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Quantitation of Intratumoral Thymidylate Synthase Expression Predicts for Resistance to Protracted Infusion of 5-fluorouracil and Weekly Leucovorin in Disseminated Colorectal Cancers: Preliminary Report from an Ongoing Trial

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A clinical trial for patients with measurable, disseminated colorectal cancer is being conducted to determine: (1) if intratumoral expression of thymidylate synthase (TS) affects response to protracted-infusion 5-fluorouracil (5FU); and (2) whether intratumoral expression of TS increases when clinical resistance is found after response to 5-FU. Polymerase chain reaction technology is employed to determine TS expression. Using β-actin as an internal standard, TS expressions for 26 patients range from 0.5×10^{-3} to 22.6×10^{-3}. Currently, 22 patients are evaluable for response and TS quantitation of their measurable tumour. 8 patients (36%) have had partial responses; 3 responding patients had been previously treated with 5-FU. A strong statistical association between TS expression and resistance to therapy has been found ($P = 0.004$). No patient with TS expression of 4.0×10^{-3} or greater has responded. On average, patients previously treated with 5-FU have slightly higher levels of TS expression in their measurable tumours ($P = 0.4$). Whether responding patients will develop increased expressions of TS upon clinical progression of their cancer remains to be determined. Confirmation of these results in a larger cohort could lead to a scientific rationale for deciding upon specific therapy for patients with disseminated colorectal cancers.

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INTRODUCTION

FOR THE past three decades, 5-fluorouracil (5-FU)-based chemotherapy has been the mainstay of treatment for patients with disseminated large bowel cancer. With the introduction of biochemical and biological modifiers of 5-FU, there has been an apparent increase in the response rates for patients with measurable tumours [1–3]. Leucovorin, a reduced folate, is the biochemical modulator which has undergone the most extensive testing and its mechanism of enhancing 5-FU action is well understood. Within the cell, the reduced folate enhances 5-FU cytotoxicity by allowing tight binding of FdUMP, the active metabolite of 5-FU, and thymidylate synthase (TS), the enzyme responsible for converting uracil to thymidine [4–6].

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The role of intratumoral TS quantitation as a determinant of 5-FU sensitivity or resistance has been suggested by several investigators. Spears and associates have demonstrated that sensitivity of particular tumour cells to 5-FU is closely associated with the drug's ability to inhibit thymidylate synthase completely [7]. Lu and associates and Washtien found that the intratumoral level of TS has a demonstrable effect on 5-FU response in cell culture systems [8, 9]. Berger and colleagues and Swain and associates have postulated that acquired resistance to 5-FU is caused by overproduction of TS as a result of gene amplification [10, 11]. Chu and associates, in a study of human breast and colon cancer cell lines exposed to 5-FU, noted that resistance correlated with *increased* levels of TS. This group postulated the increase in TS was a transcriptional or post-transcriptional regulatory event [12]. Thus, the quantity of TS within a tumour cell, or a group of tumour cells, may be the most important determinant of whether 5-FU will be an effective cytotoxic agent against that tumour.

The ability to measure TS in human tumour biopsies by the radioligand sensitive binding assay stimulated pharmacodynamic research, regarding the association between TS tumour concentration and resistance to fluorinated pyrimidines [7, 13, 14]. Using the radioligand assay for TS quantitation,

Spears and colleagues have demonstrated a striking relationship between baseline (total) TS and quality of response for patients with advanced, refractory breast cancer treated with 5-FU and leucovorin. Upon clinical progression, the TS protein within the tumour biopsies increased above baseline levels [15].

The polymerase chain reaction (PCR) is a highly sensitive and efficient method of amplifying specific DNA segments present at very low concentrations. By quantifying mRNA converted from amplified cDNA, PCR can be used to measure the expression of specific genes in tumour cells. Thus, PCR provides an alternative approach for determining the quantity of specific enzymes within tumours by measuring mRNA [16]. While gene expression is not a direct measure of enzyme activity, Curt and colleagues have shown that when gene amplification takes place, it is closely related to increases in enzyme levels, gene copy number and mRNA levels [17].

Our laboratory reported an alternative PCR quantitation strategy for comparing expressions of genes in tumours [18]. Essentially, this methodology involves: (1) use of an internal standard gene (β-actin) for measurement of relative gene expression by determining a ratio between the amplified internal standard cDNA and target cDNA within a linear range; and (2) the incorporation of the T7 RNA polymerase promotor sequence on the 5′ primer of each gene which gives a further 500-fold amplification. This PCR-based quantitation method is capable of measuring low abundance genes, such as TS, in biopsy specimens obtained by routine endoscopy of the upper and lower gastrointestinal tract.

As we had developed PCR as a tool for measurement of TS expression, we also designed a clinical protocol to test the hypothesis that quantitation of the intratumoral expression of TS within a measurable metastatic colorectal lesion is associated with 5-FU sensitivity and/or resistance. The clinical portion of the protocol uses a protracted-infusion schedule of 5-FU administration with weekly i.v. bolus leucovorin for patients with disseminated and measurable colorectal cancers [19]. This is the first report of this ongoing trial.

PATIENT ELIGIBILITY

Each patient must have disseminated and measurable colorectal cancer with, at least, one measurable, biopsy-accessible lesion from which tumour confirmation and TS quantitation is determined. Patients must have a SWOG Performance Score of 2 or less. They may have had previous therapy with one 5-FU regimen as long as the regimen did not include a protracted infusion of 5-FU (two or more consecutive weeks).

Patients must demonstrate adequate haematological function, with total granulocytes greater or equal to 1500/mm^3, platelets greater or equal to 100 000/mm^3, haemoglobin greater or equal to 10 g/l (transfusion permitted). A serum bilirubin less than or equal to 2.5 mg/dl is required. Patients must be 18 years or older and capable of understanding the experimental nature of this treatment plan. Pregnant women are excluded from this trial, as are patients who have had a second neoplasm within the past 5 years (exclusive of non-melanomatous skin cancers).

TS quantitation

Each biopsy from the metastatic colorectal cancer is divided into a fresh specimen sent to pathology for diagnostic analysis and a portion of the tissue frozen at −70°C for PCR analysis of TS gene expression. The isolation of RNA is based on the method of Chomczynski and Sacchi [20]. RNA from the tumour samples is isolated and converted to cDNA using random hexamers. The PCR-based method described above is used to quantitate the TS gene expression level. Expression of the β-actin gene within the sample serves as an internal standard. For each sample, a linear range of amplification for TS and β-actin cDNA is established. Relative gene expressions are calculated by determining the ratio between the amount of the radiolabelled PCR product within the linear amplification range of the TS gene and the β-actin gene. The method is accurate to less than 2-fold difference in expression levels [18].

The primers listed below are synthesised for the PCR using an Applied Biosystem Model 391 PCR-Mate DNA synthesiser by the phosphoramidite method. Each 5′ primer has the T7 polymerase promoter sequence TAA TAC GAC TCA CTA TA attached to its 5′ end (denoted by the symbol T7), and in addition a T7 polymerase binding sequence shown in parentheses.

TS 60: GATGTGCGCAATCATGTACGTGAG
(bases 697–720 of the TS coding sequence) [21]

TS 61: T7-"GGGAGA"
GGAGTTGACCAACTGCAAAGAGTG
(bases 469–492 of the TS coding sequence) [21]

BA 67: T7-"GGAGA"GCGGGAAATCGTGCGTGACATT
(bases 2104–2127 of the β-actin genomic sequence, located in exon 3) [22]

BA68: GATGGAGTTGAAGGTAGTTTCGTG
(bases 2409–2432 of the β-actin genomic sequence, located in exon 4) [22].

PCR-based TS quantitations are derived without knowledge of the clinical course of the patient. The clinicians are required to code responses without knowledge of the TS determinations. The TS quantitation data on a particular patient become available to the clinical team only after the patient has been declared to have had a "response" or "progressive cancer". Similarly, the laboratory investigators become aware of the clinical course only after the TS quantitation is completed.

Chemotherapy

5-FU is administered intravenously (i.v.) as a protracted-infusion at 200 mg/m^2/day for 28 days followed by a 1 week rest period. At the beginning of each week of therapy (day 1, 8, etc.), the patient is given leucovorin 20 mg/m^2 by i.v. bolus. The second cycle of therapy begins on day 35 with identical subsequent cycles until progression. The treatment is administered through an access capable of maintaining venous patency while low infusion rates of 5-FU are given and during the "rest periods" while no therapy is administered. We had previously reported a 46% response rate for this regimen in 41 patients treated at the University of Southern California [19]. A subsequent randomised trial conducted by the Southwest Oncology Group reported a 29% response rate in 84 patients treated [23]. Dose modification for this regimen for clinical toxicities has been previously described [19].

RESPONSE DEFINITIONS AND STATISTICAL METHODS

The clinical measurements of the lesion sampled for TS quantitation determine "response" or "progression". TS-sampled tumours are called "indicator lesions". A complete

response is defined as one in which the indicator lesion and all others become undetectable by clinical and X-ray criteria. A partial response requires a 50% decrease in the sum of the products of the perpendicular diameters of the indicator lesion without growth of other lesions or the appearance of new lesions. To continue on-study, a patient must have no greater than a 25% growth in the indicator lesions and no new lesions appearing. Two cycles of therapy are required for a patient to be considered evaluable for a response. All measurable responses must last for 8 weeks. Those patients whose indicator lesions have progressed at the end of 8 weeks are taken off study. Those with responsive or stable disease are continued on protocol treatment until progression is documented. Whenever possible, patients relapsing after a response will have their measurable tumour assayed again for TS expression.

The logarithm of the observed TS level is used to compare patients with or without prior 5-FU therapy and to compare patients who did or did not respond to 5-FU therapy. The two-sided t-test is being used to compare two groups of patients; geometric means are calculated; 95% confidence intervals are constructed by taking the anti-log of the limits of the parametric confidence intervals based on the logarithm of the TS levels. To identify a "most effective" cut-off point in the TS level for predicting response, a sequence of 2×2 tables is constructed. To test whether, at the selected TS cut-off point, the association between response and TS level is significant, the distribution of the maximal chi-square is estimated by simulation [24, 25].

RESULTS

Following tissue acquisition for TS level, 26 patients with disseminated colorectal cancer have been treated with protracted-infusion 5-FU plus weekly leucovorin. All patients signed informed consent for tissue acquisition. 4 patients have started on therapy within the past 8 weeks and are not yet evaluable for response. The first 5 patients treated satisfied the eligibility requirements of the protocol, but were given therapy as part of a pilot programme initiated prior to the formal opening of this protocol. The next 21 patients were registered and treated in this protocol. 22 patients have completed at least two cycles of therapy and are evaluable for correlation of response to therapy with TS expression within the measurable tumour. Table 1 describes the demographics of the 22 patients for whom response evaluation and TS quantitation within the measurable lesion are known. In summary, 13 men and 9 women, ranging in age from 43 to 80 years have been treated. 9 patients (41%) had no previous exposure to 5-FU.

Clinical response data

8 patients (36%) have had partial responses to therapy lasting 3–12 months or more. 5 of the 9 patients (56%) with no previous 5-FU responded. 3 patients, with progressive disease after treatment with 5-FU bolus therapy have responded to the protracted infusion regimen used in this protocol. 6 responding patients (with response durations from 3 to 12 months) have progressed. 4 of these previously responding patients have had measurable tumour re-analysed for TS quantitation; 2 could not undergo another procedure for TS quantitation. 2 responding patients remain on protocol treatment. All responses reported in this manuscript were reviewed and agreed upon in a Protocol Review Meeting of the Division of Medical Oncology at the University of Southern California.

TS quantitation data

Pretreatment TS/β-actin quantitations on measurable tumours range from $0.5–22.6 \times 10^{-3}$. Intratumoral TS quantitations for the 8 responding patients ranged from 0.5×10^{-3} to 3.5×10^{-3}. TS quantitations for the 14 patients who did not respond ranged from 1.7×10^{-3} to 22.6×10^{-3}. On average, patients who responded had lower baseline TS levels compared to those who did not experience an objective response ($P < 0.001$, based on a two-sided t-test.) Means and confidence intervals for the TS quantitations are given in Table 2. Furthermore, inspection of the results showed that 8 of 12 (67%) with TS quantitation less than 4.0×10^{-3} in their measurable tumour had a response, and all 10 patients with TS quantitation greater than 4.0×10^{-3} had progressive disease. This TS cut-off point significantly classifies responders and non-responders ($P = 0.015$, based on the distribution of a maximal chi-square statistic, which accounts for the fact that the cut-off of 4.0×10^{-3} was chosen after the results were observed).

TS quantitations for the 9 patients previously untreated with 5-FU range from 0.5×10^{-3} to 19.8×10^{-3}. The 3 responding patients who were previously treated with 5-FU in the adjuvant setting had low TS expressions (1.0, 1.2, and 1.4×10^{-3}) in their indicator lesions. Means and confidence intervals for TS quantitation for those with previous exposure to 5-FU versus no previous exposure are expressed in Table 2. On average, the 13 patients who had been previously treated with 5-FU had only slightly higher TS levels as compared with the 9 who had never received it ($P = 0.59$, based on a two-sided t-test).

DISCUSSION

5-FU alone or with biochemical or biological modulation, induces response rates for less than half the patients treated with disseminated colorectal cancer. The identification of patients with these tumours displaying innate resistance to 5-FU would allow treatments to be designed on a scientific, rather than an empirical basis.

The preliminary data from this trial, which uses a protracted infusion schedule of 5-FU to treat patients with metastatic colorectal cancer, suggest that intratumoral TS quantitation will accurately predict which lesions will display resistance and which lesions are likely to respond. The definition of an elevated intratumoral TS quantitation or a quantitative "cut-off point" which predicts for absolute resistance to 5-FU for this population of patients remains to be determined. This trial is continuing to accrue patients and a national programme to be conducted by the Southwest Oncology Group has been designed to determine the true "cut-off point" for resistance.

The interaction of previous 5-FU therapy and intratumoral TS quantitation also needs further study. Whether responding patients with relatively low TS quantitations develop a higher TS quantitation once the tumour is found to be resistant to protracted-infusion 5-FU will be answered as more patients are accrued to this trial.

A relatively low level of TS expression within a measurable tumour may enhance the chance for response but does *not* guarantee it. On a strictly anatomical basis, an inadequate or irregular blood supply to recurrent tumours in the pelvis, peritoneum and retroperitoneum can result in poor penetration of 5-FU into the tumour bed. On a molecular level, intratumoral TS expression is only one of several factors that determine response to 5-FU. For example, the conversion of 5-FU to the active metabolite FdUMP may be suboptimal in some tumours.

Currently, the most important role for 5-FU in the treatment

Table 1. Demographics, response and TS/β-actin quantitation data for 19 patients treated with protracted-infusion 5-FU + leucovorin

Patient No.	Age/Sex	Lesion (measured by)	Response Y/N	TS/β-actin (10^{-3})	Previous 5-FU Y/N
1	66/F	Liver (CT)	Y	0.5	N
2	69/F	Liver (CT)	Y	1.0	Y
3	57/M	Peritoneal implants (laparoscopy)	Y	1.2	Y
4	58/F	Liver (CT)	Y	1.4	Y
5	43/F	Liver (CT)	N	1.7	N
6	59/M	Liver (CT)	Y	1.8	N
7	55/F	Peritoneal implants (CT)	N	1.8	Y
8	80/M	Liver (CT)	Y	2.1	N
9	53/F	Pelvic recurrence (CT)	N	2.5	Y
10	69/M	Liver (CT)	N	2.7	Y
11	65/M	Liver (CT)	Y	2.8	N
12	53/M	Left supraclavicle lymph node	Y	3.5	N
13	55/M	Retroperitoneal lymph node (CT)	N	4.2	Y
14	72/F	Left supraclavicle lymph node	N	4.3	N
15	55/F	Peritoneal mass (laparoscopy)	N	5.2	Y
16	79/M	Liver (CT)	N	5.9	Y
17	43/M	Peritoneal implant (laparoscopy)	N	8.0	Y
18	64/M	Liver (CT)	N	9.1	N
19	55/M	Pelvic recurrence (CT)	N	9.8	Y
20	68/F	Liver (CT)	N	11.6	Y
21	53/M	Liver (CT)	N	19.8	N
22	55/M	Lymph node (left femoral)	N	22.6	Y

Table 2. TS quantitation for responding versus non-responding patients and for patients who received previous 5-FU therapy versus without prior 5-FU

Patient group	Number of patients	Mean TS/β-actin expression*	95% Confidence interval for mean TS
Partial response	8	1.54×10^{-3}	(0.92, 2.58)
No response	14	5.42×10^{-3}	(3.51, 8.38)
No prior 5-FU	9	3.00×10^{-3}	(1.44, 6.25)
Prior 5-FU	13	3.76×10^{-3}	(2.13, 6.63)

*Geometric mean.

of large bowel tumours is in the context of adjuvant therapy [26, 27]. Unfortunately, tumour recurrence after adjuvant therapy remains a common event. A recent presentation by the National Surgical Adjuvant Breast Project (NSABP) evaluated the role of TS within a primary rectal tumour in predicting relapse. Patients with the greatest levels of TS in primary lesions showed the greatest risk for relapse [28]. Thus, it is possible that TS quantitation within a tumour will predict for both the biological behaviour of the *primary* tumour and for resistance of a metastatic lesion to fluorinated pyrimidines.

Whether intratumoral differences in TS quantitation will be found in primary tumours versus tumours within draining lymph nodes and distant metastases remains to be determined. The biological and therapeutic implications of such differences could be quite important. Strategies to prevent clinical resistance to 5-FU will depend on whether initially sensitive human colorectal tumours become clinically resistant because of gene amplification, changes of structure or specific proteins within the enzyme or heterogeneity of TS quantitation within a tumour. If further data support our current conclusions, intratumoral TS quantitation will become a necessary part of the treatment decision regarding therapy for patients with disseminated colorectal cancer.

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