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Expression of Dihydropyrimidine Dehydrogenase, Thymidylate Synthase, p53 and p21 in Metastatic Liver Tumor from Colorectal Cancer after 5-Fluorouracil-based Chemotherapy

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Abstract. *Background: The expression of genes thought to be related to 5-FU chemosensitivity has been extensively investigated. However, little data is available on the expression patterns of these genes after chemotherapy. Patients and Methods: We investigated the expression of four genes, DPD, TS, p53 and p21, in the metastatic liver lesions obtained from colorectal cancer patients who had been treated with hepatic arterial infusions of 5-fluorouracil(5-FU)-based chemotherapy. Results: Expression of DPD, TS and p53 in the metastatic liver lesions was significantly higher in the chemotherapy-response group than in the no response group. In the response group, viable cancer cell nests were seen in confined spaces surrounded by fibrous tissue. It was of interest that these cancer cells in the response group showed conspicuous immunoreactivity of DPD, TS and p53. Conclusion: An analysis of genes involved in 5-FU sensitivity revealed that surviving tumor cells exhibited resistance characteristics, indicating that the chemotherapy regimen should be altered, even in partially responding cases, unless the response is pathologically complete.*

Pharmacogenetic markers of tumor cells have been intensively investigated using molecular biology technologies to predict chemosensitivity to 5-fluorouracil (5-FU) in colorectal cancer patients. Among them, *dihydropyrimidine dehydrogenase* (DPD) and *thymidylate synthase* (TS) are the most promising genes that have been used clinically in gastrointestinal cancer treatment. However, none of these genes are absolute predictors of 5-FU sensitivity.

Neoadjuvant chemotherapy has been proposed as an alternative approach to conventional surgery as an initial management strategy with the aim of improving the outcome of cancer patients. Strategies aimed at downstaging large or multifocal tumors and the control of micrometastases to enable curative resection by neoadjuvant chemotherapy have attracted much attention. Recently, in the field of breast cancer research, a sub-analysis of the National Surgical Adjuvant Breast and Bowel Project B-18 (NSABP B-18) trial revealed that a pathological complete response was the only reliable marker for selecting cases that were sensitive to a specific drug, resulting in an improved survival period (1).

In our institution, the resection of liver metastases for colorectal cancer has been actively performed after 5-FU-based chemotherapy *via* hepatic artery infusion. Although the survival advantage of hepatic arterial infusion over systemic therapy has been debated, the efficacy of this treatment with regard to tumor reduction was shown to be advantageous. We have used this procedure to treat patients with primarily unresectable metastases confined to the liver.

In this study, we examined the expression of *DPD, TS, p53* and *p21* in surgical specimens of liver metastases obtained from patients after chemotherapy.

Patients and Methods

Samples. Surgical specimens of synchronous or metachronous bilobular multiple liver metastatic tumors from 12 patients were obtained at Yokohama City University, Japan. The patients comprised 5 males and 7 females with a median age of 57.8 years (range, 41-74 years) (Table I). Since none of the 12 patients exhibited metastases at sites other than the liver, surgical resection was performed after 5-FU-based chemotherapy *via* hepatic arterial infusion. The treatment regimen was as follows: an infusion of 5-FU (500 mg/body) or 5-FU (500 mg/ body) + l-Leucovorin (150 mg/

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Table I. Patient characteristics.

Primary tumor	Age	Gender	Response	Depth	n	DPD pri	DPD meta	TS pri	TS meta	p53 pri	p53 meta	p21 pri	p21 meta
rectum	59	F	PD+NC	se	1	2	0	0	0	0	0	1	0
rectum	41	F	PD+NC	ss	4	2	0	0	0	4	0	0	0
colon	55	M	PD+NC	se	1	0	2	0	0	0	0	0	0
colon	45	M	PD+NC	se	1	2	0	2	0	6	0	0	0
colon	51	F	PD+NC	mp	1	-	3	-	1	-	3	-	0
colon	74	M	PD+NC	si	0	1	3	1	1	0	0	0	0
colon	67	F	PR	ss	2	2	6	1	4	0	0	2	2
colon	65	F	PR	se	4	0	4	0	2	12	8	0	0
colon	54	F	PR	ss	2	1	4	1	1	9	9	2	1
rectum	69	M	PR	ss	0	0	4	2	6	9	12	6	1
colon	59	M	PR	ss	2	0	9	1	1	9	6	1	0
colon	55	F	PR	ss	1	4	4	0	3	0	0	1	0

pri: primary lesion
meta: metastatic lesion

body) + Cisplatinum (10 mg/ body) was administered every day for 5 days, and this cycle was repeated every 2 weeks for up to 4 cycles. None of the patients had received any other chemotherapy or radiotherapy treatments prior to the hepatic arterial infusions of 5-FU. Paraffin-embedded archival samples of their primary colorectal lesions were also examined. The study was approved by the institutional review board of the Yokohama City University School of Medicine, Japan.

Clinical evaluation. The chemotherapy response was determined by comparing the volume of the liver metastases before and after chemotherapy. A CT scan was performed after the 4 treatment cycles had been completed, and the results were evaluated using the World Health Organization (WHO) criteria (2). A complete response (CR) was defined as the complete disappearance of all intrahepatic tumor formation, and a partial response (PR) was defined as a reduction in the tumor volume by 50% or more, measured as the sum of the products of the two largest perpendicular diameters of all visible lesions. No change (NC) was defined as a reduction in tumor volume of less than 50% or an increase of less than 25%. An increase in tumor volume of 25% or more or the appearance of new liver lesions was defined as progressive disease (PD).

Antibodies. Rabbit anti-recombinant human *DPD* polyclonal antibody (dilution=1:500; The Second Cancer Laboratory, Taiho Pharmaceutical Co, Saitama, Japan.), *TS* polyclonal antibody (RTSSA, dilution=1:800; The Second Cancer Laboratory, Taiho Pharmaceutical Co.), mouse monoclonal antibody against *p53* protein (DO-7, dilution = 1:100; DAKO, Glostrup, Denmark) and *p21* protein (OP64, dilution=1:100; Oncogene Research Products, Cambridge MA, USA) were used as the primary antibodies for the immunohistochemical staining.

Immunohistochemistry

(i) DPD. Tissue sections (4 μm thick) were cut from each block, deparaffinized in xylene, rehydrated with graded ethanol and immersed in TBS. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in distilled water for 15 minutes. *DPD* protein expression was evaluated using the avidin-biotin complex immunohistochemical technique and a rabbit polyclonal antibody to recombinant human *DPD*. To block the nonspecific binding of the primary antibody, a normal rabbit serum (DAKO X901) dilution in TBS was used for 20 minutes. After removing the blocking solution, the *DPD* antibody (2 mg/ml) was applied for 60 minutes in a humidified chamber at room temperature. The sections were then incubated with biotin-conjugated swine anti-rabbit immunoglobulins for 20 minutes (DAKO-E353), followed by avidin-biotinylated peroxidase complex for 30 minutes. After developing the color reaction product with a freshly prepared 3,3'-diaminobenzidine chromogen solution for 5 minutes, the sections were counterstained with light hematoxylin for 10 seconds, dehydrated in a series of ethanols, cleared in xylene, mounted and covered with glass coverslips. Positive and negative controls were included in each experiment.

(ii) TS, p53 and p21. Tissue specimens (4 μm thick) were fixed in formalin and embedded in paraffin wax. After dewaxing, the sections were treated with 3% hydrogen peroxidase solution in methanol for 20 minutes to block endogenous peroxidase activity. The sections were then heated in a 0.01 M citrate buffer (pH 6.0) for 3-minute periods in a microwave oven for antigen retrieval. Non-specific antibody bindings were blocked using 10% normal bovine serum in PBS at 37˚C for 15 minutes for the *p53* and *p21* staining procedures and normal goat serum for the *TS* staining procedure. The sections were then incubated with the primary antibodies described in the previous section. The sections were incubated at room temperature for 10 minutes with a byotinylated anti-mouse IgG + IgA + IgM (for monoclonal primary antibody) for *p53* and *p21* staining, and with a biotinylated anti-rabbit IgG for *TS* staining. The sections were then incubated at room temperature with peroxidase conjugated streptavidin and Elite ABC solution, respectively. The peroxidase reaction was developed using a 3,3'-deaminobenzidine tetrahydrochloride solution (Sigma Chemical Co, St. Louis, MO, USA) and 0.03% hydrogen peroxide. The sections were counterstained with hematoxylin, dehydrated and mounted in a routine fashion. Positive controls and negative controls were always included in all experiments.

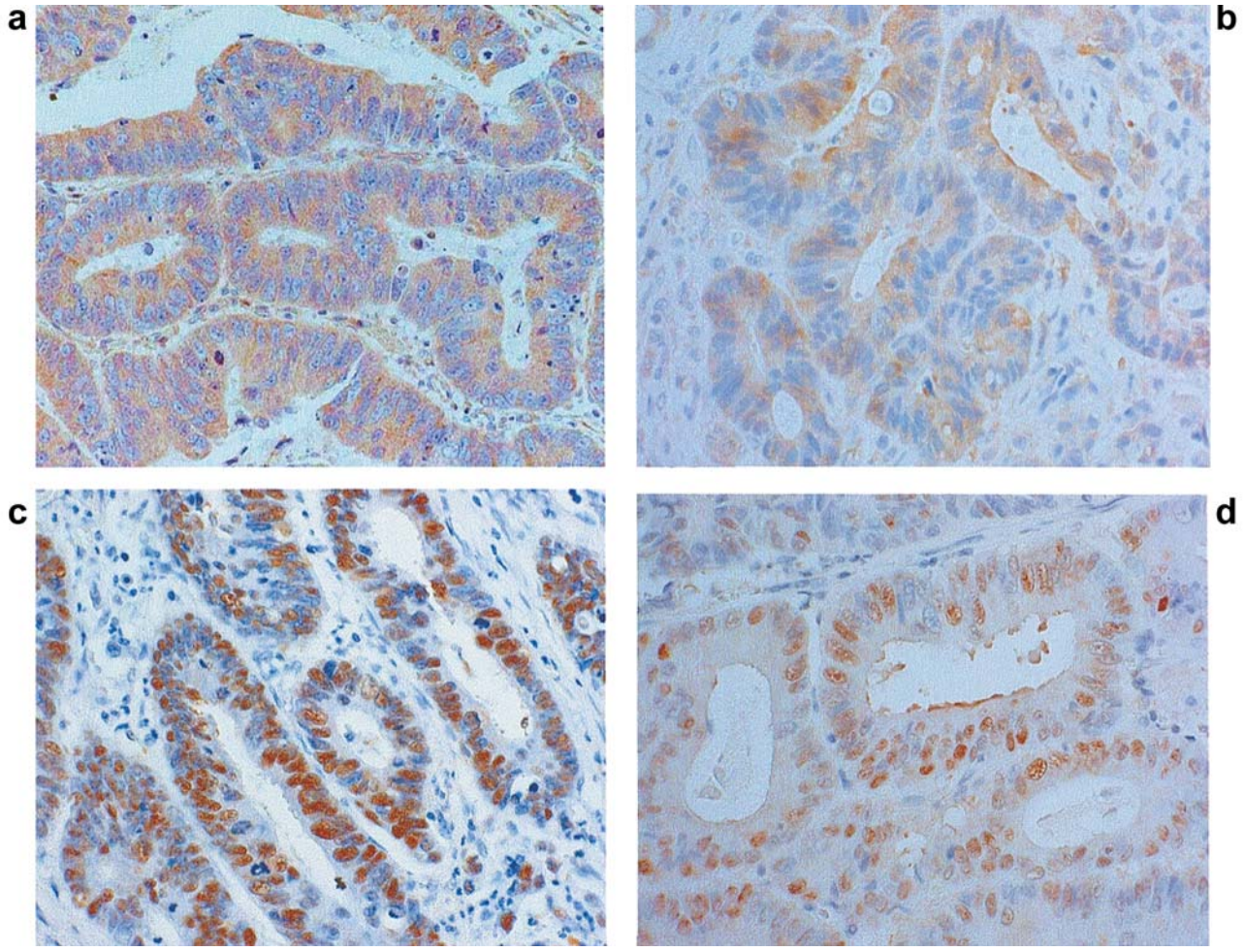

Figure 1. *Typical expression of DPD, TS, p53 and p21 genes after chemotherapy in surgical specimens of liver metastases. A: Expression of DPD, B: Expression of TS, C: Expression of p53, D: Expression of p21 (magnification, x400).*

(iii) Quantitation. Three representative fields were examined, more than 1000 tumor cells were randomly selected and the number of positive cells was counted at magnification x200. The expression of these proteins were evaluated according to the method described by Sinicrope *et al.* (3). In brief, positive-staining tumor cells were expressed as a percentage of the total number of tumor cells and assigned to one of the following five categories: class 0, ≤5%; class 1, 5% to 25%; class 2, 25% to 50%; class 3, 50% to 75%; and class 4, ≥75%. The intensity of the immunostaining was scored as follows: 1, weak; 2, moderate; 3, intense. These two scores were then multiplied. When heterogeneous levels of protein expression were found within a tumor (in multiple sections from different paraffin-embedded blocks of the same tumor), the highest protein expression score obtained for that lesion was used.

Statistical analyses. Dr. SPSS software for Windows was used for the statistical analyses; statistical significance was defined as $p<0.05$.

Results

Chemotherapy *via* hepatic arterial infusion was successfully performed in all 12 cases with no severe complications. The total 5-FU dosage was 3200±1500 mg (mean±SD). Clinical evaluations revealed that the liver tumors in 6 of the 12 patients partially responded to the treatment, although no complete responses occurred. In the remaining 6 cases, the tumors did not change in size or progressed after treatment (Table I).

Immunohistochemistry for the 4 genes was successfully performed in all 12 cases. *DPD* and *TS* were clearly observed in the cytoplasm of the cancer cells, while *p53* and *p21* were observed in the nuclei of the cancer cells. Representative cases are shown in Figure 1. Since half of the patients exhibited partial responses to the treatment,

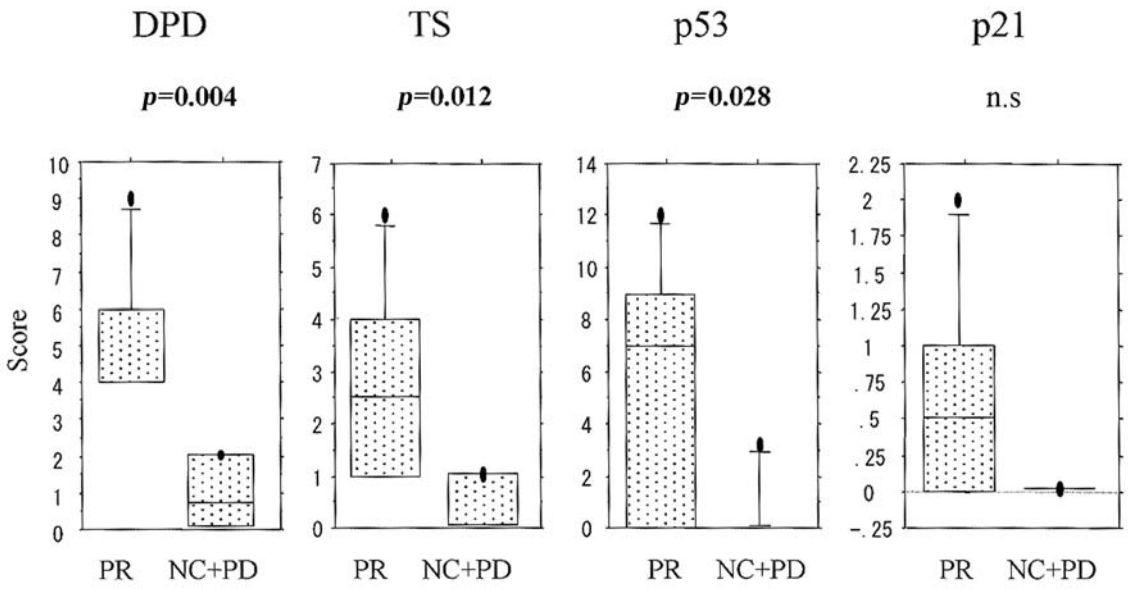

Figure 2. *Comparison of metastatic liver lesions in the PR group and the NC+PD group. The expression levels of DPD, TS and p53 were significantly higher in the PR group than in the NC+PD group (p<0.05).*

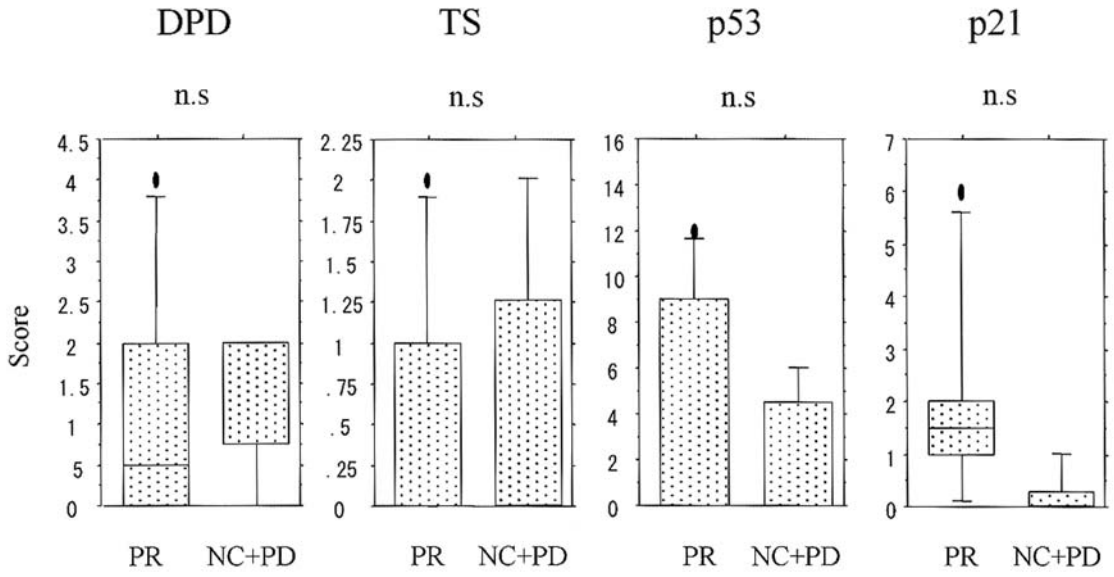

Figure 3. *Comparison of primary lesions in the PR and NC+PD groups. No significant differences in the expression levels of the 4 genes were seen between the PR and NC+PD groups.*

we compared the expression levels of these 4 genes in the chemotherapy response and no response groups. The mean scores for *DPD, TS* and *p53* immunoreactivity were significantly higher in the response group than in the no response group. All of the differences were statistically significant, as shown in Figure 2. However, the scores for *p21* were not significantly different between the two groups.

In the response group, viable cancer cell nests were seen in confined spaces surrounded by fibrous tissue. It was of interest that these cancer cells in the response group showed conspicuous immunoreactivity of *DPD, TS* and *p53*.

When the primary colorectal lesions from the archival samples were examined, no significant differences in the scores for any of the 4 genes were found between the response and no response groups (Figure 3).

Discussion

The expression of genes thought to be related to 5-FU chemosensitivity has been extensively investigated in the hope that methods for predicting 5-FU sensitivity might be established. However, little data is available on the expression patterns of these genes after chemotherapy. In our institute, the hepatic arterial infusion of 5-FU-based chemotherapy is routinely performed in patients with multiple liver metastases to improve the curative resection rate. Using surgical specimens, we examined the expression of 4 genes in liver tumors obtained from patients after chemotherapy. Although the number of cases in this study was limited, the expression rates of *DPD, TS* and *p53* were significantly higher in the response group than in the no response group. These findings suggest that the surviving tumor cells have both malignant and 5-FU-resistant characteristics.

DPD is the initial, rate-limiting enzyme in the catabolism of fluoropyrimidines, through which more than 80% of administered 5-FU is eliminated. Thus, the activity of this enzyme limits the efficacy of 5-FU treatment and is associated with tumor resistance to 5-FU (4,5). The intratumoral expression level of *TS* is considered a prognostic factor for survival in patients with colorectal cancer (6-8), although the ability of this marker to predict 5-FU chemosensitivity is controversial (9). The main pathway by which anticancer drugs induce apoptosis is a *p53*-dependent pathway (10,11). Normal *p53* protein has tumor-suppressing properties, and mutations in the *p53* gene result in the disruption of critical growth-regulating mechanisms (12-14). *p53* is also related to the malignancy of tumors and/or tumor resistance to chemotherapy. We previously reported that *p21* expression was correlated with the inhibiting activity of 5-FU (15), suggesting that *p21* may be a marker of 5-FU sensitivity.

These findings suggest two hypotheses: i) cells that are sensitive to 5-FU undergo apoptosis, but those that are resistant survive after chemotherapy, and ii) 5-FU exposure induces a mechanism that leads to drug resistance. The first hypothesis is feasible, but no direct evidence has been obtained to support this idea. However, Michael *et al.* examined *TS* expression in colorectal liver metastases after chemotherapy and found that previous fluorouracil exposure seemed to increase the resistance of the tumor cells to regional floxuridine *via TS* up-regulation (16). Nishiyama *et al.* performed an *in vitro* study on 5-FU exposure to examine changes in the expression of various genes, including *TS, DPD* and *MRP*. Although the results were very complicated, making their interpretation difficult, *DPD* and *TS* expression tended to increase in 5-FU-resistant cell lines after exposure to 5-FU (17). The mechanism of 5-FU chemoresistance is impossible to explain using the results of the present study alone.

However, the present findings may support the data obtained by the NSABP B-16 study on preoperative chemotherapy in patients with breast cancer (1). The outcome of chemotherapy was better in women whose tumors showed a pathological complete response than in women whose tumors exhibited a clinical partial response or a clinical no-response (relapse-free survival rates, 85.7%, 76.9% and 63.9%, respectively). Unless the cancer cells are totally killed by the drugs, remnant tumor cells survive and the prognosis of the patient does not improve. These findings strongly suggest that the initial chemotherapy treatment should be changed to one with a different mechanism, such as switching anthracycline to taxane, in patients with breast cancer who exhibit anything but a pathological complete response.

We also examined the expression of the 4 genes in the primary colorectal cancers obtained from this patient series, because liver metastases specimens obtained before chemotherapy were not available. No significant differences in the immunoreactivity of the 4 genes were seen between the response and the no response groups. Some differences in gene expression between the primary colorectal tumor cells and the metastatic liver tumor cells may exist. Therefore, it is not clear whether the 5-FU administration altered the expression of the 4 genes in the metastatic liver tumors after chemotherapy in the 2 groups.

Cancer chemotherapy has gradually improved with the production of new drugs exhibiting unique mechanisms and modifications. Hepatic resection after chemotherapy provides useful information enabling second-line chemotherapy treatments to be optimized. The results of this study suggest that a partial response may not be sufficient to improve the prognosis of colorectal cancer. Single-drug use limits the efficacy of treatment, while combination or sequential usage, like modified 5-FU, camptothecin and taxane regimens, may improve the prognosis of colorectal cancer patients.

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