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5-Fluorouracil-related Gene Expression in Primary Sites and Hepatic Metastases of Colorectal Carcinomas

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Abstract. *The aim of this study was to investigate the correlation of the mRNA expressions of 5-fluorouracil (5FU)-related genes in the primary sites and liver metastases of colorectal carcinomas. Patients and Methods: Patients with liver metastases from colorectal carcinomas were included (n=43). The expression ratios to β-actin of mRNA of thymidine synthase (TS), dihydropyrimidine dehydrogenase (DPD), thymidine phosphorylase (TP) and orotate phosphoribosyl transferase (OPRT) were measured in primary and liver metastases of colorectal carcinomas by laser-captured microdissection and real time PCR. Results: The ratios for the expression of TS, DPD, TP and OPRT mRNAs were significantly correlated between paired primary sites and liver metastases. The mRNA expression ratios of DPD and TP showed a significant correlation both in primary sites and in liver metastases. Conclusion: Enzymes of the primary colorectal carcinomas can be used in predicting the therapeutic efficacy of 5FU against liver metastases.*

Metastasis is the most important event that determines the prognosis of patients with advanced colorectal carcinoma (CRC). The liver is the most common target of metastases from CRCs. Surgical resection alone can result in a significant prolongation of survival in patients with favorable prognostic factors (1, 2). Systemic or regional chemotherapy regimens that include 5-fluorouracil (5FU) have been used to treat hepatic metastases in CRC patients when surgical resection cannot be performed (3-5).

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5FU metabolism is regulated *in vivo* mainly by enzymes such as thymidine synthase (TS), dihydropyrimidine dehydrogenase (DPD), thymidine phosphorylase (TP), and orotate phosphoribosyl transferase (OPRT). TS acts to catalyze the methylation of 2'-deoxyuridine-5'-monophosphate (dUMP) to 2'-deoxythymidine-5'-monophosphate (dTMP), which is an important process for DNA synthesis (6, 7). 5-Fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP), a 5FU metabolite, forms a complex with TS and folic acid, which inhibits the *de novo* synthesis of dTMP from dUMP. The 5FU metabolite 5-fluorouridine-5'-triphosphate (FUTP) inhibits the synthesis of mRNA (8). The detailed mechanism by which FUTP inhibits mRNA synthesis has not been clearly defined. TP, also known as platelet-derived endothelial cell growth factor, plays an important role in the angiogenesis of carcinomas.

It has been reported that enzymes involved in 5FU metabolism, such as TS and DPD are important predictors of the therapeutic efficacy of 5FU (9, 10). It was reported that a high level of TP gene expression in CRC is associated with non-responsiveness to 5FU (11). However, in these studies, the enzymes which were reported to be responsible for the antitumor effects of 5FU were examined in primary sites of CRCs. The expression of enzymes involved in 5FU metabolism in metastatic site has not been examined. It is necessary to examine the relationship between the enzyme expression in primary and metastatic sites of CRCs.

The aim of this study was to investigate the correlation of the expression of TS, DPD, TP and OPRT mRNAs in primary sites and liver metastases of CRCs. The expression of TS, DPD, TP and OPRT genes was examined by a newly developed technique using laser-captured microdissection (LCM) combined with RNA extraction from paraffin-embedded specimens and RT-PCR (12-15). The LCM method made it possible to remove the contamination of adjacent normal tissue surrounding the carcinoma tissue and to purify the samples.

Table I. *Patient characteristics.*

Characteristic	No. of patients
Gender	
Male	28
Female	15
Age (years; average)	62.0
Onset of liver metastasis	
Synchronous	27
Metachronous	16
Histology of primary colorectal carcinoma	
Well	11
Moderate	31
Poor	1
pTNM of primary colorectal carcinoma	
pT	
1	0
2	3
3	36
4	4
pN	
0	13
1	21
2	9
pM	
0	13
1	30

pTNM classification: a pathological classification for malignant tumors defined by UICC (International Union of Cancer).

Patients and Methods

Patients. Patients with synchronous or metachronous liver metastases originating from colorectal carcinomas were included (n=43). Their primary colorectal carcinomas and liver metastases were resected surgically. Patients who received preoperative irradiation were excluded. The patients characteristics are described in Table I. Written informed consent was obtained from all patients.

Microdissection. Four 10 μm-thick sections of the primary colorectal carcinomas and adjacent normal mucosa were prepared from the paraffin-embedded blocks. One 4 μm-thick section was prepared and stained with hematoxylin and eosin (HE). A representative formalin-fixed, paraffin-embedded (FFPE) tumor specimen was selected by a pathologist after examination of the HE-stained slides. Sections 10 μm in thickness were stained with neutral fast red to enable visualization of histology for LCM (PALM Microlaser Technologies AG, Munich, Germany), which was performed to ensure that only tumor cells were studied.

RNA extraction and cDNA synthesis. The RNA was isolated from the FFPE specimens using a novel, proprietary procedure (Response Genetics, Los Angeles, CA, USA) (9). The tissue samples to be extracted were placed in a 0.5 mL thin-walled tube containing 400 μl of 4 M dithiothreitol (DTT)-GITC/sarcosine (4 M guanidinium isothiocyanate, 50 mM Tris-HCl (pH 7.5), 25 mM EDTA) (Invitrogen, Carlsbad, CA, USA; No. 15577-018). The samples were homogenized and an additional 60 μl of GITC/sarc solution was added. They were heated at 92˚C for 30 min and then transferred to a 2 mL centrifuge tube. Fifty microliters of 2 M sodium acetate (pH 4.0) were added, followed by 600 μl of freshly prepared phenol/chloroform/isoamyl alcohol (250:50:1). The tubes were vortexed for 15 s, placed on ice for 15 min and then centrifuged at 13,000 rpm for 8 min in a chilled (8˚C) centrifuge. The upper aqueous phase was carefully removed and placed in a 1.5 mL centrifuge tube. Glycogen (10 μl) and 300-400 μl of isopropanol were added and the samples were vortexed for 10-15 s. The tubes were chilled at −20˚C for 30-45 min to precipitate the RNA. The samples were then centrifuged at 13,000 rpm for 7 min in a centrifuge of 8˚C. The supernatant was poured off and 500 μl of 75% ethanol were added. The tubes were again centrifuged at 13,000 rpm for 6 min in a chilled (8˚C) centrifuge. The supernatant was then carefully poured off, so as not to disturb the RNA pellet, and the samples were quick-spun for another 15 s at 13,000 rpm. The remaining ethanol was removed and the samples were left to air-dry for 15 min. The pellet was resuspended in 50 μl of 5 mM Tris-HCl (pH 8.0). After RNA isolation, cDNA was derived from each sample according to a previously described procedure (12).

Table II. *Median mRNA expression ratio of thymidine synthase (TS), dihydropyrimidine dehydrogenase (DPD), thymidine phosphorylase (TP) and orotate phosphoribosyl transferase (OPRT) in primary site and liver metastases.*

	Primary site	Liver metastases	*p*-value
TS	3.19 (0.73-8.35)	3.98 (0.34-18.5)	0.26
DPD	0.46 (0.09-1.41)	0.45 (0.08-1.44)	0.80
TP	3.16 (0.81-8.17)	2.72 (0.69-9.59)	**0.02**
OPRT	2.00 (0.63-4.24)	2.16 (0.45-5.51)	0.24

Expression ratio is shown as median value (range).

PCR quantification of mRNA expression. Target cDNA sequences were amplified by quantitative PCR using a fluorescence-based real-time detection method (ABI PRISM 7900 Sequence Detection System, TaqMan®; Perkin-Elmer (PE) Applied Biosystems, Foster City, CA, USA) as described elsewhere (16, 17). The PCR reaction mixture (25 μL) contained 600 μmol/L of each primer, 200 nmol/L each of dATP, dCTP and dGTP, 400 μmol/L dUTP, 5.5 mmol/L $MgCl_2$ and 1x TaqMan buffer A containing a reference dye (all reagents were supplied by Applied Biosystems). The primer and probe sequences used were as follows: TS primers: GCCTCGGTGTGCCTTTCA and CCCGTGATGTGCGCAAT, probe 6FAM–TCGCCAGCTACGCCCTGCTCA; DPD primers: AGGACGCAAGGAGGGTTTG and GTCCGCCGAGTCCTTAC TGA, probe 6FAM-CAGTGCCTACAGTCTCGAGTCTGCCAGTG; TP primers: CCTGCGGACGGAATCCT and GCTGTGATGAG TGGCAGGCT, probe 6FAM-CAGCCAGAGATGTGACAGC CACCGT; OPRT primers: TAGTGTTTTGGAAACTGTTGAGGTT and CTTGCCTCCCTGCTCTCTGT, probe 6FAM-TGGCATCA GTGACCTTCAAGCCCTCCT; β-actin primers: TGAGCGCG GCTACAGCTT and TCCTTAATGTCACGCACGATTT, probe 6FAM-ACCACCACGGCCGAGCGG.

PCR was performed at 50˚C for 10 s and 95˚C for 10 min, followed by 42 cycles at 95˚C for 15 s and 60˚C for 1 min. Gene expression values (relative mRNA levels) are expressed as ratios (differences between the Ct values) between the gene of TS, DPD,

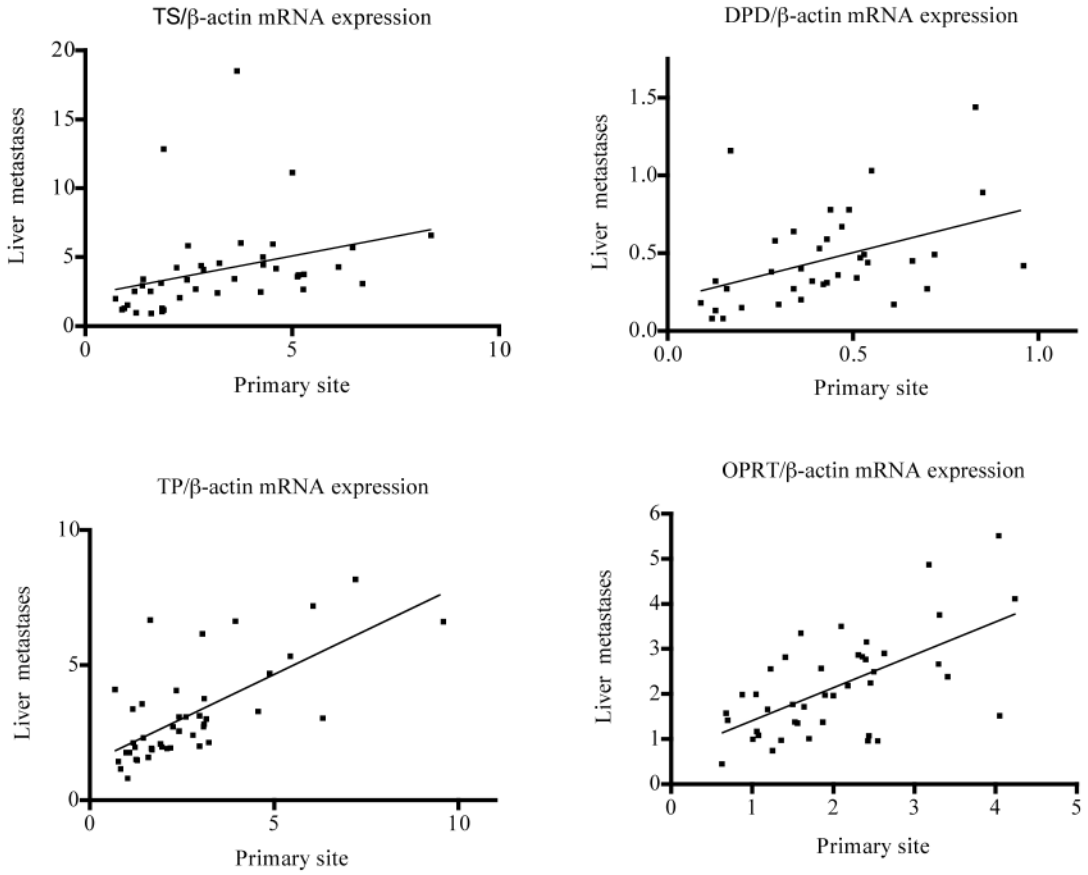

Figure 1. *Expression ratios of thymidine synthase (TS), dihydropyrimidine dehydrogenase (DPD), thymidine phosphorylase (TP) and orotate phosphoribosyl transferase (OPRT) mRNA to β-actin in primary sites and liver metastases of colorectal carcinomas (TS, r=0.62, p<0.0001; DPD, r=0.50, p=0.0009; TP, r=0.65, p<0.0001; OPRT, r=0.50, p=0.0003).*

TP or OPRT and the internal reference gene β-actin. This reference gene provides a baseline measurement for the amount of RNA isolated from a specimen.

Statistical analysis. Differences in the mRNA expression ratios of TS, DPD, TP and OPRT in the primary sites and liver metastases were determined by the Wilcoxon signed rank test. Correlations between the mRNA levels of TS, DPD, TP and OPRT were assessed using Spearman's rank correlation. A value of $p<0.05$ was considered statistically significant. GraphPad Prism version 4.0 for Macintosh (San Diego, CA, USA) was used for the analyses.

Results

Gene expression levels in primary sites and liver metastases of CRCs. Median mRNA expression ratios of TS, DPD, TP and OPRT to β-actin are given in Table II. TP expression was significantly higher in primary sites than in their corresponding liver metastases. TS, DPD and OPRT did not differ significantly between primary sites and liver metastases.

Correlation of mRNA expression between primary sites and liver metastases of CRCs. The mRNA expression ratios of TS, DPD, TP and OPRT to β-actin in primary sites were significantly correlated to those in the liver metastases of CRCs (Figure 1).

Correlation between TS, DPD, TP and OPRT mRNA expressions in primary sites or liver metastases of CRCs. The mRNA expression of DPD and TP showed a significant correlation in both primary sites and in liver metastases (Figure 2).

Discussion

Our study demonstrated that the mRNA expression ratios of TS, DPD and OPRT in primary sites did not differ significantly from those in liver metastases. Only TP expression was significantly higher in primary sites than in liver metastases. There have been several studies which examined 5FU-related gene expression in primary and corresponding liver metastases from CRCs. However, their results were controversial. Inokuchi *et al*. reported that the

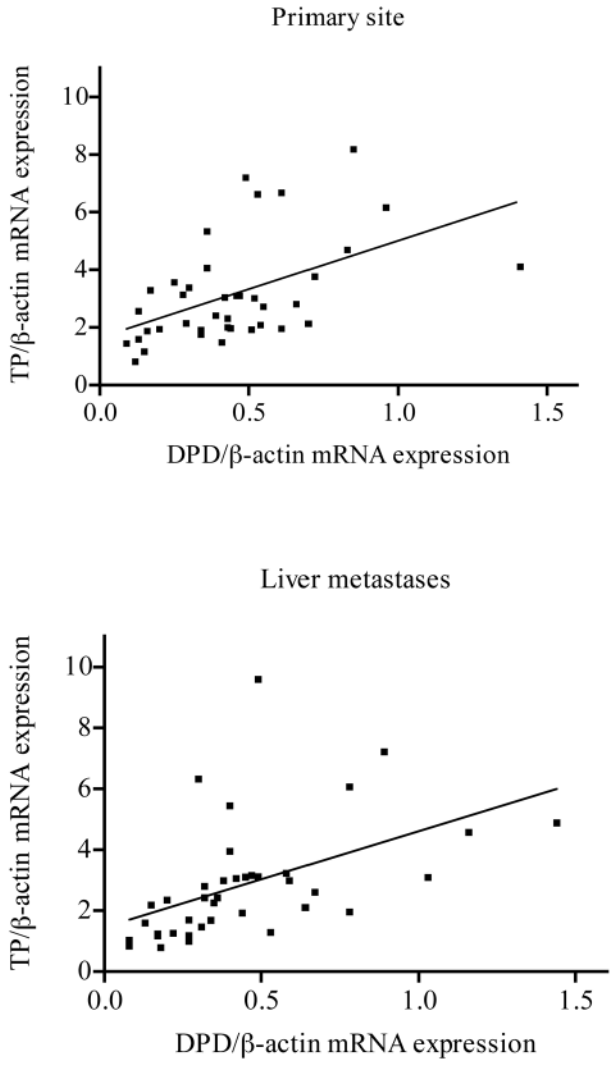

Figure 2. *Relationship between DPD and TP expression ratios to β-actin in primary sites (r=0.54, p=0.0004) and liver metastases (r=0.68, p<0.0001) of colorectal carcinomas.*

DPD, OPRT and TP mRNA levels were significantly higher in liver metastases than in primary tumors and that TS mRNA levels did not differ significantly (18), however, they did not use the LCM method to purify tissue. DPD, TP and OPRT were reported to show higher expression levels in normal liver tissue than in the liver metastases (19). The contamination of normal liver tissue with metastatic liver tissue may have affected their results. Kuramochi *et al*. reported no significant differences between median mRNA expression levels of TS, DPD, TP and OPRT in primary carcinoma and those in corresponding liver metastases (19). Their TP expression levels were lower in the metastatic liver site than in the primary sites, but not significantly.

We also demonstrated that the mRNA expression levels of TS, DPD, TP and OPRT in liver metastases were significantly correlated to those in primary sites of CRCs. Kuramochi *et al*., using the LCM method, reported a significant correlation for TS mRNA expression between primary carcinomas and corresponding liver metastases and no correlation for DPD, TP or OPRT (19). Their method was similar to ours. These different results may be due to the sample condition or sample size.

Among the four genes that were studied, a significant correlation was observed between DPD and TP both in the primary sites and the liver metastases. Inokuchi *et al*. and Kuramochi *et al*. reported similar results (18, 19). Mori *et al*. reported a positive correlation between DPD and TP protein levels in colorectal, pancreatic, esophageal, bladder, cervical, hepatic and gastric carcinomas (20). It was reported that DPD and TP gene expression in CRCs were associated with tumor progression (21, 22). A high level of TP gene expression is reported to be associated with non-responsiveness to 5FU (11). TP is supposed to play as an important role in tumor progression and 5FU sensitivity as TS or DPD.

The expression of 5FU-related enzymes has been used to predict the therapeutic efficacy and survival of 5FU-treated patients. It has been reported that the clinical response and survival rates in response to 5FU-based chemotherapy for patients with CRC are related to the expression of TS, DPD and TP (9, 23). We also reported that the expression levels of DPD and TP mRNAs in primary CRCs was significantly predictive of the therapeutic response to hepatic arterial infusion of 5FU (24). The expression of these enzymes is important for guiding the rational selection of chemotherapeutic regimens. Physicians should consider using a regimen that includes irinotecan (CPT-11) for patients who show a high expression of TS, DPD and TP in their carcinomas. In this study, we showed the positive correlation of gene expression of TS, DPD, TP and OPRT between primary carcinomas and liver metastases. Analysis of primary carcinomas can be used to predict the gene expression level in the liver metastases. Our results confirm the idea that the levels of gene expression in primary carcinomas can be used for directing the strategies of the chemotherapy against metastases. We have not examined the gene expressions in extrahepatic metastases such as lung metastases. Further studies are required for metastases from other organs.

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