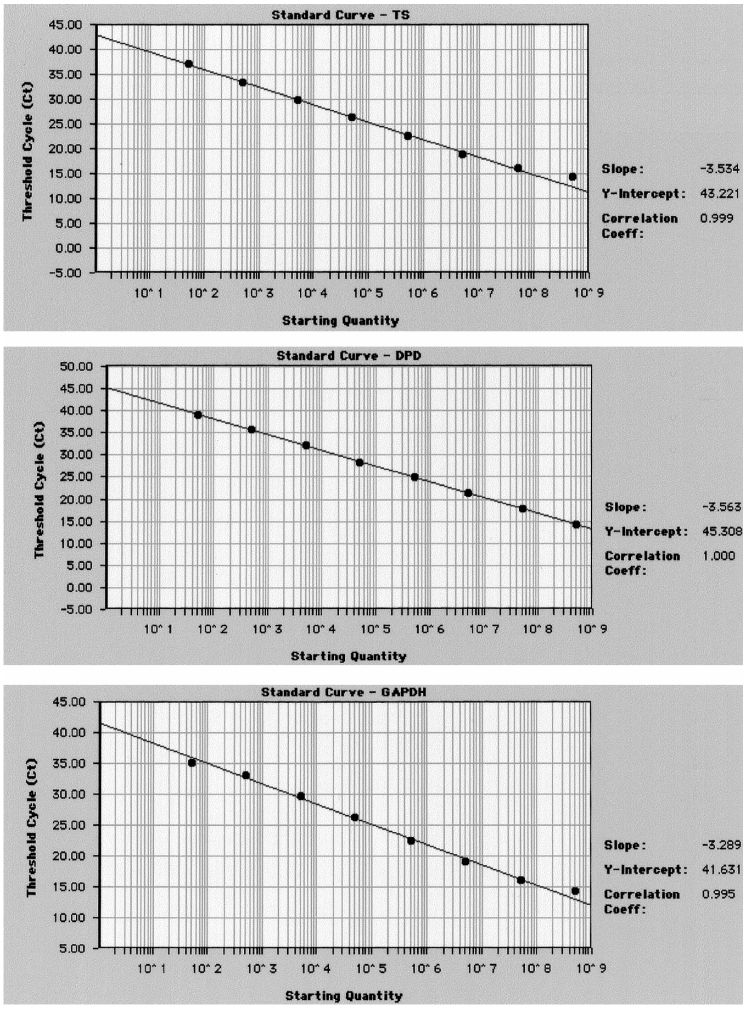

Fig. 1. Linear relationship between threshold and logarithm of the starting copy number of TS (top), DPD (middle) and GAPDH (bottom) cDNA plasmid. TS, $y=-3.534x+43.221$ ($r=0.999$); DPD, $y=-3.563x+45.308$ ($r=1.000$); GAPDH, $y=-3.289x+41.631$ ($r=0.955$).

Table III. TS and DPD Levels and Sensitivity to 5-FU of Human Gastric Cancer Xenografts

	MKN-1	MKN-28	MKN-74	GCIY	GT3TKB
IR%[a)]	55.4	63.8	71.9	34.2	15.2
TS content[b)] (pmol/mg protein)	0.160±0.056	0.258±0.067	0.094±0.044	0.311±0.141	2.621±2.241
TS mRNA level[b, c)]	0.00567 ±0.03405	0.00200 ±0.00029	0.00123 ±0.00285	0.00564 ±0.00577	0.02904 ±0.02310
DPD activity[b)] (pmol/mg protein/min)	9.6±7.3	20.3±10.3	6.0±4.6	277.8±70.2	287.8±14.3
DPD mRNA level[b, d)]	0.00533 0.00850	0.01332 0.01278	0.00018 0.00009	0.10504 0.09079	0.07155 0.04313

a) IR% was calculated as follows: (1−average ATP level in treatment group/average ATP level in untreated control group)×100.
b) Values represent means and standard deviation of results from five tumors.
c) TS mRNA levels are expressed as ratios of TS to GAPDH RT-PCR products.
d) DPD mRNA levels are expressed as a ratio of DPD to GAPDH RT-PCR products.

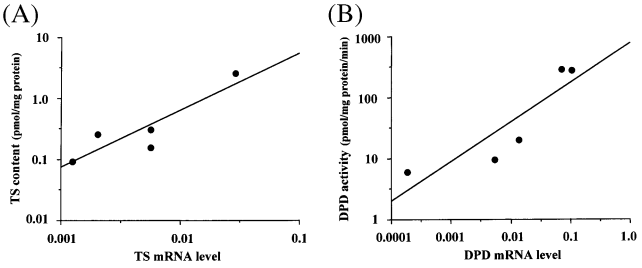

Fig. 2. Correlation between mRNA levels and TS content (A) and DPD activity (B) in five human gastric cancer xenografts. The tumor samples showed a correlation in both cases. A, r_s=0.700, P=0.1615; B, r_s=0.900, P=0.0719.

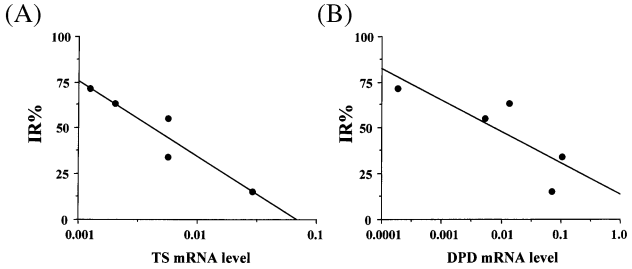

Fig. 3. Correlation between TS (A) and DPD (B) mRNA levels and sensitivity to 5-FU of five human gastric cancer xenografts. The data show an inverse correlation in both cases. A, r_s=−0.900, P=0.0719; B, r_s=−0.800, P=0.1096.

TS content and DPD activity were detectable in all of the gastric cancer xenografts, and corresponding mRNA levels of both TS and DPD were also detected. MKN-74 showed the lowest values for TS and DPD mRNA levels; GT3TKB showed the highest TS mRNA level; and GCIY showed the highest DPD mRNA level.

Correlations between TS content and DPD activity and the mRNA levels are shown in Fig. 2. A correlation was found between TS content and TS mRNA level (correlation coefficient 0.700; Fig. 2A), and between DPD activity and DPD mRNA level (correlation coefficient 0.900; Fig. 2B).

Correlations between sensitivity to 5-FU and mRNA levels of TS and DPD are shown in Fig. 3. Sensitivity to 5-FU was inversely correlated with both TS and DPD mRNA levels (correlation coefficients: −0.900 and −0.800, respectively; Fig. 3, A and B).

Sensitivity to 5-FU and mRNA levels of TS and DPD in clinical samples In the 85 surgical samples, the TS mRNA level ranged from 0.001 to 3.500, with a mean of 0.226±0.496 and a median of 0.064, while the DPD mRNA expression levels ranged from 0.001 to 8.519, with a mean of 0.803±1.726 and a median of 0.134. Several clinicopathological factors were compared with the mRNA levels of both TS and DPD. There was no correlation between the clinicopathological factors and these mRNA levels (Table IV).

Of the 85 samples, *in vitro* ATP assay was applied to 71 (83.5%) samples, and 58 (81.6%) of the 71 samples were considered to be available for evaluation of drug response.

Table IV. Comparison of Clinicopathologic Factors and mRNA Levels of TS and DPD

Clinicopathologic factors[a]		TS mRNA level[b, c]	Statistics[d]	DPD mRNA level[b, e]	Statistics[d]
Gender	M	0.212±0.500	P=0.2008	0.868±1.870	P=0.9852
	F	0.274±0.541		0.644±1.573	
Depth of invasion	T1	0.310±0.836	P=0.7421	0.648±1.185	P=0.2695
	T2	0.164±0.233		1.098±2.135	
	T3	0.269±0.525		0.917±2.050	
	T4	0.172±0.286		0.116±0.139	
Lymph node metastasis	N0	0.328±0.818	P=0.7275	0.839±1.421	P=0.3799
	N1	0.169±0.250		0.323±0.507	
	N2	0.221±0.320		0.971±2.279	
	N3	0.128±0.107		1.292±2.846	
Peritoneal metastasis	P (–)	0.360±0.687	P=0.7873	0.784±2.025	P=0.9194
	P (+)	0.203±0.463		0.793±1.719	
Hepatic metastasis	H (–)	0.109±0.088	P=0.9640	1.314±2.836	P=0.5422
	H (+)	0.246±0.53		0.737±1.638	
Stage	Ia	0.337±0.992	P=0.6939	0.665±1.365	P=0.7613
	Ib	0.153±0.225		1.176±2.003	
	II	0.096±1.01		0.432±0.608	
	IIIa	0.243±0.334		0.688±1.356	
	IIIb	0.268±0.399		1.227±2.778	
	IV	0.261±0.529		0.839±2.124	
Histological type[f]	dif.	0.310±0.665	P=0.4832	0.770±1.823	P=0.7744
	undif.	0.142±0.200		0.817±1.724	

a) Clinicopathologic factors are determined according to Japanese Classification of Gastric Carcinoma.[26]
b) Values represent means and standard deviation of results from five tumors.
c) TS mRNA levels are expressed as ratios of TS to GAPDH RT-PCR products.
d) Mann-Whitney test between the two groups; Kruskal-Wallis test among more than three groups.
e) DPD mRNA levels are expressed as a ratio of DPD to GAPDH RT-PCR products.
f) dif., differentiated type; undif., undifferentiated type.

The correlations between mRNA level and sensitivity to 5-FU were calculated (Fig. 4). Tumors with high TS or DPD mRNA level tended to show relatively high resistance to 5-FU, and there were significant inverse correlations between sensitivity to 5-FU and TS and DPD mRNA levels (correlation coefficient −0.481 and −0.458, respectively; Fig. 4, A and B).

According to our criteria, 28 samples (48.2%) were judged as sensitive to 5-FU.[3] Correlations between sensitivity to 5-FU and distributions of TS and DPD mRNA levels indicated that all tumors sensitive to 5-FU showed both TS and DPD mRNA levels in the low range, while most 5-FU-resistant tumors showed high levels of either TS or DPD mRNA (Fig. 5). When the cut-off level was set at the mean value for each mRNA level, 5-FU sensitivity could be predicted from the mRNA levels with a sensitivity of 100% (28/28), a specificity of 73.3% (22/30), and an overall predictive accuracy of 86.2% (50/58).

DISCUSSION

We performed quantitative assay of mRNA expression for tumor TS and DPD to assess the feasibility of using their levels to predict the therapeutic effectiveness of 5-FU in gastric cancer. The results of this study in nude mouse xenografts of human gastric cancer cell lines showed close correlations between TS mRNA level and TS content, and between DPD mRNA levels and DPD activity. Ishikawa and colleagues[16] used the semiquantitative RT-PCR method and also reported good correlations between enzyme activity and TS and DPD mRNA level, and our results support their findings. The TS content and DPD activity within the tumor were well predicted by the respective mRNA levels, indicating the feasibility of using mRNA measurements to predict the activity of the corresponding enzyme protein. The mRNA level was also correlated closely with sensitivity to 5-FU. Although these

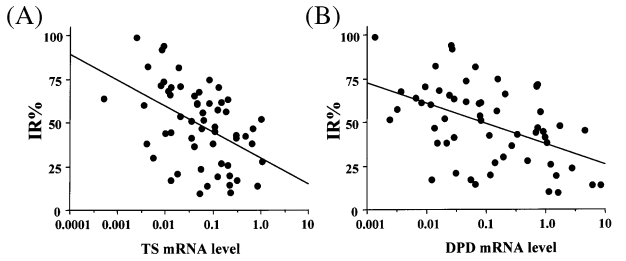

Fig. 4. Correlation between TS (A) and DPD (B) mRNA levels and the sensitivity to 5-FU of 58 clinical samples. There was a significant inverse correlation in each case. A, $r_s=-0.481$, $P=0.0003$; B, $r_s=-0.458$, $P=0.0005$.

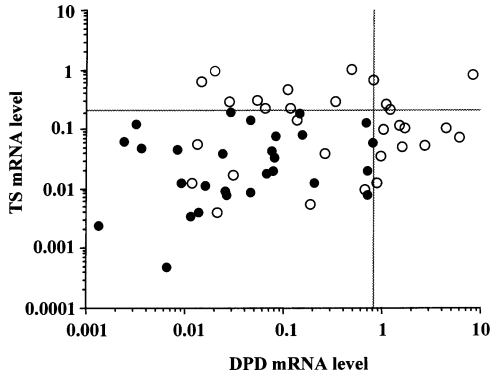

Fig. 5. Correlation between 5-FU sensitivity and TS and DPD mRNA levels in 58 clinical samples. All of the sensitive tumors showed low levels of mRNA for both TS and DPD. Resistant tumors showed high mRNA levels for either TS or DPD. ● sensitive to 5-FU, ○ resistant to 5-FU.

experiments were performed in human gastric cancer xenografts, the results suggested that sensitivity to 5-FU in humans might also be predictable on the basis of TS and DPD mRNA level. This basic research showed the importance of measurements of both targeting and degradative enzymes to predict sensitivity to 5-FU, and these results led us to investigate the correlation between TS and DPD mRNA levels and sensitivity to 5-FU in clinical samples.

Real-time RT-PCR provides a wide dynamic range for measurement of TS and DPD mRNA expression in comparison with semiquantitaitve RT-PCR measurement.[28] These findings indicate that real-time RT-PCR provides greater measurement sensitivity than conventional methods and confirms that quantitative measurement can be performed by this method across a broad activity range.

Our assessment of the relation between the sensitivity to 5-FU and mRNA levels of both enzymes in the clinical samples revealed a significant inverse correlation between both the TS and DPD mRNA levels and sensitivity to 5-FU, although the correlation coefficients were lower for the clinical samples than in our basic research.

Several investigators have reported that high intratumoral DPD level is associated with low antitumor activity of 5-FU due to increased 5-FU inactivation.[9, 15–17, 20] The role of TS in sensitivity to 5-FU, however, is still controversial. TS protein or gene expression has been reported to be strongly associated with response to 5-FU treatment and patient survival after chemotherapy,[11, 12, 20, 31, 32] but the correlation between sensitivity to 5-FU and TS activity has been reported to be relatively poor in panels of human tumor cell lines,[9, 10] and the absence of any correlation between TS activity and sensitivity to 5-FU has been documented in several recent reports.[16, 18, 33] The inconsistent results concerning the association between TS expression and response to 5-FU may in part be attributable to the different measurement procedures. The role of TS in sensitivity to 5-FU may be more accurately demonstrated by real-time RT-PCR than by enzyme activity, because it provides a very broad dynamic range. Our results on clinical samples support the idea that TS mRNA expression levels are associated with response to 5-FU treatment and patient survival after 5-FU-based chemotherapy.

We also undertook a study of the correlations between sensitivity to 5-FU and TS and DPD mRNA levels in order to investigate the feasibility of predicting sensitivity to 5-FU on the basis of combined mRNA expression. Only tumors showing low mRNA level for both TS and DPD were sensitive to 5-FU. Almost all tumors with high levels of at least one of these forms of mRNA showed resistance to 5-FU. We investigated sensitivity to 5-FU in this study by using the mean value as our cut-off level for each form of mRNA level, and the results showed a high overall predictive accuracy of approximately 86%. Based on these results, we concluded that sensitivity could be assessed more accurately on the basis of a combination of TS and DPD mRNA levels than on the basis of TS or DPD alone.

In conclusion, we found inverse correlations between sensitivity to 5-FU and TS and DPD mRNA levels, and concluded that quantitative measurement of both could be used to predict the therapeutic effectiveness of 5-FU in gastric cancer. We also found that real-time RT-PCR allows highly sensitive assessment of TS and DPD mRNA levels, quickly and easily, in ultra-low-volume samples, and it could prove extremely useful in a clinical setting. Even in cancers judged to be resistant to 5-FU, gene expression can provide clues to the mechanism of drug resistance, and this may be useful in applications such as the utilization of DPD inhibitors to control high levels of DPD expression.[14] We believe that this approach will find broad application in the selection of chemotherapy agents for the treatment of non-resectable cancers and for preoperative cytoreduction.

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