Relationship between Intratumoral Dihydropyrimidine Dehydrogenase Activity and Gene Expression in Human Colorectal Cancer

Hiroyuki Uetake,1 Wataru Ichikawa, Teiji Takechi, Masakazu Fukushima, Zenro Nihei, and Kenichi Sugihara
Second Department of Surgery, Tokyo Medical and Dental University, Tokyo 113-8519 [H. U., W. I., Z. N., K. S.], and Taiho Pharmaceutical Co., Ltd., Cancer Research Laboratory-2, Saitama 357-8527 [T. T., M. F.], Japan

ABSTRACT
Dihydropyrimidine dehydrogenase (DPD) is the ratelimiting enzyme for 5-fluorouracil catabolism. In this study, both the enzymatic activity and mRNA level of DPD were estimated in the tumor tissue and adjacent normal mucosa of 51 patients with colorectal cancer. Although no significant difference in enzymatic activity was observed between tumor tissue and normal mucosa (70.4 and 70.7 pmol/min/mg protein, respectively), the mRNA level in normal mucosa was significantly higher than that in tumor tissue (1.37 and 0.39, respectively; P < 0.01). A linear relationship was noted between DPD activity and the DPD mRNA level in cancerous tissue (r = 0.714, P < 0.001). Thus, the DPD mRNA level as determined by reverse transcription-PCR can be used to indicate the DPD activity of colorectal cancers.

INTRODUCTION
5-FU2 is widely used in the treatment of advanced gastrointestinal tumors. After administration, the 5-FU dose is degraded mainly in the liver in a three-step pathway, initially catalyzed by the rate-limiting enzyme DPD (EC 1.3.1.2; Ref. 1). DPD activity is found in most human tissues, with the highest levels in the liver and lymphocytes (2). In addition, DPD activity is detected in some human cancer cell lines and surgically resected tumor specimens, such as head and neck cancers (3) and colorectal cancers (4). Recently, determination of tumoral DPD activity has been of clinical interest because elevated intratumoral DPD activity can influence tumor response to 5-FU therapy through increased inactivation in tumor cells. Etienne et al. (3) determined the DPD activity of biopsy specimens from head and neck cancer patients before the start of 5-FU-based therapy. Among 52 tumors assessable for clinical response, the tumoral:nontumoral DPD activity ratio tended to be higher in the nonresponding patients than in those achieving a partial or complete response.

Measurement of DPD activity in tumors may be a promising parameter to predict 5-FU sensitivity. Recently, biochemical modulation to enhance the antitumor activity of 5-FU by inhibiting intratumoral DPD activity has been attempted. Milano et al. (5) demonstrated that 5-ethyluracil, which is a potent irreversible DPD inhibitor (6, 7), enhances 5-FU cytotoxicity in five human cancer cell lines that express high basal DPD activity. Takechi et al. (8) also demonstrated the same effect of uracil and 5-chloro-2,4, dihydroxypyridine (9) on DPD.

With such a high level of interest in tumoral DPD activity, alternative methods for DPD activity determination that are more convenient than a conventional radio enzymatic assay and may apply to small specimens such as biopsies are required. In the present study, both the catalytic activity and mRNA level of DPD were measured in the tumor tissue and adjacent normal mucosa of 51 patients with colorectal cancer. We describe a semiquantitative RT-PCR method for determination of DPD mRNA expression that may be used for small specimens without the need for radioisotopes. The aim of the present study was to assess the correlation between DPD enzymatic activity and expression of DPD mRNA in human colorectal cancer and normal tissue.

MATERIALS AND METHODS

Patients and Samples. We examined tumor specimens from 51 consecutive patients (28 males and 23 females; average age, 64.0 years) with primary colorectal cancers. Twenty-nine colonic and 22 rectal cancers that had been surgically resected between January 1997 and February 1998 were studied. No one had received previous 5-FU chemotherapy. Eight patients had Duke’s A cancer, 10 had Duke’s B, 24 had Duke’s C, and 9 had Duke’s D. All samples were obtained between 11 a.m. and 2 p.m. The average of the greatest diameter of the tumors was 5.2 cm. The normal mucosa sample was obtained 3 cm apart from the edge of the tumor.

Enzyme Assay. The enzyme assay used a modification of the method of Naguib et al. (10) and Ikenaka et al. (11) and has been described previously (9). Briefly, tumor tissue or normal mucosa specimens were homogenized with four volumes of homogenization buffer [20 mM potassium phosphate (pH 8.0) containing 1 mM β-mercaptoethanol]. Each homogenate was then centrifuged at 105,000 g for 1 h at 4°C, and the resultant supernatant fluid (cytosol) was collected as the enzyme source. Internal substrate including uracil and thymine, which might inhibit DPD activities (9, 11), was removed from the
The DPD activity and expression of DPD mRNA in tumoral and normal tissues is shown. There was no significant difference (ND) of DPD activity between tumor and adjacent normal mucosa. However, the level of DPD mRNA expression in normal mucosa was statistically higher than in tumor ($P < 0.01$).

### Table 1  Enzyme activity and expression of mRNA in cancerous tissue and normal mucosa

<table>
<thead>
<tr>
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<th>Enzyme activity (pmol/min/mg protein)</th>
<th>Expression of mRNA (DPD–GAPDH mRNA ratio)</th>
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<tbody>
<tr>
<td>Cancerous tissue</td>
<td>73.4 ± 33.7</td>
<td>0.39 ± 0.28</td>
</tr>
<tr>
<td>Normal mucosa</td>
<td>73.7 ± 21.9</td>
<td>1.37 ± 1.07</td>
</tr>
</tbody>
</table>

Semiquantitative RT-PCR. The reliability and validity of this method has been described previously in detail by Takechi et al. (12) and Ishikawa et al. (13). As is reported in recent studies aimed at developing quantitative RT-PCR methods, the target gene expression should be normalized relative to an internal standard gene that is expressed at a constant per-cell level, because it might be difficult to measure precisely the quantity of isolated RNA (14). To minimize the tube-to-tube variation, the internal standard gene should be coamplified in the same tube with the mRNA of interest (12, 15, 16). Thus, in the present study, DPD mRNA and GAPDH (as internal standard) mRNA were coamplified in the same tube.

The total RNA of the sample was isolated as outlined by the manufacturer in the RNeasy mini kit (Qiagen, Inc., Chatsworth, CA). The purity and amount of total RNA were estimated spectrophotometrically by measuring the absorbance of an aliquot at 260 and 280 nm. The integrity of the rRNA bands was checked by agarose gel electrophoresis in the presence of formaldehyde. Reverse transcription using up to 10 μg of total RNA was carried out in a total volume of 100 μl containing 250 pmol oligo(dT)$_{18}$, 80 units of RNasin (Promega, Madison, WI), and 500 units of Molony murine leukemia virus reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD) in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl$_2$, 10 mM DTT, and 0.5 mM deoxyribonucleotide triphosphate solution. Initially, the total RNA solution mixed with oligo(dT)$_{18}$ was heated at 70°C for 10 min and immediately chilled on ice. First-strand cDNAs were obtained after 15 min at 30°C and 60 min at 42°C. PCR was carried out in a final volume of 50 μl containing DNA, 40 pmol of each DPD primer, 2 pmol of each GAPDH primer, and 1.25 units of Ex Taq (TaKaRa, Shiga, Japan) in 5 μl of 10X Ex Taq buffer (TaKaRa) and 0.2 mM deoxyribonucleotide triphosphates, using a thermal cycler (TaKaRa PCR Thermal Cycler MP). For accurate quantification using this method, measurements have to be taken in the linear phase of PCR, where the cDNA concentration is directly proportional to signal intensity; hence, we used three different cDNA concentrations to determine whether this linear phase had been covered (17). The PCR profile consisted of a 3-min initial denaturation at 94°C, followed by 30 cycles of 1 min denaturation at 94°C, 1 min of annealing at 60°C, and 2 min of polymerization at 72°C, followed by a final 10-min extension at 72°C.

PCR products were separated by 2.0% agarose gel electrophoresis. Gels were stained with ethidium bromide, visualized on a UV transilluminator, and photographed on Type 667 film (Polaroid, Cambridge, MA). Positive results were scanned using an image scanner (JX-330; Sharp, Mahwah, NJ) and analyzed using the Image Master 1D software package (Pharmacia Biotech). The relative amount of DPD mRNA was expressed as the DPD mRNA:GAPDH mRNA ratio.

#### Statistical Analysis.

Comparisons of DPD activity or mRNA expression in matched tumor and normal tissue were performed using the Wilcoxon test. Comparisons of DPD activity or mRNA expression between gender, age, location of the tumor, and size of tumor were performed using the Mann-Whitney U test. The relationship between activity and mRNA in tumors or normal mucosa was assessed using Spearman’s rank test. Statistical significance was established at the $P < 0.05$ level.

### RESULTS

DPD activity and expression of DPD mRNA were measurable in all tumoral and paired nontumoral samples. Table 1 shows the DPD activity and expression of DPD mRNA in tumoral and normal tissues. No significant difference in DPD activity between tumor and paired normal mucosa was observed. The distribution of DPD activity was wide and asymmetrical, as shown in Fig. 1, with an 8.0-fold range (20.1–159.6 pmol/min/mg protein, with a median value of 61.8) in variability among tumor tissue samples and a 4.6-fold range (25.1–116.1 pmol/min/mg protein, with a median value of 75.0) among normal mucosa samples.

The level of DPD mRNA expression in normal mucosa was statistically higher than that in tumor tissue ($P < 0.001$; Table 1), the coefficient of variation was 72 for the level of DPD mRNA in tumoral tissue and 78 for normal mucosa.

A statistically significant correlation between DPD activity and mRNA expression was observed ($r = 0.714, P < 0.001$) in tumor tissue but not in normal mucosa ($r = -0.148, P = 0.3205$; Fig. 2).
DISCUSSION

This is the first study to compare both expression of DPD mRNA and enzymatic activity between human colorectal cancer tissue and paired normal mucosa. Although determination of DPD levels in tumor tissue is important to predict the clinical therapeutic efficacy of 5-FU, routine measurement of enzyme activity is not technically feasible in many centers. Accordingly, we have developed a semiquantitative RT-PCR method for determination of DPD mRNA expression, which is extremely useful for the measurement of small specimens, biopsies (18), without the need for radioisotopes. In the present study, a statistically significant correlation was observed ($r_s = 0.714, P < 0.001$) between tumoral DPD activity and mRNA expression. Thus, determination of DPD mRNA expression by semiquantitative RT-PCR does reflect the DPD activity of colorectal tumor tissue, which should in turn allow prediction of the sensitivity of the tumor to 5-FU.

In a recent study, McLeod et al. (4) found that the median DPD activity was 54.7 for colorectal tumor tissue and 73.5 for normal mucosa. Similarly in the present study, the median DPD activity was 61.8 and 75.0 in tumor and normal mucosa, respectively. In our previous study, DPD activity of the cytosol increased after Microspin column treatment in a human cancer xenograft and a dog liver specimen (data are not shown). Therefore, we treated the cytosol through Microspin column, although the DPD activity was almost the same as the previous report (4). Furthermore, McLeod et al. (4) also reported that tumor DPD activity was highly variable in human colorectal cancers. The 8.0-fold range in tumor DPD activity observed in the present study was also similar to this [7.9-fold range; McLeod et al. (4)] and to the range for a series of head and neck cancers [14.8-fold range; Etienne et al. (3)].

Although DPD activity was highly variable on any tumor tissue specimens (8.0-fold range), DPD activity was not quite as
variable among normal mucosa (4.6-fold). On the other hand, the DPD mRNA expression in normal mucosa was higher than that in tumor samples ($P < 0.01$) and was not in proportion to enzyme activity. These findings suggest that in normal mucosa, there is a regulatory mechanism that maintains DPD activity within some limit. McLeod et al. (4) suggested the possibility of down-regulation of DPD activity in culture cells. Takechi et al. (12) reported the possibility of a posttranscriptional regulation mechanism in DPD activity in some tumor cell lines. It was reported that ferritin and thymidylate synthase mRNA translation is regulated by its protein product (19, 20). The mechanism of this regulation for DPD remains unclear, but it was shown in the present study that in human colorectal cancer tissue, the DPD mRNA level reflects the DPD activity.

REFERENCES


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