Regulation of Dihydropyrimidine Dehydrogenase in Colorectal Cancer[1]

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ABSTRACT

Dihydropyrimidine dehydrogenase (DPD) is responsible for degradation of the pyrimidines uracil and thymine and the inactivation of the chemotherapeutic agent 5-fluorouracil. DPD activity is highly variable in cancer populations, and this variation may influence the antitumor efficacy of 5-fluorouracil. However, little is known about the regulation of DPD mRNA expression in any tissues. Using a reverse transcription competitive PCR assay, we quantified DPD mRNA levels in 10 matched colorectal tumors and adjacent normal mucosae and 7 colorectal liver metastases and adjacent normal livers. Lower levels of DPD mRNA expression were observed in colorectal tumor compared with adjacent normal colon mucosa (median, 0.01 *versus* 0.37 amole/µg total RNA, $P = 0.02$). DPD mRNA expression was also lower in metastases than adjacent normal liver tissue (median, 0.11 *versus* 1.17 amole/µg total RNA, $P = 0.001$). DPD mRNA expression was higher in normal liver than normal colonic mucosa (median, 1.17 *versus* 0.37 amole/µg total RNA, $P = 0.02$). A significant relationship was observed between DPD mRNA and catalytic activity ($r_s = 0.66$, $P < 0.001$). The tumor:normal ratio for DPD mRNA, protein, and activity was relatively stable in liver (0.25, 0.55, and 0.51, respectively) but varied considerably in colon (0.085, 0.9, and 1.25, respectively), consistent with enhanced translation of DPD transcript in primary colorectal tumor. This suggests that DPD can be regulated at the levels of both transcription and translation.

INTRODUCTION

The increased utilization of uracil by tumor tissue has been described for over 40 years and led to the rational design of 5FU[3] as an anticancer agent (1, 2). 5FU is the third most commonly prescribed anticancer agent and is used in the treatment of colorectal, breast, and head and neck malignancies (3). Although most investigations have focused on the activation of 5FU to cytotoxic nucleotides (*e.g.*, fluoro-dUMP), the majority (~85%) of an administered dose is degraded to inactive metabolites by the cytosolic enzyme DPD (4). DPD activity is highly variable in both normal and cancer populations (5–7). Impaired DPD activity, secondary to gene mutations, which has been described previously (8), results in severe toxicity after administration of 5FU. In addition, the ratio of DPD activity in paired tumor-normal tissue samples from head and neck cancer patients was higher in those with stable or progressive disease than it was in those achieving an objective response to 5FU therapy (9). This suggests that intratumoral 5FU degradation is a mechanism of 5FU resistance.

The molecular mechanisms responsible for the observed variations in DPD activity are as yet undefined but may involve alterations in gene transcription. A 3.4-kb human DPD gene transcript has been isolated and cloned (10). However, initial mRNA analysis by Northern hybridization suggests that DPD is a rare transcript that cannot easily be detected by standard methodologies (11).[4] Therefore, a cPCR assay was developed to provide quantitative analysis of DPD mRNA. Because previous studies have demonstrated that cell culture and xenograft models are inappropriate for evaluating DPD (12, 13), the cPCR assay was used in human tissue. The assay has been validated and used to evaluate variable mRNA expression of DPD in colorectal tumor. The relationships among DPD activity, protein expression, and mRNA expression were then determined in human liver, colon, and tumor tissues to establish insights into the *in vivo* regulation of this clinically relevant enzyme.

MATERIALS AND METHODS

Materials. [^{14}C]5FU (54 mCi/mmol), mouse antirabbit antibodies, and enhanced chemiluminescence substrate for Western blotting were purchased from Amersham International (Little Chalfont, United Kingdom). Taq DNA polymerase, 10× buffer, $MgCl_2$, and JM109 competent cells were obtained from Promega (Southhampton, United Kingdom). Superscript II reverse transcriptase and 5× buffer were obtained from Life Technologies (Paisley, United Kingdom). The Bigger Prep Kit was obtained from 5'→3' Inc. (Boulder, CO). All other chemicals were obtained from Sigma Chemical Co. (Poole, Dorset, United Kingdom).

amoles of competitor template

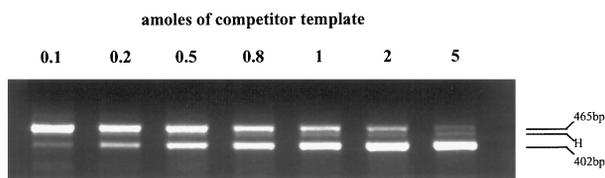| 0.1 | 0.2 | 0.5 | 0.8 | 1 | 2 | 5 |

465bp
H
402bp

Fig. 1 cPCR analysis of DPD mRNA. The ratio of competitor to DPD template increased as the concentration of competitor increased. DPD mRNA concentration was calculated as the point at which equal amounts of competitor and DPD PCR products were formed.

Table 1 Median DPD mRNA expression and enzyme activity in liver, liver metastases, colon, and colon tumor[a]

Tissue	DPD mRNA (amole/μg total RNA)	DPD activity (pmol/min/mg protein)
Liver	1.17 (0.56–2.14)	266 (210–380)
Liver metastasis	0.37 (0.02–0.66)	125 (100–210)
Colon	0.11 (0.01–0.33)	47 (20–70)
Colon tumor	0.01 (0.01–0.22)	50 (20–50)

[a] Numbers in parentheses represent ranges.

Tissue Banking. Viable tumor and adjacent normal tissue from surgical resection specimens were dissected immediately postsurgery by an experienced pathologist and snap-frozen in liquid nitrogen. The same pathologist also carried out histological examination to assess the degree of tumor cellularity in the samples. Only patients with metastatic disease had previously received 5FU chemotherapy. All samples were stored at $-80°C$ until they were required. The study was approved by the University of Aberdeen and Grampian Health Board Joint Ethical Committee.

RNA Extraction and First Strand cDNA Synthesis. Total RNA was extracted from tissues using a guanidium isothiocyanate phenol-chloroform method (14) and quantified by UV spectrophotometry. RNA integrity was assessed by electrophoresis through 1.2% denaturing formaldehyde gels, and any samples exhibiting degradation were not used. First strand cDNA was prepared in 20-μl reactions containing 10 μg of total RNA, 0.2 μg/μl random hexamers, 20 units of RNase inhibitor, 50 mM Tris-HCl (pH 8.3), 49 mM KCl, 6 mM $MgCl_2$, 1 mM DTT, 500 μM dNTP mix, and 300 units of Superscript II reverse transcriptase. The reactions were allowed to proceed for 60 min at 37°C before heat inactivation. The cDNA was stored in aliquots at $-20°C$ until required. The integrity of each cDNA sample was evaluated by amplification of a 543-bp fragment of human β-actin cDNA.

Quantification of DPD cDNA by cPCR. cPCR was performed with the sense primer QF (5'-CACTCCTATT-GATCTGGTGGAC-3') and the antisense primer QR (5'-CATTCCTCTTTCTCCCATGC-3') to produce a 465-bp product (cDNA nucleotides 1656–2121). The PCR product spanned intronic boundaries and did not produce a PCR product from genomic DNA (data not shown). The competitor template was a truncated fragment of DPD cDNA sequence engineered to contain identical primer binding sites to wild-type DPD but have a 63-bp deletion. Coamplification of target DPD cDNA and competitor fragment yielded 465- and 402-bp products, respectively. For each sample, PCRs were performed in which a series of known concentrations of competitor template were coamplified with a fixed amount of sample cDNA in 50-μl reactions (Fig. 1). Each assay was carried out in triplicate.

Analysis of PCR Products. The PCR products were resolved by electrophoresis through 2.5% agarose gels and visualized by ethidium bromide staining. The images were scanned using an UV Products gel documentation system (Cambridge, United Kingdom) and analyzed by densitometry using Molecular Analyst software (Bio-Rad, Richmond, CA). To correct for differences in PCR product size, we multiplied the density of the competitor band by a correction factor of 1.16 (465/402). The logarithm of corrected ratio of target:competitor was plotted against the logarithm of the concentration of the competitor template.

Validation of cPCR Assay. The assay was validated by quantifying DPD mRNA levels in preparations from normal liver. Accuracy and linearity were determined by assaying serial dilutions of liver cDNA. DPD mRNA could be quantified at concentrations as low as 0.01 amole/μg total RNA, and the assay was linear from 0.01 to 10 amole/μg total RNA ($r^2 = 0.99$). The intra-assay coefficient of variation was 4.2% ($n = 10$). The efficiency of cDNA preparation and reverse transcription was assessed by assaying cDNA samples prepared from the same liver RNA sample on 10 separate occasions. The resulting coefficient of variation was 11%.

DPD Activity Measurement. DPD activity was determined using a previously described method (15). Tissue cytosol (50 μl) was incubated in a reaction mix containing 125 μM NADPH, 20 μM [^{14}C]5FU, 35 mM potassium phosphate buffer (pH 7.4), 2.5 mM $MgCl_2$, and 10 mM β-mercaptoethanol in a final volume of 125 μl. The reaction mix was incubated at 37°C for 45 min (liver) or 60 min (colon and tumor). The reaction was stopped by the addition of an equal volume of ice-cold 99% ethanol. The supernatant was then assayed for 5FU catabolites using a previously described high-performance liquid chromatography method (15). Each sample was analyzed in triplicate, and a negative control (no NADPH) was included for each sample. DPD activity was expressed as pmol of catabolite formed per minute and was normalized for cytosolic protein concentration (pmol/min/mg protein).

Immunoblot Analysis. A rabbit polyclonal antibody was generated against human DPD using a peptide for amino acids 1006–1020. This antibody was specific for a M_r 105,000 band in human tissues. Fifty μg of cytosolic protein from tissue lysates were resolved on a 7.5% polyacrylamide gel. The resolved proteins were transferred to a nitrocellulose membrane (Immobilon-P; Millipore, Bedford, MA) and blocked for 2 h at room temperature with 5% milk in TBST (Tris-buffered saline-0.1% Tween) before incubation with the primary antibody (1:2000 dilution in 5% milk-TBST) for 2 h at room temperature. The membrane was then incubated with a horseradish peroxidase-linked antirabbit antibody, followed by detection by chemiluminescence. The intensity of DPD protein expression was then determined using densitometry. The ratio of tumor and
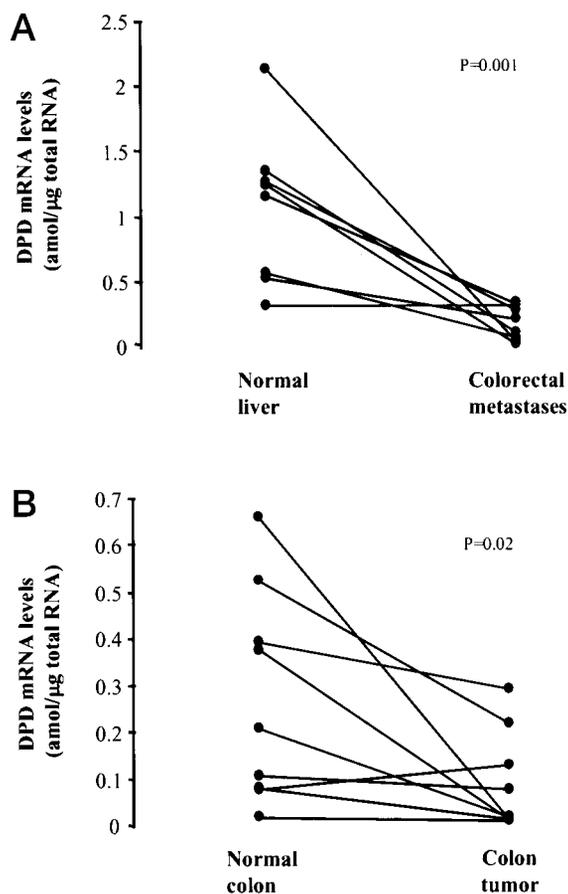
Fig. 2 DPD mRNA levels in paired tumor and normal tissue for liver (*A*) and colorectal tissue (*B*).

Fig. 3 Correlation between DPD mRNA and enzyme activity in normal (○) and tumor (●) tissue.

Tumor/normal ratio	1	2	3	4
mRNA	0.026	0.015	0.42	0.097
Protein	0.90	0.71	0.45	1.70
Activity	0.57	1.25	0.71	1.25

Fig. 4 Representative blot of DPD protein expression in colon mucosa (*Lanes N*) and matched colorectal tumor (*Lanes T*) from four patients. Corresponding DPD mRNA and activity values are shown below each lane. DPD mRNA expression is clearly reduced in the tumor tissues, whereas protein and activity remain unchanged.

normal tissue protein content was used as a determinant of relative DPD protein expression. Paired samples were analyzed on the same membrane.

Statistical Analysis. Comparisons of mRNA expression, DPD catalytic activity, and protein content between matched tumor and normal tissue were made using the Wilcoxon test. Comparisons of DPD mRNA expression, protein content, and activity between liver and colon tissues were performed using the Mann-Whitney test. The relationship between DPD mRNA expression and catalytic activity was assessed by the Spearman's rank test.

RESULTS

Characterization of DPD mRNA in Colorectal Tumors. DPD mRNA levels were quantified in 10 pairs of colorectal tumors and adjacent normal mucosae and 7 matched colorectal liver metastases and adjacent nonmalignant liver tissues (Table 1). DPD mRNA was expressed in a tissue-specific manner, with significantly higher levels in normal liver than in normal colon mucosa (median, 1.17 *versus* 0.37 amole/μg total RNA, $P = 0.02$). DPD mRNA levels were significantly lower in tumor than in matched normal tissue for both liver and colon (liver: median, 0.11 *versus* 1.17 amole/μg total RNA, $P = 0.001$; colon: me-
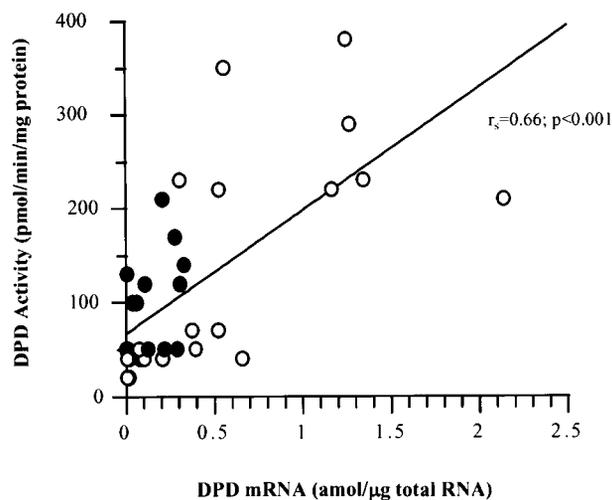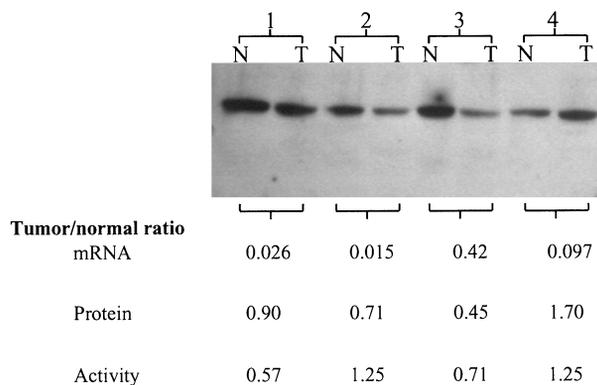
dian, 0.01 *versus* 0.37 amole/μg total RNA, $P = 0.02$; Fig. 2). Median DPD mRNA expression in colorectal metastases was greater than that in primary colon tumor (0.11 *versus* 0.01 amole/μg total RNA, $P = 0.01$; Fig. 2). DPD mRNA expression was correlated with enzyme activity for both normal tissue ($r_s = 0.67$, $P = 0.005$) and tumor tissue ($r_s = 0.67$, $P = 0.006$) and for all tissues combined ($r_s = 0.66$, $P < 0.001$; Fig. 3).

DPD Transcription and Translation in Colorectal Tumors. DPD activity was significantly higher in normal liver than in normal colon mucosa (median activity, 266 *versus* 47 pmol/min/mg protein, $P = 0.001$). DPD activity and protein content were significantly lower in colorectal metastases than in matched normal liver (median activity ratio, 0.51, $P < 0.001$; median protein ratio, 0.55, $P = 0.035$; Fig. 4). However, DPD activity and protein content were similar in colorectal tumor and normal colon mucosa (median activity ratio, 1.25, $P = 0.89$; median protein ratio, 0.9, $P = 0.52$; Fig. 3). In contrast, the

median tumor:normal tissue ratios of DPD mRNA were 0.25 in liver and 0.085 in colon.

DISCUSSION

This study provides a description of DPD mRNA expression in human tissue and, more specifically, colorectal tumor. DPD mRNA levels were 37- and 10.6-fold (median values) lower in tumor than they were in adjacent normal liver and colon, respectively. Decreased tumor uracil catabolism has previously been reported in colorectal and hepatocellular carcinoma with lower DPD activity than normal tissue (12, 16). Down-regulation of DPD expression may create a favorable environment for tumor growth. One possible advantage is decreased catabolism of uracil and thymine by DPD, leading to an increase in pyrimidine nucleotide pools for use in DNA and RNA biosynthesis in tumor cells (2). Although uridine is generally accepted as the preferential substrate for nucleic acid synthesis, enhanced utilization of uracil *in vivo* is consistently found and was the original hypothesis for the development of fluoropyrimidine anticancer agents (1, 2). The down-regulation of DPD in tumor is in direct contrast with the overexpression of enzymes of the pyrimidine salvage pathway (thymidine kinase, uridine phosphorylase, and thymidine phosphorylase), which is observed in colorectal tumor compared with normal mucosa (17). Similar findings have also been observed in lung tumor, with the activities of uridine kinase, deoxythymidine phosphorylase, deoxythymidine kinase, and uridine phosphorylase being higher in tumor than in normal tissue (18). Coregulation of pyrimidine metabolic pathways has been described previously in regenerating rat liver and rat hepatoma with increased anabolism and decreased catabolism of thymine (19). This may suggest a general mechanism by which pyrimidine nucleotide biosynthesis and degradation are coregulated to maintain a growth advantage in the tumor. There may also be other unrecognized roles for DPD or its natural substrates (uracil and thymine), which lead to lower mRNA expression in tumor.

DPD activity has previously been reported to correlate with protein level in human lymphocytes (20). Here, we demonstrate a correlation between catalytic activity and DPD mRNA expression, supporting a relationship between *in vivo* transcription and translation of DPD. However, there was a large degree of variation in the mRNA-activity relationship, suggesting that DPD mRNA levels may not be a robust marker for DPD activity. In addition, there was a discrepancy in the DPD mRNA and activity relationship between normal and tumor tissue. DPD mRNA levels were significantly lower in colorectal tumor than in normal mucosae (ratio = 0.085), whereas catalytic activity (ratio = 1.25) and protein level (ratio = 0.9) did not differ significantly in the samples evaluated. This discrepancy may represent down-regulation of DPD mRNA expression in tumor tissue relative to normal tissue. Dysregulation between DPD activity and mRNA expression has recently been observed in human tumor xenografts transplanted into nude mice (13). Low levels of DPD activity and high levels of mRNA expression were observed in exponentially growing cell lines. The authors postulated that translation of DPD mRNA is somehow suppressed. In our study, it appears that the suppression of translation has been removed in the tumor tissue, with low mRNA levels yielding protein levels that are similar to those in normal tissue. However, these findings still support the regulation of DPD at the posttranscriptional level. A decrease in DPD-mediated thymine degradation has been described in regenerating rat liver and hepatoma, suggesting a link between DPD regulation and cell proliferation (19). However, lower transcript number may be required to maintain the level of DPD catalytic activity needed for homeostasis. The biochemical or molecular basis for discordance in DPD mRNA and activity now needs to be more thoroughly examined. This is made difficult by the recognition that neither human cancer cell lines (12) nor human xenografts (13) are adequate models of human tumor, preventing classical approaches to assessment of mRNA stability (*e.g.*, pulse-chase studies).

Tissue specificity in DPD was observed at all levels, with higher mRNA, activity, and protein in liver than in normal colon. Variability in DPD activity between tissues has been demonstrated in both rat and human tissue with highest activity in liver and lymphocytes (21, 22). The tissue-specific differences in both DPD mRNA expression and catalytic activity demonstrate that DPD is under transcriptional control. DPD mRNA expression, catalytic activity, and protein content are all lower in liver metastases than in normal liver, suggesting a role for hepatocyte-specific transcriptional control, rather than a generalized effect of the liver environment.

In conclusion, a cPCR assay was developed and used in the analysis of DPD mRNA expression in colorectal tumor. DPD mRNA levels correlated well with DPD catalytic activity. Although DPD mRNA expression was down-regulated in tumor compared with matched normal tissue, this difference was not observed at the level of protein expression or catalytic activity, indicating possible translational enhancement of DPD within the tumor. This opens up new avenues of research into the overall regulatory control of nucleotide synthesis and the mechanisms of regulation of DPD at the levels of both transcription and translation.

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