Thymidylate Synthase Protein Expression in Primary Colorectal Cancer Compared with the Corresponding Distant Metastases and Relationship with the Clinical Response to 5-Fluorouracil

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ABSTRACT

Thymidylate synthase (TS) expression in colorectal cancer metastases has been shown to predict for the clinical response to 5-fluorouracil. Because primary tumors may easily provide accessible sources of tissue for marker analysis, we have investigated the stability of TS expression between primary colorectal cancer and the corresponding distant metastases and compared their relative ability to predict response to chemotherapy on a series of 27 patients homogeneously treated with biochemically modulated fluorouracil for advanced disease. By immunohistochemistry, high levels of TS expression were observed in 19 of 27 (70%) primary tumors and in 13 of 27 (48%) metastatic samples. Overall, TS levels observed in primary tumors did not correlate with those measured in the corresponding metastases (\(r = 0.30, P = 0.13\)), with higher TS levels in primary tumors in 8 of 10 discordant cases. Accordingly, the degree of TS immunoreactivity was significantly higher in primary tumors compared with the corresponding metastases (mean TS score 3.8; median, 4 versus 2.8; median 3; \(P = 0.001\)). Response rates after chemotherapy for metastatic disease were similar for patients with low and high TS levels in their primary tumors (37% versus 53%, \(P = 0.47\)). In contrast, response rates were 71% and 23% in patients with low and high TS in metastatic samples (\(P = 0.012\), respectively). In summary, TS levels measured in primary colorectal cancer do not reflect those observed in the corresponding metastases and cannot be used to predict their response to chemotherapy. The basis for the higher TS content of primary colorectal cancer compared with the corresponding metastases needs clarification.

INTRODUCTION

TS, a rate-limiting enzyme in the DNA synthetic pathway, is a critical target for the medical treatment of colorectal cancer. FUra, a TS-inhibitor drug, has remained the only standard agent for the treatment of large bowel tumors for nearly four decades (1). During this time, infusional schedules have been developed (2), and LV has been combined with the fluoropyrimidine (3) to prolong and potentiate the TS-inhibitory activity of this antimetabolite. More recently, pure TS inhibitors were introduced for the treatment of this disease (4, 5), and oral fluoropyrimidines (6) were developed to simulate continuous infusion of FUra so that long-term TS inhibition is achieved without requiring infusion pumps and central venous lines.

Given the central role of this enzyme in the medical treatment of colorectal cancer, considerable efforts have been directed at identifying groups of patients unresponsive to TS inhibitors. Initially, the strategy behind these studies was to avoid useless toxicity, because the response rate to FUra does not exceed 30% even with complex regimens using LV, infusional schedules, and/or high FUra doses (7–10). Similarly, pure TS inhibitors under clinical testing are not more active (11, 12). More recently, non-TS-targeted antineoplastic agents with clinical activity in colorectal cancer have emerged that could be offered to patients who are unresponsive to TS inhibitors. In particular, the DNA cross-linking agent Oxaliplatin (13–15) and the topoisomerase I inhibitor Irinotecan (16–20) have consistently shown substantial activity in patients with advanced disease, whereas antibody therapy is being compared with FUra in the adjuvant setting (21). Therefore, the identification of patients unlikely to respond to TS inhibitors may now also address the choice between different treatment options that were not available until a few years ago.

Since preclinical studies (22–24) and preliminary observations on small groups of patients (25–27), TS mRNA expression measured in biotic samples from colorectal cancer metastases has been identified recently as a predictor of the clinical response to a regimen of infusional FUra (28). We have developed a rabbit polyclonal antibody to recombinant human TS, and we have obtained similar results with immunohistochemical TS.
quantitation on paraffin sections of metastatic colorectal cancer (29). Other studies have then confirmed the relationship between the level of TS expression in colorectal cancer metastases and the clinical response to fluoropyrimidine-based chemotherapy (30–32). However, a considerable number of patients with advanced colorectal cancer will have metastases that are inaccessible for biopsy, whereas the primary cancer may provide an ample source of tissue for marker analysis. Thus the prediction of the clinical response to FUra for patients with metastatic disease on the basis of TS quantitation would be much easier if the level of this enzyme could be measured in the primary tumor.

In this study, we have therefore used an immunohistochemical method suitable for archival material to analyze the level of TS expression in the primary tumor and the corresponding metastases from a series of patients included in two consecutive Phase II trials of biochemically modulated FUra conducted at our institute (33, 34). The main aim was to compare the levels of TS expression between primary colorectal cancer and the corresponding distant metastases. An additional objective was to examine their relative ability to predict response to FUra-based chemotherapy.

**PATIENTS AND METHODS**

**Patients and Sample Preparation.** Paraffin-embedded archival samples of the primary tumors and the corresponding distant metastases derived from 27 patients with advanced colorectal cancer were used for this study (12 males and 15 females; median age 59, range 43–72 years). These represent all of the patients from two consecutive Phase II trials of a hybrid regimen alternating bolus FUra + methotrexate with continuous-infusion of FUra + LV (33, 34) for which tissue sections from both the primary tumor and at least one distant metastatic lesion were available. In detail, the treatment consisted of two biweekly cycles of bolus FUra modulated by methotrexate (24 h earlier) alternating (14 days later) with a 3-week continuous infusion of FUra modulated by LV (on the 1st day of each week of infusion). Additional details, along with data on the outcome after chemotherapy with this regimen, have been published previously (33, 34).

**TS Immunohistochemical Analysis.** Two-μm-thick tissue sections were cut from each block, deparaffinized in xylene, rehydrated with graded ethanol, and immersed in TBS. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in distilled water for 15 min.

TS protein expression was evaluated with the avidin-biotin complex immunohistochemical technique (35) using a rabbit polyclonal antibody to recombinant human TS, as described previously (29).

The tissue sections were heated in a microwave oven at 300 W for 10 min, cooled, and stored in TBS (pH 7.6). To block nonspecific binding of the primary antibody, a normal rabbit serum (DAKO X901) dilution in TBS was used for 20 min. After removing the blocking solution, the TS antibody (2 mg/ml) was applied for 60 min in a humidified chamber at room temperature. Negative control studies were performed without applying the primary antibody. The sections were then incubated with biotin-conjugated swine antirabbit immunoglobulins for 20 min (DAKO-E353) and then with the avidin-biotinylated peroxidase complex for 30 min. After developing the color reaction product with a freshly prepared 3,3'-diaminobenzidine chromogen solution for 5 min, the sections were counterstained with light hematoxylin for 1 min, dehydrated in a series of ethanol, cleared in xylene, and mounted with glass coverslips. Sections known to stain positively were included in each run as positive controls.

Slides were then examined under a light microscope and scored independently by two of the authors (D. D. and G. T.) who were blinded to both the clinical and the pathological data. Only tumor cells with cytoplasmatic staining were counted as positive. TS expression was quantitated using a visual grading system based on the intensity of staining and classified into five groups ranging from 0 (undetectable staining) to 4 (very-high intensity of staining). Intensity levels of 0–2 were grouped together and considered low expression, whereas levels of 3 and 4 were considered high expression. The agreement in TS evaluation between the two observers was >90%. In the two cases of disagreement, a final score was determined by consensus after reexamination. When heterogeneous levels of TS expression were found within a tumor (in multiple sections from different paraffin-embedded blocks of the same tumor), the level of TS expression of that lesion was defined according to the highest TS score that was recorded.

**p53 Immunohistochemical Analysis.** Tissue sections were prepared as described for TS analysis. p53 protein expression was assessed with the same immunohistochemical technique (35) using the mouse monoclonal antibody D07 (Dako, Glostrup, Denmark). This reagent recognizes an epitope in the NH2 terminus of the human p53 protein so that it reacts with both wild-type and mutant forms of p53 protein.

The tissue sections were heated in a microwave oven at 300 W for 10 min and then incubated with D07 (1:800 dilution) in a humidified chamber at room temperature for 30 min. After washing, the slides were incubated with rabbit antimouse immunoglobulins for 25 min (DAKO-E354) and then with avidin-biotinylated peroxidase complex for 30 min. Finally, the color reaction product was developed for 5 min with a freshly prepared 3,3'-diaminobenzidine solution. The sections were counterstained with light ematoxylin for 1 min. Positive controls, which are cases known to show >85% positive staining, and negative controls, in which the primary antibody was omitted, were always included. Slides were then observed under a light microscope, and the pattern of immunoreactivity was scored according to both the percentage of cell nuclei that stained positively (from 1 to 3) and the intensity of staining (+ to +++). Cases in which <10% of the tumor cells displayed nuclear staining were considered negative.

**Statistics.** The correlation between TS levels measured in primary colorectal cancers and in the corresponding metastases was analyzed with the Spearman rank test. Intrapatient comparison of TS scores was performed using the Student t test for paired samples. The association between the levels of TS expression and patient characteristics as well as the clinical response to chemotherapy were analyzed using the χ² or Fisher's exact test, as indicated.
RESULTS

Twenty-two of 27 primary tumors analyzed were colon cancers and 5 were rectal adenocarcinomas. The site and timing of metastases, age, sex, and prior treatment for each of the 27 patients are listed in Table 1. Fifteen of 27 primary tumor specimens were classified as moderately differentiated, and 2 samples were well to moderately differentiated. Four specimens were classified as well-differentiated and four were classified as poorly differentiated. Differentiation was not described in two specimens.

Table 1 Patient and tumor characteristics, TS and p53 immunostaining, and clinical outcome

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<th>Site/timing of metastases</th>
<th>Adjuvant treatment</th>
<th>TS level* (scorea)</th>
<th>p53 expression*</th>
<th>Clinical response*</th>
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*a M, male; F, female.
*b Synch, synchronous; metach, metachronous.
*c L, low (i.e., intensity of staining, 0–2); H, high (intensity of staining, 3–4).
*d Intensity of TS immunostaining from 0 to 4.
*e NEG, 1, 2 and 3, = <5%, 5–25%, 25–70%, and >70% nuclear reactivity, respectively; +, intensity of staining.
*f Response to FU-based chemotherapy: MR, minor response; SD, stable disease; P, progression.

Table 1. Patient and tumor characteristics, TS and p53 immunostaining, and clinical outcome.

The difference in TS levels between primary tumors and metastases could not be correlated to the use of adjuvant FURa because only five patients received chemotherapy after resection of the primary tumor, and four of those showed the same pattern of TS expression in both the primary tumor and daughter metastases (Table 1). Similarly, the timing of the development of the secondary lesions did not affect the variations in TS status between primary tumors and metastases. A discordant pattern of TS expression was in fact observed in 7 of 17 cases (41%) with synchronous metastases and in 3 of 10 cases (30%) with metachronous metastases (Table 1).

TS overexpression has been associated with p53 alterations (36). In this series, however, the nuclear level of p53 protein expression, measured immunohistochemically with the mouse monoclonal antibody DO7, was identical in primary tumors and the corresponding metastases (Table 1; positive nuclear staining in 16 of 27 cases, 59%), and no correlation was found between p53 nuclear accumulation and TS overexpression (high TS levels, with and without p53 overexpression: 69% versus 73%, χ² = 0.05, P = 0.82 in primary tumors; and 37% versus 64%, χ² = 1.784, P = 0.18 in metastatic samples). In addition, the proportion of cases with a discordant pattern of TS staining between primary tumors and metastases was similar in patients with or without p53 overexpression [5 of 16 (31%) and 3 of 11 (45%), respectively].

Fig. 2 shows that the variation in TS expression between primary and metastatic colorectal cancer translates into a differ-
ent predictivity of the clinical response to FUra (Fig. 2). Indeed, no correlation was found between TS levels in primary tumors and response to chemotherapy of the corresponding metastases (response rate, 37% versus 53%, with low and high TS, respectively; $\chi^2 = 0.516; P = 0.47$). In contrast, when TS immunoreactivity was measured in metastatic tumor samples, the combined CR + PR rate was 71% and 23% in patients with low and high TS levels, respectively ($\chi^2 = 6.312; P = 0.012$).

**DISCUSSION**

This study is the first where TS levels in primary colorectal cancer and the corresponding metastases were compared and their relative ability to predict response to chemotherapy analyzed. The results show that TS levels measured in primary colorectal cancer do not reflect those observed in the corresponding metastases and fail to predict their response to palliative, FUra-based chemotherapy. These findings are consistent with the results of a previous study on a large cohort of patients with disseminated colorectal cancer, which failed to demonstrate a correlation between tumor response and TS expression measured immunohistochemically in primary tumors (37). In contrast, when TS immunoreactivity was measured in metastatic tumor samples, a significant correlation with response to chemotherapy was found in both this series of 27 patients and in a larger group of patients treated with the same chemotherapy regimen (29).

This information may have a major clinical relevance. TS expression was identified as a predictor of response to fluoropyrimidines in multiple recent studies (28–32). Targeted treatment based on TS immunophenotype may thus be attractive, similar to the use of hormonal therapy based on estrogen receptor determination in breast cancer. Intrapatient variations in TS levels as found in this study may challenge the application of this concept to colon cancer patients. Estrogen receptor quantitation in primary breast cancer is in fact informative as to the hormonal status of the corresponding metastases and predicts the response to hormonal manipulation (38). Also, receptor status usually remains the same in different metastatic lesions over long time periods (39). At variance, our results clearly show that TS levels determined in primary colorectal cancer are neither related to TS expression in the corresponding metastases nor predictive of their response to chemotherapy. This is unfortunate, because a considerable number of patients with advanced colorectal cancer will have metastases that are inaccessible for biopsy, whereas the primary cancer provides an ample source of tissue for marker analysis.

Higher levels of TS expression in pelvic (32) or lung (40) metastases compared with liver metastases from colorectal cancer have been recently reported both by other investigators and by our own group. In the present study, significant differences in TS levels between different metastatic sites could not be found, probably because of the limited number of patients analyzed. However, in a subset of cases for which multiple metastatic samples or multiple sections from the same metastasis were available, both intrapatient and intratumor variations in TS expression were observed (data not shown). The variation in TS levels between primary colorectal cancer and the corresponding metastases may thus be part of a more general intrapatient heterogeneity in TS expression, reflecting a dynamic mechanism of TS regulation based on the actual rate of cell proliferation and DNA synthesis in a specific tumor lesion.

Of interest, this heterogeneity is consistent with the short
duration of the clinical response to FUra in advanced colorectal cancer. Specific cell populations with high TS levels may in fact have a growth advantage during FU-based chemotherapy. The different pattern of TS expression in different tumor sites and different tumor areas also provides a rationale for the use of combination chemotherapy, including non-TS-targeted drugs, in advanced colorectal cancer. The enhanced activity obtained with the incorporation of DNA damaging agents (Mitomycin C, Oxaliplatin, Irinotecan) in different FUra regimens lends support to this hypothesis (41–44).

Our findings may also explain the paradoxical association of high levels of TS expression with a greater efficacy of adjuvant FUra-based chemotherapy after radical resection of high-risk rectal cancer (45). TS status may have in fact changed in the micrometastases that are the target of adjuvant chemotherapy compared with the tumor of origin. Studies are in progress to test whether TS quantitation in the regional lymph nodes may improve the predictivity for the efficacy of adjuvant FUra-based chemotherapy. This may be of crucial clinical importance at a time when novel, non-TS-targeted agents are being proposed for the adjuvant treatment of large bowel tumors.

REFERENCES


Clinical Cancer Research

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