5-Fluorouracil-related Gene Expression in Hepatic Artery Infusion-treated Patients with Hepatic Metastases from Colorectal Carcinomas

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Abstract. *Aim: To predict the therapeutic efficacy of hepatic arterial infusion (HAI) with 5-fluorouracil (5FU) for patients with liver metastases from colorectal carcinomas, 5FU-related gene expressions were examined in primary colorectal carcinomas. Patients and Methods: Thirty-eight patients with liver metastases from colorectal carcinoma received HAI of 5FU. The expressions of the mRNAs for thymidine synthase (TS), dihydropyrimidine dehydrogenase (DPD), thymidine phosphorylase (TP), and oroteta phophoribosyl transferase (OPRT) in primary colorectal carcinomas were measured by RT-PCR. Results: The response rate was 52.6% (20/38). The overall median survival time was 29.1 months. DPD and TP expression was significantly higher in the progressive disease (PD) group than in the complete response (CR) or partial response (PR) group (p=0.032, p=0.014), respectively. The levels of DPD and TP mRNAs showed a significant correlation (r=0.76, p=0.0001). Conclusion: The expression of DPD and TP mRNAs in primary colorectal carcinomas was significantly predictive of the therapeutic response to 5FU HAI.*

Hepatic metastasis is one of the most important factors that determines the prognosis of patients with advanced colorectal carcinoma. Surgical resection alone can result in significant prolongation of survival in patients with favorable prognostic factors (1, 2). Systemic chemotherapy regimens that include 5-fluorouracil (5FU) have been used to treat hepatic metastases in colorectal carcinoma patients when surgical resection cannot be performed (3, 4).

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Hepatic artery infusions (HAIs) have also been performed as regional chemotherapy for liver metastases arising from colorectal carcinomas. Randomized trials evaluating HAI therapy for the treatment of unresectable hepatic metastases have demonstrated higher response rates (31%-50%) than those achieved with systemic chemotherapy (8%-20%), but no survival benefit was reported (5, 6). Recently, Kemeny and associates have reported the results of a randomized trial comparison between HAI using floxuridine and systemic chemotherapy using 5FU and leucovorin (7). The overall survival was significantly longer for HAI than the systemic treatment (median, 24.4 *vs.* 20 months).

In a previous study, we administered 5FU by HAI to patients with liver metastases from colorectal carcinoma after radiological placement of the infusion lines, and found that HAI significantly improved the median survival time (MST) and response rate (8). We also reported that lymph node metastases in primary carcinoma and the pre-treatment serum CEA level were prognostic factors for MST in HAI-treated patients.

However, the response rate was not influenced by the histological features or lymph node metastases of the primary colorectal carcinomas, nor was it influenced by the synchronous/metachronous status of the liver metastases, the number of hepatic metastases, or the pre-treatment serum CEA levels.

It has been reported that enzymes involved in 5FU metabolism, such as thymidine synthase (TS), dihydropyrimidine dehydrogenase (DPD), thymidine phosphorylase (TP) and oroteta phophoribosyl transferase (OPRT) are important predictors of the therapeutic efficacy of 5FU (9, 10). TP, also known as platelet-derived endothelial cell growth factor, plays an important role in the angiogenesis of carcinomas. It has been reported that the clinical response and survival rates in response to 5FU-based chemotherapy for colorectal carcinomas are related

to the expressions of TS, DPD and TP and that a high level of TP gene expression in colorectal carcinomas is associated with non-responsiveness to 5FU (9, 11, 12). The expression of these enzymes is important for guiding the rational selection of chemotherapeutic regimens. The expression of TS, DPD, TP, and OPRT genes has been examined by a newly developed technique using laser-captured microdissection combined with RNA extraction from paraffin-embedded specimens (13-16).

The expression of enzymes involved in 5FU metabolism has not been examined in patients with liver metastases treated using HAI. The aim of this study was to investigate the correlation between the clinical response to HAI and the expression of TS, DPD, TP and OPRT mRNAs in primary colorectal carcinomas.

Patients and Methods

Patients. Patients with liver metastases originating from colorectal carcinomas were included (n=38). Patients characteristics are described in Table I. Their primary colorectal carcinomas had been resected surgically and were histologically confirmed. Patients with extrahepatic metastases were excluded. The patients received no other chemotherapy prior to HAI. Informed consent was obtained from all patients.

Table I. *Patients characteristics.*

Characteristics	No. of patients	Characteristics	No. of patients
		pTNM of primary	
Gender		colorectal carcinoma	
Male	25	pT	
Female	13	pT1	0
Age (average)	65.6	pT2	0
Onset of liver metastases		pT3	35
Synchronous	25	pT4	3
Metachronous	13		
Histology of primary colorectal		pN	
carcinoma		pN0	11
Well	11	pN1	15
Moderate	25	pN2	12
Poor	1	pM	
Mucinous	1	pM0	13
		pM1	25

Catheter placement and HAI procedure. Catheter placements in the hepatic artery were performed radiologically by interventional radiologists using the distal fixation method (17). The catheter was inserted *via* the right femoral artery and connected to the infusion port (Infuse-a-Port, Strato Medical Corp., Beverly, MA, USA). The HAI treatment was performed weekly or every 2 weeks at an outpatient chemotherapy facility. The 5FU (1,000-1,500 mg) was dissolved in 200 ml of physiological saline and loaded into a portable infusion pump (Intermate LV; Baxter Healthcare Corp., Deerfield, IL, USA). HAI was performed continuously for 5 h at an infusion rate of 50 ml/h (8).

Clinical response and survival evaluation. The patients scheduled for HAI received a chest and abdominal computed tomography (CT) scan before the start of treatment. Tumor status was assessed by chest and abdominal CT scans after every 10 infusions. The therapeutic response was evaluated according to the Response Evaluation Criteria In Solid Tumors (RECIST) guideline (18) as complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD). Actuarial survival curves were computed by the Kaplan-Meier method, using GraphPad Prism version 4.0 for Macintosh (San Diego, CA, USA).

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 laser capture microdissection (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 Bl of 4 M dithiothreitol (DTT)- GITC/sarc (4 M guanidinium isothiocyanate, 50 mM Tris-HCl, pH 7.5, 25 mM EDTA) (Invitrogen; No. 15577-018). The samples were homogenized and an additional 60 Bl 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 was added at pH 4.0, followed by 600 Bl of freshly prepared phenol/chloroform/isoamyl alcohol (250:50:1). The tubes were vortexed for 15 sec, 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 Bl) and 300-400 Bl of isopropanol were added and the samples were vortexed for 10-15 sec. 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 an 8˚C centrifuge. The supernatant was poured off and 500 Bl of 75% ethanol was 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 sec 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 Bl of 5 mM Tris. After RNA isolation, cDNA was derived from each sample according to a previously described procedure (13).

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 previously described (19, 20). The PCR reaction

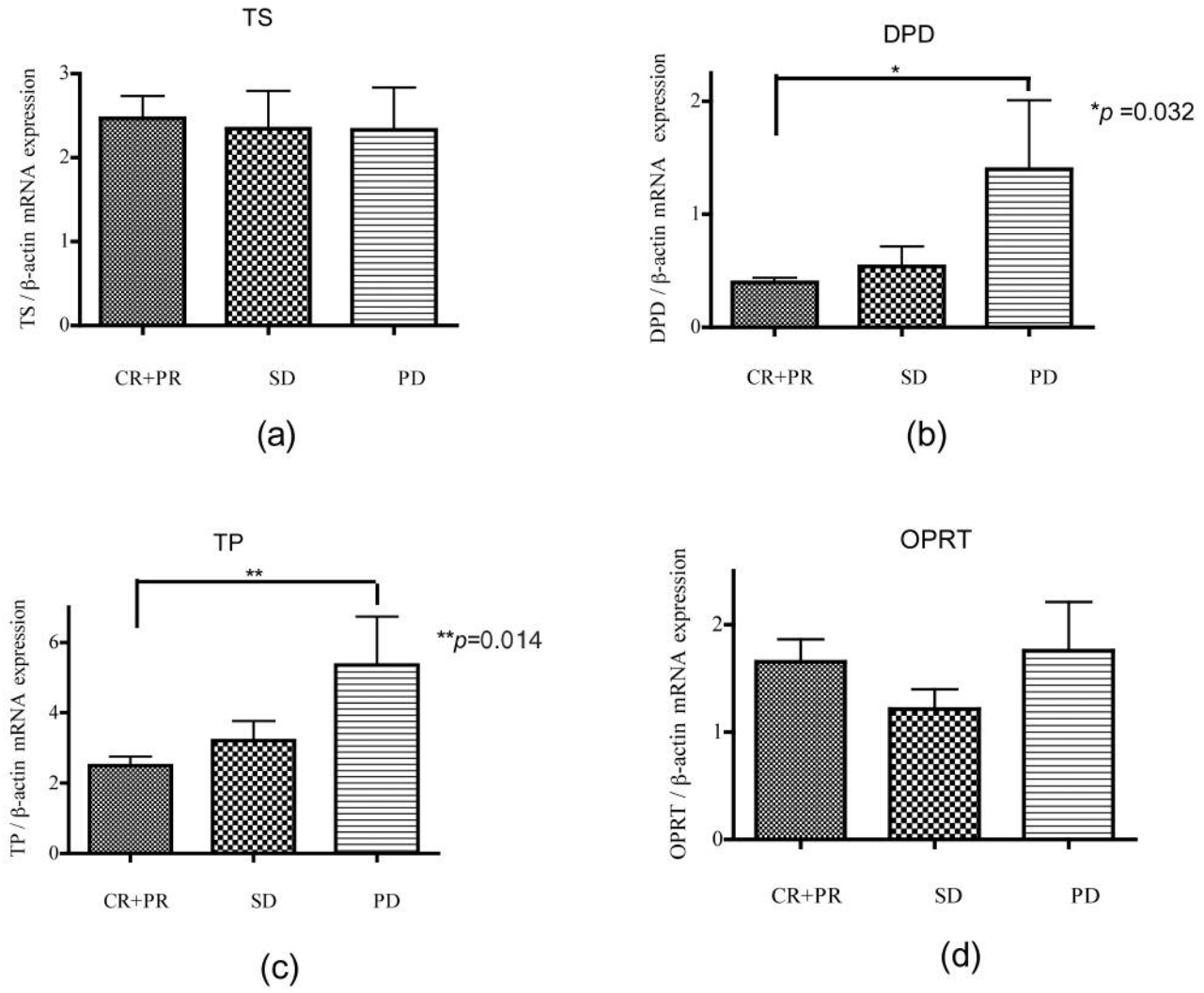

Figure 1. *mRNA expression ratio of thymidine synthase (TS) (a), dihydropyrimidine dehydrogenase (DPD) (b), thymidine phosphorylase (TP) (c), and oroteta phophoribosyl transferase (OPRT) (d) to β-actin in HAI-treated patients. CR=complete response, PR=partial response, SD=stable disease, PD=progressive disease.*

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 primers and probes sequences used were as follows: TS primers: GCCTCGGTGTGCCTTTCA and CCCGTGATGTGCGCAAT, probe 6FAM - TCGCCAGCTACGCCCTGCTCA; DPD primer: AGGACGCAAGGAGGGTTTG and GTCCGCCGAGTCCTTA CTGA, probe 6FAM - CAGTGCCTACAGTCTCGAGTCTG CCAGTG; TP primers: CCTGCGGACGGAATCCT and GCTG TGATGAGTGGCAGGCT, probe 6FAM - CAGCCAGAGATG TGACAGCCACCGT; OPRT primers: TAGTGTTTTGGAAA CTGTTGAGGTT and CTTGCCTCCCTGCTCTCTGT, probe 6FAM - TGGCATCAGTGACCTTCAAGCCCTCCT; β-actin primers: TGAGCGCGGCTACAGCTT and TCCTTAATGTCA CGCACGATTT, probe 6FAM - ACCACCACGGCCGAGCGG. PCR was performed at 50˚C for 10 sec and 95˚C for 10 min, followed by 42 cycles at 95˚C for 15 sec 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, TP or OPRT and an 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 expression of TS, DPD, TP, and OPRT between the CR/PR group, SD and PD groups were determined by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Correlations between the mRNA levels of TS, DPD, TP and OPRT were assessed using Spearmans's rank correlation. A value of $p<0.05$ was considered statistically significant. GraphPad Prism version 4.0 for Macintosh was used for the analyses.

Results

Therapeutic response and survival of patients treated by HAI. A CR in 5 patients, PR in 15 patients, SD in 9 patients, and PD in 9 patients were found. The overall response rate was 52.6%. The overall MST was 29.1 months.

5FU-related gene expression in HAI-treated patients. DPD and TP expression was significantly higher in the PD than in the CR/PR group ($p=0.032$, $p=0.014$, respectively) (Figure 1). There was no significant difference in the expression of TS or OPRT between the 3 subgroups. MST was not related to the expression of TS, DPD, TP, or OPRT. The mRNA levels of DPD and TP showed a significant correlation (r=0.76, $p=0.0001$) (Figure 2).

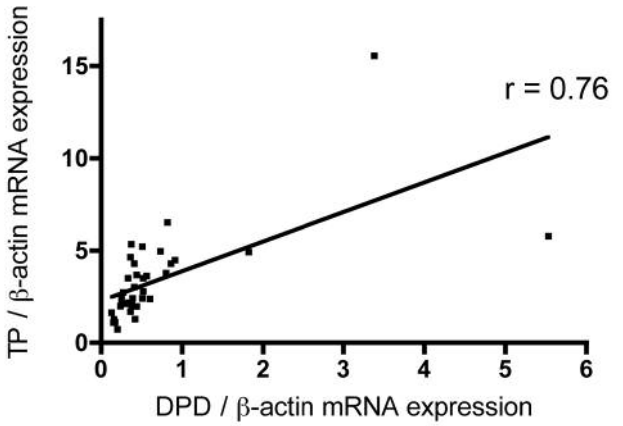

Figure 2. *Correlation between mRNA expression ratio of dihydropyrimidine dehydrogenase (DPD) and thymidine phosphorylase (TP) to β-actin in HAI-treated patients.*

Discussion

In the present HAI study, the expression of DPD and TP mRNAs were significantly lower in responders than in the PD group. Furthermore, DPD and TP expressions showed a significant correlation. DPD and/or TP were thus predictive factors for the therapeutic efficacy of HAI treatment. It has also previously been reported that DPD and TP expression in liver metastases of colorectal carcinomas correlated (21).

In the present study, TS expression did not vary significantly between the responding and non-responding groups. TS has been described as a key marker for predicting the therapeutic efficacy of 5FU-based systemic chemotherapy (9). The hepatic concentration of 5FU is much higher in patients treated by HAI than by systemic infusion. The mechanism of the antitumor effects of 5FU in HAI may be different from that in systemic chemotherapy and it may be more cytotoxic when administered by HAI than when given systemically. The antitumor effects of 5FU mainly involve two pathways: the inhibition of DNA synthesis and the inhibition of mRNA synthesis (22, 23). 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 (22, 24). The 5FU metabolite 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP) forms a complex with TS and folic acid, which inhibits the de novo synthesis of dTMP from dUMP. In contrast, the pathway for inhibition of mRNA synthesis is not associated with TS. The 5-FU metabolite 5-fluorouridine-5'-triphosphate (FUTP) inhibits the synthesis of mRNA (25). The detailed mechanism by which FUTP inhibits mRNA synthesis has not been clearly defined. It is reported that bolus injection can be considered to be more effective with respect to RNA damage in tumor tissue (26, 27). As HAI in our study was performed with high dose 5FU in 5h, it is close to bolus injection more than continuous injection. The anti-tumor effect of HAI may be mainly due to the inhibition of mRNA. Physicians should consider CPT-11–based treatment for patients who show high TS gene expression levels prior to systemic chemotherapy generally (9, 10). However, according to our data, high TS gene expression would not be a limiting factor with HAI treatment.

DPD or TP, or both but not TS were demonstrated to be predictive factors of response to HAI treatment. No relationship between 5FU-related enzymes and survival time was found. Additional prospective studies will be required to determine whether the expression of these enzymes can be used to predict the prognosis of patients treated by HAI.

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