

Thymidylate Synthase Protein Expression in Advanced Colon Cancer: Correlation with the Site of Metastasis and the Clinical Response to Leucovorin-modulated Bolus 5-Fluorouracil

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ABSTRACT

Recently, we have demonstrated that thymidylate synthase (TS) protein expression predicts for the clinical response to a regimen of infusional 5-fluorouracil (5FU) in advanced colorectal cancer patients. Previous studies by other groups that showed a correlation between TS gene expression and response to the fluoropyrimidine also involved infusional regimens. Considering the putatively different mechanism of action of bolus compared with continuous infusion of 5FU, the aim of the present study was to test whether the correlation between TS expression and the clinical response to 5FU is valid for bolus regimens. A secondary aim was to compare TS levels between liver metastases and abdominal recurrences from colon cancer, because these sites have a distinctly different responsiveness to 5FU chemotherapy. The study population consisted of 41 patients (25 males and 16 females; median age, 60 years) with unresectable metastatic or recurrent colon cancer, homogeneously treated with 5FU (420 mg/m² i.v., days 1–5) and leucovorin (20 mg/m² i.v., days 1–5); cycles were repeated every 28 days. Twenty-seven patients (66%) showed high levels of TS expression as defined by TS scores equal to 3 and 4. The proportion of cases with high levels of TS expression was significantly higher in abdominal recurrences (18 of 22, 82%) compared with liver metastases (9 of

19, 47%; $P = 0.02$). Intratumoral TS protein expression was inversely correlated with response to chemotherapy (response rate: 7 of 14, 50%, versus 0 of 27 in patients with low and high levels of TS expression, respectively; $P = 0.0001$). These results confirm that the level of TS protein expression predicts for response to 5FU, even with a bolus schedule. The higher TS levels observed in abdominal compared with liver metastases may account for their different responsiveness to 5FU chemotherapy. Immunohistochemical quantitation of TS protein levels may thus allow us to change the therapeutic approach to advanced colorectal cancer from a general to an individual treatment strategy at a time when new non TS-targeted drugs have become available for this disease.

INTRODUCTION

Metastatic colorectal cancer remains a significant health care problem in Western countries. Following the demonstration of a small but statistically significant advantage in survival and symptom control, biochemically modulated 5FU² has become the standard first line treatment of this disease. However, the response rate to the fluoropyrimidines does not exceed 30%, even using biochemical modulators, infusional schedules, and/or high 5FU doses (1). If patients with tumors sensitive to 5FU can be identified before the initiation of therapy, 5FU-based treatment could be targeted to this group, whereas patients with tumor resistant to fluoropyrimidines could receive alternative drugs working with a different mechanism of action, such as CPT-11 or oxaliplatin.

The importance of TS in determining fluoropyrimidine cytotoxicity has been established in both preclinical and clinical studies (2, 3). After preliminary observations in small numbers of patients (4, 5), Leichman *et al.* (6) showed a statistically significant association between TS gene expression and the clinical response to infusional 5-FU in disseminated colorectal cancer. We have used a polyclonal antibody to recombinant human TS to measure TS protein expression immunohistochemically and obtained similar results in a series of patients treated by alternating bolus and continuous infusion 5-FU (7).

In the present study, our aim was to investigate whether the correlation between TS expression and response to 5FU is valid for bolus 5FU regimens. The schedule of 5FU administration was in fact shown to influence the mechanism of action of this agent, and incorporation into RNA may be the dominant mech-

Received 3/9/99; revised 5/26/99; accepted 6/2/99.

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² The abbreviations used are: 5FU, 5-fluorouracil; LV, leucovorin; TS, thymidylate synthase.

Table 1 Patient characteristics

No. of patients	41
Age	
Median	60
Range	44–80
Sex	
M/F	25/16
Primary tumor	
Colon	41
Biopsy site	
Liver	19
Abdominal masses	22
Chemotherapy	
5FU/LV	41

anism of cytotoxicity with bolus administration (8). The predictivity of TS expression for the clinical response may thus be limited with this schedule. Because we have observed substantial inpatient variation in TS levels (between primary tumors and the corresponding metastases and even between different metastatic lesions in the same patient) and other authors have found higher TS levels in lung compared with liver metastases (9), a secondary aim of this study was to compare TS expression between abdominal recurrences and liver metastases.

PATIENTS AND METHODS

The study population consisted of 41 patients (25 males and 16 females; median age, 61.5 years) with unresectable, metastatic, or recurrent colon cancer enrolled in a GISCAD (Italian Group for the Study of Digestive Tract Cancer) phase III trial that compared high and low doses of LV combined with 5FU between 1992 and 1994 (10). These 41 cases represent all of the patients who received the same treatment as first line chemotherapy for advanced disease (5FU at the dose of 420 mg/m² i.v., days 1–5, and leucovorin at the dose of 20 mg/m² i.v., days 1–5; cycles were repeated every 28 days) and for which archival metastatic tumor samples were available (Table 1).

After three cycles of treatment, measurable disease was reassessed by the initial method for tumor response. Only lesions stained for TS were used as measurable indices for response. Response evaluation was based on WHO criteria (11).

Immunohistochemistry

Sample Preparation. Paraffin-embedded archival samples derived from diagnostic biopsies of abdominal recurrences or liver metastases from colon cancer were used for this study. Six tissue sections (each 1 μ m in thickness) were cut from each block, deparaffinized in xylene, rehydrated with graded ethanol, and immersed in Tris-buffered saline (TBS). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in distilled water for 15 min.

TS Analysis. TS protein expression was evaluated by the avidin-biotin complex immunohistochemical technique, using a rabbit polyclonal antibody to recombinant human TS. The antibody was produced in the laboratory of Dr. Frank Maley (Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, NY) and used previously to localize and quantitate normal and mutant TS in human

sarcoma cells lines by Western blotting (12) and to measure TS expression in human tumor samples with results consistent with those obtained by mRNA quantitation (9). The tissue sections were heated in a microwave oven at 300 W for 10 min, cooled, and stored in TBS at 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. Under these conditions, a complete interexperiment reproducibility in TS assessment by the same investigator (*i.e.*, TS expression repeatedly measured on the same sample in different experiments) was obtained. The sections were then incubated with biotin-conjugated, swine anti-rabbit immunoglobulins for 20 min (DAKO-E353), followed by the avidin-biotinylated peroxidase complex for 30 min. After developing the color reaction product with a freshly prepared diaminobenzidine chromogen solution for 5 min, the sections were counterstained with light hematoxylin for 1 min, dehydrated in a series of ethanols, cleared in xylene, and mounted with glass coverslips using Permount. 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), blinded to both the clinical and pathological data. Only tumor cells with cytoplasmic 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 from 0 (undetectable staining) to 4 (very high intensity of staining). For the purpose of correlation with clinical data, intensity levels 0 to 2 were grouped together and considered low expression, whereas levels 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 levels of TS expression of that lesion were defined according to the highest TS score that was recorded.

RESULTS

Twenty-seven patients (66%) showed high levels of TS expression as defined by TS scores equal to 3 and 4 in 19 and 8 cases, respectively. Among the patients with a low level of TS expression (14 of 41, 34%), 5 exhibited a completely negative staining, 4 had a staining score of 1, and 5 had a score of 2.

The proportion of cases with high levels of TS expression was significantly higher in abdominal recurrences (18 of 22, 82%) compared with liver metastases (9 of 19, 47%; $P = 0.02$). Accordingly, the degree of TS immunoreactivity tended to be higher in abdominal masses compared with liver metastases (median TS score, 3 *versus* 2, $P =$ not significant).

Intratumor TS protein expression was inversely correlated with response to chemotherapy (Table 2; $\chi^2 = 16.32$; $P = 0.001$, Mantel test for linear association). All of the patients achieving an objective response had low levels of TS expression (scores 0–2), whereas 18 of 23 failures occurred in patients with

Table 2 Clinical response according to TS expression

TS	Patients	Complete response		Partial response		SD		P	
		n	%	n	%	n	%	n	%
Low	14	2	14	5	35	2	14	5	36
High	27	0		0		9	33	18	67

high TS levels (scores 3–4). The difference in the proportion of objective responses between patients with low (complete response + partial response: 7 of 14, 50%) and high (complete response + partial response: 0 of 27) TS levels was statistically significant ($P = 0.0001$). The relationship between TS levels and clinical response remained significant when TS expression was graded from 0 to 4 (response rate: 80, 25, 40, 0, and 0%, with a TS score of 0, 1, 2, 3, and 4, respectively; $\chi^2 = 26.18$; $P = 0.01$, Mantel test for linear association).

The degree of TS immunoreactivity was significantly lower in responding patients compared with those unresponsive to 5FU chemotherapy: mean TS score, 0.7 (median, 0) versus 2.8 (median, 3), respectively ($P = 0.000004$, Student's *t* test for independent samples). In contrast, there were no differences in TS immunoreactivity between those patients who immediately progressed after the first cycle and those who achieved stable disease (mean TS score, 2.9 versus 2.8).

DISCUSSION

These results demonstrate that intratumoral TS protein expression is inversely correlated with the clinical response to a bolus regimen of 5FU/LV, in the same way as reported previously for infusional regimens (5, 6, 13). Remarkably, although a subset of patients with low TS expression failed to achieve a clinical response, no response was observed in patients with high TS levels. This strong association between high TS levels and resistance to bolus 5FU plus LV indirectly indicates that, under clinical conditions, incorporation into RNA does not play a substantial role with this schedule of 5FU administration, probably due to the ability of LV modulation to shift the mechanism of action toward TS inhibition. In a previous study, we have in fact reported a 25% response rate in patients with high TS levels treated with a regimen including methotrexate followed by bolus 5FU (7). Our results also support the notion that TS expression is variable among different metastatic sites; higher levels of TS expression were observed in abdominal recurrences as compared with liver metastases (82% versus 47%, $P = 0.02$). This difference is substantially similar to that observed by Gorlick *et al.* (9) between pulmonary and hepatic metastases, and it may account for the different responsiveness to 5-FU chemotherapy of abdominal recurrences compared with liver metastases. This finding, if confirmed in prospective trials, may have important clinical implications. Drugs targeting TS may be appropriate for liver metastases but not for other sites such as pulmonary or abdominal, where alternative drugs (CPT-11 or oxaliplatin) may be more active. Thus, additional studies are required to determine whether TS expression needs to be measured separately in each metastatic site if this parameter has to be used to predict for the clinical response to

5FU-based chemotherapy in colon cancer. We have in fact observed previously a substantial inpatient heterogeneity in TS levels between primary colorectal cancer and even between different metastatic lesions in the same patient.³ Consistently, TS levels measured on archival samples of previously resected primary tumors failed to predict for response to chemotherapy of advanced disease (14).

Although high TS expression effectively predicted resistance to 5FU, in our study low TS expression did not necessarily result in a clinical response; all responding patients had low TS expression, but a subset of patients with low TS did not respond to treatment. Of note, an ~50% failure rate has been reported also among patients with low TS mRNA. These patients may have other mechanisms of resistance that the favorable condition of low TS is insufficient to overcome.

In conclusion, these results confirm the ability of TS protein expression to predict for response to 5FU, even using a bolus schedule, and provide further evidence for the existence of different patterns of TS expression among different metastatic sites. This could have a significant impact on the choice of drugs and the design of new treatments in advanced colon cancer, changing the therapeutic approach from a general to an individual treatment strategy.

ACKNOWLEDGMENTS

We thank Dr. Frank Maley for kindly providing polyclonal antibody to TS.

REFERENCES

- Seymour, M. T. Colorectal cancer: treatment of advanced disease. *Cancer Treat. Rev.*, 24: 119–131, 1998.
- Peters, G. J., van der Wilt, C. L., van Triest, B., Codacci-Pisanelli, G., Johnston, P. G., van Groeningen, C. J., and Pinedo, H. M. Thymidylate synthase and drug resistance. *Eur. J. Cancer*, 31A: 1299–1305, 1995.
- Leichman, L., Lenz, H. J., Leichman, C. G., Groshen, S., Danenberg, K., Baranda, J., Spears, C. P., Boswell, W., Silberman, H., Ortega, A., Stain, S., Beart, R., and Danenberg, P. 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. *Eur. J. Cancer*, 31A: 1306–1310, 1995.
- Peters, G. J., van der Wilt, C. L., van Groeningen, C. J., Smid, K., Meijer, S., and Pinedo, H. M. Thymidylate synthase inhibition after administration of fluorouracil with or without leucovorin in colon cancer patients: implications for treatment with fluorouracil. *J. Clin. Oncol.*, 12: 2035–2042, 1994.

³ C. Aschele, personal communication.

5. Johnston, P. G., Lenz, H. J., Leichman, C. G., Danenberg, K. D., Allegra, C. J., Danenberg, P. V., and Leichman, L. Thymidylate synthase gene and protein expression correlate and are associated with response to 5-fluorouracil in human colorectal and gastric tumors. *Cancer Res.*, 55: 1407–1412, 1995.
6. Leichman, C. G., Lenz, H. J., Leichman, L., Danenberg, K., Baranda, J., Groshen, S., Boswell, W., Metzger, R., Tan, M., and Danenberg, P. Quantitation of intratumoral thymidylate synthase expression predicts for disseminated colorectal cancer response and resistance to protracted-infusion fluorouracil and weekly leucovorin. *J. Clin. Oncol.*, 15: 3223–3229, 1997.
7. Aschele, C., Debernardis, D., Casazza, S., Antonelli, A., Tunesi, G., Baldo, C., Lionetto, R., Maley, F., and Sobrero, A. Immunohistochemical quantitation of thymidylate synthase expression in colorectal cancer metastases predicts for clinical outcome to fluorouracil-based chemotherapy. *J. Clin. Oncol.*, in press, 1999.
8. Aschele, C., Sobrero, A., Faderan, M. A., and Bertino, J. R. Novel mechanism(s) of resistance to 5-fluorouracil in human colon cancer (HCT-8) sublines following exposure to two different clinically relevant dose schedules. *Cancer Res.*, 52: 1855–1864, 1992.
9. Gorlick, R., Metzger, R., Danenberg, K. D., Salanga, D., Miles, J. S., Longo, G. S. A., Fu, J., Banerjee, D., Klimstra, D., Jhanwar, S., Danenberg, P. V., Kemeny, N., and Bertino, J. R. Higher levels of thymidylate synthase gene expression are observed in pulmonary as compared with hepatic metastases of colorectal adenocarcinoma. *J. Clin. Oncol.*, 16: 1465–1469, 1998.
10. Labianca, R., Cascinu, S., Frontini, L., Barni, S., Fiorentini, G., Comella, G., Zaniboni, A., Gottardi, O., Arnoldi, E., Oliani, C., Duro, M., Pavanato, G., Martignoni, G., Raina, A., Piazza, E., Dallavalle, G., Valsecchi, R., Pancera, G., and Luporini, G. High *versus* low dose levo leucovorin as a modulator of 5-fluorouracil in advanced colorectal cancer: A GISCAD phase III study. *Ann. Oncol.*, 8: 169–174, 1997.
11. Miller, A. B., Hoodgstraten, B., Staquet, M., and Winkler, A. Reporting results of cancer treatment. *Cancer (Phila.)*, 47: 207–214, 1981.
12. Tong, Y., Liu-Chen, X., Ercikan-Abali, E. A., Capioux, G. M., Zhao, S. C., Banerjee, D., and Bertino, J. R. Isolation and characterization of thymitaq (AG337) and 5-fluoro-2-deoxyuridilate-resistant mutants of human thymidylate synthase from ethyl methanesulfonate-exposed human sarcoma HT1080 cells. *J. Biol. Chem.*, 273: 11611–11618, 1998.
13. Benson, A. B., Catalano, P. J., Rao, S., Haller, D. G., O'Dwyer, P. J., Allegra, C. J., and Johnston, P. G. Thymidylate synthase expression as a predictor for response to a 5-fluorouracil-based therapy and survival for patients with resected colon or advanced colorectal cancer. *Proc. Am. Soc. Clin. Oncol.*, 16: 917, 1997.
14. Findlay, M. P. N., Cunningham, D., Morgan, G., Clinton, S., Hardcastle, A., and Aherne, G. W. Lack of correlation between thymidylate synthase levels in primary colorectal tumours and subsequent response to chemotherapy. *Br. J. Cancer*, 75: 903–909, 1997.