Combination of Dihydropyrimidine Dehydrogenase and Thymidylate Synthase Gene Expressions in Primary Tumors as Predictive Parameters for the Efficacy of Fluoropyrimidine-based Chemotherapy for Metastatic Colorectal Cancer

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ABSTRACT

Dihydropyrimidine dehydrogenase (DPD) and thymidylate synthase (TS) gene expressions in metastatic colorectal cancer have been reported to be predictive parameters for the efficacy of fluoropyrimidine-based chemotherapy. In this study, we investigated the association between both DPD and TS expressions in primary colorectal tumor and the antitumor effect in patients with metastatic colorectal cancer when treated with a fluoropyrimidine-based protocol. DPD and TS expressions were measured by reverse transcription-PCR in surgically resected materials of primary colorectal tumors from 37 patients who went on to receive oral treatment of uracil and tegafur and leucovorin for either synchronous or metachronous metastatic diseases. Relative mRNA amounts of DPD or TS were expressed as the ratios of targeted gene to glyceraldehyde-3-phosphate dehydrogenase reverse transcription-PCR products. Median values of DPD mRNA expressions were 0.30 and 0.65 for responding tumors and nonresponding ones, respectively, with a statistical significance ($P < 0.0001$). No responding tumor had a DPD mRNA expression ≥ 0.5. A total of 19 tumors had low DPD mRNA expressions of <0.5, and 63% of them showed response. There was no responding tumor with both high DPD and high TS (TS mRNA expression ≥ 1.0). However, the response rate was 75% in tumors with both low DPD and low TS. The median survival time was 16.3 months in patients with both low DPD and low TS *versus* 8.4 months in patients with high DPD or high TS mRNA expression. In conclusion, the combination of DPD and TS mRNA expressions in the primary tumor might be useful as predictive parameters for the efficacy of fluoropyrimidine-based chemotherapy for metastatic colorectal cancer.

INTRODUCTION

5-FU[2] is an important anticancer agent that is widely used in the treatment of colorectal cancers (1). 5-FU is catabolized to 2-fluoro-β-alamine by the first and rate-limiting enzyme, DPD (2). The main mode of action of 5-FU is thought to be through its active metabolite, FdUMP (3, 4). FdUMP suppresses TS, an essential DNA synthetic enzyme that catalyzes the methylation of dUMP to dTMP. FdUMP and TS form covalent ternary complexes with 5,10-methylenetetrahydrofolate that subsequently inhibit DNA synthases (3, 4). Pharmacogenetic variability in these 5-FU-related enzymes, such as DPD and TS, might be a major determinant of variations in the outcome for colorectal cancer patients treated with 5-FU.

DPD activity *in vitro* in tumor cells is significantly related to 5-FU sensitivity: the lower the enzymatic activity, the greater the cytotoxicity (5). Recent studies in human cancer xenografts demonstrated that the efficacy of fluorouracil correlated very well with DPD enzyme activity (6). The role of tumoral DPD activity was recently evaluated in the clinical setting. For head and neck cancer patients, DPD activity was detectable in all tumor samples. Patients with complete response to 5-FU-based induction chemotherapy exhibited lower tumoral DPD activities than partial or nonresponding patients (7).

The use of biochemical assays is often not practical, particularly with the unavailability of sufficient clinical samples (*e.g.*, needle biopsies). An alternative approach was described recently using molecular methodology (*i.e.*, quantification of DPD mRNA by RT-PCR). We previously described that DPD mRNA levels correlated with enzyme activities in human colorectal cancer samples (8). Ishikawa *et al.* (6) reported that high DPD mRNA was associated with low sensitivity to 5-FU in human tumor xenografts in nude mice. In the clinical setting, Danenberg *et al.* (9) described an inverse relationship between DPD mRNA expression in metastatic tumors and the antitumor effect of 5-FU in colorectal cancer.

Table 1 Patient characteristics

No. of patients	37
Age (yr)	
Median	62
Range	38–80
Sex	
Male/Female	24/13
Primary tumor	
Colon/Rectum	27/10
Adjuvant chemotherapy	
Yes/No	20/17
Performance status	
0/1/2	25/10/2
Metastasis	
Synchronous/Metachronous	9/28
Metastatic sites	
Liver	12
Lung	6
Lymph node	5
Liver and lung	2
Liver and lymph node	8
Lung and lymph node	4

TS expression in colorectal cancer metastases has also been identified as a predictor of response to 5-FU in clinical settings (9–14). On the other hand, TS expression in the primary tumor did not predict disease response among patients receiving 5-FU-based chemotherapy for advanced colorectal cancer (15–17).

The aim of this study was to examine intratumoral DPD and TS expression in primary colorectal cancer as a possible determinant of the response of metastatic tumors to fluoropyrimidine-based chemotherapy, as well as survival.

MATERIALS AND METHODS

Clinical Methods.   The study population consisted of 37 patients with metastatic colorectal cancer from July 1998 to December 2000 at the Department of Digestive Surgery, Tokyo Medical and Dental University (Tokyo, Japan). This study was approved by the Institutional Review Board of Tokyo Medical and Dental University, and all patients gave written consent.

These 37 cases represent all patients during that period who received the same treatment as first line chemotherapy for metastatic disease after resection of primary tumor. Eligible patients had (*a*) performance status score of 2 or better on the Zubrod scale [Eastern Cooperative Oncology Group (18)]; (*b*) at least one measurable lesion; (*c*) adequate hematological, hepatic, and renal function; and (*d*) life expectancy over 3 months. In patients treated with adjuvant chemotherapy, there was a washout period of at least 4 weeks. Table 1 outlines patient characteristics. Nine patients had synchronous metastatic tumors at the time of resection of primary tumors and the other 28 patients had metachronous metastasis after resection of the primary tumors. The treatment regimen was described previously (19). Briefly, treatment consisted of oral UFT (400 mg/m²/day; in two doses q 12 h) and LV (15 mg/body/day; in three doses q 8 h) for 5 days, followed by a 2-day rest period for four times during one 28-day cycle.

Before the treatment and after every two cycles (8 weeks) of treatment, measurable disease was reassessed by computed tomography. Response evaluation was based on standard Union

International Contre Cancer guidelines, as CR, PR, NC, or PD (20). There were 2 CRs, 10 PRs, 16 NCs, and 9 PDs, with a 32.4% response rate (95% confidence interval, 18.0–49.8%). All patients in this study are now off treatment, 36 of 37 have died from cancer. One patient is still alive with CR at 23.0 months. The median follow-up time was 14.0 months.

Archival fresh frozen samples were obtained from primary colorectal tumors at the time of surgery. No patient had received 5-FU chemotherapy preoperatively. Immediately after resection, the tumor sample was divided into two equal portions of at least 500 mg each, after removal of necrotic tissues. One portion was fresh frozen in liquid nitrogen until the time of RNA extraction, and the other portion was embedded in paraffin to confirm histologically that it contained <5% contamination of normal tissues, necrotic tissues, and lymphocytes.

Laboratory Methods.   Semiquantitative RT-PCR was performed using a previously described method, the reliability and validity of which have been previously described in detail by Ishikawa *et al.* (6), Uetake *et al.* (8), and Takechi *et al.* (21).

Briefly, total RNA for each sample was isolated using the RNeasy mini kit (Qiagen Inc., Chatsworth, CA) according to the manufacturer's instructions. Reverse transcription using 10 μg of total RNA was performed in a total volume of 100 μg containing 250 pmol oligo $(dT)_{18}$, 80 units of RNasin (Promega, Madison, WI), and 500 units Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, and 0.5 mM deoxynucleotide triphosphates solution.

Initially, RNA and oligo $(dT)_{18}$ were heated at 70°C for 10 min and immediately chilled on ice, and the remaining reagents added and incubated for 15 min at 30°C and 60 min at 42°C.

Three different concentrations of transcribed RNA were used as PCR templates. For accurate quantification, we confirmed that the PCR amplification was in the linear phase. PCRs were carried out at a final volume of 50 μl containing cDNA template, oligonucleotide primers for DPD (40 pmol of each primer), TS (10 pmol of each primer), and GAPDH (2 pmol of each primer), 1.25 units of Ex Taq (Takara, Shiga, Japan) in 10× Ex Taq (Takara) in 10× Ex Taq buffer (Takara), and 0.2 mM deoxynucleotide triphosphates, using a thermal cycler (Takara PCR Thermal Cycler MP). The PCR profile consisted of an initial 3-min degeneration at 94°C, followed by 30 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 60°C, and 2 min of polymerization at 72°C and a final 10 min extension at 72°C. PCR products were separated by 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized on a UV transilluminator. Gels were photographed on Type 667 films (Polaroid, Cambridge, MA), and images were scanned with an image scanner (JX-330; Sharp, Mahwah, NJ) and analyzed with an Image Master 1D (Pharmacia Biotech, Uppsala, Sweden). Relative amounts of DPD mRNA and TS mRNA were expressed as the ratios of DPD to GAPDH RT-PCR products (henceforth termed DPD mRNA expression) and TS to GAPDH RT-PCR products (henceforth termed TS mRNA expression), respectively.

Statistics.   The Mann-Whitney U test was used to compare the responders and nonresponders in terms of the related
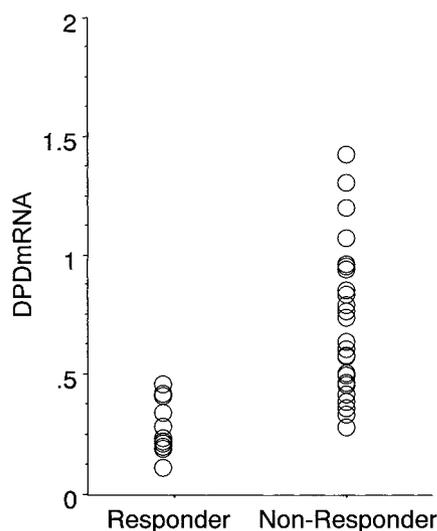
Fig. 1 DPD mRNA in 37 primary colorectal tumors grouped according to nonresponse and response. Response evaluation was based on Union International Contre Cancer criteria. CR and PR were categorized into the response group, and NC and PD were categorized into the nonresponse group.

Fig. 2 DPD mRNA expression in 37 primary colorectal tumors and response to chemotherapy. An inverse relationship between DPD mRNA expression and clinical response categories was observed ($P <$ 0.0001; Kruskal-Wallis test).

Fig. 3 DPD expression plotted against TS expression in 37 primary colorectal cancer. The *dotted lines* indicate the nonresponse cutoff values for each gene expression. There was no responder in patients with high DPD expression tumor. Response rates were 75% (9 of 12) and 43% (3 of 7) in patients with low DPD and low TS tumors, and low DPD and high DPD tumors, respectively.

gene expressions. The association between gene expressions and the proportion of responses was assessed using the Kruskal-Wallis test for trends. The Spearman correlation was used to evaluate the association between the expression of DPD and TS. To evaluate the association with response, the two-sided Fisher's exact test was used. Survival was calculated from the onset of chemotherapy until death. One patient without any event was censored at the date of last follow-up. The overall survival curve was constructed using the Kaplan-Meier method, and differences were assessed by the log-rank test.

RESULTS

Both relative DPD and TS mRNA expressions were determined by semiquantitative RT-PCR in 37 specimens obtained from the primary colorectal cancer. Median values of DPD and TS mRNA expressions were 0.49 (range: 0.15–1.44) and 1.02 (range: 0.21–1.59), respectively. Median values, 0.5 for DPD and 1.0 for TS, were selected for cutoff values, which separated high and low gene expression of DPD and TS.

Tumors were categorized as either responding or not responding to a regimen of UFT and LV. Median values of DPD mRNA expressions were 0.30 (range: 0.15–0.50) and 0.65 (range: 0.29–1.44) for responding tumors and nonresponding tumors, respectively, with the difference being statistically significant ($P < 0.0001$; Mann-Whitney U test; Fig. 1). No responding tumors had DPD mRNA expression of 0.5 or more (Fig. 1). A total of 19 tumors had low DPD mRNA expressions < 0.5 with a corresponding response rate of 63% (12 of 19).

Median values of DPD mRNA expression were 0.19, 0.35, 0.56, and 0.95 for CR, PR, NC, and PD, respectively. DPD mRNA expression inversely correlated with the proportion of responses to chemotherapy (Fig. 2; $P < 0.0001$; Kruskal-Wallis
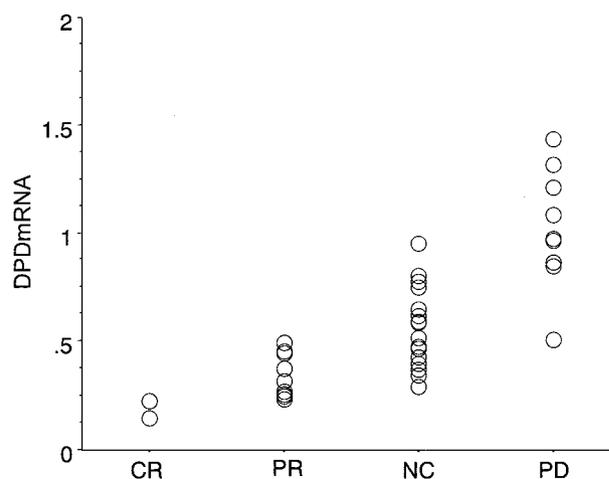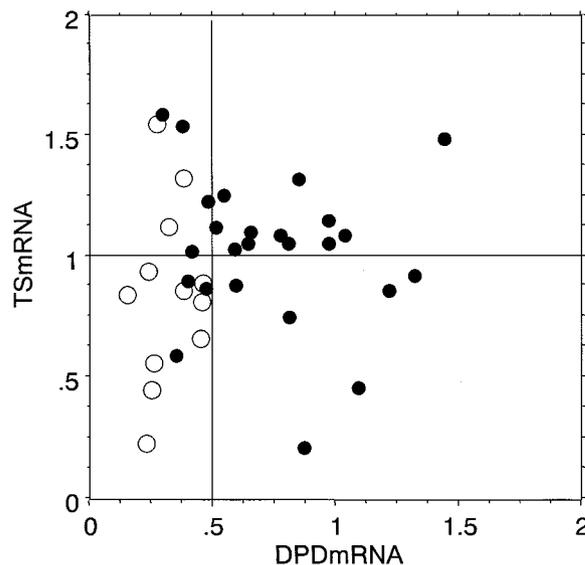
test). On the other hand, there was no relationship between TS mRNA expression and response categories (data not shown).

A plot of DPD against TS mRNA expression showed no correlation between the expression of these genes (Spearman rank correlation coefficient 0.058; $P = 0.733$), with a number of low DPD tumors having high TS (≥ 1.0) and high DPD tumors having low TS expressions (< 1.0) *vice versa* (Fig. 3). There were no responding tumors with high DPD expression. The response rates were significantly higher ($P = 0.0382$; two-sided

Table 2 Summary of response data for tumors with different expressions of DPD and TS genes

Gene expression status	No. of responding patients	No. of nonresponding patients	P
DPD < 0.5 (n = 19)	12	7	<0.0001
DPD ≥ 0.5 (n = 18)	0	18	
TS < 1 (n = 18)	9	9	
TS ≥ 1 (n = 19)	3	16	0.0382
DPD < 0.5 and TS < 1 (n = 12)	9	3	
DPD ≥ 0.5 or TS ≥ 1 (n = 25)	3	22	0.0003

Fig. 4 Cumulative survival curves (Kaplan-Meier) for patients with DPD expressions above or below the nonresponse cutoff and for patients with TS expressions above and below the response cutoff.

Fisher's exact test) in tumors with low TS expression (50% responding) than in those with high TS expression (16%). Fig. 3 additionally shows that 9 of 12 tumors with both low DPD and low TS responded (response rate: 75%), but only 3 of 7 tumors with low DPD and high TS responded (response rate: 43%). The response data are summarized in Table 2. The response rate was 25% in tumors with high DPD or high TS, statistically lower than those with both low DPD and low TS ($P = 0.0003$; two-sided Fisher's exact test).

The median survival was 14.4 months (range: 8.7–28.3 months) in patients with low DPD expression, but only 7.4 months (range: 3.0–11.2 months) in patients with high expression ($P < 0.0001$; log-rank test; Fig. 4). Patients with low expression of TS mRNA survived longer than those with high TS expression (median: 8.4 months ranging from 3.3 to 17.3 months for high expression *versus* 12.3 months ranging from 3.1 to 28.3 months for low expression, $P = 0.0069$; log-rank test; Fig. 5). The median survival time was 16.3 months (range: 8.7–28.3 months) in patients with both low DPD and low TS mRNA expression, which was significantly longer than the 8.4 months (range: 3.1–17.3 months) in patients with other combinations of DPD and TS mRNA expression level ($P < 0.0001$; log-rank test; Fig. 6).

DISCUSSION

Our data demonstrate that there is a significant correlation among the combination of DPD and TS expression levels in primary colorectal cancer lesions, and both the response to UFT and LV and survival in patients with synchronous or metachronous metastatic colorectal cancer. These data reflect Danenberg's report that combination of two genes, TS and DPD, in metastatic tumors was related to the antitumor effect in terms of tumor shrinkage and survival in patients treated with a 5-FU and LV regimen (9).

In this study, DPD mRNA expression statistically correlated with response, the proportion of responses to chemotherapy, and survival. This is the first report to indicate a positive relationship between the antitumor effect and DPD expression in the primary tumor, although the expression of DPD in metastatic tumor has already been reported to reflect the antitumor effect such as tumor shrinkage and survival (9). The heterogeneity in DPD expression between primary and metastatic colorectal tumors is still largely unknown. McLeod *et al.* (22) showed that both DPD mRNA expression and catalytic activity
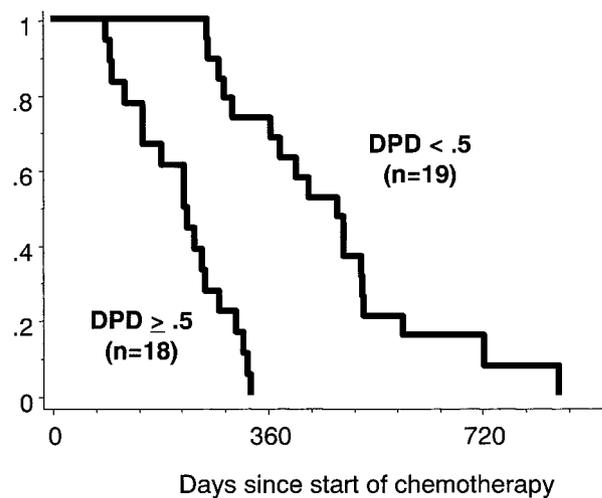
were higher in liver metastasis than in primary colorectal tumors. However, their sample size was quite small: 10 colorectal tumors and 7 liver metastases. Additional studies are needed to clarify the association of DPD expression among primary tumors and their metastatic tumors.

In this study, when categorizing into high and low TS tumors with a cutoff value, low TS expression was significantly related to both tumor shrinkage and extended survival. All studies on TS expression in metastatic tumors showed that responders had significantly lower TS expression than nonresponders, even when evaluating TS expression by immunohistochemical methods or PCR methods (10–14). However, TS expression levels in primary tumor determined by immunohistochemical methods did not correlate with tumor response and survival (15–17). The basis for the discordant results remains unclear.

Immunohistochemical studies have only assessed cytoplasmic expression of TS, although the nuclear staining is also present (23). Cytoplasmic TS levels measured in primary colorectal cancer do not reflect those observed in the corresponding metastases. Aschele *et al.* (17) reported that cytoplasmic TS expression in primary tumors and in corresponding metastases did not correlate. However, Wong *et al.* (24) reported that there was a strong positive correlation between nuclear TS expression in primary and metastatic colorectal cancer in the same cases. The degree of TS nuclear expression correlated closely with TS mRNA expression, and higher nuclear TS expression in primary tumor was associated with poorer response to protracted 5-FU venous infusion therapy. The discrepancy could therefore be explained by the difference in methodology to measure TS expression.

Another possible explanation might be the difference in the treatment schedule such as 5-FU bolus regimen or protracted infusion regimen. When treated with 5-FU bolus based regimens, TS expression in primary tumor had no impact on tumor shrinkage or survival (16, 17). However, TS expression in
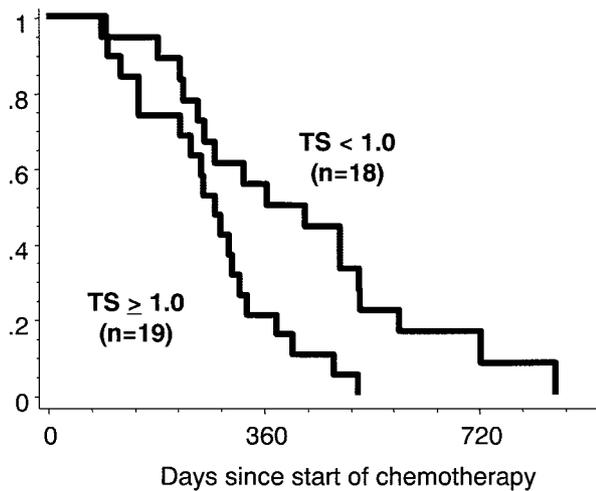
Fig. 5 Cumulative survival curves (Kaplan-Meier) for patients with TS expressions above or below the nonresponse cutoff and for patients with TS expressions above and below the response cutoff.
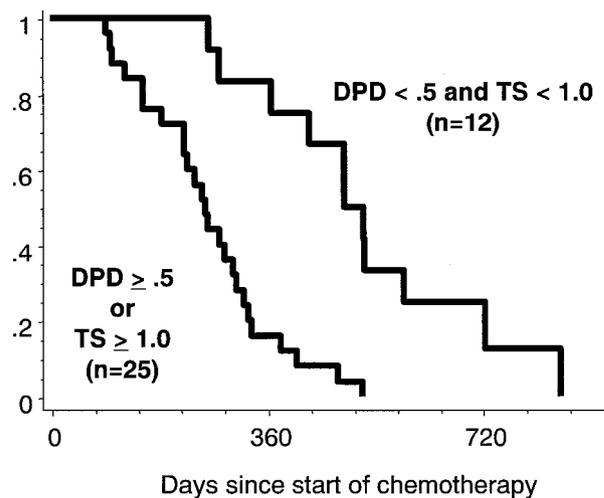

Fig. 6 Cumulative survival curves (Kaplan-Meier) for patients with both low DPD and low TS expressions and those with high DPD or high TS expressions.

primary tumor affected the antitumor effect when treated with a 5-FU protracted infusion regimen (24). The schedule of 5-FU administration was in fact shown to influence the mechanism of action of this agent, the incorporation into RNA, not TS inhibition, may be the dominant mechanism of cytotoxicity with bolus administration (3). When given by only protracted infusion, FdUMP, the active metabolite of 5-FU, may inhibit TS activity (3) and TS expression in tumor has the predictability of antitumor effect. Plasma 5-FU concentrations with oral administration of UFT are similar to equimolar doses of 5-FU administrated as a continuous infusion (25). Lower TS expression in primary tumor was associated with better response in UFT + LV therapy for metastatic colorectal cancer, as well as 5-FU protracted infusion regimen.

When combined DPD and TS mRNA expressions were examined, both response and survival could be predicted more precisely than on the bases of only one gene expression. There was no correlation between DPD and TS mRNA expressions. If DPD and TS coregulated, there would be little or no additional benefit for response prediction from measuring both gene expressions. No tumor with both high DPD and high TS expression responded to UFT and LV chemotherapy but not even all tumors with both low DPD and low TS expression responded to the therapy, having a response rate of 75%. These data suggested combined evaluation of other gene expression such as thymidine phosphorylase (9) is needed to more accurately predict response. In terms of survival, patients with tumor with both low DPD and low TS survived longer than patients with tumor having the other patterns of TS and DPD expression. Among the other three combination pattern, patients with both high DPD and high TS expressed had the worst outcome (data not shown).

Our data may have major clinical relevance. A considerable number of patients with advanced colorectal cancer will have metastases that are usually inaccessible for biopsy, whereas the primary cancer may provide an ample source of tissue for marker analysis. Thus the prediction of the clinical response to fluoropyrimidine therapy for patients with meta-

static disease on the basis of combination of DPD and TS would be much easier if the level of expression could be measured in the primary tumor. In the past, measurement of intratumoral gene expression was labor intensive and only possible when fresh frozen tumor tissue was available. Recently, a new method for isolating mRNA and measuring TS gene expression from paraffin-embedded tissue section using real-time reverse transcript PCR method has been reported (26).

We find that combined evaluation of DPD and TS gene expressions in primary colorectal cancer can be used to predict tumor shrinkage in metastatic tumor and survival when treated with UFT and LV chemotherapy. Our study is a first step toward the goal of individualized cancer chemotherapy based on the fluoropyrimidine-related molecular characteristics of the tumor. However, the conclusions are drawn from a limited retrospective study. Prospectively randomized translational treatment trials are needed to confirm our results and to define the best DPD and TS cutoff points.

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