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ORIGINAL ARTICLE

Correlation between thymidylate synthase and dihydropyrimidine dehydrogenase mRNA level and in vitro chemosensitivity to 5-fluorouracil, in relation to differentiation in gastric cancer

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Abstract
Purpose It has been suggested that the gene expression levels of thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD) help in the prediction of the response to 5-fluorouracil (5-FU) in vivo and in vitro in gastric cancers.
Methods In this study, intratumoral TS and DPD gene expressions were evaluated with real time reverse transcriptional polymerase chain reaction technique to determine the correlation between the expression of these two genes and in vitro sensitivity to 5-FU, assessed by the histoculture drug response assay on 87 patients with gastric adenocarcinoma.
Results The sensitivity to 5-FU did not show any difference in clinicopathological groups. DPD gene level was higher in undifferentiated ($n = 39$) than differentiated ($n = 48$) tumors ($P = 0.043$). In differentiated tumors, TS gene expression levels were higher in the tumors with relative resistance to 5-FU, while in undifferentiated cases, DPD mRNA levels were higher in tumors that showed resistance to 5-FU in vitro ($P = 0.043$ and 0.007, respectively). DPD also had significant predictive value for 5-FU sensitivity in undifferentiated cases [$R(S) = -0.401$, $P = 0.011$]. TS and DPD gene expression levels were more highly correlated in undifferentiated compared to differentiated cases [$R(S) = 0.515$ and 0.359, respectively].
Conclusions Different gene expression might be responsible for 5-FU sensitivity in gastric cancers of different histologic origin.

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Keywords Thymidylate synthase · Dihydropyrimidine dehydrogenase · 5-Fluorouracil · HDRA · RT-PCR · Gastric · Cancer

Introduction

Adenocarcinoma of the stomach was the leading cause of malignancy-related death worldwide through most of the twentieth century [22]. In Japan, despite a decreasing trend in its incidence, the prognosis for this disease remains poor and it is the second most common cause of cancer-related death in males and the most common cause in females [4]. Nevertheless, no standard therapy thus far has been established either for advanced disease or for curatively resected gastric cancers [3, 53]. Efforts to improve the prognosis of the disease have focused on developing effective pre- and post-operative systemic adjuvant therapies [37, 46, 55]. The 5-fluorouracil (5-FU) is a drug widely used in the treatment of gastrointestinal carcinomas and is considered to be one of the most effective drugs against gastric cancer as either monotherapy or a part of a combination regimen [35], despite its single agent response rate of 10–30% [43].

In vitro drug-response assays have been developed for the few past decades in order to individualize chemotherapy for cancer patients [18, 45]. One of these assays is histoculture drug assay (HDRA) with the end point of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H tetrazolium bromide (MTT), where fresh surgical specimens maintain their cell-to-cell contact and three-dimensional native tissue architecture in culture for 7 days in exposure to 5-FU. The data of this assay have been shown to correlate strongly with clinical resistance, clinical sensitivity, and survival of patients whose tumors were tested [14, 30, 51]. The in vitro efficacy of 5-FU with this method has been shown to be 18.6% for

102 gastric cancer samples [13]. The mechanisms of resistance to 5-FU are broad and include decreased activation to increased elimination. Two important mechanisms are the alterations in the target enzyme Thymidylate Synthase (TS), which is an essential DNA de novo synthetic enzyme, and Dihydropyrimidine Dehydrogenase (DPD), which rapidly catabolyzes 5-FU to the inactive metabolite [25, 52, 56].

Both basic and clinical researchers have focused on identifying the biochemical response determinants of 5-FU to improve the clinical outcome of gastric cancer therapy with the application of a wide range of techniques from immunohistochemistry to quantitative reverse-transcription polymerase chain reaction (RT-PCR) [19, 33]. Ichikawa et al. [19] have shown that the TS and DPD mRNA expressions are different in relation to differentiation of gastric cancer. In a clinical study of 5-FU-based chemotherapy for gastric cancer, the response rates have been different between the differentiated type compared to undifferentiated type [28]. If such determinant can be measured before chemotherapy along with predicting the susceptibility of tumor by the use of HDRA, patients with favorable 5-FU response indices will anticipate a higher than average probability of response and in the case of unfavorable response, understanding the underlying mechanism may help to plan the adjuvant chemotherapy. Thus, we investigated the potential association of intratumoral mRNA expression levels of DPD and TS with clinicopathological variables including histologic type and in vitro chemosensitivity to 5-FU assessed with HDRA.

Materials and methods

Drugs

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

Patients and samples

The surgically resected specimens used for this study were obtained from 87 patients who were clinically diagnosed to have gastric cancer during open or laparoscopic surgery at the Department of General and Gastroenterological Surgery, Osaka Medical College Hospital between 2000 and 2004. None of the patients had received chemotherapy or radiotherapy before surgery and all of them had given written informed consent. The patients (62 males, 25 females) were 23–91 years old with a median age of 65. The clinicopathological features were assessed according to the Japanese Classification of Gastric Carcinoma, second English Edition [26]. However, regarding the histologic types in this study, well- and moderately differentiated adenocarcinomas were categorized as differentiated, and poorly differentiated adenocarcinomas, signet ring cell carcinomas, and mucinous carcinomas were categorized as undifferentiated type as previously described by Kuniyasu et al. [33]. Sufficient volumes of cancer tissues were obtained to perform HDRA and gene analysis. Carcinoma tissues were identified by the naked eye and cut into pieces of ~2 to 3 mm in size. Only the specimens that were histologically proved to be carcinoma by microscopic staging of the tumors were included in the study. Samples from patients who received surgery for other type of gastric cancers were excluded from the study.

HDRA with MTT as the end point

Each HDRA was performed according to the method described previously with slight modifications [13]. Resected specimens were stored in Hank's balanced salt solution (GIBCO, Gaithersburg, MD, USA) containing 100 IU penicillin (GIBCO), 100 μg of streptomycin (GIBCO), and 0.25 μg of amphotericin B (GIBCO) per ml and brought directly to the lab. Collagen sponge gels (Gelfoam) were purchased from Pharmacia & Upjohn Inc., Kalamazoo, MI, USA. The cancerous portions of surgically resected specimens were scissor-minced into pieces and then placed on prepared collagen surfaces in 24-well plates. The 5-FU purchased from Kyowa Hakko Pharmaceutical Co. Ltd., New York, NY, USA was dissolved in RPMI-1640 medium containing 20% fetal calf serum. Each tumor piece was then immersed in 750 μl of complete medium and 250 μl of diluted 5-FU. The in vitro 5-FU concentration used in this study was 300 μg/ml as reported previously [13]. For each tumor specimen the drug was assessed in triplicate. Two or three pieces of tumor specimen were incubated in complete medium without any 5-FU as control. The plates were then incubated for 7 days at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. After 7 days of incubation, 100 μl of RPMI 1640 medium containing 0.06% collagenase (type 1: Sigma, St. Louis, MO, USA) and 100 μl of 0.2% MTT (Sigma) solution were added to each well. The plates were then incubated for an additional 24 h. The supernatant in each well was carefully aspirated after centrifugation and 500 μl of DMSO (Wako Pure Chemicals Co. Ltd., Osaka, Japan) was added to solubilize the MTT-formazan. After another 4 h of incubation, 100 μl of the MTT-formazan solution from each well was transferred to the wells of a 96-well microplate and the absorbance of each well was read at a wavelength of 540 nm on a BIO-RAD Model 550 microplate reader. The inhibition rate (IR) was calculated as follows: IR (%) = (1 − mean absorbance per gram of tumor specimen

in the drug treated wells/mean absorbance per gram of tumor specimen in the non-drug treated control wells) × 100.

Real time reverse transcriptional-polymerase chain reaction (RT-PCR) quantification

Samples from each patient were immediately frozen and kept in −80°C for later gene expression analysis. Total RNA was extracted by an acid guanidinium-phenol-chloroform method of Chomczynski and Sacchi [6] using ISOGEN (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. Afterwards, the total RNA was purified using DNase 1 (GIBCO-BRL). Extracted total RNA pellets were dissolved with RNase free diethyl pyrocarbonate (DEPC)-treated water. Reverse transcription with up to 2 µg of total RNA was carried out with the use of High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions with the application of random priming method. Absolute quantitation of the cDNA of the genes of interest and an internal reference gene [glyceraldehydes-3-phosphate dehydrogenase (GAPDH)] was conducted using a fluorescence-based real time detection method (ABI Prism 7700 Sequence Detection System (TaqMan); Perkin-Elmer Applied Biosystems) as described previously [15, 17]. The PCR was carried out in the final 50 µl reaction mixture containing 25 µl of "2 X TaqMan Universal PCR Master Mix" with AmpErase UNG purchased from Applied Biosystems, 22.5 µl of diluted cDNA sample equivalent to 20 ng of total RNA of appropriately diluted cDNA plasmid sample and 2.5 µl of TaqMan® Gene Expression Assay primer and probe sets of Hs99999905_m1, Hs00559279_m1, Hs00426591_m1 for GAPDH, DPD, and TS, respectively. Thermal cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 45 cycles at 95°C for 15 s and 60°C for 1 min. A standard curve was constructed with seven points representing tenfold serial dilution of each cDNA plasmid purchased from Invitrogen life technologies (San Diego, CA, USA). The value of each point was equivalent to the value of each cDNA plasmid PCR product from 10 to 10^7 copies. The standard curves of GAPDH, DPD, and TS are shown in Fig. 1. A relative target gene mRNA expression value was obtained by division of the target gene expression by the value for GAPDH as an internal reference gene. All the reactions were done in triplicate.

Statistical analysis of data

Chi-square test was used for analyses of the distributions of chemosensitive and chemoresistant cases among clinicopathological groups. Because normalized DPD and TS levels exhibited asymmetrical distributions, non-parametric tests were used in the statistical analysis. To evaluate the association between the two continuous variables, linear regression analysis was performed to calculate Spearman's rank correlation coefficient. Statistical differences were evaluated between two groups using Mann–Whitney test and for three or more groups using the Kruskal–Wallis test. $P < 0.05$ was considered to be statistically significant.

Results

Correlation between clinicopathologic variables and HDRA result and TS and DPD gene expressions

Eighty-eight gastric tumor specimens were evaluable for measurement of mRNA from 2000 to 2004 for which HDRA data had been previously collected on fresh specimens. Among them one sample was excluded due to insufficient GAPDH expression (<5 copies). These 87 samples showed chemosensitivity to 5-FU from no sensitivity to IR of 77.5%. At the cut-off value of IR = 60%, efficacy rate of 5-FU was 18.4% (16/87) which is similar to the previous report [13]. Samples were divided into two groups (IR ≥ 60% vs. IR < 60%) regarding this cut-off value. The range and median values of relative expressions of DPD and TS mRNA determined by real-time RT-PCR and in-vitro chemosensitivity are shown in Table 1, with reference to clinicopathologic features including gender, age, histological type, tumor size, depth of tumor invasion, lymphatic invasion, venous invasion, lymph node metastasis, any other local or distant metastasis, and staging. Chi-square test was used to test the frequency difference of sensitive versus resistant cases among clinicopathologic groups. To compare both TS and DPD gene expressions with clinocopathological variables, Mann–Whitney and Kruskal–Wallis tests were used. DPD gene expression showed a statistically significant difference between the differentiated and undifferentiated type (Mann–Whitney U-Test, $P = 0.043$, Table 1). There was no other statistically significant association between any of the gene expressions and clinicopathologic features (Table 1). There was no statistically significant difference between the cases with IR < 60% and IR ≥ 60% in relation to clinicopathological background factors tested with chi-square test (Table 1).

Association of DPD and TS gene expression levels and sensitivity to 5-FU in relation to differentiation group

At the cut-off level of IR = 60% where 5-FU efficacy was 18.8% in the differentiated group and 17.9% in the undifferentiated group, the resistant tumors ($N = 39$) showed a higher TS level in the differentiated group compared to the sensitive samples ($N = 9$)(median, 0.0123 vs. 0.0049,

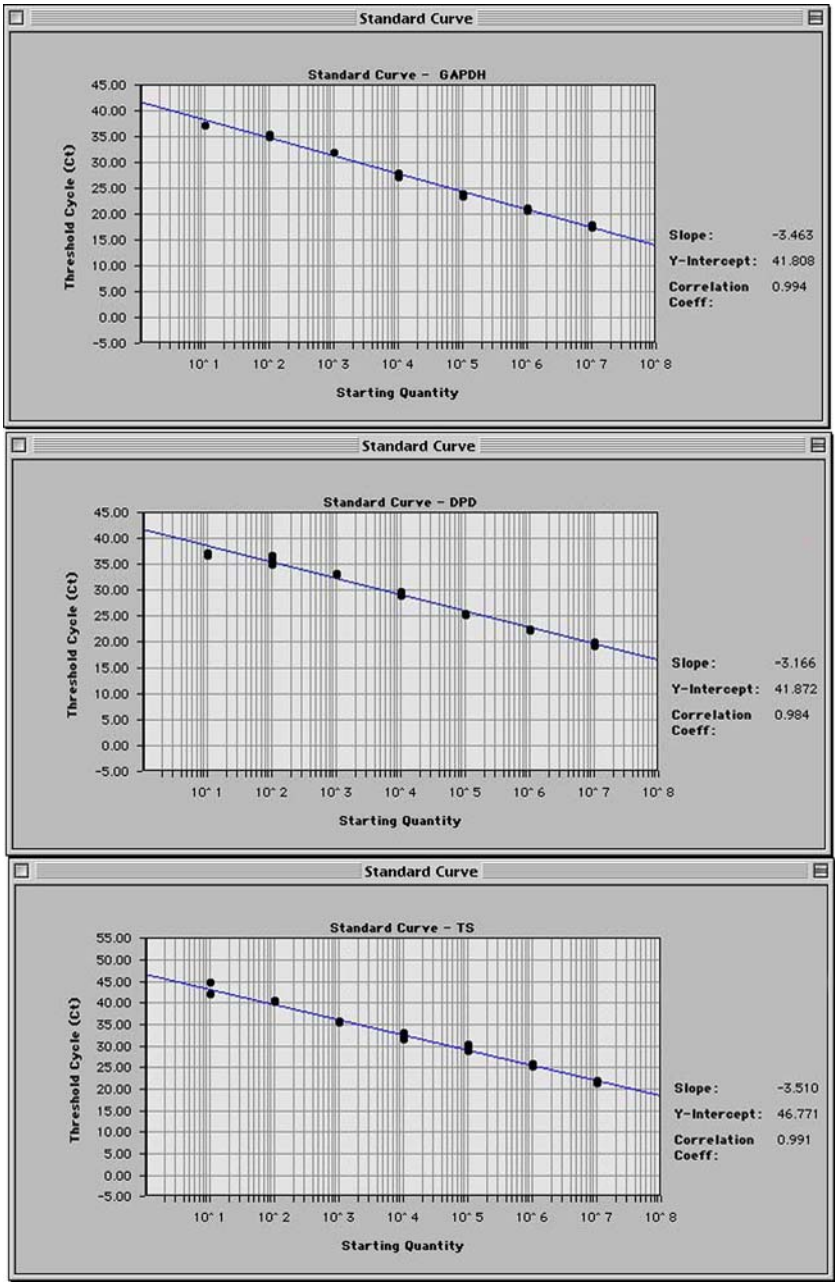

Fig. 1 Linear relationship between threshold and logarithm of the starting copy number of GAPDH (*top*), TS (*middle*), and DPD (*bottom*) cDNA plasmid. Correlation coefficients are more than 0.95 in all three genes. GAPDH, $= -3.463x + 41.808$; TS, $y = -3.51x + 46.771$; DPD, $y = -3.166 + 41.872$

respectively; $P = 0.043$, Mann–Whitney U-Test) while in the undifferentiated group DPD the mRNA level was higher in resistant tumors ($N = 32$, median, 0.02) compared to sensitive ones ($N = 7$, median 0.0013)($P = 0.007$, Mann–Whitney U-Test)(Figs. 2, 3). No significant correlation was observed between TS mRNA expressions and in vitro sensitivity to 5-FU in either of the histologic groups (Fig. 4a, b). Tumors with higher DPD mRNA level tended to show relatively high resistance to 5-FU in the undifferentiated group (correlation of coefficient = −0.401; Fig. 5a), but not in the differentiated group (correlation of coefficient = 0.126; Fig. 5b). In undifferentiated group, DPD and TS mRNAs had higher correlation (correlation coefficient = 0.515) than in differentiated group (correlation coefficient = 0.359) (Fig. 6a, b).

Discussion

A remarkable number of gastric cancer patients who have undergone curative resection surgery will relapse. Adjuvant chemotherapy is regularly attempted to improve the treatment outcome. To overcome the interpatient variability and to individualize chemotherapy for patients with similar histopathologic tumors, in vitro drug response assays have been developed [30]. The Japan Society for Appropriate Cancer

Table 1 Correlation between clinicopathological variables and TS and DPD gene expressions and 5-FU sensitivity

Variable	Number of patients	DPD				TS				5-FU	Sensitivity	*P*-value
		Median	Minimum	Maximum	*P*-value	Median	Minimum	Maximum	*P*-value	IR < 60% (*n* = 71)	IR ≥ 60% (*n* = 16)	
Gender					0.851				0.177			0.512
Male	62	0.01	0.00029	0.0738		0.012	0.00148	0.1013		51	11	
Female	25	0.008	0.00038	0.0621		0.009	0.00195	0.0385		20	5	
Age					0.683				0.792			0.334
<65	42	0.0116	0.00031	0.0705		0.0116	0.00195	0.072		33	9	
≥65	45	0.008	0.00029	0.0738		0.009	0.00148	0.1013		38	7	
Tumor size (mm)					0.551				0.296			0.6
<60	27	0.008	0.00123	0.0738		0.012	0.00204	0.072		22	5	
≥60	60	0.0123	0.00029	0.0705		0.009	0.00148	0.1013		49	11	
Histological type					0.043				0.219			0.574
Differentiated	48	0.0077	0.00029	0.0738		0.0115	0.00321	0.1013		39	9	
Undifferentiated	39	0.015	0.00031	0.0705		0.0095	0.00148	0.0528		32	7	
Serous Invasion					0.212				0.191			0.334
No	42	0.0079	0.00038	0.0738		0.012	0.00204	0.1013		33	9	
Yes	45	0.014	0.00029	0.0705		0.008	0.00148	0.0385		38	7	
Lymphatic invasion					0.964				0.052			0.547
Ly0, Ly1	29	0.0079	0.0014	0.0738		0.0075	0.00195	0.0481		24	5	
Ly2, Ly3	58	0.0116	0.00029	0.0643		0.0139	0.00148	0.1013		47	11	
Venous invasion					0.068				0.647			0.176
v0, v1	32	0.0139	0.00105	0.0738		0.01	0.00195	0.0481		24	8	
v2, v3	55	0.008	0.00029	0.0643		0.011	0.00148	0.1013		47	8	
Lymph node metastasis					0.918				0.179			0.308
N0, N1	51	0.008	0.00038	0.0738		0.012	0.00204	0.1013		22	5	
N2, N3	36	0.012	0.00029	0.0537		0.008	0.00148	0.0528		49	11	
Other metastasis					0.115				0.094			0.394
No	71	0.0115	0.00058	0.0738		0.012	0.00195	0.1013		57	14	
Yes	16	0.007	0.00029	0.0621		0.007	0.00148	0.0385		14	2	
Stage					0.509				0.125			0.214
I	12	0.007	0.0014	0.0519		0.008	0.00204	0.0631		11	1	
II	21	0.0138	0.0015	0.0738		0.018	0.00325	0.1013		16	5	
III	24	0.007	0.00058	0.0537		0.009	0.00321	0.0528		17	7	
IV	30	0.012	0.00029	0.0621		0.008	0.00148	0.0385		27	3	

Among each clinicopathologic variable group, chi-square test was used to evaluate the frequency difference of sensitive versus resistant. Statistical difference of TS and DPD gene expressions in each clinicopathological variables group was tested by Mann–Whitney between two groups and by Kruskal–Wallis among three groups or more

Chemotherapy summarized the status of chemosensitivity testing for antitumor agents in Japan in 2000 [7]. Data showed that patients treated with drugs testing in the "sensitive" range were nearly seven times more likely to respond to chemotherapy than were patients treated with drugs testing in the "resistant" range (47% vs. 7%). They concluded that chemosensitivity testing was being widely applied in Japan and that it has a high-accurate predictive value for advanced carcinomas. Thus, the chemosensitivity testing was approved by the Japanese Ministry of health, Welfare, and Labor as "advanced clinical medicine". Our laboratory is one of the few approved institutes in Japan (http://www.mhwl.go.jp/topics/bukyoku/isei/sensiniryo/ikikan02.html). In order to study the usefulness of in vitro drug sensitivity testing for gastric cancer adjuvant chemotherapy, two clinical trials have just been started in Japan by the Japan Clinical Cancer Research Organization (JACCRO, Tokyo, Japan) [31] and by the Japan Clinical Oncology Group (JCOG) [42]. Among

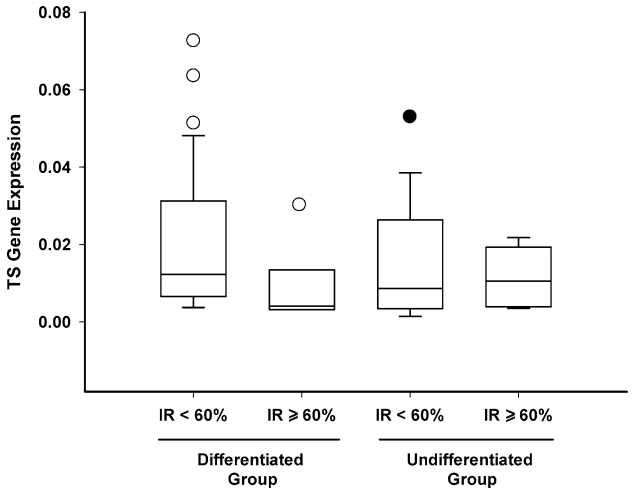

Fig. 2 Distribution of TS gene expression in sensitive versus resistant cases in relation to differentiation. Cut-off level of IR = 60% was applied for sensitivity. *Boxes* indicates the first and third quartile (median inside); *bars* represent the range of values falling within 1.5-fold the interquartile range. *Open* and *closed circles* represent outliners (>1.5-fold the interquartile range) for differentiated and undifferentiated type cases, respectively. The resistant tumors showed a higher TS level only in differentiated group compared to sensitive ones (median, 0.0123 vs. 0.0049, respectively; $P = 0.043$, Mann–Whitney *U*-Test)

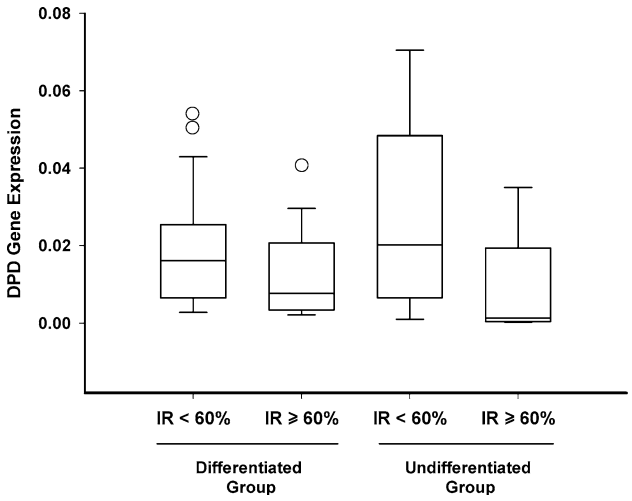

Fig. 3 Distribution of DPD gene expression in sensitive versus resistant cases in relation to differentiation. Cut-off level of IR = 60% was applied for sensitivity. *Boxes* indicates the first and third quartile (median inside); *bars* represent the range of values falling within 1.5-fold the interquartile range. *Open circles* represent outliners (>1.5-fold the interquartile range) for differentiated type cases. Only in undifferentiated group DPD mRNA level was higher in resistant tumors (median 0.02) compared to sensitive ones (median 0.0013) ($P = 0.007$, Mann–Whitney *U*-Test)

these in vitro assays, HDRA with MTT endpoint has been demonstrated to have a high rate of evaluability, often more than 80% in previous studies [9, 14, 54]. It is believed that HDRA allows tumor cells to maintain their native three-

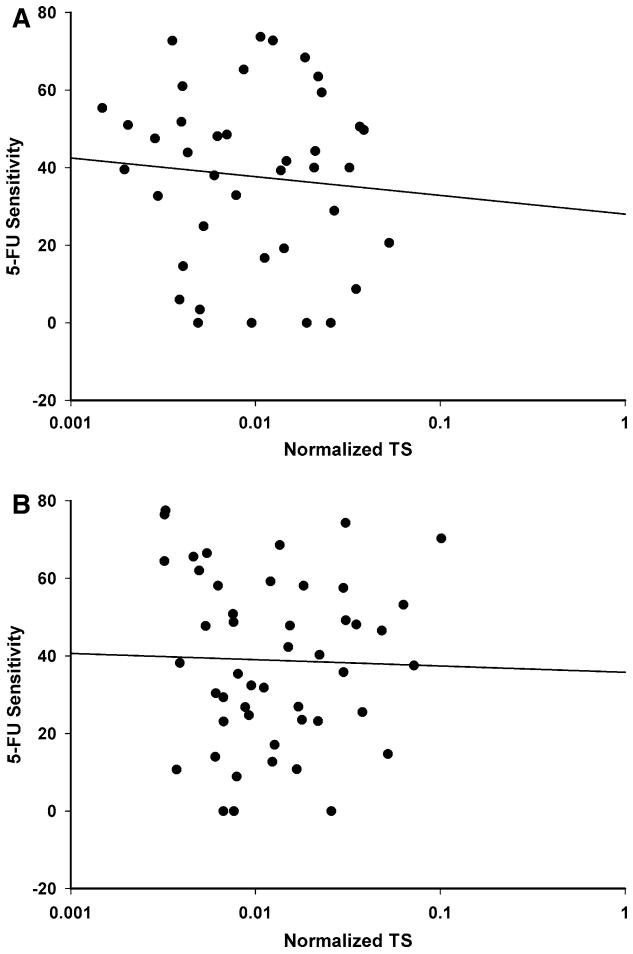

Fig. 4 Correlation between tumoral TS levels and 5-FU sensitivity in undifferentiated (a) and differentiated group (b). *Plots* show the correlation between TS: GAPDH RT-PCR product ratios of biopsied specimens and sensitivity to 5-FU measured by the MTT assay using formula: IR(%) = (1 − mean absorbance per gram of tumor specimen in the drug treated wells/mean absorbance per gram of tumor specimen in the non-drug treated control wells) × 100. Spearman's rank correlation coefficient neither in undifferentiated group ($r = -0.082$) nor in differentiated group ($r = -0.074$) was statistically significant

dimensional tissue architecture and in intact tissue with cell-to-cell contact have a high survival rate and viability [9, 54]. MTT endpoint evaluates total tumor cell viability and is more appropriate since many cells in gastrointestinal tumors may be in the resting stage of the cell cycle. In those studies, HDRA demonstrated a very high rate of evaluability (96.3%) and 5-FU had an in vitro efficacy rate of 18.6% against gastric cancers [13, 14].

In the present study, at the cut-off value of IR = 60%, in vitro efficacy rate of 5-FU was shown to be 18.4% in 87 gastric carcinomas which is similar to the previous studies [13, 30]. At this cut-off value, histologic type did not show any impact on sensitivity to 5-FU; 9 of 43 cases in differentiated, and 7 of 39 cases in the undifferentiated group were sensitive to 5-FU. This result is consistent with the in-vitro

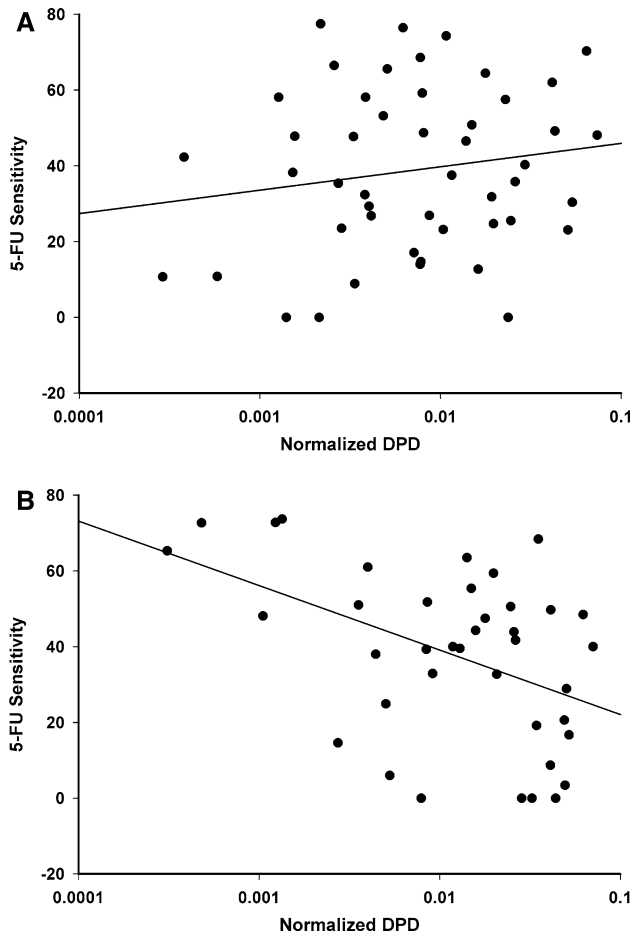

Fig. 5 Correlation between tumoral DPD levels and 5-FU sensitivity in undifferentiated (**a**) and differentiated group (**b**). *Plots* show the correlation between DPD: GAPDH RT-PCR product ratios of biopsied specimens and sensitivity to 5-FU measured by the MTT assay using formula: IR(%) = (1 − mean absorbance per gram of tumor specimen in the drug treated wells/mean absorbance per gram of tumor specimen in the non-drug treated control wells) × 100. Spearman's rank correlation coefficient was $r = -0.401$ in undifferentiated group and $r = 0.126$ in differentiated group. DPD was a predictive factor for sensitivity only in undifferentiated group ($P = 0.011$). The correlation was not statistically significant in differentiated group

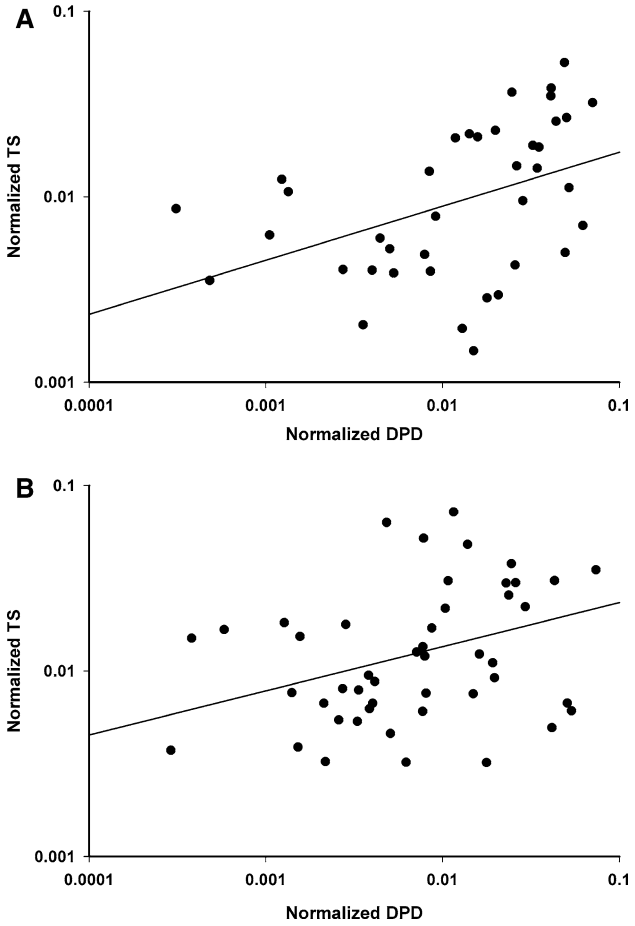

Fig. 6 Correlation between TS and DPD gene expression in undifferentiated (**a**) and differentiated group (**b**). The correlation coefficient in undifferentiated group ($r = 0.515$, $P = 0.001$) was higher than differentiated group ($r = 0.359$, $P = 0.012$)

study of Noguchi et al. on 485 gastric cancer lesions where the chemosesnitivity in differentiated cancer was equivalent to that of the undifferentiated variant (median IR of 70.3% vs. 65.9%, respectively) [41]. On the other hand, it is not in agreement with the study by Kubota et al. They reported that the differentiated gastric tumors were more sensitive (14/52) than undifferentiated tumors (6/67) in HDRA with [^{3}H]thymidine end point measured by audiography where tumors were exposed for 24 h to 5-FU [30]. No association was found between in vitro chemosensitivity and other histopathological variables in our study. The inconsistency of these data with other reports might be described by the different evaluability of in vitro chemosensitivity methods encountered [10, 38].

Dihydropyrimidine dehydrogenase is the first and rate-limiting enzyme in the catabolism of 5-FU. The 5-FU is enzymatically inactivated to dihydrofluorouracil by DPD [35]. Although the liver expresses the highest level of DPD in the body, this enzyme is widely distributed in other tissues including the gastrointestinal mucosa and thus tumor cells. It has been previously shown that the DPD mRNA level is significantly correlated with enzyme activity in gastric cancer [23]. TS, an essential DNA synthetic enzyme that catalyses the methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) is the main cellular target of 5-FU [35]. Several basic and clinical studies suggest that high-TS expression will result in 5-FU resistance in gastrointestinal malignancies [27, 34, 40]. The methods used for the measurement of TS expression in those studies vary greatly including biochemical assays, immunohistochemistry, enzyme-linked immunoabsorbant assay (ELISA) and RT-PCR methods. A close linear relationship was noted between TS protein expression and TS gene expression in colorectal and gastric tumors

studied by Johnston et al. [27]. The data from study by Ishikawa et al. on gastric tumors showed the correlation between enzymatic activity of TS and its mRNA expression [24]. In the present study, high-DPD mRNA expression was associated with the undifferentiated gastric tumors. This result is comparable with Ishikawa et al. report that DPD mRNA level studied by RT-PCR in fresh-frozen specimens was shown to be statistically higher in the undifferentiated type cases [26]. DPD gene was more highly expressed in the undifferentiated gastric adenocarcinomas studied by Ichikawa et al. who used RT-PCR on laser-captured microdissected tissues from paraffin-embedded materials (mean value 1.23 in undifferentiated type versus 0.99 in differentiated type) [19]. However, other studies on gastric cancer did not show any relationship between histologic type and DPD expression [11].

In this study, TS mRNA level did not show a statistically significant relationship with histologic type, though it had a higher trend in differentiated cases. These results are in agreement with several immunohistochemical and RT-PCR studies on TS in gastric cancer who failed to show any relationship between TS expression and histologic type [11, 50]. Ichikawa et al. reported a higher expression of TS gene level in differentiated gastric adenocarcinoma [19]. Their result is in contrast with Choi et al. who reported that poorly differentiated gastric carcinoma was more common in the high-TS group [5]. Sakamoto et al. who investigated TS enzyme activity for pyrimidine nucleotide synthesis in 57 gastric carcinoma, reported higher activity of TS enzyme in poorly differentiated than well-differentiated adenocarcinomas [47]. Thus, the relation between TS expression and histologic type seems to be a controversial issue. In the present study, TS mRNA level was not a predictive factor for in vitro sensitivity to 5-FU in any histologic group, cases which showed in vitro sensitivity to 5-FU in the differentiated carcinomas had lower TS gene expressions. On the other hand, DPD mRNA level was a predictive factor for in-vitro sensitivity to 5-FU in the undifferentiated cases. In this group, the non-sensitive cases to 5-FU had shown higher levels of DPD mRNA level. These data are consistent with the phase II study of S-1 drug for advanced gastric cancer [29]. S-1 is a new oral fluorinated pyrimidine, consisting of tegafur (FT), a prodrug of 5-FU, and 5-chloro-2,4-dihydroxypyridine (CDHP), a potent inhibitor of DPD and potassium oxonate (Oxo) [49]. In the report by S-1 Cooperative Gastric Study Group, 52% (13/25) of diffuse type of metastatic gastric cancers responded to S-1 while the response rate was 28% (7/25) for intestinal type carcinomas. Fujiwara et al. reported that the antitumor activity of S-1 was more potent against tumors with higher DPD activity [12]. The data from Adjuvant Chemotherapy Trial of S-1 for Gastric Cancer (ACTS-GC) on 1034 patients with stage II or III gastric cancer has shown that S-1 is safe and highly effective, and can be considered as the standard adjuvant chemotherapy for stage II/III gastric cancer patients after potentially D2 resection [48]. The mortality rate of cases with undifferentiated cancer has decreased more than cases with differentiated cancers (8.4% vs. 5.7%, respectively) when S-1 was administered after surgery compared to surgery alone group. Therefore, the difference in response rate of S-1 for different histologic type might be explained by their different DPD content. The predictive value of intratumoral TS gene expression in 5-FU based treatment of gastric cancer has been well studied. TS mRNA level in responders to combination treatment of irinotecan (CPT-11), a DNA topoisomerase I inhibitor [1, 32], plus S-1 was higher than non-responders in 26 primary gastric cancers (median value, 4.26 and 2.11, respectively) [20]. Ichikawa et al. also reported a positive relation between TS and Topo-I mRNA expression in 49 colorectal tumors (correlation coefficient = 0.513) [21]. While phase III French clinical trial on 260 patients has not shown any significant survival benefit with cisplatin-based adjuvant chemotherapy neither in diffuse nor in intestinal type [2], the median survival time of patients with diffuse type was shorter in comparison to intestinal type (291 days vs.472 days, respectively) when treated with combination chemotherapy of irinotecan plus cisplatin [57]. Thus, this difference might be explained with different TS gene expression levels. Evident differences in pathogenetic and bio-molecular characteristics have been shown between diffuse and intestinal type of gastric cancer in several studies [16, 36]. A multicenter Italian study by Italian Research Group for Gastric Cancer on 412 surgically treated gastric cancer patients, has shown that peritoneal recurrence occurred more in diffuse-type compared to intestinal-type [39]. In another study by the same group on 584 patients who underwent D2 gasterectomy, the multivariate analysis has shown that the risk of lymph node metastasis was associated with diffuse type [8]. TS, an essential enzyme for intracellular de novo source of thymidylate, and DPD, which catabolize thymine, are important enzymes for DNA biosynthesis. Therefore, the closer relation of DPD and TS in undifferentiated carcinomas in the present study might be explained with distinct different biologic behavior, cellular origin, growth pattern and site of metastasis of differentiated, and undifferentiated gastric adenocarcinomas [44].

With the introduction of new TS inhibitors like Tomudex and Thymitag that are more specific and potent TS inhibitors and with the availability of S-1, identification of responsible target of possible 5-FU resistance along with HDRA in different histologic gastric cancer, will help clinicians to select more appropriate drug agents for individualized chemotherapy.

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References

1. Andoh T, Ishii K, Suzuki Y, Ikegami Y, Kusunoki Y, Takemoto Y, Okada K (1987) Characterization of a mammalian mutant with a camptothecin-resistant DNA topoisomerase I. Proc Natl Acad Sci USA 84:5565–5569
2. Bouche O, Ychou M, Burtin P, Bedenne L, Ducreux M, Lebreton G, Baulieux J, Nordlinger B, Martin C, Seitz JF, Tigaud JM, Echinard E, Stremsdoerfer N, Milan C, Rougier P, On behalf of the fédération francophone de cancérologie digestive group (2005) Adjuvant chemotherapy with 5-fluorouracil and cisplatin compared with surgery alone for gastric cancer: 7-year results of the FECD randomized phase III trial (8801). Ann Oncol 16:1488–1497
3. Brennan MF (2005) Current status of surgery for gastric cancer: a review. Gastric Cancer 8:64–70
4. Cancer Statistics in Japan (2003) National Cancer Center, Tokyo, Japan
5. Choi J, Lim H, Nam DK, Kim HS, Cho DY, Yi JW, Kim HC, Cho YK, Kim MW, Joo HJ, Lee KB, Kim KB (2001) Expression of thymidylate synthase in gastric cancer patients treated with 5-fluorouracil and doxorubicin-based adjuvant chemotherapy after curative resection. Br J Cancer 84:186–192
6. Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159
7. Do TK, Kubota T, Tanimura T, Yamaue H, Akiyama S, Maehara Y, Tanigawa N, Kitajima M, Takagi H (2000) Cummulative results of chemosensitivity tests for antitumor agents in Japan. Anticancer Res 20:2389–2392
8. Folli S, Morgagni P, Roviello F, De Manzori G, Marrelli D, Saragoni L, Di Leo A, Gaudio M, Nanni ScD O, Carli A, Cordiano C, Dell'Amore D, Vio A (2001) Risk factors for lymph node metastases and their prognostic significance in early gastric cancer (EGC) for the Italian Research Group for Gastric Cancer (IRGGC). Jpn J Clin Oncol 31:495–499
9. Freeman AE, Hoffman RM (1986) In vivo-like growth of human tumors in vitro. Proc Natl Acad Sci USA 83:2694–2698
10. Fujita K, Kubota T, Matsuzaki SW, Otani Y, Watanabe M, Teramoto T, Kumai K, Kitajima M (1998) Further evidence for the value of the chemosensitivity test in deciding appropriate chemotherapy for advanced gastric cancer. Anticancer Res 18:1973–1978
11. Fujiwara H, Terashima M, Irinoda T, Takagane A, Abe K, Kashiwaba M, Oyama K, Takahashi M, Maesawa C, Saito K, Takechi T, Fukushima M (2002) Quantitative measurement of thymidylate synthase and dihydropyrimidine dehydrogenase mRNA level in gastric cancer by real-time RT-PCR. Jpn J Cancer Res 93:1342–1350
12. Fujiwara H, Terashima M, Irinoda T, Takagane A, Abe K, Nakaya T, Yonezawa H, Oyama K, Takahashi M, Saito K, Takechi T, Fukushima M, Shirasaka T (2003) Superior antitumour activity of S-1 in tumours with a high dihydropyrimidine dehydrogenase activity. Eur J Cancer 39:2387–2394
13. Furukawa T, Kubota T, Hoffman RM (1995) Clinical applications of the histoculture drug response assay. Clin Cancer Res 1:305–311
14. Furukawa T, Kubota T, Watanabe M, Takahara T, Yamaguchi H, Takeuchi T, Kase S, Kodaira S, Ishibiki K, Kitajima M, et al (1992) High in vitro-in vivo correlation of drug response using sponge-gel-supported three-dimensional histoculture and the MTT end point. Int J Cancer 51:489–498
15. Gibson UE, Heid CA, Williams PM (1996) A novel method for real time quantitative RT-PCR. Genome Res 6:995–1001
16. Hamilton JP, Meltzer SJ (2006) A review of the genomics of gastric cancer. Clin Gasteroenterol Hepatol 4:416–425
17. Heid CA, Stevens J, Livak KJ, Williams PM (1996) Real time quantitative PCR. Genome Res 6:986–994
18. Hoffman RM (1991) In vitro sensitivity assays in cancer: a review, analysis, and prognosis. J Clin Lab Anal 5:133–143
19. Ichikawa W, Takahashi T, Suto K, Nihei Z, Shirota Y, Shimizu M, Sasaki Y, Hirayama R (2004) Thymidylate synthase and dihydropyrimidine dehydrogenase gene expression in relation to differentiation of gastric cancer. Int J Cancer 112:967–973
20. Ichikawa W, Takahashi T, Suto K, Yamashita T, Nihei Z, Shirota Y, Shimizu M, Sasaki Y, Hirayama R (2004) Thymidylate synthase predictive power is overcome by irinotecan combination therapy with S-1 for gastric cancer. Br J Cancer 91:1245–1250
21. Ichikawa W, Uetake H, Niheil Z, Matsuo K, Fujita H, Yamada Y (1999) Topoisomerase-I (Topo-I) expression correlates to thymidylate synthase (TS) expression in colorectal cancer (CRC). Proc Am Soc Clin Oncol 18:246a
22. International Agency for Research on Cancer Publication (2003). In: Kleihves P, Stewart B.W (eds) World cancer report, pp 194–197
23. Ishikawa Y, Kubota T, Otani Y, Watanabe M, Teramoto T, Kumai K, Kitajima M, Takechi T, Okabe H, Fukushima M (1999) 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
24. Ishikawa Y, Kubota T, Otani Y, Watanabe M, Teramoto T, Kumai K, Takechi T, Okabe H, Fukushima M, Kitajima M (2000) Dihydropyrimidine dehydrogenase and messenger RNA levels in gastric cancer: possible predictor for sensitivity to 5-fluorouracil. Jpn J Cancer Res 91:105–112
25. Ishikawa Y, Kubota T, Otani Y, Watanabe M, Teramoto T, Kumai K, Takechi T, Okabe H, Fukushima M, Kitajima M (1999) Thymidylate synthetase and dihydropyrimidine dehydrogenase levels in gastric cancer. Anticancer Res 19:5635–5640
26. Japanese Gastric Cancer Association (1998) Japanese Classification of Gastric Carcinoma–2nd English Edition. Gastric Cancer 1:10–24
27. Johnston PG, Lenz HJ, Leichman CG, Danenberg KD, Allegra CJ, Danenberg PV, Leichman L (1995) 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
28. Kawai H, Ohtsu A, Boku N, Hamamoto Y, Nagashima F, Muto M, Sano Y, Mera K, Yano T, Doi T, Yoshida S (2003) Efficacy and safety profile of S-1 in patients with metastatic gastric cancer in clinical practice: results from a post-marketing survey. Gastric Cancer 1:19–23
29. Koizumi W, Kurihara M, Nakano S, Hasegawa K (2000) Phase II study of S-1, a novel oral derivative of 5-fluorouracil, in advanced gastric cancer. Oncology 58:191–197
30. 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 RM, The chemosensitivity study group for the histoculture drug-response assay (1995) Potential of the histoculture drug-response assay to contribute to cancer patient survival. Clin Cancer Res 1:1537–1543
31. Kubota T (2006) Chemosensitivity test for adjuvant cancer chemotherapy of gastric cancer (JACCRO GC-04). Igaku no Ayumi (J Clin Exp Med):217, 9:869–873

32. Kunimoto T, Nitta K, Tanaka T, Uehara N, Baba H, Takeuchi M, Yokokura T, Sawada S, Miyasaka T, Mutai M (1987) Antitumor activity of 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothec in, a novel water-soluble derivative of camptothecin, against murine tumors. Cancer Res 47:5944–5947
33. Kuniyasu T, Nakamura T, Tabuchi Y, Kuroda Y (1998) Immunohistochemical evaluation of thymidylate synthase in gastric carcinoma using a new polyclonal antibody: the clinical role of thymidylate synthase as a prognostic indicator and its therapeutic usefulness. Cancer 83:1300–1306
34. Lenz HJ, Leichman CG, Danenberg KD, Danenberg PV, Groshen S, Cohen H, Laine L, Crookes P, Silberman H, Baranda J, Garcia Y, Li J, Leichman L (1996) Thymidylate synthase mRNA level in adenocarcinoma of the stomach: a predictor for primary tumor response and overall survival. J Clin Oncol 14:176–182
35. Longley DB, Harkin DP, Johnston PG (2003) 5-fluorouracil: mechanisms of action and clinical strategies. Nat Rev Cancer 3:330–338
36. Lynch HT, Grady W, Suriano G, Huntsman D (2005) Gastric cancer: new genetic developments. J Surg Oncol 90:114–133
37. Macdonald JS (2005) Role of post-operative chemoradiation in resected gastric cancer. J Surg Oncol 90:166–170
38. Maehara Y, Anai H, Kusumoto H, Sugimachi K (1987) Poorly differentiated human gastric carcinoma is more sensitive to antitumor drugs than is well differentiated carcinoma. Eur J Surg Oncol 13:203–206
39. Marrelli D, Roviello F, de Manzoni G, Morgagni P, Di Leo A, Saragoni L, De Stefano A, Folli S, Cordiano C, Pinto E, for The Italian Research Group for Gastric Cancer (2002) Different patterns of recurrence in gastric cancer depending on Lauren's histological type: longitudinal study. World J Surg 26:1160–1165
40. Metzger R, Leichman CG, Danenberg KD, Danenberg PV, Lenz HJ, Hayashi K, Groshen S, Salonga D, Cohen H, Laine L, Crookes P, Silberman H, Baranda J, Konda B, Leichman L (1998) ERCC1 mRNA levels complement thymidylate synthase mRNA levels in predicting response and survival for gastric cancer patients receiving combination cisplatin and fluorouracil chemotherapy. J Clin Oncol 16:309–316
41. Noguchi K, Iwahashi M, Tani M, Nakamura M, Nakamori M, Nakatani Y, Ueda K, Ishida K, Naka T, Ojima T, Hotta T, Mizobata S, Yamaue H (2005) Evaluation of chemosensitivity testing with highly purified tumor cells in 435 patients with gastric carcinoma using an MTT assay. Anticancer Res 25:931–937
42. Nomura E, Tanigawa N (2006) JCOG clinical trial for evaluation of the utility of in vitro chemosensitivity test for cancer. Igaku no Ayumi (J Clin Exp Med):217, 9:875–879
43. Ohtsu A (2005) Status and future prospects of chemotherapy for metastatic gastric cancer: a review. Gastric Cancer 8:95–102
44. Rosai J (2004) Rosai and Ackerman's surgical pathology, 9th edn. Mosby, St. Louis pp 662–666
45. Saikawa Y, Kubota T, Furukawa T, Suto A, Watanabe M, Kumai K, Ishibiki K, Kitajima M (1994) 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
46. Sakamoto J, Morita S, Kodera Y, Rahman M, Nakao A (2004) Adjuvant chemotherapy for gastric cancer in Japan: global and Japanese perspectives. Cancer Chemother Pharmacol 54:S25–S31
47. Sakamoto S, Kawachi Y, Konishi T (1993) Pathological features and pyrimidine nucleotide synthesis in human gastric carcinomas. Anticancer Res 13:879–881
48. Sasako M, Yamaguchi T, Kinoshita T, Fujii M, Nashimoto A, Furukawa H, Nakajima T, Ohashi Y, Sakuromoto S, Imamura H, for the ACTS-GC group (2007) Randomized phase III trial comparing S-1 monotherapy versus surgery alone for stage II/III gastric cancer patients after curative D2 gasterectomy (ACTS-GC study) presented in 2007 Gastrointestinal Cancers Symposium (ASCO-GI) January 19–21, 2007 Orlando World Center Marriott, Orlando, FL, USA
49. Shirasaka T, Shimamato Y, Ohshimo H, Yamaguchi M, Kato T, Yonekura K, Fukushima M (1996) Development of a novel form of an oral 5-fluorouracil derivative (S-1) directed to the potentiation of the tumor selective cytotoxicity of 5-fluorouracil by two biochemical modulators. Anticancer Drugs 7:548–557
50. Suda Y, Kuwashima Y, Tanaka Y, Uchida K, Akazawa S (1999) Immunohistochemical detection of thymidylate synthase in advanced gastric cancer: a prognostic indicator in patients undergoing gastrectomy followed by adjuvant chemotherapy with 5-fluoropyrimidines. Anticancer Res 19:805–810
51. Suto A, Kubota T, Shimoyama Y, Ishibiki K, Abe O (1989) MTT assay with reference to the clinical effect of chemotherapy. J Surg Oncol 42:28–32
52. Terashima M, Irinoda T, Fujiwara H, Nakaya T, Takagane A, Abe K, Yonezawa H, Oyama K, Inaba T, Saito K, Takechi T, Fukushima M (2002) Roles of thymidylate synthase and dihydropyrimidine dehydrogenase in tumor progression and sensitivity to 5-fluorouracil in human gastric cancer. Anticancer Res 22:761–768
53. van de Velde CJ, Peeters KC (2003) The gastric cancer treatment controversy. J Clin Oncol 21:2234–2236
54. Vescio RA, Redfern CH, Nelson TJ, Ugoretz S, Stern PH, Hoffman RM (1987) In vivo-like drug responses of human tumors growing in three-dimensional gel-supported, primary culture. Proc Natl Acad Sci USA 86:2013–2017
55. Wohrer SS, Raderer M, Hejna M (2004) Palliative chemotherapy for advanced gastric cancer. Ann Oncol 15:1585–1595
56. Yeh KH, Shun CT, Chen CL, Lin JT, Lee WJ, Lee PH, Chen YC, Cheng AL (1998) High expression of thymidylate synthase is associated with the drug resistance of gastric carcinoma to high dose 5-fluorouracil-based systemic chemotherapy. Cancer 82:1626–1631
57. Yoshida M, Boku N, Ohtsu A, Muto M, Nagashima F, Yoshida S (2001) Combination chemotherapy of irinotecan plus cisplatin for advanced gastric cancer: efficacy and feasibility in clinical practice. Gastric Cancer 4:144–149