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ORIGINAL ARTICLE

Expression level of thymidylate synthase mRNA reflects 5-fluorouracil sensitivity with low dose and long duration in primary colorectal cancer

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Abstract
Objectives To investigate the prognostic marker for the adjuvant chemotherapy of primary colorectal carcinoma.
Methods Primary colorectal cancer tissue from 24 patients was investigated to evaluate the relationship between the mRNA expression level of several 5-fluorouracil (5-FU)-related metabolic enzymes (thymidylate synthase, TS; dihydropyrimidine dehydrogenase, DPD; and thymidine phosphorylase, TP) and chemosensitivity to two different 5-FU doses and duration (1: 5-FU concentration 1.0 μg/mL (7.68 μM), 24 h exposure and 2: 5-FU concentration 0.3 μg/mL (2.30 μM), 144 h exposure). Chemosensitivity and mRNA expression levels were measured using collagen gel droplet embedded culture drug sensitivity tests and real-time quantitative reverse transcription-polymerase chain reaction. Clinicopathological features and chemosensitivity were also compared.
Results The TS mRNA expression level was significantly higher in the 5-FU resistant group (T/C > 60%) compared with the 5-FU sensitive group (T/C < 60%) in both 5-FU regimens (1: 5.03 ± 0.92 vs. 1.58 ± 0.76, $p < 0.01$, 2: 4.88 ± 0.91 vs. 0.96 ± 0.20, $p < 0.001$). The group with the higher TS mRNA expression level (>3.83, the average) were more resistant to both 5-FU regimens than those with lower TS mRNA (<3.83) (1: T/C = 80 vs. 66%, $p = 0.11$, 2: T/C = 89 vs. 64%, $p < 0.005$). The TS mRNA expression level inversely correlated with the sensitivity to the latter 5-FU regimen ($R = 0.577$, $p < 0.01$). There were no relationships between chemosensitivity to 5-FU and the mRNA expression level of DPD and TP and clinicopathological factors.
Conclusions The TS mRNA expression level might be a good marker of chemosensitivity to 5-FU in primary colorectal cancer, especially the sensitivity to low dose 5-FU with a long duration.

Keywords Colorectal cancer · 5-FU metabolic enzymes · Chemosensitivity · Low dose 5-FU

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Introduction

For about 50 years, 5-fluorouracil (5-FU) has been used to treat various cancers and is accepted worldwide as a first-line anticancer drug for colorectal carcinoma [1]. Although the response rate as a single agent is usually less than 20%, biochemical modulation by leucovorin (LV) enhances the anti-cancer effects of 5-FU and increased its response rate to 20–30% [2]. Recently, irinotecan or oxaliplatin showed a higher response rate of nearly 50% when it was used with 5-FU and LV for the treatment of metastatic colorectal cancer [3, 4]. These facts suggest that it is important to understand the mechanism of the anti-tumor effect of 5-FU and to determine the factors that affect 5-FU sensitivity in order to improve or predict the response rate. When 5-FU is predicted as not being effective, another anti-cancer drug should be selected or combined with 5-FU.

There are some 5-FU regimens that are used to treat colorectal cancer. The most accepted ones are the 5-FU i.v. bolus + low dose LV (Mayo regimen [5]), 5-FU i.v. bolus + high dose LV (RPMI regimen [6]) or 5-FU i.v. bolus + high dose LV + 5-FU continuous i.v. (de Gramont regimen [7]). Recently, oral drugs such as uracil/tegafur + LV

were approved and as effective as 5-FU + LV i.v. regimens in patients with previously untreated metastatic colorectal cancer [8, 9]. The cell-killing mechanism of 5-FU was indicated to depend on the cell cycle and thus the exposure time [10]. Therefore, it is important to compare the sensitivity to different 5-FU doses and duration because there might be some difference between them.

Several 5-FU-related metabolic enzymes have been studied and thought to correlate with the sensitivity to 5-FU [11–16]. Two main modes of action have been proposed for 5-FU through its active metabolites, 5-fluoro-2•deoxyuridine-5•-monophosphate (FdUMP) and 5-fluoro-uridine-5•-triphosphate (FUTP) [16]. FdUMP suppresses thymidylate synthase (TS) by forming a covalent ternary complex with 5, 10-methylenetetrahydrofolate, and thus indirectly affects DNA synthesis [17]. Hence, the expression levels of TS have been thought to correlate with sensitivity to 5-FU. This pathway has been pointed out to depend on time, long continuous exposure conditions [18]. 5-Fluoro-uridine-5•-triphosphate is incorporated into cellular RNA, resulting in RNA dysfunction [19]. This pathway has been indicated to depend on high 5-FU doses with short duration [18]. 5-Fluorouracil (5-FU) is catabolized to 2-fluoro-βalanine in the liver and other tissues by dihydropyrimidine dehydrogenase (DPD). The DPD, which is the first and rate-limiting enzyme, rapidly eliminates over 85% of the administered 5-FU [20,21]. Since 5-FU is catabolised quickly by DPD, and DPD shows a circadian pattern, the concentration of 5-FU is predicted to show an inverse circadian pattern [22]. Recently, this catabolism has been thought to have a meaningful role in the resistance to 5-FU. To activate 5-FU into its nucleotides, phosphorylation pathways and enzymes are known [23]. Thymidylate phosphorylase (TP) metabolizes 5-FU indirectly to FdUMP by 5-fluoro-2•-deoxyuridine (FUdR).

In gastric and colorectal cancers, high expression levels of TS mRNA and protein were correlated with unresponsiveness to 5-FU [11]. In colorectal cancers, low levels of DPD mRNA expression were seen in all of the 5-FU responders compared with much higher levels in the 5-FU nonresponders [12]. A few studies demonstrated that high levels of TP mRNA correlated with the resistance to 5-FU [12, 13].

In this study, we investigated the correlation between the expression levels of TS, DPD, TP mRNA and the sensitivity to two different 5-FU doses and duration in primary colorectal cancer for the help of adjuvant 5-FU therapy. The data from this study indicated that the expression level of TS mRNA might reflect chemosensitivity to 5-FU in primary colorectal cancer, especially the sensitivity to low dose 5-FU with a long duration.

Materials and methods

Collection of tumor tissues

Tumor tissues were collected from surgically resected primary colorectal cancer from 24 patients between September 2001 and October 2004 at the Department of Surgery, Shiga University of Medical Science, Shiga, Japan. The clinicopathological features of the 24 patients who partici-

Table 1 Patients and tumor characteristics

Age (average)	64.1
Sex	
M	19
F	5
Histology	
Wel	9
Mod	11
Por	2
Sig	1
Muc	1
T	
T1	2
T2	5
T3	15
T4	2
N	
N0	14
N1	4
N2	6
M	
M0	22
M1	2
TNM stage[a]	
I	4
II	7
III	11
IV	2
Site	
R	8
L	9
Rectum	7
Sensitivity[b]	
24 h	71.7
144 h	75.9

[a] TNM were categorized according to TNM 2002 classification in colorectal carcinoma

[b] Sensitivity; 24 h: 5-FU concentration 1.0 μg/mL (7.68 μM), 24 h exposure, 144 h: 5-FU concentration 0.3 μg/mL (2.30 μM), 144 h exposure

pated in this study are listed in Table 1 Briefly, seven patients had rectal cancer, five patients were women, the average age was 64 years. None of the patients had received preoperative treatment.

The samples were immediately cut into pieces. One portion was snap frozen in liquid nitrogen and stored at −80°C until the extraction of RNA, and the other portion was preserved in culture medium at 4°C until sensitivity tests were performed. This study was approved by the Institutional Review Board of Shiga University of Medical Science. All patients gave written consent.

Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using Trizol Reagent (Invitrogen, Inc., Carlsbad, CA) and an RNeasy mini kit (Qiagen, Inc., Chatsworth, CA) and DNA-free (Ambion, Inc., Austin, TX) according to the manufacturer's instructions. The purity and amount of total RNA were estimated spectrophotometrically by measuring the absorbance of an aliquot at 260 and 280 nm. The integrity of the rRNA bands was checked by agarose gel electrophoresis. One microgram of the prepared total RNA was reverse-transcribed to synthesize cDNA using the oligo$(dT)_{12–18}$ primer and Superscript II (Invitrogen) according to the manufacturer's instructions. Expression levels of TS, DPD and TP mRNA were measured by real-time quantitative RT-PCR (LightCycler; Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. The PCR reaction mixture for TS, DPD and TP mRNA consisted of 500 nM of each primer, 4 mM $MgCl_2$ and 1 μL LightCycler-FastStart DNA Master SYBR Green I (Roche Diagnostics GmbH) to a final volume of 20 μL. The sequences and annealing temperatures were as follows: DPD: 5′-TGTTC GGACAGAGCA AGATG-3′,5′-TAGAAATGGCCGGAT TGAAG-3′, 55°C; and TP: 5′-CCTGCGGACGGAATC CT-3′, 5′-AGCCTGCCACTCATCACAGC-3′, 60°C, TS: 5′-CACACTTTGGGAGATGCACA-3′, 5′-CTTTGAAA GCACCCTAAACAGCCAT-3′, 55°C followed by 40 cycles. The GAPDH Primer Set was obtained from Search LC (GmbH, Heidelberg, Germany) and used for the internal control.

Collagen gel droplet embedded culture drug sensitivity test (CD-DST)

Chemosensitivity to 5-FU was analyzed by using the CD-DST method according to the manufacturer's instructions [24, 25]. Briefly, each sample was treated with Dispersion Enzyme Cocktail EZ (Nitta Gelatin, Inc., Osaka, Japan). The cell suspension obtained was inoculated into collagen-coated flasks (CG-flask, Nitta Gelatin, Inc.) and cultured in preculture medium (PCM-1) at 37°C overnight. Collagen gel was digested with Dispersion Enzyme Cocktail EZ, and viable cancer cells were obtained. The prepared cancer cell suspension was added to a collagen solution (Collagen Gel Culture Kit, Nitta Gelatin, Inc.) with the final density being 1×10^5 cells/mL. Three drops of the collagen-cell mixture (30 μL/droplet) were placed in each well of six-well plates on ice and allowed to gel at 37°C in a CO_2 incubator. DF medium containing 10% fetal bovine serum was overlaid on each well 1 h later and incubated overnight.

The 5-FU was purchased from Kyowa Hakko Kogyo, Co., Ltd. (Tokyo, Japan). We performed two 5-FU regimens. (1) 5-FU was added to the medium at a final concentration of 1.0 μg/mL (7.68 μM), and the plates were incubated for 24 h. After removal of the medium containing 5-FU, each well was rinsed twice with 3 mL of Hank's balanced salt solution, overlaid with 4 mL of PCM-2 medium (serum-free medium, Nitta Gelatin, Inc.), and incubated for an additional 7 days. (2) 5-FU was added to the medium at a final concentration of 0.3 μg/mL (2.30 μM), and the plates were incubated for 144 h. After removal of the medium containing 5-FU, each well was rinsed twice with 3 mL of Hank's balanced salt solution, overlaid with 4 mL of PCM-2 medium (serum-free medium, Nitta Gelatin, Inc.), and incubated for an additional 1 day. At the end of the incubation, neutral red was added to each well at a final concentration of 50 μg/mL and colonies of cancer cells in the collagen gel droplets were stained for 2 h. Each collagen droplet was fixed with 10% neutral-buffered formalin, washed in water, air-dried and quantified using image analysis. The growth rates of control incubations were calculated as the image density on day 7 divided by the image density on day 1. Cases with growth rates greater than 0.8 were regarded as evaluable cases. The in vitro sensitivity was expressed as the percentage T/C ratio, where T was the image optical density of the treated group and C that of the control.

Statistical analysis

Student's t test was used to compare the sensitivity to 5-FU and each mRNA expression level of primary colorectal cancer. Linear regression analysis was used to evaluate the correlation between the sensitivity to 5-FU and mRNA expression level of 5-FU-related metabolic enzymes. Correlation between the clinicopathological factors and 5-FU sensitivity were analyzed using the Pearson χ^2 test. Statistical significance was established at the $p < 0.05$ level for each analysis.

Results

The quality of mRNA isolated from 24 patients could be used for further RT-PCR analysis. Chemosensitivity to two

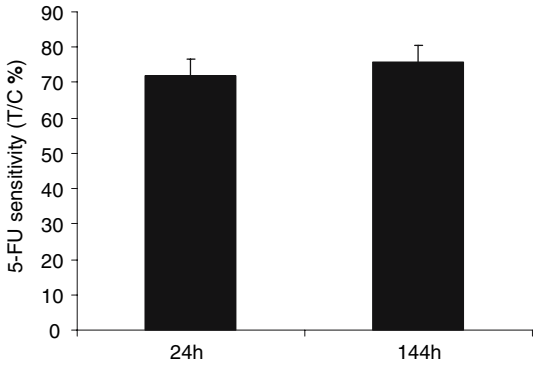

Fig. 1 Sensitivity to 5-FU measured using CD-DST method [24 h: 5-FU concentration 1.0 μg/mL (7.68 μM), 24 h exposure, 144 h: 5-FU concentration 0.3 μg/mL (2.30 μM), 144 h exposure] of colorectal cancer from 24 patients (average ± SE, 24 h: 71.7 ± 4.74%, 144 h: 75.9 ± 4.68%, n.s.)

different 5-FU regimens (1: 5-FU concentration 1.0 μg/mL (7.68 μM), 24 h exposure, 2: 5-FU concentration 0.3 μg/mL (2.30 μM), 144 h exposure) were measured using the CD-DST method. The data from 23 and 21 patients each could be analyzed. The sensitivities to 5-FU (T/C %) were 71.7 ± 4.74% and 75.9 ± 4.68%, respectively (average ± SE, Fig. 1).

The TS mRNA expression level was significantly higher in the 5-FU resistant group (1: $n = 16$, 2: $n = 17$, T/C > 60%) compared with the 5-FU sensitive group (1: $n = 7$, 2: $n = 4$, T/C < 60%) in both 5-FU regimens (average ± SE, 1: 5.03 ± 0.92 vs. 1.58 ± 0.76, $p < 0.01$, Fig. 2a, 2: 4.88 ± 0.91 vs. 0.96 ± 0.20, $p < 0.001$, Fig. 2b). No significant changes of the mRNA expression level of DPD and TP were detected between the resistant group and sensitive group in both regimens. (DPD—1: 2.17 ± 0.66

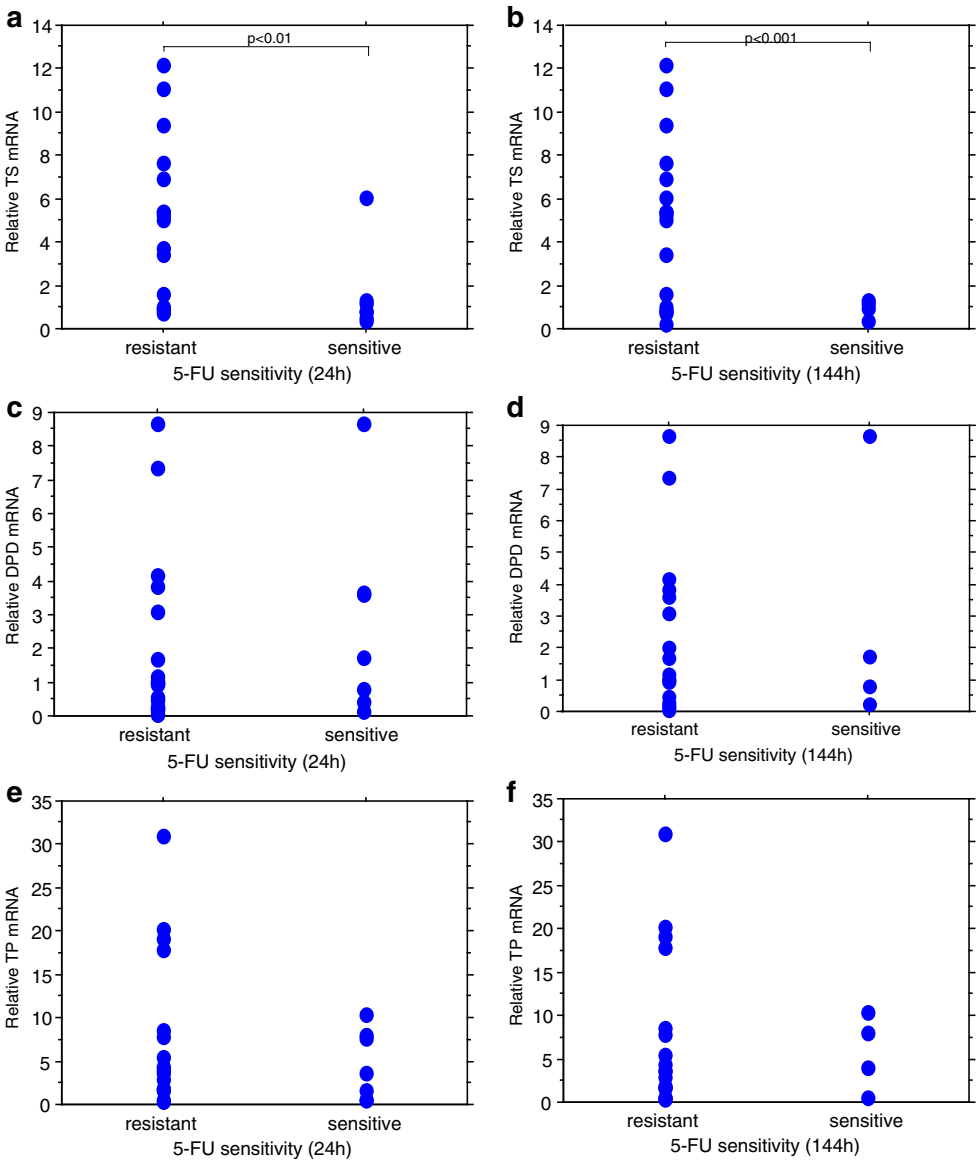

Fig. 2 The expression level of TS, DPD and TP mRNA in 24 colorectal cancers (**a**–**f**). The expression level of TS mRNA was significantly higher in the 5-FU resistant group (T/C > 60%) compared with the 5-FU sensitive group (T/C < 60%) in both 5-FU regimens (1: 5.03 ± 0.92 vs. 1.58 ± 0.76, $p < 0.01$, **a**, 2: 4.88 ± 0.91 vs. 0.96 ± 0.20, $p < 0.001$, **b**)

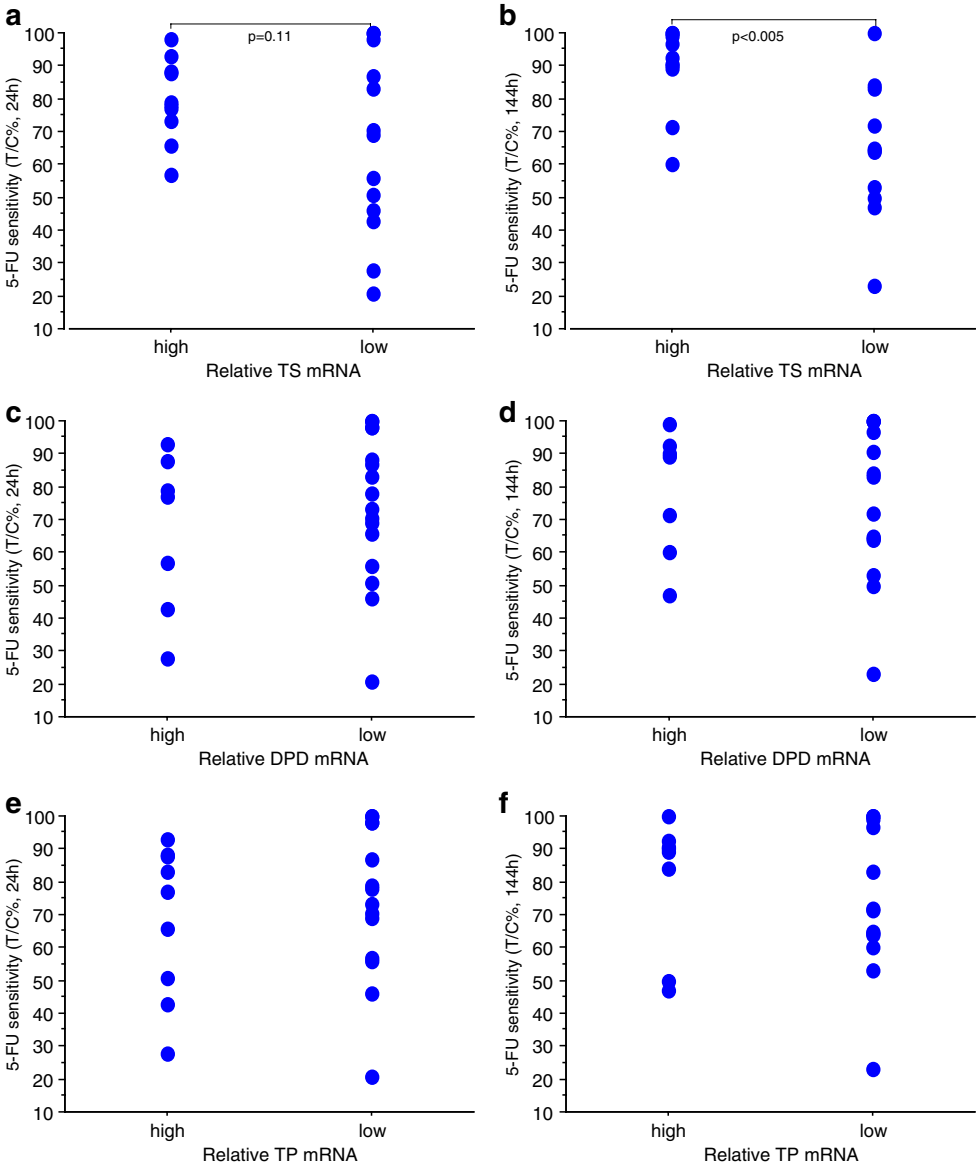

Fig. 3 Sensitivity to 5-FU (**a**–**f**) of 24 colorectal cancers. The group with the higher TS mRNA expression level (>3.83, the average) was more resistant to both 5-FU regimens than the one with the lower TS mRNA (< 3.83) in both regimens (1: T/C = 80 vs. 66%, $p = 0.11$, **a**, 2: T/C = 89 vs. 64%, $p < 0.005$, **b**)

vs. 2.72 ± 1.13, n.s., Fig. 2c, 2: 2.36 ± 0.58 vs. 2.85 ± 1.96, n.s., Fig. 2d, TP—1: 8.17 ± 2.25 vs. 4.63 ± 1.51, n.s., Fig. 2e, 2: 7.72 ± 2.16 vs. 5.69 ± 2.16, n.s., Fig. 2f)

Two groups were divided by the average of each mRNA expression level. The group with the higher TS mRNA expression level (>3.83, the average, 1: $n = 10$, 2: $n = 10$) were more resistant to both 5-FU regimens than that with the lower TS mRNA (<3.83, 1: $n = 13$, 2: $n = 11$) (1: T/C = 79.7 vs. 65.6%, $p = 0.11$, Fig. 3a, 2: T/C = 88.9% vs. 64.1%, $p < 0.005$, Fig. 3b). No significant differences of 5-FU sensitivity were found between the group with higher DPD (>2.31, the average, 1: $n = 7$, 2: $n = 7$) or TP (>6.76, the average, 1: $n = 9$, 2: $n = 8$) mRNA expression level and the one with the lower level (DPD < 2.31, 1: $n = 16$, 2: $n = 14$, TP < 6.76, 1: $n = 14$, 2: $n = 13$) in both regimens (DPD—1: T/C = 64.4 vs. 74.1%, n.s., Fig. 3c, 2: T/C = 78.4 vs. 74.7%, n.s., Fig. 3d, TP—1: T/C = 63.5 vs. 73.8%, n.s., Fig. 3e, 2: T/C = 80.4 vs. 73.2%, n.s., Fig. 3f). The TS mRNA expression level inversely correlated with the sensitivity to the latter 5-FU regimen ($R = 0.577$, $p < 0.01$, Fig. 4b).

No correlation was found between the clinicopathological factors and both 5-FU sensitivities (Table 2).

Discussion

The dose and duration of 5-FU (1.0 μg/mL (7.68 μM), 24 h exposure) for CD-DST was followed by several previous reports [24, 25]. The plasma peak concentration (C max) was determined 10 μg/ml and when the dose was 1/10 C max and the duration was 24 h, the anticancer effect of

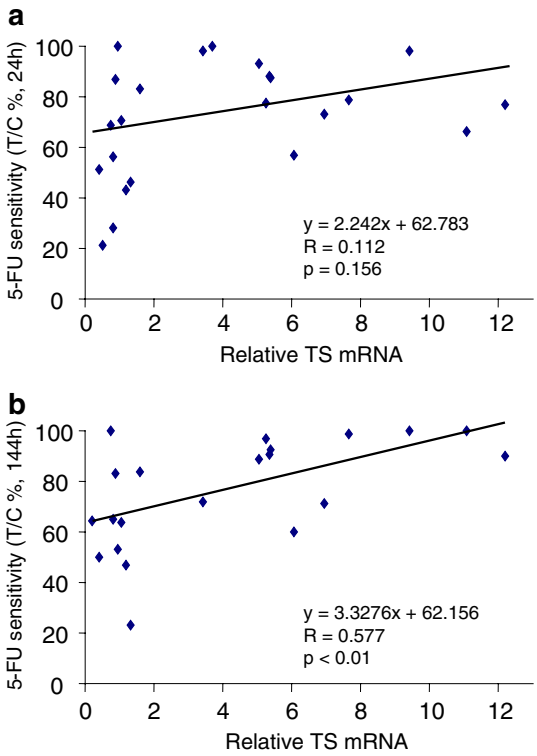

Fig. 4 Correlation between the expression level of TS mRNA and sensitivity to 5-FU (**a**, **b**) of colorectal cancer. The expression level of TS mRNA was significantly inversely correlated with the sensitivity to the 5-FU (5-FU concentration 0.3 μg/mL (2.30 μM), 144 h exposure) (**b**)

5-FU in the test was shown to correlate with response rates of clinical materials and nude mice model in vivo [26, 27]. It was indicated the cell-kill mechanism of 5-FU dependent on the cell cycle and 5-FU was a time dependent anticancer agent. For this reason, long-term frequent administration of divided doses, continuous infusion, or intermittent administration have been recommended [28]. In this study, we compared two different doses and duration time in vitro, one was the standard dose and duration of 5-FU (1.0 μg/mL (7.68 μM), 24 h exposure) and the other was low dose and long duration of 5-FU (0.3 μg/mL (2.30 μM), 144 h exposure). However, there were no differences between their 5-FU sensitivity.

Several reports have indicated that TS expression was significantly related to the response to 5-FU in gastric and colorectal cancer patients and that high intratumoral expression of TS mRNA or protein was one cause of resistance to 5-FU [11]. The TS gene expression had been demonstrated that it did not vary within the tumor specimens and that there was no evidence of significant tumor heterogeneity [29]. Hence there have been many reports that show expression level of TS mRNA could be a good marker for 5-FU sensitivity in addition to its merit: it could be measured from low amounts of materials. In the present study, the expression levels of TS mRNA were significantly higher in primary colorectal cancer, which is resistant to both different 5-FU doses and duration. However, the level was significantly inversely correlated with low concentration of 5-FU with longer duration. In Japan, oral 5-FU drugs, such as UFT, doxifluridine, capecitabine and S-1, are recognized and frequently used [30]. Uracil/tegafur + oral LV was reported to have an equivalent survival compared with Mayo regimens in metastatic colorectal cancer [8, 9]. Recently, 5-FU administration by such oral drugs was indicated to be as effective as continuous 5-FU infusion for adjuvant therapy of colon cancer [31]. Low concentrations of 5-FU have been indicated to exert a DNA-directed action with long exposure time [10]. Our study supports that the TS mRNA expression level might be a good prognostic marker in such oral treatment, which uses low dose 5-FU with a long duration as adjuvant therapy of primary colorectal cancer. In this study, we could realize that 5-FU plus LV or UFT plus LV would be a better adjuvant therapy because a high expression level of TS mRNA correlated with resistance to 5-FU, and LV, which was a mixture of stereoisomers ([6R,S]-5-formyltetrzhydrofolate), could increase the intracellular concentration of 5–10 methylene tetrahydrofolate (CH_2FH_4), and stabilize the ternary complex that inhibits TS.

Several studies reported that 5-FU catabolism by DPD was a probable determining factor for resistance to 5-FU [12, 16, 23] and that the expression level of TP correlated with resistance to 5-FU [12, 13]. However, results from the present study did not correspond with previous reports that tumors with higher expression levels of DPD or TP mRNA showed low sensitivity to 5-FU for colorectal cancer [12]. We chose primary colorectal cancer to measure the mRNA expression level of 5-FU-related metabolic enzymes and chemosensitivity for the help of the adjuvant therapy. DPD or TP mRNA expression was shown to change between primary and metastatic colorectal cancer [32], and several reports state that not in primary but in metastatic colorectal cancer DPD, TP and TS affect the response rate or chemosensitivity to 5-FU [12, 13]. As for primary colorectal cancer, only TS might be a strong affector to 5-FU sensitivity and DPD or TP might not.

Since the environment changes between in vitro and in vivo, this may influence gene expression. The advantage of CD-DST from other in vitro methods is that cells are cultured in the three-dimensional collagen gel droplets which might reflect better in vivo circumstances. Lung cancer cells grew in collagen gels with a three-dimensional spherical morphology, which mimics their in vivo characteristics [24, 25].

We conclude that TS is a good marker for chemosemsitivity to 5-FU in primary colorectal cancer, especially the

Table 2 5-FU sensitivity and clinicopathological characteristics

	Sensitivity (24 h)<60	Sensitivity (24 h)>60		Sensitivity (144 h)<60	Sensitivity (144 h)>60	
M	6	12	n.s.	3	13	n.s.
F	1	4		1	4	
Age	60.3	65.9	n.s.	68.8	63.1	n.s.
R	3	5	n.s.	1	5	n.s.
L	3	6		2	6	
Rectum	1	5		1	6	
T1	1	1	n.s.	0	1	n.s.
T2	1	3		0	4	
T3	4	11		4	10	
T4	1	1		0	2	
N0	4	9	n.s.	3	9	n.s.
N1,2	3	7		1	8	
M0	7	14	n.s.	4	15	n.s.
M1	0	2		0	2	
I	1	2	n.s.	1	3	n.s.
II	2	5		2	4	
III	4	7		1	8	
IV	0	2		0	2	
wel	2	6	n.s.	2	7	n.s.
mod	3	8		3	6	
por	2	0		0	1	
Sig	0	1		0	1	
muc	0	1		0	1	

Sensitivity; 24 h: 5-FU concentration 1.0 μg/mL (7.68 μM), 24 h exposure, 144 h: 5-FU concentration 0.3 μg/mL (2.30 μM), 144 h exposure

n.s., not significant

sensitivity to low dose 5-FU with a long duration. By using CD-DST method or measuring TS mRNA expression, we might be able to obtain more information about adjuvant 5-FU therapy such as continuous infusion or oral drugs after the resection of primary colorectal cancer. When 5-FU is predicted to not be effective, other anti-cancer drugs would be better to be selected or combined with 5-FU.

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