Colorectal Tumors Responding to 5-Fluorouracil Have Low Gene Expression Levels of Dihydropyrimidine Dehydrogenase, Thymidylate Synthase, and Thymidine Phosphorylase

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ABSTRACT

We had previously shown that high gene expressions (mRNA levels) of thymidylate synthase (TS; Leichman et al., J. Clin. Oncol., 15: 3223–3229, 1997) and thymidine phosphorylase (TP; Metzger et al., Clin. Cancer Res., 4: 2371–2376, 1998) in pretreatment tumor biopsies could identify tumors that would be nonresponsive to 5-fluorouracil (5-FU)-based therapy. In this study, we investigated the association between intratumoral gene expression of the pyrimidine catabolism enzyme dihydropyrimidine dehydrogenase (DPD) and the response of colorectal tumors to the same 5-FU-based protocol. DPD expressions were measured by quantitative reverse transcription-PCR in 33 pretreatment biopsies of colorectal tumors from patients who went on to receive treatment with 5-FU and leucovorin (LV). The range of DPD gene expression in those tumors that were nonresponsive to 5-FU was much broader than that of the responding tumors. None of the tumors with basal-level DPD expressions above a DPD:β-actin ratio of 2.5 × 10⁻³ (14 of 33) were responders to 5-FU/LV therapy, whereas those patients with DPD gene expressions below DPD:β-actin ratio of 2.5 × 10⁻³ had a response rate of 50%. There was no correlation among DPD, TS, and TP expression values in this set of colorectal tumors, which indicated that these gene expressions are independent variables. All of the tumors that responded to 5-FU therapy (11 of 33) had expression values of all three of the genes, TS, TP, and DPD, below their respective nonresponse cutoff values, whereas, in each of the nonresponding tumors, at least one of these gene expressions was high. The patients with low expression of all three of the genes had significantly longer survival than patients with a high value of any one of the gene expressions. The results of this study show that intratumoral gene expression level of DPD is associated with tumor response to 5-FU and that the use of more than one independent determinant of response permits the identification of a high percentage of responding patients.

INTRODUCTION

5-FU³ has been used for more than 40 years for the treatment of various cancers and remains the standard first-line treatment for colorectal cancer, although the response rate as a single agent is usually less than 20% (1). Over the years, various strategies have been attempted to increase the antitumor efficacy of 5-FU by modulation of its intracellular metabolism and biochemistry (2). We have been pursuing another approach for improving therapy with 5-FU by identifying the biochemical response determinants of this drug. If such determinants could be measured in tumors before treatment, patients who are judged unlikely to respond to 5-FU would then have the option to be treated, instead, with another agent to which they might respond, whereas those patients with favorable 5-FU response indices could anticipate a higher than average probability of response. We had previously found that the intratumoral levels of expression of the mRNA for the 5-FU target enzyme TS (3) and the catabolic enzyme TP (4) were predictors of the sensitivity of colorectal tumors to 5-FU-based protocols. TS and TP expression values that were above a certain threshold identified a subset of tumors not responding to 5-FU, whereas expression values below the nonresponse cutoff value predicted an appreciably higher response rate but did not specifically identify responding tumors. However, we also noted that tumors with both low TS and low TP had a higher response rate than tumors with only one or the other gene expression being low (4); that is, by combining response determinants, it was possible to achieve a considerably better response prediction than with just one determinant. This observation suggested that, with yet a third independent response determinant, it might be possible to identify a set of tumors that would have a very high, if not 100%, response rate to 5-FU-based protocols.

³ The abbreviations used are: 5-FU, 5-fluorouracil; TS, thymidylate synthase; DPD, dihydropyrimidine dehydrogenase; LV, leucovorin; RT, reverse transcription; TP, thymidine phosphorylase.
In this study, we examined intratumoral DPD expression as a possible additional determinant of the response of colorectal tumors to 5-FU treatment. DPD reduces the 5,6 double bond of uracil (as well as that of 5-FU) and is the rate-limiting enzyme for 5-FU catabolism (5–7). DPD activity is highly variable in normal tissues (8) and, thus, by sufficiently influencing the bioavailability of 5-FU, could affect its pharmacokinetics, toxicity, and antitumor activity. DPD levels not only vary among individuals but also within the same individual on a Circadian rhythm basis (9). Patients with DPD levels within the normal range rapidly eliminate over 80% of administered 5-FU as 2-fluoro-β-alanine, whereas patients who are deficient in DPD retain 5-FU over a much longer half-life and excrete mostly unchanged 5-FU in the urine (10). In such patients, 5-FU can cause profound toxicity (11). Because inactivation of 5-FU by DPD seemed to be a mechanism of clinical resistance to 5-FU, vigorous efforts have been made to design inhibitors of DPD.

The most successful of these, 5-ethynyluracil, markedly improved the efficacy and therapeutic index of 5-FU in rats (12). In addition to its role in normal tissue toxicity of 5-FU, DPD expression specifically in tumors has been studied as a possible determinant of tumor response to 5-FU. DPD expression is variable among tumors also, and, in head-and-neck tumors, DPD levels have been inversely associated with response to 5-FU (13).

The above-cited studies, along with previous studies showing that DPD and TS levels both were significantly associated with sensitivity to 5-FU (14), suggested to us that DPD expression could be a useful 5-FU response determinant in colorectal tumors along with TS and TP expression provided that: (a) intratumoral basal DPD level was associated with clinical efficacy of FU; (b) TS, TP, and DPD expressions were independent variables; and (c) there was sufficient variation among individuals in the expression of this gene. Accordingly, we used quantitative RT-PCR to measure DPD expression (relative mRNA levels) in a group of 33 colorectal tumors in which we had already determined TS and TP expressions, and we correlated these expressions with response to 5-FU-based therapy.

MATERIALS AND METHODS

Clinical Methods. Eligibility criteria for these trial-and-tumor-response-evaluation parameters were described previously in detail (3). In brief, eligible patients had (a) a diagnosis of disseminated or recurrent colorectal cancer with a measurable lesion accessible for biopsy; (b) a Southwest Oncology Group performance status of 0 to 2 with adequate hematological, hepatic, and renal function; (c) no prior infusional 5-FU; and (d) a lesion that was bidimensionally measurable either by physical or radiological examination. After placement of an indwelling venous access, patients were treated with 5-FU (200 mg/m²/day) as a continuous infusion administered by ambulatory infusion pump (Pharmacia Deltec Cadd pump, Minneapolis, MN) with LV (20 mg/m²) by i.v. push weekly. The initial cycle was given for 4 weeks, followed by a 1-week rest, with subsequent cycles consisting of 3 weeks continuous therapy and a 1-week rest. Response rates and toxicity profiles for this regimen have been published previously (15).

After two cycles (8 weeks) of treatment, measurable disease was reassessed. Response criteria were the standard definitions used for national cooperative group trials (16). To be classified as a responder, a tumor had to have a 50% reduction in the sum of the products of the perpendicular diameters of the indicator lesion without growth of other disease or the appearance of new lesions. Those with responses or stable disease were continued on protocol until progression was documented.

Liver metastases represented the most common site of disease on which a biopsy was performed and assessed for response, whereas, in some cases, nodal metastases or peritoneal metastases were the disease sites evaluated. All of the specimens were obtained by core-needle biopsy. This technique uses a coaxial system in which fine-needle aspiration is used to confirm cytological evidence of cancer within moments of the aspiration. The fine needle is withdrawn from a sheath and a core needle is inserted through the sheath without losing position. The core-needle material is used for gene expression analysis. Specimens were examined by pathologists and were not used for analysis unless they were judged to consist of >80% tumor tissue.

Laboratory Methods. The procedure for RT-PCR quantitation of gene expression has been described in detail previously (17, 18). In brief, the method involves isolation of mRNA from each tumor, preparation of cDNA using reverse transcriptase and random hexamers and PCR amplification of the specific cDNA of interest in a range of concentrations that gives rise to a linear curve of the resulting PCR products. An internal standard gene (for example, β-actin) from the same cDNA solution is PCR-amplified separately. We found previously that with β-actin as the reference gene, a good linearity is obtained between gene expression values determined by RT-PCR and protein content determined by immunohistochemistry (19). Once the concentration ranges for linear amplification are established for the cDNA of the target gene and the reference gene, the ratio of the slopes generates an empirical number proportional to the amount of mRNA of interest in the tissue normalized to total RNA. Gene expression values are reported only if the PCR of serial dilutions of the cDNA solution generates a set of distinct bands with intensities that are linear with

Fig. 1 DPD gene expressions in 33 colorectal tumors grouped according to response (>50% tumor shrinkage) or nonresponse (<50% tumor shrinkage).
the concentration of cDNA. Slopes of the lines are obtained from at least three data points, so that each reported gene expression value represents the average of a minimum of three separate PCR reactions within the linear amplification range. When the initially chosen cDNA concentrations for a particular determination give PCR products clearly outside of the proportional linearity region, the determination is repeated with adjusted cDNA concentrations until the data points are in the linear range, and the correlation coefficient for linearity for a set of at least three consecutive serial dilutions plus the zero point is greater than 0.90. This method has been used by us (3, 19) as well as by others (20) to quantify various gene expressions in tumor biopsy specimens. Gene expression values are expressed as a ratio of PCR products of the gene of interest to that of the internal reference gene β-actin.

For RT-PCR quantitation of DPD, the following primers were designed based on the sequence of the DPD gene (GenBank accession no. U20938): -1740F, T7-GGTCTTGCTAGCG-CAACTCC (“T7” designates the T7 RNA polymerase clamping end), and -1989R, CCTTTAGTTGACTGACCTTGA. These primers were designed to give an amplicon of 250 bp, spanning positions 1740 to 1989 of the genomic sequence. β-actin primers BA67 and BA68 were described previously (17). The reaction was performed as described previously (17, 18), except that cycling conditions were modified to be optimal for the DPD primers. The cycling conditions for DPD amplification were 15 s at 96°C, 30 s at 65°C, and 30 s at 72°C for 31 cycles.

### Statistical Methods

To evaluate the association of DPD with response and with survival, DPD was categorized into a low and a high value. To determine this cutoff value, the maximally selected χ² method of Miller and Halpern (21, 22) was adapted. For each observed DPD value, patients were classified as falling below or equal to that value, or above that value. The Pearson χ² test statistic was used to compare the response rates of the two resulting groups of patients (below or equal to the value versus above the value). The DPD value that yielded the largest χ² test statistic (the maximal χ² statistic) was selected as the optimal cut-point. To determine the P-value associated with the maximal χ² statistic, we performed 2000 bootstrap-like simulations. For each simulation, a randomly selected DPD value was drawn (with replacement) from the set of observed DPD values and assigned to each of the observed responses; the maximal χ² test statistic was calculated based on this set of randomly matched DPD values and responses. The corrected P-value was calculated as the proportion of the 2000 simulated maximal statistics that was larger than the original maximal χ² test statistic; the calculated corrected value was 0.033, compared with the uncorrected P of 0.0041. This analysis was repeated using the log-rank test to compare survival. For this analysis, the calculated corrected value was 0.03, compared with the uncorrected P of 0.0015. These corrected P-values account for the fact that all of the possible DPD values were examined before selecting the optimal cut-point. In both analyses, the optimal DPD cut-point was 2.5. The rest of the analysis was descriptive, and P-values were calculated to reflect the magnitude of associations rather than to perform formal testing. The cutoff values for TS and TP expressions were based on the highest level in the group of responders (therefore, no patient with a TS ≥ 4.1 or a TP ≥ 18 was a responder). To examine the joint association of TS, TP, and DPD with either response or survival, the 2×2 × 2 = 8 groups of patients were examined. The group of patients with TS ≤ 4, TP ≤ 18, and DPD ≤ 2.5, was the largest (n = 11 patients) and also identified a subset with a longer survival; the outcome of this subset was compared with the others. To evaluate the association with response, the two-sided Fisher’s exact test was used; the Spearman correlation was used to evaluate the association between DPD and TS; the log-rank test was used to measure the association between TS, TP, and DPD, and survival. The Ansari-Bradley test was used to compare the responders and nonresponders in terms of the variability of DPD gene expression (23).

### RESULTS

Relative DPD mRNA content was determined by quantitative RT-PCR (17, 18) in biopsy specimens from 33 colorectal cancer patients analyzed previously for TS expression levels (3). Tumors were categorized as either responding or not responding...
to a regimen of 5-FU and LV (see “Materials and Methods” for definition of clinical response criteria). Fig. 1 shows the distributions of DPD:β-actin PCR product ratios (henceforth termed “expressions” for convenience) for the two groups. The range of DPD expressions among the responding tumors was relatively narrow (0.60 × 10⁻³ to 2.5 × 10⁻³, 4.2-fold) compared with that of the nonresponders (0.2 × 10⁻³ to 16 × 10⁻³, 80-fold). This difference in the range of gene expression between the responding and nonresponding groups was significant (P = 0.019, Ansari-Bradley rank test), as was the lack of responding tumors with DPD expressions < 2.5 × 10⁻³ (Table 1). A total of 22 tumors had DPD expressions < 2.5 × 10⁻³ with a corresponding response rate of 50% (11 of 22).

For tumor response prediction, it was of interest to determine the relationship between these gene expressions. For example, if TS and DPD were coregulated, there would be little or no additional benefit for response prediction from measuring two gene expressions, whereas if the genes were independently regulated, some portion of the low-TS patients might be identified as nonresponders by their high DPD expression. A plot of DPD against TS expressions (Fig. 2) showed no correlation between the expressions of these genes (r², 0.01), with a number of low-DPD tumors having high TS and high-TS tumors having low DPD expressions. As Fig. 3 further shows, only 1 of 12 tumors falling within the area designated by the nonresponse cutoff values of TS and DPD (i.e., having both low TS and low DPD) was a nonresponder to 5-FU/LV, but this one tumor (Fig. 3, tumor 152) had a TP expression value above its cutoff. Thus, all of the responding tumors could be identified by low expression values of DPD, TS, and TP (Fig. 3). The response data are summarized in Table 1.

Longer periods of survival have been observed for colorectal cancer patients whose tumors respond to chemotherapy (24). In the present study also, survival for those patients with DPD values below the nonresponse threshold of DPD < 2.5 × 10⁻³ was significantly longer than for those patients with DPD above this value (Fig. 4, upper panel). The greatest increase in survival was observed for the group of patients with all of the three genes, DPD, TS, and TP below the respective nonresponse cutoff values (Fig. 4, lower panel).

**DISCUSSION**

In this study, we have shown a significant correlation between DPD expression levels in colorectal tumors, response to 5-FU, and patient survival. The pattern of DPD expressions as a function of response or nonresponse resembled that of TS and TP expressions determined previously (3, 4) in that: (a) the range of gene expressions among the nonresponders was considerably broader than among the responders; and (b) there was a cutoff value of DPD expression (DPD:β-actin, 2.5 × 10⁻³) above which there were no 5-FU-responsive tumors, whereas the set of tumors with expressions below the cutoff value comprised both responders and nonresponders. Thus, low DPD expressions do not uniformly identify responding tumors but do predict a high response rate of 50%. However, because TS and DPD gene expressions are independently regulated (as indicated by the lack of correlation between them in Fig. 2), 10 of these low-DPD tumors could be positively identified as nonresponders by TS expression values > 4.1 × 10⁻³ (the nonresponse cutoff value for TS expression). Thus, tumors having both TS < 4.1 × 10⁻³ and DPD < 2.5 × 10⁻² in this group of patients had a 92% response rate. Adding TP expression as a third determinant allowed all of the responders in this group of
tumors to be identified by low expression of all of the three genes (Fig. 3). These data show how combining more than one independent response determinant can increase predictive power for clinical outcome.

It is striking to see in Fig. 3 that the patterns of expression of TS, DPD, and TP vary over a wide range and are different in every one of the nonresponsive tumors. This almost chaotic variation of gene expressions among the individual tumors of the nonresponsive group compared with the responding group, suggests that the transition from a drug-sensitive tumor to one that is insensitive is accompanied or preceded by a substantial degradation of the ability of the tumor cells to regulate their gene expressions.

This study also shows that, because response of colorectal tumors to chemotherapy is associated with a significant survival benefit (24), measuring these gene expressions not only can predict response to 5-FU but also can identify patients with a better survival prognosis. If these results can be confirmed in a clinical trial with a larger set of patients, data on TS, DPD, and TP expressions in tumors would permit more rational decisions on whether or not to proceed with 5-FU-based therapy as first-line treatment. If the 5-FU response indices seem unfavorable, the alternative drug CPT-11 is now available for treatment of colorectal cancer patients. Preliminary results show that tumors with TS expression levels that would put them into the group not expected to respond to 5-FU, or indeed have already been treated and have failed 5-FU, do respond to CPT-11 (25). If patients destined for 5-FU failure can be identified before the start of treatment, benefits will of course be gained by avoiding the toxicity associated with the drug, but, moreover, it is possible that patients will be more likely to respond to CPT-11 when it is given as primary therapy, compared with its use after 5-FU failure. Thus, the assignment of patients for either 5-FU or CPT-11 treatment by taking into consideration their TS, DPD, or TP levels may provide a way of achieving a substantial increase in the overall response rate to chemotherapy using currently available agents.

**REFERENCES**

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