Quantitation of dihydropyrimidine dehydrogenase (DPD) mRNA expression levels in normal colon and colorectal cancer tumor paraffin-embedded tissue specimens

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5-Fluorouracil (5-FU) has been used for more than 40 years in the treatment of neoplastic disease, and remains the standard first-line treatment for colorectal cancer in combination with irinotecan and leucovorin. Previous studies indicated that measurement of dihydropyrimidine dehydrogenase (DPD) gene expression before treatment was valuable in determining the potential benefit of and toxicity to 5-FU treatment. In this study, we investigated the association between intratumoral DPD gene expression and the adjacent normal tissue DPD gene expression and DPD mRNA expression level in non-paired colon tumor and normal colon tissue specimens. In addition, we have compared the difference of DPD gene expression at three different RNA concentrations from the same specimen (180, 100 and 5 ng/reaction, respectively). DPD expression was measured by quantitative RT-PCR using a LightCycler instrument in a total of 31 specimens. Gene expression values were expressed as a ratio of target gene (DPD) to the internal reference gene (G6PDH). Our study revealed no statistically significant difference ($p = 0.23$) between tumor tissues and matched normal tissue in DPD expression. In contrast, the data on DPD mRNA expression in non-paired colon tumor and normal tissue specimens revealed a significant difference ($p = 0.0004$) between the tumor group and the normal group. In the three RNA concentration groups, there was no significant difference ($p = 0.55$) in gene expression at the different RNA concentrations from the same donor. These results demonstrate that intratumoral gene expression levels of DPD do not correlate with tumor cell percentage or with RNA concentration. Thus, DPD mRNA expression appears to be a valid sensitivity test for 5-FU in spite of a varying density of tumor cells and RNA yield in specimens submitted for analysis. *Anti-Cancer Drugs* 14:563–567 © 2003 Lippincott Williams & Wilkins.

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Introduction

Fluorouracil has been a standard of cancer treatment for over four decades [1]. Introduced as a chemotherapy agent over 40 years ago, 5-fluorouracil (5-FU) remains one of the most widely prescribed cancer chemotherapy drugs in the US and worldwide for the treatment of several malignancies, including carcinomas of the colon, rectum and breast [2,3]. The amount of 5-FU available for anabolism is determined by the extent of its catabolism. In humans, 80–90% of an administered dose of 5-FU is inactivated and eliminated through the catabolic pathway, where dihydropyrimidine dehydrogenase (DPD) is the initial enzyme and the rate-limiting step [4,5]. DPD catalyzes the first reaction in the three-step catabolic pathway, which enables the reduction of uracil and thymine to 5,6-dihydrouracil and 5,6-dihydrothymine, respectively [6,7]. Pharmacokinetic studies have shown that decreased DPD activity (DPD deficiency) can result in decreased drug clearance, increased half-life and a concurrent increase in 5-FU anabolism, ultimately leading to increased toxicity [8]. Complete DPD deficiency is a very rare event occurring in about 3% of unselected patients undergoing 5-FU chemotherapy [9–13]. Previous studies demonstrate that cancer patients with decreased DPD activity are at increased risk to experience serious adverse reactions following 5-FU-based chemotherapy [14]. Symptoms include severe and potentially life-threatening gastrointestinal toxicity, diarrhea, stomatitis, mucositis, myelosuppression, neurotoxicity and, in some cases, death [15]. These results suggest that a small, yet significant, proportion of the population may benefit from measurement of DPD activity before 5-FU treatment [16].

The activity of DPD can be detected in various normal and neoplastic human tissues [17]; however, no definitive pattern between enzyme activity in the normal and in corresponding neoplastic tissues have been established. On the other hand, investigators found that DPD activity was detectable in all samples (tumoral and non-tumoral biopsies obtained before chemotherapy) and that tumoral DPD activity was not influenced by the tumor stage. The

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most recent studies examining gene expression of the DPD gene (i.e. DYPD) in normal and tumor tissue have demonstrated that DPYD mRNA levels correlate with enzyme activity. Thus, DPD enzyme activity can be regulated at the level of transcription. Heretofore, there has been a lack of quantitative DPD mRNA data in normal tissue and in matched neoplastic tissue. In the present study we investigate the association between DPD gene expression at various concentrations of tumor cells.

Materials and methods

Specimen procurement

Paraffin-embedded tissue specimens were obtained from Cooperative Human Tissue Network (CHTN) Western Division. Specimens were inspected by a pathologist to assess the presence and percentage of tumors.

Chemicals

All chemicals were provided by Roche Molecular Biochemicals (Mannheim, Germany) including High Pure RNA Paraffin Kit (#3270289) and LightCycler DPD mRNA Quantification Kit (#3136957).

Isolation of total RNA from paraffin-embedded tissue specimens

RNA was isolated from tissue sections using the method outlined by Roche Diagnostics. In brief, the principle of the method is that tissue sections (5–10 μm in thickness) are disrupted during an overnight incubating with proteinase K. The released nucleic acids then bind, in the presence of a chaotropic salt, to the surface of glass fibers pre-packed in the High Pure Purification filter tube. The binding reaction occurs within seconds due to the disruption of the organized structure of water molecules and the interaction with nucleic acids. The binding process is specific for nucleic acids in general, but the binding conditions are optimized for RNA. Bound RNA is purified in a series of rapid 'wash-and-spin' steps to remove cellular components. After elution from the column, residual DNA is digested by incubation the eluate with DNase I. A second incubation step with proteinase K improves the purity of RNA. Finally, a low salt elution releases the RNA from the glass fibers. The process does not require RNA precipitation or organic solvent extractions and yields high-purity, intact RNA.

Reverse transcription of RNA

The reverse transcription process requires that cDNA is prepared from total RNA using random hexamers serving as primers. Only one cDNA preparation is required for both DPD and for G6PDH analysis. The volumes indicated in tabular form below are calculated for a single 20 μl standard reaction. RNA amounts of around 0.2–20 ng/μl can be used directly for RT-PCR without further dilution steps (Table 1).

Table 1 Preparation of the reverse transcription reaction mixture per tube

Component	Volume (μl)	Final concentration
H_2O sterile, PCR grade	2.2	
Reverse transcriptase reaction mix, 10x	2	1x
Random hexamers $p(dN)_6$	2	0.08 A_{260}
Deoxynucleotide triphosphate (dNTP) mix	2	0.4 mM (dATP, dCTP, dGTP), 1.2 mM (dUTP)
RNase inhibitor	1	40 U
Reverse transcriptase	0.8	20 U
RNA sample	10	2–200 ng
Total reaction volume per tube	20	

All components are from the LightCycler DPD mRNA Quantification Kit (Roche).

Table 2 The temperature profile of the Thermocycler for the reverse transcription reaction

Step	Time (min)	Temperature (°C)
Annealing	10	25
Reverse transcription	60	42
Inactivation	5	94

This protocol is from the LightCycler DPD mRNA Quantification Kit insert (Roche).

The number of reactions required equals the number of samples plus one, which is for the calibrator RNA. The calibrator in this kit is typically a positive sample with a stable ratio of target to reference and is used to normalize all samples within one run, as well as to provide a constant calibration point between LightCycler runs. The RT^- control mixture is prepared by replacing the reverse transcriptase with an equal volume of H_2O. The mixture was incubated in a Thermocycler, and the reverse transcription reaction initiated and arrested using the summarized temperature protocol in Table 2.

LightCycler PCR

The LightCycler instrument was run using three separate programs as summarized in Table 3. As DPD and G6PDH are analyzed in separate capillaries and the carousel capacity is 32, there is a maximum of 13 capillaries available for each gene. Four controls, $2 \times RT^-$ controls and PCR controls (for target and reference gene), and two calibrator reactions, for the target and reference gene, are included with each run. The mixture was prepared for LightCycler PCR as described in Table 4.

Quantification of the transcription product

By using the LightCycler DPD mRNA Quantification Kit, the amount of mRNA encoding for DPD is expressed as a relative ratio to the reference gene (G6PDH). The calculation of the DPD mRNA expression level is determined from the crossing point of one particular sample. The crossing point is the cycle at which PCR begins its exponential amplification phase and is considered to be proportional to the initial concentration. In the first step, the amount of DPD is calculated as a ratio

Table 3 The LightCycler instrument programming protocol

Cycle program data	Program 1 (Denaturation)	Program 2 (PCR amplification)			Program 3 (Cooling)
Number of cycles	1	50			1
Analysis mode	none	quantification			none
Temperature targets	segment 1	segment 1	segment 2	segment 3	segment 1
Target temperature (°C)	95	95	62	72	40
Incubation time (h:min:s)	0:05:00	0:0:10	0:0:10	0:0:10	0:0:30
Temperature transition rate (°C/s)	20	20	20	20	20
Secondary target temperature (°C)	0	0	0	0	0
Step size (°C)	0	0	0	0	0
Step delay (cycles)	0	0	0	0	0
Acquisition mode	none	none	single	none	none

This protocol is from the LightCycler DPD mRNA Quantification Kit package insert (Roche).

Table 4 The preparation of the LightCycler PCR reaction mixture per tube

Component	Volume (μl)
FastStart reaction mix	2.0
DPD detection mix or reference detection mix	6.0
$MgCl_2$ (25 mM)	1.6
H_2O sterile, PCR grade	6.4
cDNA template	4
Total reaction volume	20

All components are from the LightCycler DPD mRNA Quantification Kit (Roche).

of the amount of target (T) gene to the amount of reference (R) gene (G6PDH). In the second step, the ratio of target to reference (DPD: G6PDH) of the sample is divided by the T:R ratio of the calibrator RNA. The final result expresses the ratio of T:R in the sample, relative to the ratio of T:R in the calibrator.

Results

Variation of DPD mRNA expression in paired specimens

Relative DPD mRNA content was determined by quantitative RT-PCR in paraffin-embedded tissue sections from 10 specimens, five containing tumor and five uninvolved adjacent tissues. Figure 1 is a collage of normal and tumor cell photomicrographs of the specimens studied. In the five patients studied, the normalized ratio of DPD:G6PDH divided by calibrator DPD:G6PDH revealed no significant difference ($p = 0.23$) between enzyme activity in tumor tissue and in adjacent uninvolved tissue (Table 5).

Variation of DPD mRNA expression in non-paired specimens

In the twenty specimens, 10 tumor tissues and 10 normal tissue samples, the normalized ratio of DPD:G6PDH divided by calibrator DPD:G6PDH was significantly different ($p = 0.0004$). between the tumor and normal groups (Table 6). Variation of DPD mRNA expression at different RNA concentrations with the same specimen. We tested DPD expression at three different RNA concentrations: 180, 100 and 5 ng/reaction, respectively. The results are summarized in Table 7. There was no significant difference ($p = 0.55$) in DPD mRNA expression at the three RNA concentrations studied. (All RNA concentrations are in the linear measuring range.)

Discussion

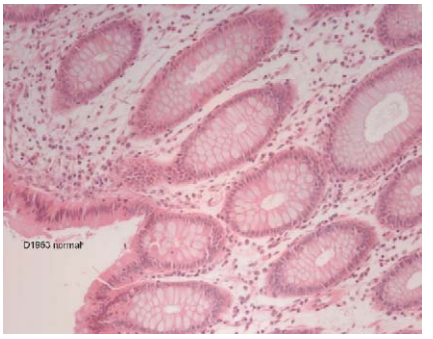
DPD is an important catabolic enzyme for 5-FU in man. In this study, we used quantitative RT-PCR to measure the DPD mRNA expression levels in three groups of colon tissue specimens. First, we compared the DPD mRNA expression level in paired colon tumor tissue with adjacent uninvolved tissue specimens, then in non-paired colon tumor tissue specimens with normal tissue specimens, and finally at three different RNA concentrations using the same colon tumor tissue specimen.

This study reveals no significant difference of DPD mRNA expression between colon tumor tissue specimen and adjacent normal tissue specimens ($p = 0.23$). By using an internal reference gene expression (G6PDH) and positive control, DPD mRNA expression values are normalized to expression per cell. By reporting relative ratios, not absolute values, the values are comparable between specimens regardless of the number of tumor cells analyzed. Previous work by Leichman *et al.* [18] revealed no significant variation of thymidylate synthase (TS):β-actin (reference gene) ratios by RT-PCR from different sites within the same colon cancer specimen. However, DPD mRNA expression has not been previously investigated in paired specimens of neoplastic and normal tissue prior to this study. Our data demonstrates that DPD mRNA expression level can be measured in paired tissue specimens of normal and neoplastic tissue to assess the potential for 5-FU responsiveness and for toxicity prior to initiating therapy.

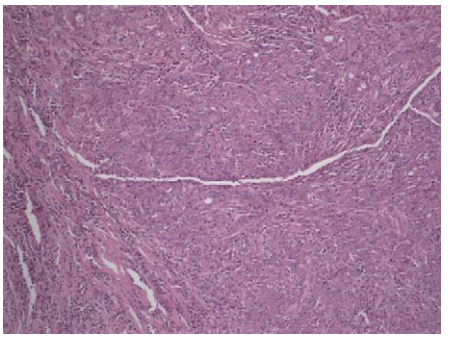
Based on population studies, approximately 3–5% of unselected colon cancer patients are at risk for 5-FU toxicity [9,10]. As response to 5-FU chemotherapy is associated with a significant survival benefit, measuring DPD gene expression can be used to both predict response to 5-FU and also identify patients with a better overall survival. Our study revealed a significant difference between DPD mRNA expression in colon tumor tissue and in adjacent uninvolved tissue ($p = 0.0004$). If

Fig. 1

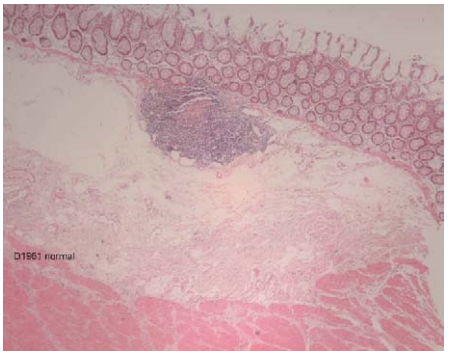
(a) D1963-Normal (10×)



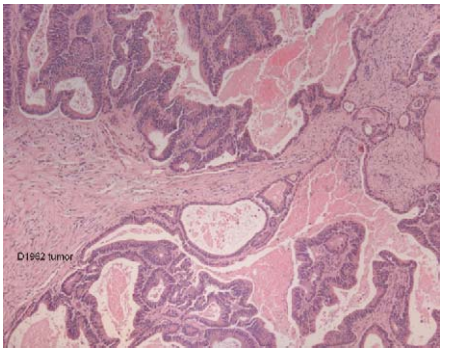
(b) D1964-CA, 75% (10×)



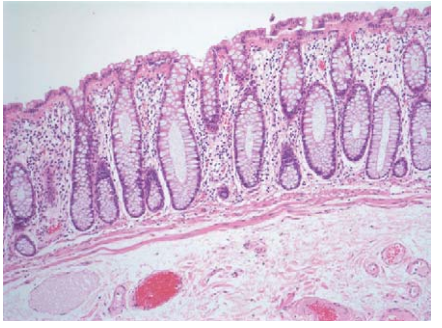
(c) D1961-Normal (4×)



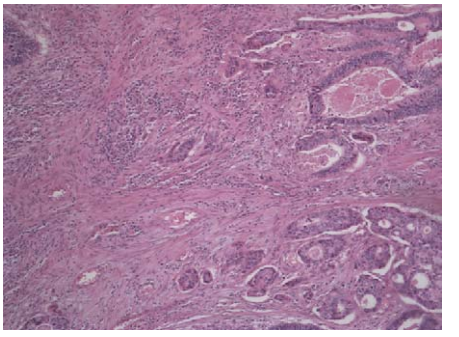
(d) D1962-CA, 50% (10×)



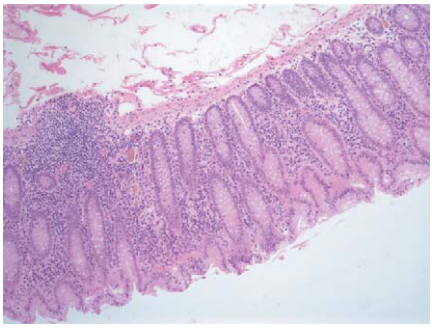
(e) D2162-Normal (10×)



(f) D2163-CA, 40% (10×)



(g) D2160-Normal (10×)



(h) D2161-CA, 30–40% (10×)

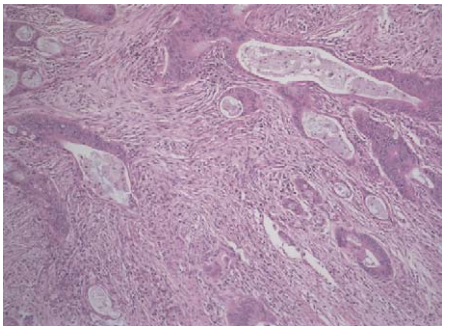


Table 5 Summary of DPD mRNA expression normalized ratio with the same RNA concentration (100 ng/reaction) per RT-PCR reaction in paired tumor and adjacent tissue specimens

	No. tested	DPD normalized ratio		*p* value
		Mean	Range	
Tumor tissue	5	0.85	0.83–0.88	0.23[a]
Adjacent tissue	5	0.87	0.85–0.92	

[a]ANOVA: single factor. $p > 0.05$.

Table 6 Summary of mRNA expression normalized ratio with same RNA concentration (100 ng/reaction) per RT-PCR reaction in non-paired normal and tumor tissue specimens

	No. tested	DPD normalized ratio		*p* value
		Mean	Range	
Normal tissue	10	0.85	0.82–0.86	0.0004[a]
Tumor tissue	10	0.93	0.88–0.99	

[a]*t*-test: paired two sample for means.

Table 7 Summary of DPD mRNA expression normalized ratio with different RNA concentration per RT-PCR reaction with the same tumor tissue specimen

RNA concentration (ng/reaction)	No. tested	DPD normalized ratio		*p* value
		Mean	Range	
180	11	0.86	0.85–0.88	
100	11	0.86	0.85–0.89	0.55[a]
5	11	0.86	0.84–0.89	

[a]ANOVA: single factor.

these results can be confirmed in a larger patient population, then a potential cut-off value can be established that would permit a better-informed decision as to whether or not to proceed with 5-FU-based therapy as first-line treatment in colorectal cancer.

Our study also shows that, there is no significant difference of DPD mRNA expression at several RNA concentrations in the same colon cancer patient ($p = 0.55$). All of the RNA concentrations in our study are in the linear measuring range (0.2–20 ng/μl). These data confirm that the LightCycler RT-PCR method for DPD mRNA expression assay is useful in clinical specimens where the number of tumor cells and the yield of RNA isolation may be variable.

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The H & E staining of colon tumor tissue and adjacent tissue slides. A + B, C + D, E + F and G + H are paired specimens. The tumor cell density is 75, 50, 40 and 30–40%, respectively.