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Intratumoral Dihydropyrimidine Dehydrogenase Messenger RNA Level Reflects Tumor Progression in Human Colorectal Cancer

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Background: Determination of intratumoral dihydropyrimidine dehydrogenase (DPD) is of clinical interest because increased DPD levels can influence the tumor response to 5-fluorouracil–based chemotherapy through increased inactivation of the agent in tumor cells.

Methods: DPD messenger RNA (mRNA) levels were evaluated in 80 consecutive patients undergoing surgery for primary colorectal cancer and 12 cases of liver metastasis.

Results: Higher DPD mRNA levels were associated with higher pathologic classification, corresponding to the T categories ($r = .267$; $P = .003$). The DPD mRNA level was statistically higher in tumors with microscopic lymph node metastasis than in those without ($P = .002$). Hence, the DPD mRNA level increased in accordance with Dukes' classification ($r = .387$; $P = .0001$). The DPD mRNA level of the liver metastasis from colorectal cancer was significantly higher than that of primary lesions ($P = .002$). In eight patients, the DPD mRNA level of the liver metastasis was significantly higher than that of the matched primary tumor ($P = .017$).

Conclusions: Increases of the DPD mRNA level in cancerous tissue seem to reflect tumor progression. High DPD mRNA levels in liver metastasis and advanced colorectal cancer may have clinical importance for 5-fluorouracil–based chemosensitivity.

Key Words: Dihydropyrimidine dehydrogenase—5-Fluorouracil—Reverse transcription-polymerase chain reaction—Messenger RNA level—Colorectal cancer—Metastatic liver tumors.

5-Fluorouracil (5-FU) is one of the most commonly used anticancer agents in gastrointestinal cancer chemotherapy, whereas dihydropyrimidine dehydrogenase (DPD; Enzyme Commission 1.3.1.2) is the rate-limiting enzyme for 5-FU catabolism.[1] Recently, determination of intratumoral DPD activity has been of clinical interest because increased intratumoral DPD activity can influence the tumor response to 5-FU chemotherapy through increased inactivation of the agent in tumor cells.[2–4] Measurement of DPD activity in head and neck cancer patients before 5-FU chemotherapy revealed that the tumor:normal DPD activity ratio was higher in nonresponding patients than in responders, and this suggests that increased intratumoral catabolism can influence tumor response to 5-FU chemotherapy by decreasing the amount of drug available to form cytotoxic nucleotides.[5]

The development of polymerase chain reaction (PCR) technology has provided alternative methods for estimating enzyme activity.[6] Because routine measurement of DPD enzyme activity is not technically feasible in many institutes, the semiquantitative reverse transcription-PCR (RT-PCR) method can be used to determine DPD levels in small specimens, such as biopsy specimens, without the need for a radioisotope.[7] We have already reported a significant linear correlation between DPD activity and DPD messenger RNA (mRNA) levels in human colorectal cancer, indicating that the DPD mRNA level determined by semiquantitative RT-PCR reflects DPD activity.[8] Johnston et al.[9] reported the same positive relationship between DPD mRNA level and activity by

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using real-time PCR methods. Moreover, the DPD mRNA level has also been shown to influence the response to 5-FU chemotherapy.[10]

Takechi et al.[11] reported that DPD activity increases in accordance with the progression of cancer cells (increases of cellularity and growth in size of the xenograft) in cancer cell lines. DPD activity has also been measured in surgical specimens from colorectal tumor and adjacent normal tissue from 63 patients, including 3 with liver metastasis.[12] The DPD activity of the liver metastasis specimens was higher than that of the primary colorectal cancer in all three cases. Hepatic metastasis is one of the main targets in chemotherapy for colorectal cancer, although the difference in DPD mRNA levels between primary and metastatic tumor tissue is unclear. Only a few reports have investigated this difference in clinical samples, and no previous study has compared DPD mRNA levels between metastatic liver tumor and the primary colorectal cancer.

In this study, we investigated 80 consecutive cases of primary colorectal cancer that were surgically resected, to clarify the relationship between intratumoral DPD mRNA levels and tumor progression in human colorectal cancer patients. Moreover, the DPD mRNA level of liver metastasis tissue was compared with that in the corresponding primary colorectal cancer without previous 5-FU–based chemotherapy.

MATERIALS AND METHODS

Patients and Samples

DPD mRNA levels were evaluated in 80 consecutive patients (47 men and 33 women; average age, 64.6 years) undergoing surgery for primary colorectal cancer and 12 patients (5 men and 7 women) with colorectal cancer with liver metastasis treated between November 1996 and October 1998 at 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. Four of the 12 liver tumors were synchronous metastases, and 8 were metachronous metastases. Thus, in 8 of these 12 patients, the DPD mRNA level could be determined in both the primary cancer and the metastasis. Four of the 12 patients had undergone resection of the primary lesion before November 1996. No patient had received 5-FU–based chemotherapy before the study.

Immediately after resection, approximately 50 mg of the tumor and adjacent normal tissue was removed, flash-frozen in liquid nitrogen, and stored at −80°C until the preparation of RNA extracts. Pathologic evaluation of the remaining specimen was performed by the gastrointestinal pathologist. No contamination of the normal colonic mucosa or liver tissue in tumor samples was histologically identified.

RT-PCR Semiquantification of DPD mRNA

RT-PCR–based semiquantification of DPD mRNA expression was performed as previously described in detail.[8,11,13] Total RNA was isolated as outlined by the manufacturer of the RNeasy™ minikit (Qiagen Inc., Chatsworth, CA). RT was performed in a total volume of 100 μl containing 250 pmol of oligo(dT)$_{18}$, 80 U of RNasin™ ribonuclease inhibitor (Promega, Madison, WI), and 500 U of Molony murine leukemia virus RT (Gibco BRL, Gaithersburg, MD) in 50 mM of Tris-HCl (pH 8.3), 75 mM of KCl, 3 mM of $MgCl_2$, 10 mM of DTT, and .5 mM of deoxynucleotide triphosphate solution. To minimize tube-to-tube variation and to normalize DPD expression relative to an internal standard gene,[11,14–16] DPD mRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, as internal standard) mRNA were coamplified in the same tube.

PCR primers were designed on the basis of the nucleotide sequence of human DPD[17] and GAPDH,[18] as described previously.[8] PCR was performed in a final volume of 50 μl containing 20 μl of complementary DNA, 40 pmol of each DPD primer, 2 pmol of each GAPDH primer, and 1.25 U of Ex Taq™ polymerase (TaKaRa, Shiga, Japan) in 5 μl of 10× Ex Taq buffer (TaKaRa) and .2 mM of deoxynucleotide triphosphate solution, by using a thermal cycler (PCR Thermal Cycler MP™; TaKaRa). For accurate quantification, three different complementary concentrations were used to determine whether amplification had occurred in the linear phase.[19] PCR products were separated by 2.0% agarose gel electrophoresis, stained with ethidium bromide, visualized under an ultraviolet transilluminator, and photographed on type 667™ film (Polaroid, Cambridge, MA). Positive results were scanned with an image scanner (JX-330™; Sharp, Mahwah, NJ) and analyzed with the Image Master 1D™ software package (Pharmacia Biotech, Piscataway, NJ). The relative amount of DPD mRNA was expressed as the ratio of DPD mRNA to GAPDH mRNA.

Statistical Analysis

The relationship between the DPD mRNA level and Dukes' staging[20] or pT number (pathologic classification, corresponding to the T categories described by the International Union Against Cancer[21]) was assessed with Spearman's rank test. Comparisons of DPD mRNA level among other clinicopathologic factors were performed with the Mann-Whitney *U*-test. Comparisons of DPD

mRNA level between liver metastasis and colorectal cancers were performed with the Mann-Whitney *U*-test. Comparisons of the DPD mRNA level between matched liver metastasis and primary colorectal cancers were made by using the Wilcoxon signed-rank test in eight cases. Statistical significance was established at the $P < .05$ level for each analysis.

RESULTS

The DPD mRNA level was assessed in 80 consecutive primary colorectal cancer patients. Table 1 shows a comparison of the DPD mRNA level in primary colorectal tumor tissue among patients' clinicopathologic factors. No significant difference in DPD mRNA level was observed among sex, age, location, histological type, and size of the tumor. Higher DPD mRNA levels were associated with higher pT numbers ($r = .267$; $P = .003$; Spearman's rank correlation). The DPD mRNA level was statistically higher in tumors with microscopic lymph node metastasis than in tumors without ($P = .002$; Mann-Whitney *U*-test). Thus, the DPD mRNA level increased in accordance with Dukes' classification.[20] The median DPD mRNA level for each Dukes' stage was as follows: Dukes A, .250; Dukes B, .332; Dukes C, .504; and Dukes D, .531 ($r = .387$; $P = .0001$; Spearman's rank correlation). In addition, the DPD mRNA level was statistically higher in tissue from tumors with lymphatic permeation than from those without ($P = .010$; Mann-Whitney *U*-test). Similarly, the DPD mRNA level was statistically higher in tissue from tumors with vessel involvement than from those without ($P = .013$; Mann-Whitney *U*-test).

TABLE 1. *A comparison of the DPD mRNA levels in tumor tissue among patients' clinicopathologic factors*

Clinicopathologic value	n	DPD mRNA	*P* value
Sex			
Male	47	.45	
Female	33	.33	NS
Age			
≤65 y	45	.43	
>65 y	35	.38	NS
Location			
Colon	48	.37	
Rectum	32	.42	NS
Histological type			
Well	38	.32	
Moderate	36	.50	
Poor	6	.48	NS
Size of tumor			
≤50 mm	39	.39	
>50 mm	41	.42	NS
Depth of invasion			
Tis	4	.23	
T1	4	.32	
T2	13	.47	.048
T3	34	.48	
T4	25	.98	
Lymph node metastases			
Negative	35	.30	
Positive	45	.50	.002

DPD, dihydropyrimidine dehydrogenase; mRNA, messenger RNA; NS, not significant.

Measurement of the DPD mRNA level was possible in all 12 cases with metastatic liver tumors. The DPD mRNA level in hepatic metastatic foci was significantly higher than that in primary lesions (median DPD:GAPDH ratio, .86 and .40, respectively; $P = .002$; Mann-Whitney *U*-test; Fig. 1). The DPD mRNA level of liver metastasis tissue was higher than that of matched primary colorectal cancer foci for all eight cases, with a median DPD:GAPDH ratio of .89 and .45, respectively ($P = .017$; Wilcoxon signed-rank test; Fig. 2). No correlation was observed between DPD mRNA levels in primary colorectal cancer and liver metastasis. The DPD mRNA level in normal liver tissue was statistically higher than in metastatic liver tumor from colorectal cancer (median DPD:GAPDH ratio, 3.188 and .831, respectively; $P = .0019$; Wilcoxon signed-rank test). No correlation was found between the DPD mRNA level in the liver metastases and paired normal liver tissue.

DISCUSSION

This study demonstrated that the DPD mRNA level in liver metastasis tissue is statistically higher than in primary colorectal cancer, similar to Johnston et al.[9], who

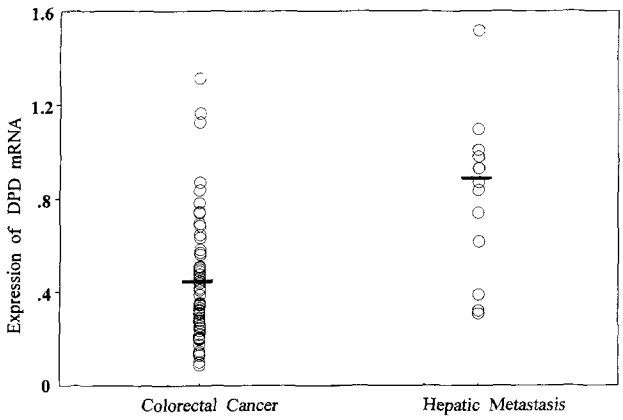

FIG. 1. A comparison of the dihydropyrimidine dehydrogenase (DPD) messenger RNA (mRNA) levels in the hepatic metastatic foci of 12 patients and primary foci in colorectal cancers of 84 patients. The DPD mRNA levels in hepatic metastatic foci were significantly higher than those in primary foci (median DPD:glyceraldehyde-3-phosphate dehydrogenase ratio, .86 and .40, respectively; $P = .0020$; Mann-Whitney *U*-test).

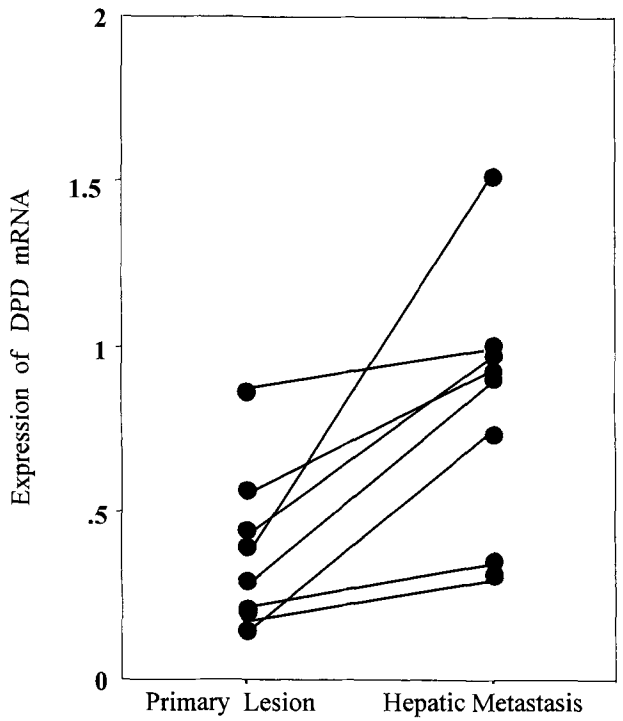

FIG. 2. A comparison of the dihydropyrimidine dehydrogenase (DPD) messenger RNA (mRNA) levels in hepatic metastatic foci and those of matched primary colorectal cancer. The DPD mRNA levels of the liver metastatic foci were significantly higher than those of the matched primary colorectal cancers (median DPD:glyceraldehyde-3-phosphate dehydrogenase ratio, .89 and .45, respectively; $P = .017$; Wilcoxon signed-rank test).

reported that the median DPD mRNA expression in colorectal liver metastasis was higher than that in primary colorectal tumor. Furthermore, significantly higher DPD activity has also been reported in liver metastasis than in primary colorectal cancer.[12] Moreover, significantly higher DPD mRNA levels of metastatic liver tumors were observed between matched primary cancerous and metastatic tissue in our study. Thus, the difference in DPD mRNA expression between primary cancer tissue and liver metastasis should be taken into account when predicting the antitumor effect of 5-FU–based chemotherapy.

DPD activity is found in most human tissues, with the highest levels in liver and peripheral mononuclear cells.[22,23] In this study, the DPD mRNA level in normal liver tissue was statistically higher than in metastatic liver tumor from colorectal cancer. No correlation was found between DPD mRNA levels in liver metastases and paired normal liver tissue; this indicates that the mechanism for regulation of DPD gene expression differs between liver metastasis and normal liver tissue. Similarly, no correlation was observed between DPD mRNA levels in primary colorectal cancer and liver metastasis. We have already reported a significant linear correlation between DPD activity and mRNA levels in human colorectal cancer.[8] However, little is known about the details of the regulation mechanism of DPD in either primary or metastatic tumors. Takenoue et al.[24] reported a poor correlation between DPD mRNA and protein levels. The regulation system of DPD mRNA and protein expression is still unclear.

In this analysis of 80 consecutive cases of colorectal cancer, higher intratumoral DPD mRNA levels were observed in tumors with a higher pT number and the presence of lymph node metastasis. Our 80 consecutive patients with surgically resected colorectal cancer included Dukes' A to D patients (not only patients with far-advanced or recurrent cancer about to be treated with 5-FU), and this enabled clarification of the relationship between intratumoral DPD mRNA levels and tumor progression in human colorectal cancer. The results suggest that increases in the intratumoral DPD mRNA level reflect tumor progression in human colorectal cancer. Thymidine phosphorylase (Enzyme Commission 2.4.2.4), another metabolic enzyme of nucleic acid expression, is also reported to correlate with tumor malignancy.[25] In addition, in 163 patients with colorectal cancer, high thymidine phosphorylase levels were associated with poor clinical outcome.[26] DPD is supposed to be one of the catalytic enzymes of nucleic acid that has higher gene expression in tumors with higher malignancy.

In summary, we have shown that a higher DPD mRNA level in tumors is associated with higher pT number and the presence of lymph node metastasis. Furthermore, a higher level of DPD mRNA was observed in liver metastasis than in primary colorectal cancers. Such high DPD mRNA levels in liver metastasis and highly advanced colorectal cancer may have clinical importance for 5-FU–based chemosensitivity, because of rapid degeneration of this anticancer agent by DPD. For highly advanced colorectal cancer, the results of this study suggest that the administration of a DPD inhibitor, perhaps containing a fluoropyrimidine, may improve the response to chemotherapy.

REFERENCES

1. Heggie GD, Sommadosi J-P, Cross DS, Huster WJ, Diasio RB. Clinical pharmacokinetics of 5-fluorouracil and its metabolites in plasma, urine, and bile. *Cancer Res* 1987;47:2203–6.
2. Porter DJ, Chestnut WG, Merrill BM, Spector T. Mechanism-based inactivation of dihydropyrimidine dehydrogenase by 5-ethynyluracil. *J Biol Chem* 1992;267:5236–42.
3. Spector T, Harrington JA, Porter DJ. 5-Ethynyluracil (766C85): inactivation of dihydropyrimidine dehydrogenase in vivo. *Biochem Pharmacol* 1993;46:2243–8.

4. Takechi T, Uchida J, Fujioka A, Fukushima M. Enhancing 5-fluorouracil cytotoxicity by inhibiting dihydropyrimidine dehydrogenase activity with uracil in human tumor cells. *Int J Oncol* 1997;11:1041–4.
5. Etienne MC, Cheradame S, Fischei JL, et al. Response to fluorouracil therapy in cancer patients: the role of tumoral dihydropyrimidine dehydrogenase activity. *J Clin Oncol* 1995;13:1663–70.
6. Eisenstein BI. The polymerase chain reaction. A new method of using molecular genetics for medical diagnosis. *N Engl J Med* 1990;322:178–83.
7. Chu E, Koeller DM, Casey JL, et al. Autoregulation of human thymidylate synthase messenger RNA translation by thymidylate synthase. *Proc Natl Acad Sci U S A* 1991;88:8977–81.
8. Uetake H, Ichikawa W, Takechi T, Fukushima M, Nihei Z, Sugihara K. Relationship between intratumoral dihydropyrimidine dehydrogenase activity and gene expression in human colorectal cancer. *Clin Cancer Res* 1999;5:2836–9.
9. Johnston SJ, Ridge SA, Cassidy J, McLeod HL. Regulation of dihydropyrimidine dehydrogenase in colorectal cancer. *Clin Cancer Res* 1999;5:2566–70.
10. Salonga D, Danenberg KD, Johnson M, et al. Colorectal tumors responding to 5-fluorouracil have low gene expression levels of dihydropyrimidine dehydrogenase, thymidylate synthase, and thymidine phosphorylase. *Clin Cancer Res* 2000;6:1322–7.
11. Takechi T, Okabe H, Fujioka A, Fukushima M. Relationship between protein levels and gene expression of dihydropyrimidine dehydrogenase in human tumor cells during growth in culture and in nude mice. *Jpn J Cancer Res* 1998;89:1144–53.
12. McLeod HL, Sludden J, Murray GI, et al. Characterization of dihydropyrimidine dehydrogenase in human colorectal tumours. *Br J Cancer* 1998;77:461–5.
13. Ishikawa Y, Kubota T, Otani Y, et al. Dihydropyrimidine dehydrogenase activity and messenger RNA level may be related to the antitumor effect of 5-fluorouracil on human tumor xenografts in nude mice. *Clin Cancer Res* 1999;5:883–9.
14. Horikoshi T, Danenberg KD, Stadlbauer THW, et al. Quantification of thymidylate synthase, dihydrofolate reductase, and DT-diaphorase gene expression in human tumors using the polymerase chain reaction. *Cancer Res* 1992;52:108–16.
15. Dukas K, Sarfati P, Vaysse N, Pradayrol L. Quantitation of changes in the expression of multiple genes by simultaneous polymerase chain reaction. *Anal Biochem* 1993;215:66–72.
16. Murphy LD, Herzog CE, Rudick JB, Fojo AT, Bates SE. Use of the polymerase chain reaction in the quantification of mdr-1 gene expression. *Biochemistry* 1990;29:10351–6.
17. Yokota H, Fernandez-Salguero P, Furuya H, et al. cDNA cloning and chromosome mapping of human dihydropyrimidine dehydrogenase, an enzyme associated with 5-fluorouracil toxicity and congenital thymine uraciluria. *J Biol Chem* 1994;269:23192–6.
18. Tokunaga K, Nakamura Y, Sakata K, et al. Enhanced expression of a glyceraldehyde-3-phosphate dehydrogenase gene in human lung cancers. *Cancer Res* 1987;47:5616–9.
19. Freemantle SJ, Jackman AL, Kelland LR, Calvert AH, Lunec J. Molecular characterisation of two cell lines selected for resistance to the folate-based thymidylate synthase inhibitor, ZD1694. *Br J Cancer* 1995;71:925–30.
20. Dukes CE. The surgical pathology of rectal cancer. *J Clin Pathol* 1949:2:95–9.
21. International Union Against Cancer. Colon and rectum. In: Sobin LH, Wittekind C, eds. *TNM Classification of Malignant Tumours*. 5th ed. New York: John Wiley & Sons, 1997:66–9.
22. Ho DH, Townsend L, Luna MA, Bodey GP. Distribution and inhibition of dihydrouracil dehydrogenase activities in human tissues using 5-fluorouracil as a substrate. *Anticancer Res* 1984;6:781–4.
23. McMurrough J, McLeod HL. Analysis of the dihydropyrimidine dehydrogenase polymorphism in a British population. *Br J Clin Pharmacol* 1996;41:425–7.
24. Takenoue T, Kitayama J, Takei Y, et al. Characterization of dihydropyrimidine dehydrogenase on immunohistochemistry in colon carcinoma, and correlation between immunohistochemical score and protein level or messenger RNA expression. *Ann Oncol* 2000;11:273–9.
25. van Triest B, Pinedo HM, Blaauwgeers JL, et al. Prognostic role of thymidylate synthase, thymidine phosphorylase/platelet-derived endothelial cell growth factor, and proliferation markers in colorectal cancer. *Clin Cancer Res* 2000;6:1063–72.
26. Takebayashi Y, Akiyama S, Akiba S, et al. Clinicopathologic and prognostic significance of an angiogenic factor, thymidine phosphorylase, in human colorectal carcinoma. *J Natl Cancer Inst* 1996;88:1110–7.