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Thymidylate Synthetase (TS) Genotype and TS/dihydropyrimidine Dehydrogenase mRNA Level as an Indicator in Determining Chemosensitivity to 5-Fluorouracil in Advanced Gastric Carcinoma

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Abstract. *Background: One of the target enzymes of 5-fluorouracil (5-FU) is thymidylate synthetase (TS). The DNA sequence of the TS gene includes either double or triple tandem 28-bp repeats within the promoter region, such that TS genotypes can be classified as homozygous 3R/3R, heterozygous 2R/3R or homozygous 2R/2R. Several recent studies have shown that TS genotype affects mRNA expression, with 3R/3R homozygotes showing higher TS mRNA expression compared to the other genotypes. Purpose: We analyzed the TS genotype and TS and dihydropyrimidine dehydrogenase (DPD) mRNA expression levels in 22 advanced gastric carcinoma patients, and analyzed results with respect to patient 5-FU chemosensitivity, as detected by the tetrazolium-based colorimetric (MTT) assay and survival outcome. Patients and Methods: Between September 2001 and April 2002, 22 Japanese patients with advanced gastric carcinoma were evaluated. Informed written consent was obtained from all patients and the study was approved by the ethical committee at our University Hospital. Fresh surgical specimens from carcinoma lesions were enzymatically dissociated and incubated with 5-FU at a concentration of 50 μg/ml for 48 hours to determine the inhibition rate as detected by MTT assay. Normal and tumor tissue and peripheral blood samples were collected and stored at -80˚C until assay for TS genotype and TS and DPD mRNA level. The TS genotype was assessed by PCR assay using peripheral monocytes, since monocyte genotypes represent the genotype of normal and tumor tissues. Quantification of TS and DPD mRNA levels was performed using real-time PCR. Survival outcome was assessed according to the disease-free survival period in cases with similar clinical backgrounds. Results: TS genotyping revealed 19 3R/3R homozygotes and 3 2R/3R heterozygotes. After analysis of normal and tumor tissues, samples from homozygote 3R/3R cases showed higher TS mRNA expression than heterozygote 2R/3R cases, which was statistically significant at $p<0.05$. We also observed a statistically significant correlation in TS mRNA levels between normal and tumor tissues, while no significant correlation was observed for DPD mRNA levels between normal and tumor tissues. While no relationship between 5-FU chemosensitivity and TS genotype or mRNA expression was observed, cases with high DPD mRNA expression were resistant to 5-FU and exhibited poor survival outcomes. Conclusion: While TS genotype affected TS mRNA expression in both normal and tumor tissues in advanced gastric cancer, there is no relationship between TS genotype or mRNA expression level and 5-FU chemosensitivity. Conclusion: Our finding, that DPD mRNA expression appears to be a factor in determining 5-FU chemosensitivity and the survival outcome of advanced gastric cancer patients, is comparable with previous reports.*

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Key Words: 5-Fluorouracil, chemosensitivity, thymidylate synthetase, dihydropyrimidine dehydrogenase, tandem repeat, gastric cancer.

Gastric cancer remains a major cause of cancer death in developed countries, despite the declining trends in morbidity and mortality. In Japan, the mortality of gastric cancer is 17.9% of all cancer types in 1998, with almost half of the gastric cancer patients in Japan expected to die of their cancer (1). The similar rates of decline in morbidity and mortality suggest that the present therapeutic strategies are not enough to reduce gastric cancer mortality in far advanced cases.

5-Fluorouracil (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. However, as the efficacy rate of 5-FU treatment alone in gastric cancer is only 10-20% (2), the clinical effects of 5-FU-based regimens are still unsatisfactory with regard to long-term survival. Recently, S-1 was introduced as a novel oral anticancer drug.

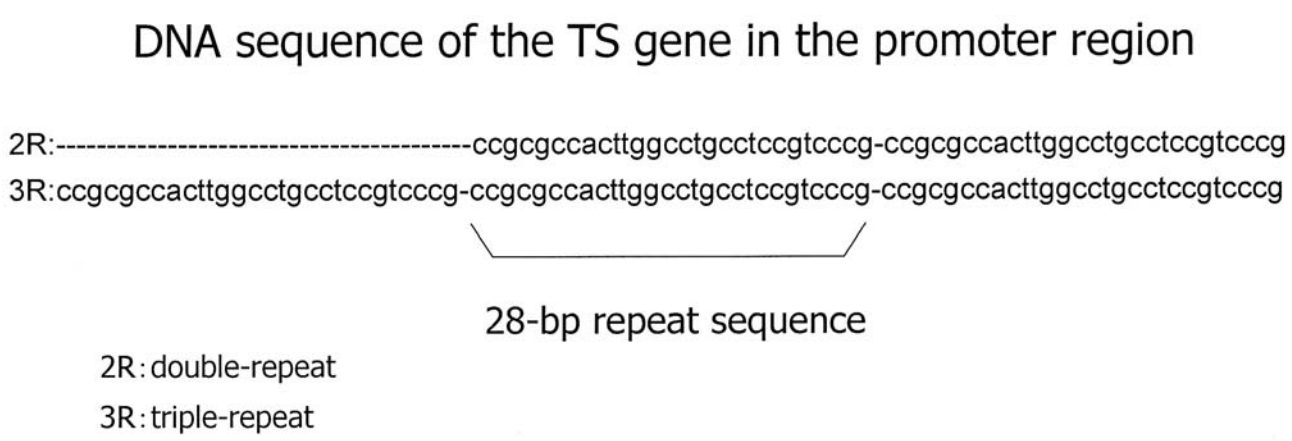

Figure 1. *DNA sequence of the thymidylate synthetase (TS) gene, including 28-bp double or triple tandem repeats in the promoter region. TS genotypes are classified as homozygous 3R/3R, heterozygous 2R/3R or homozygous 2R/2R.*

It is composed of tegafur (FT), gimestat (CDHP) and otastat potassium (Oxo) in a molar ratio of 1:0.4:1, and is based on the biochemical modulation of 5-FU. A late-phase II clinical trial of S-1 against advanced gastric cancer showed a response rate of 49% (25/51), which suggested that this drug was effective and well-tolerated in patients with advanced gastric cancer (3). In addition, a dose-escalation study of cisplatin (CDDP) combined with S-1, performed on advanced gastric cancer patients, showed a response rate of 74% (14/19) and a median survival of 383 days, which suggested that this regimen was active against advanced gastric cancer (4).

As a result, 5-FU, as an active metabolite of S-1, is considered to be a key drug in the control of advanced gastric cancer. One of the main modes of action of 5-FU is thought to be through the disruption of DNA synthesis through its active metabolite fluorodeoxyuridine monophosphate (FdUMP). FdUMP suppresses thymidylate synthetase (TS; EC 2.1.1.45) by forming covalent ternary complexes with 5,10-methylenetetrahydrofolate, which then inhibits DNA synthesis (5). Several reports have indicated that the level of tumoral TS expression is related to the response to 5-FU-based chemotherapy and patient survival in gastric and colorectal cancers (6-9), although increased TS expression is not always recognized as a determining factor for 5-FU resistance (10,11). The TS gene DNA sequence includes a 28-bp sequence that can occur as a double or triple tandem repeat in the promoter, as shown in Figure 1. Based on this tandem repeat, TS genotypes can be classified as homozygous 3R/3R, heterozygous 2R/3R, or homozygous 2R/2R (12). Several studies have recently reported that the TS genotypes influence TS mRNA expression, such that TS mRNA expression tends to be higher in 3R/3R homozygotes compared to the other genotypes (13-17).

In the present study, we analyzed the TS genotype of peripheral mononuclear cells and the TS and dihydropyrimidine dehydrogenase (DPD) mRNA expression levels in normal gastric mucosa and cancerous tissues from advanced gastric carcinoma patients. These results were then analyzed with respect to chemosensitivity, as detected by the tetrazolium-based colorimetric (MTT) assay and the survival outcome of patients receiving S-1 as an adjuvant treatment.

Patients and Methods

Drugs. 5-Fluorouracil (5-FU) was purchased from Kyowa Hakko Kogyo, Co., Ltd., Tokyo, Japan. All other chemicals used were of the highest standard grade commercially available.

Patients. Twenty-two Japanese patients with advanced gastric cancer were evaluated between September 2001 and April 2002. Written informed consent was obtained from all study participants and the study was approved by the Ethical Committee at our Hospital (#12–13). The patient group comprised 17 males and 5 females, ranging in age from 45 to 82 years old, with a mean age of 64 years. Following removal of the surgical specimens, samples were stores in Hanks' balanced salt solution (GIBCO, Gaithersburg, MD, USA) containing 100 IU/ml penicillin (GIBCO), 100 μg/ml streptomycin (GIBCO) and 0.25 mg/ml amphotericin B (GIBCO), and sent to our laboratory for MTT assay and gene analysis. MTT assay was conducted on the same day as the surgery, while samples for gene analysis were stored at -80˚C until use. After surgery, the patients were followed-up every two weeks and recurrence determined by physical findings, tumor markers, ultra-sonography and computed tomography. Survival outcome was assessed as the recurrence-free survival period from surgery to the day when recurrence was observed in patients with similar background factors.

Evaluation of antitumor activity. The *in vitro* chemosensitivity of fresh surgical gastric cancer specimens to 5-FU was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma Chemical Co., St. Louis, MO, USA) assay as described by Mosmann (18), with some modifications (19-22).

Single-cell suspensions were prepared from the specimens enzymatically by incubation with 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) for 30 minutes. After

Table I. *Primers/probes for TS, DPD and GAPDH.*

Gene (GenBank Accession No.)	Primer/probe	Sequence	Corresponding cDNA sequence
TS (X02308)	Forward primer	GAATCACATCGAGCCACTGAAA	882-1099
	Reverse primer	CAGCCCAACCCCTAAAGACTGA	
	Probe	TTCAGCTTCAGCGAGAACCCAGA	
DPD (U09178)	Forward primer	AATGATTCGAAGAGCTTTTGAAGC	1755-1862
	Reverse primer	GTTCCCCGGATGATTCTGG	
	Probe	TGCCCTCACCAAAACTTTCTCTCTTGATAAGGA	
GAPDH (M33197)	Forward primer	GAAGGTGAAGGTCGGAGTC	66-291
	Reverse primer	GAAGATGGTGATGGGATTTC	
	Probe	CAAGCTTCCCGTTCTCAGCC	

two centrifugations, tumor cells were suspended in RPMI-1640 medium (GIBCO) supplemented with 10% fetal bovine serum (FBS; CSL Limited, Australia) and then diluted to 1 x 10^5 cells/ml. Aliquots (100 μl) were plated into 96-well microplates (GIBCO) to give approximately 10^4 cells per well. Drug solutions were dissolved in RPMI-1640 and 100 μl aliquots added to each well to give a final concentration of 50 μg/ml 5-FU, as previously reported (19-22). Control wells contained 100 μl cell suspension and 100 μl RPMI-1640 with 10% FBS, while 200 μl RPMI with 10% FBS was used as a blank. Plates were incubated for 48 hours at 37˚C in a humidified atmosphere containing 95% air and 5% CO_2. A mixture of 0.4% MTT (Sigma) and 0.1 M sodium succinate (Wako Pure Chemical Ind., Ltd., Osaka, Japan) dissolved in 10 ml phosphate-buffered saline and filtered through a 0.45-mm membrane filter (Millipore, Bedford, MA, USA) was then added, and the plates incubated for an additional 3 hours at 37˚C. After the final incubation, 150 ml dimethyl sulfoxide (Nacalai Tesuque, Kyoto, Japan) was added to each well to dissolve the MTT-formazan salt, and the plates shaken mechanically for 10 minutes on a mixer (Model 250, Sonifier, Branson, MO, USA). Optical densities for each well were determined on a model Immuno Reader (Nalgen Nunc International, Rochester, NY, USA) at 540 and 630 nm. Inhibition rates (I.R.; %) were calculated using the following formula: (1 – A/B) x 100 (percentage), where A and B represent the mean absorbance of the treated and control wells, respectively.

RNA extraction and cDNA synthesis. RNA was extracted from frozen fixed carcinoma and normal tissues using the acid guanidinium-phenol-chloroform (AGPC) method. Quantification of TS and DPD mRNA was performed using real-time RT-PCR.

Reverse transcription of up to 10 μg total RNA was carried out in a total volume of 100 μl containing 250 pmol oligo $(dT)_{18}$, 80 U RNasin ribonuclease inhibitor (Promega, Madison, WI, USA) and 500 U Moloney murine leukemia virus reverse transcriptase (GIBCO BRL) in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT and 0.5 mM dNTPs. The total RNA solution was mixed with oligo (dT)18 and heated initially at 70˚C for 10 minutes, immediately chilled on ice and then the other reagents added. First-strand cDNA samples were obtained after 15 minutes at 30˚C and 60 minutes at 42˚C.

PCA procedure. Genomic DNA was extracted from peripheral monocytes using SMI TEST R&D (Genome Science Laboratories Co. Ltd., Tokyo, Japan). Sequences of primers used were TS12 5'-GTGGCTCCTGCGTTTCCCCC-3'(sense) and TS18 5'-TCCGAGCCGGCCACAGGCAT-3'(antisense). PCR was carried out as in 20 μl 2xGC buffer, 8 μl dNTP mixture, 100 μM each primer, 10 μl genomic DNA and 0.4 ml TaKaRa LA Taq in a total reaction volume of 40 μl. PCR was carried out for 37 cycles of 40 seconds at 94˚C, 1 minute at 62˚C and 40 seconds at 72˚C with a 5-minute elongation step at 72˚C after the completion of the last cycle. PCR products were analyzed by electrophoresis on 4% agarose gels. Two products of 212 bp (2R) and 240 bp (3R) were observed. Patients homozygous for the triple repeat (3R/3R) displayed only the larger fragment, while heterozygous 2R/3R patients showed both the larger and smaller fragments.

Relative quantification of gene expression. Quantification was performed using the standard curve method. Primers/probes for TS, DPD and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are shown in the Table I. Standard curves were created automatically by the ABI PRISM 7700 Detection System by plotting the threshold cycle (C_T) against each input amount (16, 4, 1, 0.25, 0.063, 0.016, or 0.0039 ng) of control total RNA (total starting RNA), prepared from MDA-MB-231 human breast tumor cells (American Type Culture Collection, Manassas, VA, USA). The coefficient of linear regression (r) for each standard curve was more than 0.990. For each unknown sample, the relative amount was calculated using linear regression analysis from the respective standard curve. A relative target gene expression value was obtained by division of the target gene value by the GAPDH value as an internal reference gene.

Table II. *TS, DPD mRNA expression and TS genotype according to clinicopathological background and sensitivity to 5-Fluorouracil.*

Case	Age	Depth	N	H,P,CY,M	5-FU sensitivity (%)	Cancerous TS mRNA	TS mRNA in normal tissue	Cancerous DPD mRNA	DPD mRNA in normal tissue	TS genotype
1	45	SE	1	0	14.4	11	10	19	0.69	3R/3R
2	75	SS	1	0	7.1	11	8.3	47	4.4	3R/3R
3	49	SE	3	H1	7.9	30	10	1.4	5.5	3R/3R
4	71	SE	3	0	8.4	6.8	N.D.	0.045	N.D.	3R/3R
5	73	SI	1	0	0	6.4	4.8	0.32	8.2	3R/3R
6	71	MP	0	0	1.3	39	40	0.67	1.7	3R/3R
7	70	SE	1	CY1	4.6	26	41	3.5	53	3R/3R
8	59	SE	2	0	81.7	8.3	4.5	0.18	1.7	3R/3R
9	71	SS,M	0	0	5.1	22	N.D.	1.7	N.D.	3R/3R
10	57	SI	1	0	0	12	17	32	1.9	3R/3R
11	70	SE	1	0	79.4	29	26	11	8.6	3R/3R
12	47	SI	0	CY1	38.2	16	27	15	5.5	3R/3R
13	57	SI	0	0	3.6	6.3	19	3.3	4.7	3R/3R
14	74	SE	0	0	21.2	13	6	3.5	12	3R/3R
15	53	SE	0	0	0	12	9.3	7.4	2.8	3R/3R
16	54	SS	0	0	N.D.	5.1	11	30	11	3R/3R
17	59	MP	0	0	N.D.	14	8.9	7.9	9.5	3R/3R
18	82	SS	0	0	N.D.	5.6	10	3.6	17	3R/3R
19	78	SS	1	0	N.D.	72	38	7.3	0.62	3R/3R
20	78	SE	1	0	6.2	N.D.	N.D.	N.D.	N.D.	3R/3R
21	63	SS	1	0	50.3	N.D.	N.D.	N.D.	N.D.	3R/3R
22	58	SE	0	0	69.5	N.D.	N.D.	N.D.	N.D.	3R/3R

a) Depth of invasion, lymph node metastasis, hepatic metastasis (H), peritoneal dissemination (P), cytological findings (CY) and distant metastasis (M) were determined according to the Japanese Classification of Gastric Carcinoma (the 13th edition). b) Sensitivity to 5-Fluorouracil was expressed as % inhibition rate (I.R.) relative to control by MTT assay. c) TS and DPD mRNA levels are shown as units $x10^6$ copy/μg-RNA.

Statistical analysis. TS and DPD mRNA expression levels were compared between normal and cancerous tissues using coefficient correlation. TS mRNA expression levels in normal and cancerous tissues were compared between 3R/3R and 2R/3R genotypes by Student's *t*-test. Sensitivity to 5-FU as detected by MTT assay was represented as an inhibition rate (I.R.; %) and compared with mRNA expression expressed as a ratio to the internal standard, GAPDH. Patient clinicopathological factors examined included age, microscopic depth of invasion (T), microscopic lymph node metastasis (N) and hepatic (H), peritoneal (P), cytological study of the peritoneal washings, or ascites (CY) and distant (M) metastases, which were classified according to the Japanese Classification of Gastric Carcinoma, 13th Edition (23). Background factors of T (≤T2 or T3≤), N (- or +) and H, P, CY and M (- or +) were compared with respect to TS and DPD mRNA levels and sensitivity to 5-FU using Student's *t*-test. T2 included MP and SS and T3 meant equal or deeper than SE. Patients greater than Stage IIIA were assessed as being either disease-free or exhibiting recurrence, and TS and DPD mRNA expression levels compared between the two cohorts in normal and cancerous tissues by Student's *t*-test. $p<0.05$ was regarded as statistically significant.

Results

TS genotyping revealed that, of the 22 patients studied, 19 were 3R/3R homozygotes and 3 were 2R/3R heterozygotes, as shown in Table II. No 2R/2R homozygotes were observed in this study. Inhibition rates (I.R.) for 5-FU ranged from 0% to 81.7% with a mean I.R. of 22.1% and a median I.R. of 25%. Only 4 cases were found to be highly sensitive to 5-FU with >50% I.R., which resulted in an 18.2% response rate (4/22). The mean TS mRNA level in cancerous tissue was $18.2\pm16.2 \times 10^6$ copies/μg RNA and 17.1±12.6 x copies/μg RNA in normal gastric mucosa. The mean DPD mRNA level in cancerous tissues was $10.3\pm13.1 \times 10^6$ copies/μg RNA; and $8.8\pm12.3 \times 10^6$ copies/μg RNA in normal gastric mucosa. There was a statistically significant correlation in TS mRNA level between normal and cancerous tissues (Figure 2, <0.05), while no significant correlation was observed for DPD mRNA between normal and cancerous tissues (data not shown). Patients were divided into cohorts on the basis of clinicopathological findings, including T (≤T2 or T3≤), N (N- or N+), and presence of metastases at other sites (H, P, CY and M + or -). These cohorts were then compared with respect to expression level of TS and DPD mRNA in normal and cancerous tissues. No statistically significant differences in gene expression level between the cohorts were observed for the clinicopathological factors tested. In addition, no significant differences were observed for sensitivity to 5-FU as expressed by I.R. between any of cohorts for the clinicopathological factors tested, as shown in Table III (Data not shown for H, P and CY).

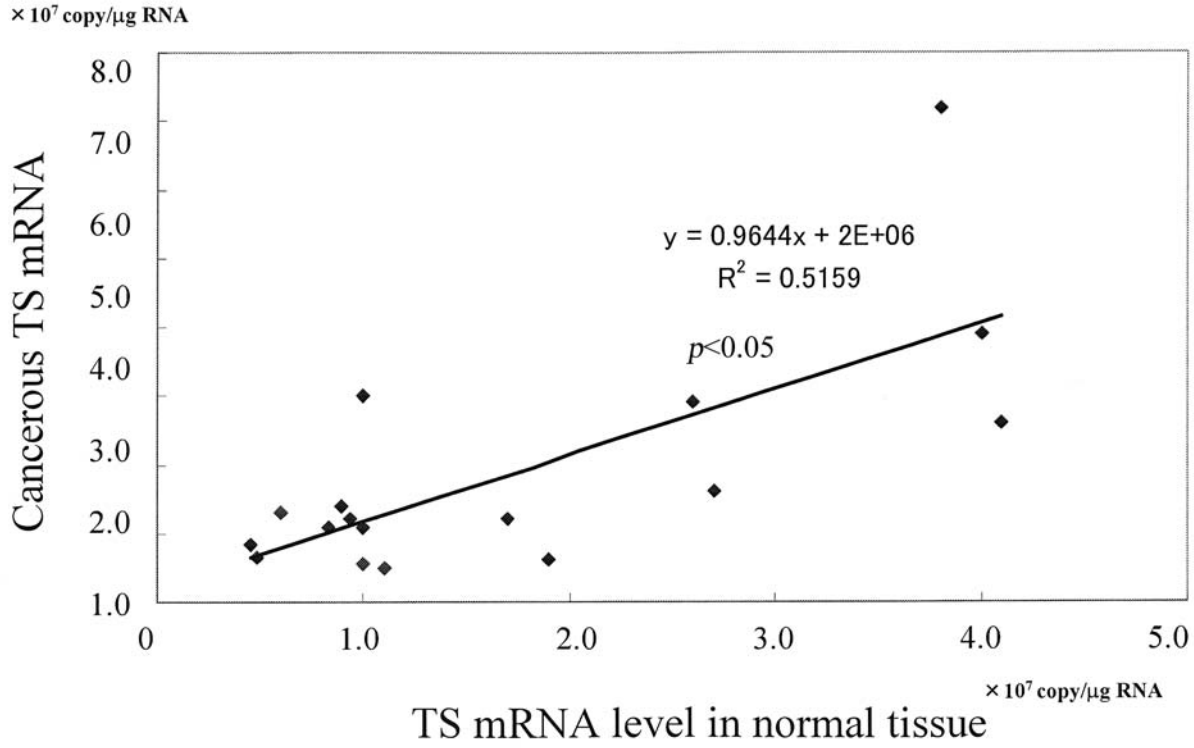

Figure 2. *Correlation between TS mRNA levels in normal and cancerous tissues. There was a statistically significant correlation in TS mRNA levels between normal and cancerous tissues ($p<0.05$).*

Table III. *TS, DPD mRNA and sensitivity to 5-Fluorouracil according to T, N and M factor.*

	Cancerous TS mRNA[a)]	TS mRNA in normal tissue	Cancerous DPD mRNA	DPD mRNA in normal tissue	5-FU I.R. (%)[c)]
≤T2[b)]	24.1±24.1	19.4±15.2	14.0±17.6	7.4±6.3	16.0±23.0
T3≤	14.7±8.7	15.9±11.5	8.1±9.6	9.5±14.8	23.9±30.5
p	NS[d)]	NS	NS	NS	NS
N(–)[b)]	14.8±10.6	16.4±11.7	8.1±9.3	8.0±5.2	19.8±25.9
N(+)	21.3±20.1	17.7±14.0	12.1±16.0	9.4±16.6	23.6±31.3
p	NS	NS	NS	NS	NS
M0	14.5±17.9	15.6±12.0	10±14.5	6.9±4.7	15.8±23.9
M1	21.0±8.8	22.0±15.0	9.7±8.6	16.2±24.7	22.6±25.3
p	NS	NS	NS	NS	NS

[a)] mRNA levels are shown as units x10^6 copy/μg-RNA
[b)] T (depth of invasion), N (lymph node metastasis) and M (distant metastasis) were determined according to the Japanese Classification of Gastric Carcinoma (13th Edition).
[c)] Sensitivity to 5-Fluorouracil is expressed as % inhibition rate relative to control by MTT assay.
[d)] NS=Not significant

Analysis of all normal and cancerous tissues showed that samples from homozygous 3R/3R cases exhibited higher TS mRNA expression levels than samples from heterozygous 2R/3R cases, which was a statistically significant difference at $p<0.05$ (Figure 3). However, there was no relationship between 5-FU chemosensitivity and TS mRNA expression (Figure 4), while the TS mRNA level in normal gastric mucosa also showed no correlation to 5-FU sensitivity (data not shown). These results suggested that, although 3R/3R homozygotes had higher TS mRNA levels compared to 3R/2R heterozygotes, TS mRNA levels in normal and cancerous tissues were not involved in the sensitivity to 5-FU. However,

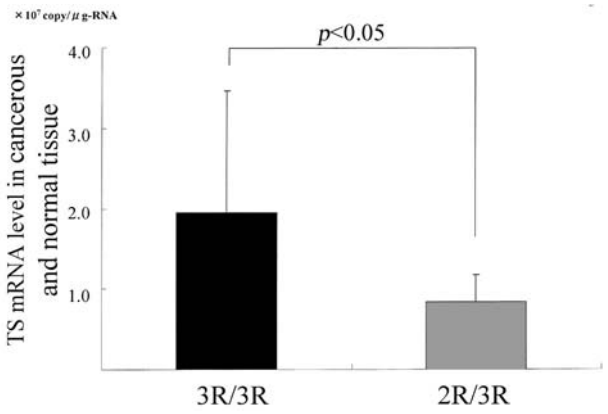

Figure 3. *TS mRNA expression in 3R/3R and 2R/3R TS genotypes. Analysis of both normal and cancerous tissues showed that samples from homozygous 3R/3R cases showed higher expression of TS mRNA compared to heterozygous (2R/3R) cases ($p<0.05$).*

cases sensitive to 5-FU all showed low DPD mRNA expression in cancerous tissues, while all the cases with high DPD mRNA level were resistant to 5-FU, as shown in Figure 5. In cases with greater than Stage IIIA gastric cancer, disease recurrence was encountered in 8 out of 14 cases after 20- to 28-month follow-up periods (median of 24 months). Both cohorts (disease-free *vs.* recurrent disease) were treated with adjuvant cancer chemotherapy of oral fluoropyrimidines, TS-1 (80 mg/m^2/day for 4 weeks with 2-week rest) for 1 year, and drug reduction or rest were performed according to the package insert. Recurrent cases showed statistically higher DPD mRNA expression levels in cancerous tissues compared to disease-free patients ($p<0.05$, Table IV). In contrast, there were no significant differences between recurrent and disease-free patients in terms of TS mRNA expression in normal and cancerous tissues and in terms of DPD mRNA expression in normal tissues.

Discussion

TS is an important target for 5-FU-based chemotherapy drugs, including related compounds such as 5-fluorodeoxyuridine (FUDR), oral 5-FU prodrugs (*e.g.* uracil/tegafur [UFT], S-1 and capecitabine) and other folate-based drugs. High levels of TS expression have been associated with poor response and survival in gastrointestinal carcinoma patients treated with 5-FU-based chemotherapies (6-9). The variable number of tandem repeats in the TS gene, either 2 repeats (2R) or 3 repeats (3R), is a genetic variation

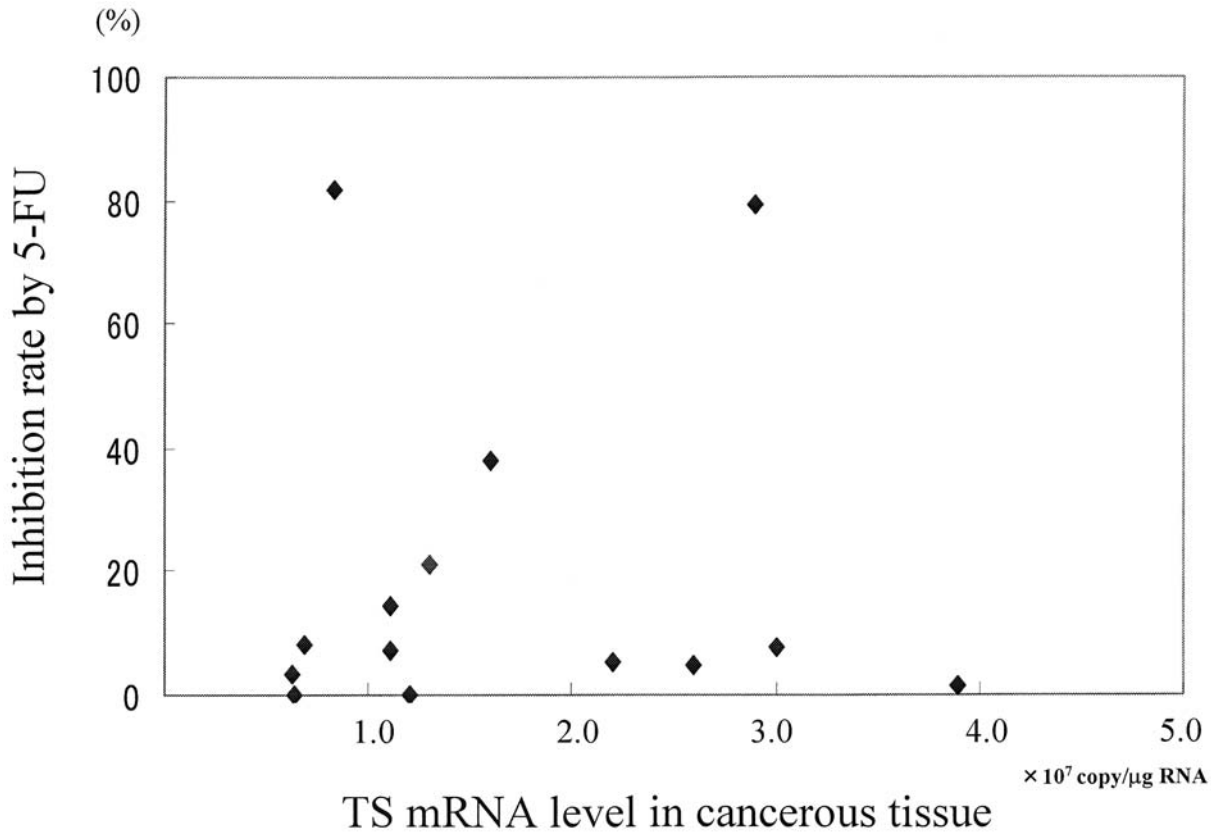

Figure 4. *TS mRNA and 5-FU sensitivity. There was no statistically significant relationship between 5-FU chemosensitivity and TS mRNA expression.*

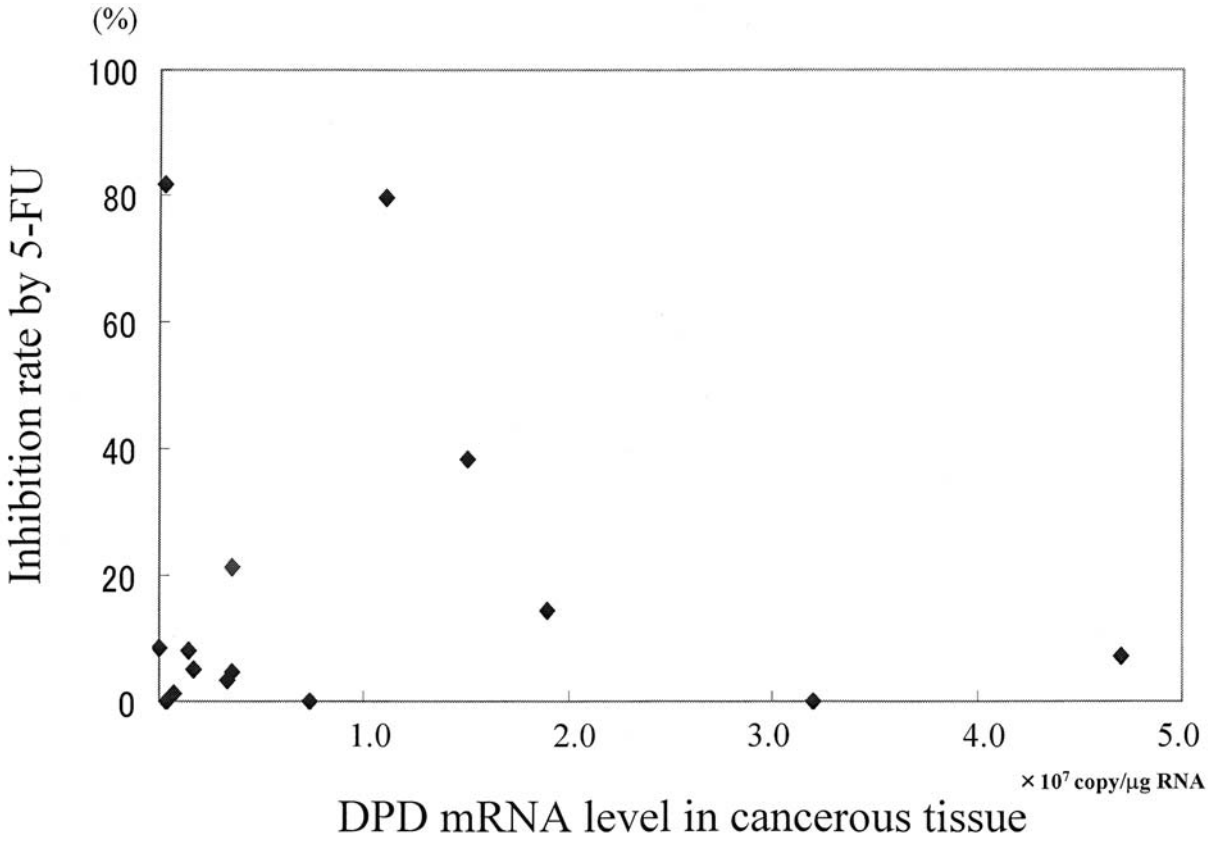

Figure 5. *DPD mRNA and 5-FU sensitivity. 5-FU sensitivity was only observed in cases with low DPD mRNA expression in cancerous tissues, while all cases with high DPD mRNA expression were resistant to 5-FU.*

that could potentially predict the effectiveness of 5-FU-based chemotherapies. Recent evidence has suggested that a polymorphism within the enhancer region of the TS gene promoter influences TS expression, with the triple repeat homozygote (3R/3R) being associated with significantly higher tumor TS levels than either the double repeat homozygote (2R/2R) or heterozygote (2R/3R) (13-17).

Iacopetta *et al.* investigated whether TS genotype was associated with the degree of survival benefit from chemotherapy in 221 Dukes' C stage colorectal cancer (CRC) patients (24). Patients with the 3R/3R polymorphism showed no significant long-term survival benefit from chemotherapy, whereas those with the 2R/2R or 2R/3R genotypes showed significant survival gains ($p=0.005$). These results demonstrate that a polymorphism within the TS gene can influence the survival benefit of 5-FU-based chemotherapy in CRC patients, probably through the polymorphism's effect on TS expression levels. Villafranca *et al.* investigated whether TS expression level and polymorphism correlated to down-staging and disease-free survival in 65 patients with rectal cancer who underwent tumor resection after pre-operative 5-FU-based chemoradiation (25). Patients homozygous for 3R/3R had a lower probability of down-staging than homozygous 2R/2R and heterozygous 2R/3R patients ($p=0.036$). Furthermore, a trend toward improved 3-year disease-free survival was detected in the 2R/2R and 2R/3R patient groups compared to the 3R/3R patient group. Thus, TS repetitive-sequence polymorphisms appear to be predictive for tumor down staging, and TR sequences in the TS promoter may be useful as a novel means of predicting the response to preoperative 5-FU-based chemoradiation.

Table IV. *TS, DPD mRNA levels between recurrent disease-free patients.*

	Recurrent disease patients	Disease-free patients	*p*
Cancerous TSmRNA	15.3±10.2	16.4±10.9	NS
TS mRNA in normal tissue	18.5±9.3	15.0±14.7	NS
Cancerous DPD mRNA	18.8±8.2	3.2±2.8	<0.05
DPD mRNA in normal tissue	6.5±4.5	16.0±21.6	NS

Units=x10^6 copy/μg-RNA

An ethnic variation in TS tandem repeat genotype frequencies has been observed, such that while 3R/3R occurs in 30% of Caucasian patients, Asian populations have

significantly higher frequencies of 3R/3R than other world populations. Indeed, in the present study, the frequency of 3R/3R homozygous gastric cancer patients was 86.4% (19/22), with no 2R/2R patients observed. Kawakami *et al.* reported TS genotypes in gastrointestinal carcinomas from 70 Japanese patients to be 3 cases with 2R/2R, 16 with 2R/3R (n=16) and 51 with 3R/3R, to give 73% 3R/3R patients (17). However, Iacopetta *et al.* reported a 3R/3R genotype frequency of 26% (58/221) (24). The higher incidence of 3R/3R in our study may be due to the difficulties in analyzing TS tandem repeats, mRNA expression, 5-FU-sensitivity as detected by MTT assay, or survival outcome. When the patients were divided into 3R/3R and 3R/2R groups, TS mRNA levels in both cancerous and normal tissues were found to be higher in the 3R/3R group than the 2R/3R group, and TS mRNA levels in respective normal and cancerous tissues correlated with each other. As TS mRNA levels in biopsied gastric cancer specimens correlated with enzyme activities in resected specimens in our previous study (26), it is highly likely that the TS 3R/3R genotypes observed in our study reflect high TS enzymatic activity in the gastric cancers. However, TS mRNA did not correlate with 5-FU-sensitivity or survival outcome. This result was comparable with our previous study, in which TS mRNA and enzymatic activity were not associated with 5-FU sensitivity in terms of human tumor-xenografts (27), MTT assay (26, 28), histoculture drug response assay (29, 30), cDNA microarray (31) and survival (32) in gastric and colon carcinoma patients.

In contrast, DPD mRNA levels were related to *in vitro* sensitivity to 5-FU, such that no tumors with high DPD mRNA levels were sensitive to 5-FU, whereas 5-FU-sensitive specimens all showed low DPD mRNA expression. 5-FU is catabolized to 2-fluoro-β-alanine mainly in the liver *via* three enzymes. DPD (EC 1.3.1.2) is the first and rate-limiting enzyme, followed by dihydropyrimidinase and β-ureidopropionase. Previous studies (33-36) examining 5-FU antitumor effects demonstrated that tumoral DPD activity varied between different human tumor cell lines and clinical samples from patients with head and neck, liver and colorectal cancers. Etienne *et al.* (34) determined tumoral/non-tumoral DPD activity ratios 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 non-responding patients. Moreover, some DPD inhibitors, such as uracil (36) and 5-chloro-2,4-dihydroxypyridine (3, 4) have been demonstrated to enhance the antitumor activity of 5-FU in human tumor cell lines. Beck *et al.* (33) measured TS and DPD enzyme activities in a panel of 19 human tumor cell lines in parallel with 5-FU responsiveness and showed that both TS and DPD activities were significantly correlated to 5-FU effectiveness, such that the greater the enzymatic activity, the higher the 5-FU IC_{50}. The findings that high DPD expression resulted in low 5-FU sensitivity were reproduced in our laboratory in experiments involving the human tumor-xenograft system (27), MTT assay of gastric (26) and colon cancer (28), histoculture drug response assay of gastric (30) and colon (29) cancer and cDNA microarray of gastric cancer (31). In the present study, the DPD mRNA level was not associated with any of the conventional clinicopathological factors examined, which suggested that the DPD expression level may be an independent prognostic factor for patients with advanced gastric cancer being treated with S-1. As S-1 was designed to inhibit DPD by CDHP, it is interesting that high DPD mRNA expression resulted in poor outcome for patients with greater than Stage IIIA gastric cancer. This suggests that further DPD inhibition may result in additional cytotoxicity of fluoropyrimidines.

Our results showed that, while the TS genotype regulated TS mRNA expression in normal and tumor tissues in advanced gastric cancer patients, there was no relationship between the TS genotype or mRNA level and 5-FU chemosensitivity. However, DPD mRNA expression was a factor that determined the 5-FU chemosensitivity and the survival outcome of advanced gastric cancer patients treated with S-1.

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