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• GASTRIC CANCER •

Correlation of thymidylate synthase, thymidine phosphorylase and dihydropyrimidine dehydrogenase with sensitivity of gastrointestinal cancer cells to 5-fluorouracil and 5-fluoro-2'-deoxyuridine

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Abstract

AIM: To determine the expression levels of three metabolic enzymes of fluoropyrimidines: thymidylate synthase (TS), thymidine phosphorylase (TP) and dihydropyrimidine dehydrogenase (DPD) in seven human gastrointestinal cancer cell lines, and to compare the enzyme levels with the sensitivity to 5-fluorouracil (5-FU) and 5-fluoro-2'-deoxyuridine (FdUrd).

METHODS: TS, TP and DPD mRNA levels were assessed by semi-quantitative RT-PCR, TP and DPD protein contents were measured by ELISA. Fifty percent inhibitory concentrations of growth (IC50), representing the sensitivity to drugs, were determined by MTT assay.

RESULTS: IC50 values ranged from 1.28 to 12.26 uM for 5-FU, and from 5.02 to 24.21 uM for FdUrd, respectively. Cell lines with lower DPD mRNA and protein levels tended to be more sensitive to 5-FU ($P<0.05$), but neither TS nor TP correlated with 5-FU IC50 ($P>0.05$). Only TS mRNA level was sharply related with FdUrd sensitivity ($P<0.05$), but TP and DPD were not ($P>0.05$). A correlation was found between mRNA and protein levels of DPD ($P<0.05$), but not TP ($P<0.05$).

CONCLUSION: DPD and TS enzyme levels may be useful indicators in predicting the antitumor activity of 5-FU or FdUrd, respectively.

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INTRODUCTION

The antimetabolite, 5-fluorouracil (5-FU), remains to be widely prescribed in the treatment of gastrointestinal carcinoma. Although it was originally synthesized 46 years ago, and the deoxyribonucleoside derivative of 5-FU, 5-fluoro-2'-deoxyuridine (FdUrd) is also applied in clinics through regional administration[1-3].

The response rate of gastrointestinal carcinoma to 5-FU as a single agent, however, is only 10%-30%, and differs greatly among patients[1,4]. So it is imperative to identify some indexes which could be applied to predict the efficacy of 5-FU in clinical settings. As a pyrimidine analog, 5-FU is metabolized *in vivo* similarly to uracil, and exerts its antitumor effects through anabolism, which is determined by the rate of catabolism. Thus, the expression level of genes coding for key enzymes in the metabolism within tumor cells may play a pivotal role in the sensitivity and efficacy of 5-FU[1,4,5].

The primary biochemical mechanism responsible for cytotoxicity of 5-FU and FdUrd is the formation of 5-fluorouridine monophosphate (FdUMP), which can bind tightly to and inhibit thymidylate synthase (TS) in the presence of 5, 10-methylene tetrahydrofolate (CH_2FH_4). TS catalyzes the reductive methylation of deoxyuridine-5'-monophosphate (dUMP) to deoxythymidine-5'-monophosphate (dTMP), which is the only pathway for *de novo* synthesis of dTMP, so the inbibition of TS by FdUMP disrupts intracellular nucleotide pools necessary for DNA synthesis[1,3]. As the main target of fluoropyrimidines, the expression level of TS is assumed to influence the response of chemotherapy, although the amount of TS is not unanimously recognized as a determinant factor of 5-FU sensitivity[6-8].

Thymidine phosphorylase (TP) is known to be elevated in tumors compared with surrounding normal tissue. When 5-FU is administered, it is anabolized to FdUMP by TP present in the tumor, and FdUrd can be converted to 5-FU by TP[9-11]. TP levels might affect the sensitivity of 5-FU, the transfection of TP cDNA into cancer cells increased their sensitivity to 5-FU[12]. The expression of TP was reported to be useful for predicting the efficacy and survival of fluoropyrimidine chemotherapy[13,14], but this tendency was not confirmed in a recent clinical trial of colorectal carcinoma[15]. The relationship between TP and the sensitivity of fluoropyrimidines needs to be further explored.

In contrast to anabolism of 5-FU, much less attention has been focused on its catabolism. In human, dihydropyrimidine dehydrogenase (DPD) is the initial and rate-limiting enzyme of 5-FU catabolism, 85% of an administered dose of 5-FU is degraded to inactive metabolites by DPD, with only 1-3% of the drug anabolized. While anabolism is essential for the antitumor activity of 5-FU, catabolism by indirectly controlling the availability of 5-FU for anabolism is a critical determinant of 5-FU cytotoxicity[4,5,16,17]. Several studies have shown a great interindividual difference in DPD activity, and suggested that DPD activity could be used as a predictive marker of 5-FU response[18-20].

We measured the expression levels of TS, DPD and TP on a panel of seven gastrointestinal cancer cell lines to probe the correlation between TS/DPD/TP and 5-FU or FdUrd sensitivity.

MATERIALS AND METHODS

Chemicals

5-FU was kindly provided by Faulding Pharmaceuticals Co,

FdUrd and MTT were obtained from Sigma-Aldrich Chemicals Co.

Cell culture

Seven cancer cell lines of human origin were adopted, including four gastric carcinoma cells (MKN45, SGC7901, MKN28 and AGS) and three colorectal carcinoma cells (SW1116, Lovo and HCT-8). Cells were routinely cultured in RPMI-1640 media (Gibco BRL), supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL), 100 u/ml penicillin and 100 ug/ml streptomycin in a humidified incubator at 37 ℃ with an atmosphere containing 5% CO_2.

Evaluation of 5-FU and FdUrd-induced cytoxicity by MTT assay

Cells were dispersed into 96-well microtitration plates, and the initial cell density was 5 000-10 000 cells per well, so that the cells were in a Log growth phase. Twenty-four hours after plating, the cells were exposed to a series of 10-fold dilutions of 5-FU or FdUrd (10^{-2}-10^{-9}M) for 72 hours. Each concentration was performed in quadruplicate, the percentage of growth inhibition was assessed by MTT assay, and determined according to the following equation: $[1-(T-T_0)/(C-T_0)]\times100\%$, where T is the absorbance of the experimental wells after 72 h of 5-FU or FdUrd exposure, and T_0 is that of background control with the same drug concentrations, C is that of cell control wells (without drug) after 72 h incubation. The 5-FU or FdUrd concentrations causing a 50% growth inhibition as compared to cell controls (IC50) were calculated by modified Kärbers method[21]: IC50=l $g^{-1}[X_k-i(\Sigma P-0.5)]$, in which X_k represents logarithm of the highest drug concentration, i is that of ratio of adjacent concentration, Σ P equals the sum of the percentage of growth inhibition at various concentrations, and 0.5 is a constant of experience. All experiments were repeated 4 times, from which we reported the mean and standard deviation of IC50.

Semi-quantification of TS/DPD/TP mRNA by RT-PCR

Total RNA from seven gastrointestinal cancer cell lines was extracted using TRIzol (Gibco BRL) and quantified by UV spectrophotometry. First-strand cDNA was synthesized from 1 μg of total RNA with oligo $(dT)_{15}$ primer and avian myeloblastosis virus reverse transcriptase using an RT-PCR kit (Promega) following the conditions of the manufacturer. PCR primers were designed based on the sequences of human TS/DPD/TP and GAPDH mRNA (internal standard), and the specificity of all primers was confirmed by DNA sequencing of the PCR products amplified with them (Table 1).

Table 1 Primers for TS, DPD, TP and GAPDH amplification

mRNA	Bases	Sequences(5'→3')	Product size (bp)
TS	613-632	accaaccctgacgacagaag	405
	998-1017	atgcggattgtacccttcaa	
DPD	1325-1344	tgttcggacagagcaagatg	400
	1705-1724	cttcaatccggccatttcta	
TP	390-408	aggagacctcggtgctgac	402
	772-791	tgagaatggaggctgtgatg	
GAPDH	109-127	gaaggtgaaggtcggagtc	226
	315-334	gaagatggtgatgggatttc	

TS/DPD/TS was co-amplified with GAPDH in 50 μl of PCR mixture containing 4 μg of cDNA template, 2.5 mM $MgCl_2$, 5 μl 10×buffer, 0.4 mM dNTP, 2.5 u Taq polymerase (Promega), 12.5pmol of each sense and anti-sense primer. The PCR profile of TS consisted of an initial 4 min denaturation at 95 ℃, followed by 25 PCR cycles (at 94 ℃ for 1 min, at 60 ℃ for 30 s, and at 72 ℃ for 1 min) and a final 7 min extension (33 cycles of amplification for DPD and 35 cycles for TP). The PCR products were separated by ethidium bromide-stained 2% agarose gel electrophoresis, the images were scanned and analyzed by densitometry using Fluro-s™ image software (Bio-Rad). The relative amount of mRNA was calculated by determining the product intensity ratio of TS/DPD/TP to GAPDH within the linear amplification range of PCR, and four separate experiments were repeated.

Protein contents of TP and DPD

Cell lines in a Log growth phase were harvested and washed twice by phosphate buffered saline (PBS, pH 7.4). After the last wash, cell pellets were resuspended in 500 μl PBS and lysed by a sonifier (pulses, 10 min), then the lysates were centrifuged at 13 000×g for 15 min, and the supernatants were carefully collected, the protein concentration of which were determined using a BCA protein assay reagent kit (Pierce).

The protein contents of DPD and TP in cell lines were determined by sandwich ELISA (Roche), according to the manufacturer' s instructions. Enzyme levels were expressed as U/mg protein, where one U of TP is an amount equivalent to 1 μg 5-FU generated in an hour, and one U of DPD is an amount equivalent to catabolizing 1pmol of 5-FU per minute.

Determination of population doubling time

As described before[22], 1-2×10^5 cells were cultured in a 25 ml flask containing 2.5 ml of RPMI-1640, and the number of cells per flask was counted every 24 hours for 7 days. When the cells were in a Log growth phase, the population doubling time (dt) was determined by the following formula: dt=l g2/l g $(Ct/C_0)\times t$, where t means the time between cell counts Ct and C_0, C_0 is the initial count, and Ct is the count after time *t*.

Statistics

Linear regression analysis and paired *t*-test were performed by SPSS software, $P<0.05$ was regarded as statistically significant.

RESULTS

Sensitivity of cell lines to 5-FU and FdUrd

Table 2 shows the parameters of interest for the whole cell line panel. After 72-hour drug exposure, the chemosensitivity of cell lines presented a marked difference, with IC50 values ranged from 1.28 to 12.26uM for 5-FU (9.57-fold), and from 5.02 to 24.21uM (4.83-fold) for FdUrd, respectively. The IC50 value of 5-FU was 2.8-fold lower than that of FdUrd ($P<0.01$), and there was no significant correlation between IC50 values of these two drugs among seven gastrointestinal cancer cell lines ($P>0.05$).

TS mRNA levels

TS mRNA was highly expressed in the seven cell lines, and the TS:GAPDH product intensity ratio varied from 0.84 to 2.69 (Figure 1A, Table 2). TS mRNA expression was significantly correlated with the sensitivity of cell lines to FdUrd ($r=0.81$, $P=0.028$), where low TS mRNA levels were associated with the high sensitivity to FdUrd (Figure 2), but the sensitivity to 5-FU was not influenced by TS mRNA levels.

DPD mRNA and protein levels

DPD mRNA expression was measurable in all cell lines but HCT-8 (Figure 1B). Although cDNA of DPD was amplified by PCR with 8 more cycles, DPD mRNA expression was much lower than TS expression, as shown in Table 2. DPD protein content, representing enzyme activity, ranged from 1.16 to

Table 2 Sensitivity to 5-FU or FdUrd and enzyme levels for cell lines

Cell line	IC50[a]		TS mRNA[b]	DPD		TP		Doubling time (hours)
	5-FU	FdUrd		mRNA[b]	protein[c]	mRNA[b]	protein[c]	
MKN45	12.26±2.13	16.85±2.28	1.13±0.22	0.57±0.17	10.13	0.72±0.13	5.11	22.9
SGC7901	8.97±1.55	12.45±1.46	1.04±0.17	0.50±0.09	9.13	0.59±0.06	1.78	25.8
MKN28	3.44±0.36	5.02±1.32	0.84±0.21	0.36±0.12	4.57	0.70±0.12	0.55	20.5
AGS	2.77±0.58	19.31±1.85	1.27±0.23	0.35±0.08	4.95	0.67±0.20	3.835	29.5
SW1116	5.45±0.47	24.21±3.26	2.69±0.36	0.47±0.11	5.99	0.57±0.11	2.60	22.2
Lovo	4.86±0.92	14.41±0.96	1.19±0.12	0.31±0.05	1.83	0.70±0.08	0.04	33.9
HCT-8	1.28±0.43	17.62±1.84	1.54±0.31	ND[d]	1.16	0.10±0.06	0.67	28.1

a: values of IC50 ($\bar{x}\pm s$), b: TS/DPD/TP mRNA levels, expressed as TS/DPD/TP:GAPDH product intensity ratio ($\bar{x}\pm s$), c: DPD/TP protein levels, in U/mg protein, d: not detectable.

10.13 U/mg protein (8.73-fold), and there was a statistically significant correlation between mRNA and protein level of DPD (r=0.88, P=0.009, Figure 3A).

Linear regression analysis showed that both mRNA (r=0.82, P=0.025) and protein level of DPD (r=0.88, P=0.009) were significantly correlated to the sensitivity to 5-FU (Figure 4). The greater the enzyme level was, the higher the IC50 of 5-FU. The most sensitive cell line (HCT-8) exhibited the lowest DPD mRNA and protein level, and the most resistant cell line (MKN45) had the greatest DPD mRNA and protein level. But the correlation between mRNA or protein level of DPD and the IC50 of FdUrd was not found (P>0.05).

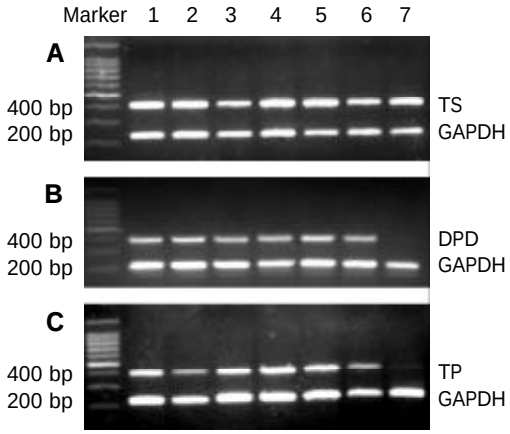

Figure 1 mRNA expression of seven gastrointestinal cancer cell lines by RT-PCR. (A) the bands of TS and GAPDH; (B) the bands of DPD and GAPDH; (C) the bands of TP and GAPDH (1-MKN45; 2-SGC7901; 3-MKN28; 4-AGS; 5-SW1116; 6-Lovo; 7-HCT-8). The relative amount of mRNA was expressed as the intensity ratio of TS/DPD/TP to GAPDH RT-PCR products, as showed in Table 2.

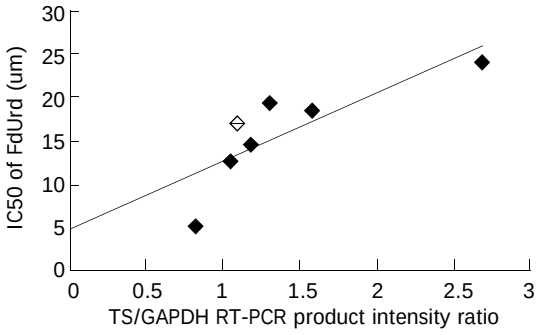

Figure 2 Linear regression for the sensitivity to FdUrd as a function of TS mRNA level (FdUrd-IC50=7.89TS mRNA+4.77, r=0.81, P=0.028). Scatter plot shows the corrlation between TS mRNA levels and IC50 of FdUrd.

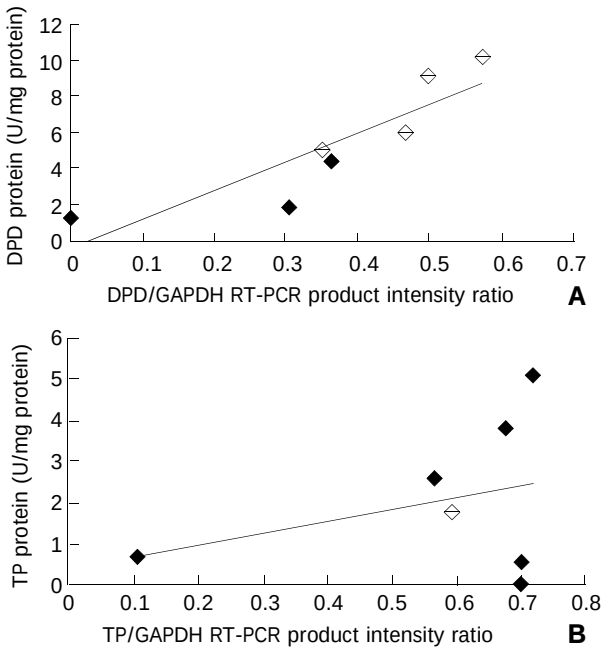

Figure 3 (A) Correlation between mRNA and protein level of DPD (r=0.88, P=0.009); (B) Correlation between mRNA and protein level of TP (r=0.33, P=0.466).

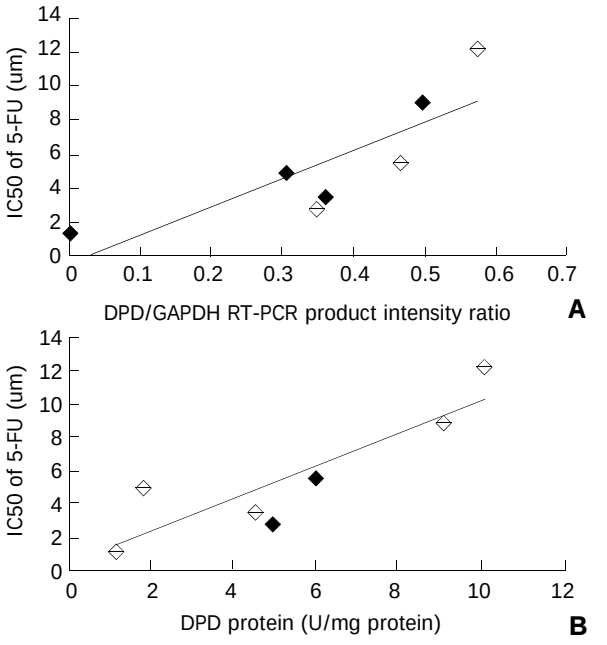

Figure 4 (A) Linear regression for the sensitivity to 5-FU as a function of DPD mRNA level (5-FU-IC50=16.77DPD mRNA-0.54, r=0.82, P=0.025). (B) Linear regression for the sensitivity to

5-FU as a function of of DPD protein level (5-FU-IC50=1.00DPD protein+0.20, r=0.88, P=0.009). Scatter plot shows the corrlation between DPD mRNA or protein levels and IC50 of 5-FU.

TP mRNA and protein levels

Both mRNA and protein of TP had lower expression levels compared to TS or DPD (Figrure 1C, Table 2). Figure 3B shows no correlation between TP/GAPDH RT-PCR product intensity ratio and TP protein level (r=0.33, P=0.466). Although it was expected that mRNA and protein level of TP would predict the sensitivity to 5-FU or FdUrd, no significant correlation was shown (P>0.05).

Doubling time of cell lines

The cell doubling time ranged from 20.5 (MKN28) to 33.9 hours (Lovo), and no correlation was demonstrated between the doubling time and IC50 of 5-FU or FdUrd. Cell doubling time did not correlate with either TS/DPD/TP mRNA or protein levels (P>0.05).

DISCUSSION

There is a high prevalence of gastrointestinal cancer, including gastric and colorectal cancer in China, but most of the diagnoses are established at advanced stages, when chemotherapy is regarded as one of the main treatments. At present, 5-FU has been considered as the principal component of chemotherapy regimen for gastrointestinal cancer in both advanced disease and the adjuvant setting[2,23], and FdUrd is also available commercially for chemotherapy, especially for intraarterial and intracavitary infusion. But the response of gastrointestinal cancer to fluoropyrimidines was still unsatisfactory and their efficacy varied greatly among individuals, so how to enhance the chemotherapeutic response of 5-FU has become a very interesting subject[24,25]. The identification of predicative markers of chemosensitivity through pharmacogenomics means could clarify which subset of patients might benefit, and enable clinicians to design individualized chemotherapy regimens[26].

It has been demonstrated in our study that the sensitivity of seven gastrointestinal cancer cell lines to 5-FU corresponded to DPD mRNA or DPD protein levels linearly. The cancer cells with lower DPD levels were more sensitive to 5-FU, and DPD mRNA was even undetectable in the most sensitive cell line HCT-8. But no correlation between TS or TP levels and sensitivity to 5-FU was found in the present study, in view of the known relevance of TS or TP as a determinant of response of 5-FU in some preclinical and clinical studies[6,7,27], therefore the results obtained from the study of seven cancer cell lines must be interpreted scrupulously.

The low expression levels of TP could partly explain our finding, besides inhibition of TS activity through anabolism to FdUMP by TP catalyzation. 5-FU exerted its antitumor activity by converting to FdUTP and FUTP for incorporating into DNA and RNA, respectively[1], thereby interfering with their normal structure and function, so there was no correlation between TS or TP levels and 5-FU sensitivity. On the other hand, Nita *et al*[28] also observed DPD expression and predicted 5-FU sensitivity in colorectal cancer cell lines. Etienne *et al*[29] confirmed this association in tumor biopsy tissues from the patients of head and neck cancers, but no relationship was demonstrated between TS activity and 5-FU response, either. Our findings, similar to these results, suggested that most of 5-FU within insensitive tumor cells was quickly catabolized by a higher DPD level, which regulated the amount of 5-FU available for anabolism, thereby affecting its cytotoxicity. In addition, these results suggested that DPD inhibitors, such as eniluracil[30], uracil[31], 5-chloro-2,4-dihydroxypyrimidine (CDHP)[32], and BOF-A2[33], might be used as a novel type of biochemical modulators for elevating the antitumor activity of 5-FU.

It has been indicated in our work that TS may contribute greatly to the sensitivity of FdUrd, and the higher the TS mRNA levels, the higher the IC50 of FdUrd. Because both TP mRNA and protein levels of this panel of cell lines were rather low, and only little amount of FdUrd could be converted to 5-FU, the suppression of TS through conversion to FdUMP was the principal mechanism of action of FdUrd[1,10,11]. Therefore TS may serve as a predictive marker of FdUrd. Given the metabolic characteristics of FdUrd, it is comprehensible that there was no correlation between TP or DPD levels and FdUrd sensitivity.

Grem *et al*[8] found cell doubling time was a potentially important variable in drug sensitivity, and cell lines with faster doubling times tended to have higher TS activities, but we did not observe the similar trend. In their study, the cytotoxicity was determined by MTT assay after 48 hours of drug exposure, whilst we did it after 72 hours by the same protocol. Since the cell doubling time, ranging from 20.5 to 33.9 hours, was relatively shorter in our study than Grem' s, the influence of doubling time on fluoropyrimidines seemed to be much weaker.

We used semi-quantitative RT-PCR and ELISA to determine DPD/TP mRNA and protein level, respectively, and found a statistically significant correlation between mRNA level and protein content of DPD. Compared to traditional radioisotopic enzyme acitivity assay[34], RT-PCR and ELISA were less laborious, less expensive, and more feasible in most laboratories. In general, even small amounts ($\leqslant$100 mg) of tissues were enough for them, and the correlation between DPD activity and mRNA or protein levels has been already confirmed in several preclinical and clinical trials[18,35-38]. But there was no such a correlation in TP, a close look showed three cell lines had extremely low TP protein contents, and even TP enzyme level of MKN45, the highest one, was only 5.11 U/mg protein. A recent clinical trial disclosed that the range of TP level in primary colorectal cancer was 13.8-196.0 U/mg protein[36], which might explain this apparent discrepancy between mRNA and protein level. Griffiths also pointed out by immunohistochemistry that the predominant cells positive for TP were macrophages and other stroma cells within tumor tissues, and the activation of fluoropyrimidines in human might rely on the paracrine of TP by these stroma cells, but not tumor cells[10]. As the low expression of TP could directly influence the sensitivity of these seven cell lines to fluoropyrimidines, the role of TP in gastrointestinal cancer cells sensitive to 5-FU and FdUrd needs to be more deeply explored.

In summary, we found that DPD and TS were potential indicators in predicting tumor sensitivity to 5-FU and FdUrd. However, the conclusions were drawn from the limited *in vitro* experiment. This study was merely a first step toward the goal of individualized fluoropyrimidine chemotherapy for gastrointestinal cancers. Controlled, prospective clinical trials are required to confirm our results and to establish the advantage of pre-treatment tumor biopsy for TS/DPD screening, which permits a more rational decision on whether to proceed a fluoropyrimidine-based therapy as first-line treatment. So patients who are unlikely to respond may spare unnecessary toxicity and can be treated with alternative drugs such as CPT-11, oxaliplatin, or with potent biochemical modulators of fluopyrimidine.

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