Decreased Folylpolyglutamate Synthetase Activity in Tumors Resistant to Fluorouracil-Folinic Acid Treatment: Clinical Data

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ABSTRACT

Thymidylate synthase (TS) is the main target for fluorouracil (FU). Optimal cellular concentrations of reduced folates in polyglutamated forms (via folylpolyglutamate synthetase [FPGS]) are necessary for achieving maximal TS inhibition. The aim of this multicentric prospective study was to analyze the link between clinical response to FU therapy for liver metastases of colorectal carcinoma and tumoral TS and FPGS activities. Forty-four advanced colorectal cancer patients (15 women and 29 men; median age 63, range, 27–78 years) receiving a standard FU-folinic acid protocol were included. A single hepatic tumoral biopsy was obtained systematically at the time of diagnosis. For 24 patients, a biopsy in the primary colon tumor was available. TS and FPGS activities were measured by radioenzymatic assays. Clinical response on hepatic metastases was 1 complete response, 12 partial responses, 14 stabilizations, and 17 progressions. In hepatic biopsies, TS activity (median, 185; range, <10–3111 fmol/min/mg protein) and FPGS activity (median, 1270; range, <400–3730 fmol/min/mg protein) exhibited a wide variability. TS activity in primary tumors (median, 461; range, 35–2565 fmol/min/mg protein) was significantly higher than in hepatic metastases. No difference was observed between primaries and metastases for FPGS. FPGS activity expressed in liver metastases was significantly correlated to that expressed in primaries. The distribution of TS activity in liver metastases was not significantly different between responsive and nonresponsive patients. However, FPGS activity measured in liver metastases was significantly higher in responsive patients (median, 1550 fmol/min/mg protein) than in nonresponsive patients (median, 1100 fmol/min/mg protein). A discriminant analysis revealed that 24 of the 25 patients exhibiting a liver FPGS activity >1100 fmol/min/mg protein and/or a liver TS >320 fmol/min/mg protein were nonresponding patients. These data establish for the first time the potential importance of tumoral FPGS activity for assessing FU-folinic acid responsiveness in the clinical setting.

INTRODUCTION

FU is among the most frequently used anticancer agents not only for treatment of colorectal cancer but also for induction chemotherapy in advanced head and neck cancer and for treatment of metastatic breast cancer. FU exerts its cytotoxic activity through different cellular pathways. As a result, the resistance mechanisms opposed to the action of FU may be numerous and complex. The main FU cellular target is the TS enzyme whose activity is inhibited through the formation of a stable ternary complex including TS, FdUMP, and CH2FH4. Inside the cells, CH2FH4 is polyglutamated by the FPGS enzyme. Both CH2FH4 cellular retention and affinity to TS are increased with CH2FH4-polyglutamated forms. Preclinical and clinical data have indicated that overexpression of tumoral TS is a predictor of FU resistance. Recently, experimental studies have suggested that a decrease in tumoral FPGS expression could be a cause of resistance to FU-based chemotherapy. We present the results of a prospective study performed in 44 advanced colorectal cancer patients. Both TS activity and FPGS activity were measured in hepatic metastases obtained before FU-FA chemotherapy. Response to treatment and tumoral TS and FPGS activities were compared. The main objective was to test the predictive value of tumoral FPGS activity in clinical resistance to FU-FA therapy. In addition, tumoral TS activity was included since it is a known factor for predicting resistance to FU-based chemotherapy.

The abbreviations used are: FU, fluorouracil; CH2FH4, 5,10-methyltetrahydrofolate; CR, complete response; FA, folinic acid; FdUMP, 5-fluoro-2'-deoxyuridine-5'-monophosphate; FPGS, folylpolyglutamate synthetase; PR, partial response; Prog, progression; Stab, stabilization; TS, thymidylate synthase.

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MATERIALS AND METHODS

Patients

Forty-four patients with liver metastases of colorectal cancer were included (15 women and 29 men; median age, 63 years, range, 27–78). Among these patients, six exhibited metastatic hepatic metastases. The remaining 38 patients exhibited synchronous hepatic metastases. Primary localizations were 2 in the transverse colon, 11 in the rectum, 8 in the right colon, and 23 in the left colon. Patients did not receive any previous chemotherapy. All patients had WHO status ≤2. For all patients, liver tumoral biopsies were obtained during laparotomy which was systematically performed at the time of anatomicopathological diagnosis. For 24 patients, a biopsy of the primary colorectal tumor was also available. At the time of sampling, biopsies were split in half: one part being used for histological examination, and the other being immediately stored in liquid nitrogen for enzyme measurement.

None of the patient study had received adjuvant chemotherapy by FU before inclusion. After surgery, all patients received a FU-FA chemotherapy protocol: 37 patients were treated by short iv. of FU (350 mg/m²/day) plus FA (200 mg/m²/day) for 5 days, 3 cycles at 4-week intervals (FUFOL A); 3 patients received a weekly 24-h continuous infusion of FU (1300 mg/m²) and FA (200 mg/m²), 12 cycles (FUFOL B); and 4 patients received a 2-h infusion of FA (200 mg/m²/day) associated with FU bolus (400 mg/m²/day) followed by a 22-h infusion of FU (600 mg/m²/day) for 2 days, 6 cycles at 2-week intervals (FUFOL C). After completion of chemotherapy cycles, clinical response on hepatic metastases was evaluated by scanography according to Union Internationale Contre le Cancer criteria: CR was defined as complete regression of all lesions; PR was defined as regression of 50% or more of all visible lesions; Stab was defined as regression of less than 50% or progression of less than 25% of all visible lesions; and Prog was defined as an increase of more than 25% of all visible lesions.

Chemicals

Purified TS from Lactobacillus casei was provided by Professor David Priest (University of South Carolina, Columbia, SC). CH₂FH₄ was prepared from tetrahydrofolate supplied by Schircks Laboratories (Jona, Switzerland). [¹³C]FdUMP labeled at position 6 (23 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA). [³H]-FdUMP labeled at position 5 (16 Ci/mmol) and [¹⁴C]glutamic acid tetrалabeled (264 Ci/mmol) were obtained from Amersham (Les Ulis, France). Aminopterin and all other chemicals were purchased from Sigma Chemical Co. (St. Quentin Fallavier, France).

Enzyme Determinations

Frozen tissues were pulverized in a liquid nitrogen-cooled Thermovac pulverizer. The resulting powders were homogenized in 10 volumes of a 10 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA, 0.5 mM DTT, and 10 mM sodium molybdate. The homogenates were centrifuged for 1 h at 105,000 × g (+4°C), and the supernatants (cytosols) were used for protein and enzymatic assays. Proteins were measured using Coomassie G-250 (Protein Assay Reagent; Bio-Rad Ltd., Paris, France) with human serum albumin as standard.

TS Activity. TS activity was measured according to the tritium release assay described by Spears and Gustavsson (17). The assay consisted of incubating 25 μl of cytosol with [³H]dUMP (1 μM final concentration) and CH₂FH₄ (0.62 mM final concentration) in a total volume of 55 μl. After 0, 10, 20, and 30 min of incubation at 37°C, the reaction was stopped on ice. The excess of [³H]dUMP was removed by adding 300 μl of activated charcoal (15%) containing 4% trichloroacetic acid (5-min centrifugation at 14,000 × g, room temperature). The ³H₂O formed during the incubation was then counted in an aliquot of the above supernatant. Results were expressed as fmol/min/mg protein. Interassay reproducibility was evaluated through repeated analysis of single-use aliquots of a pooled cytosol: n = 5; mean, 1109.95 fmol/min/mg protein; SD, 78.59 fmol/min/mg protein; coefficient of variation, 7.08%.

FPGS Activity. FPGS activity was measured according to the method described by Montero and Llorente (18) and consisted of incorporating an additional [¹⁴C]glutamic acid residue into the glutamate chain of aminopterin. Each cytosol was assayed in duplicate. The assay consisted of incubating 100 μl of cytosol with [¹⁴C]glutamic acid (isotopic dilution, 250 μM final concentration) and aminopterin (250 μM final concentration) in a total volume of 250 μl. After a 2-h incubation at +37°C, the reaction was stopped by the addition of 50 μl of 40% trichloroacetic acid. Tubes were then centrifuged for 10 min at 3000 × g. The supernatant was analyzed for the presence of aminopterin polyglutamates by high-performance liquid chromatography using an RP18 5-μm Lichrospher column and a radioactivity flow monitor (LD 506 Berthold, Wildbad, Germany). Results were expressed as fmol/min/mg protein. The limit of sensitivity was 400 fmol/min/mg protein. Interassay reproducibility was evaluated through repeated analysis of single-use aliquots of a pooled cytosol: n = 5; mean, 269.5 fmol/min/mg protein; SD, 25.5 fmol/min/mg protein; coefficient of variation, 9.45%.

Statistics

Since TS activity did not fit a Gaussian distribution, non-parametric tests were used. Intrapatient comparisons of TS and FPGS activities between liver metastases and primary were performed using Wilcoxon-paired tests. Correlations were performed using the Spearman rank test. The comparison of TS and FPGS distribution between responding (CR + PR) and nonresponding (Prog + Stab) patients was performed by means of the Mann-Whitney U test. Discriminant analyses for predicting responsiveness were performed for TS and/or FPGS by means of successive separate χ² tests, each test being performed for TS and/or FPGS with a different threshold. Statistics were performed on Statgraphics Plus software (Uniware, Paris, France).

RESULTS

TS and FPGS activity exhibited a wide interpatient variability both in liver metastases and primaries. TS activity was detectable in 87.9% of the samples and was significantly lower in liver metastases (median, 185 fmol/min/mg protein; range, <10–3110) as compared with primaries (median, 461 fmol/
Table 1: Tumoral TS and FPGS activity according to clinical response

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<th>TS activity (fmol/min/mg protein)</th>
<th>FPGS activity (fmol/min/mg protein)</th>
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<td>Liver metastasis</td>
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<td>Responding patients</td>
<td>13</td>
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<tr>
<td>Nonresponding patients</td>
<td>31</td>
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Mann-Whitney U test: responders versus nonresponders

- \( P = 0.28 \)
- \( P = 0.32 \)
- \( P = 0.046 \)
- \( P = 0.84 \)

*The tumoral target for clinical response was the liver metastases. Responding patients were defined as patients with CR or PR; nonresponding patients were defined as patients with progressive or stable disease.

*ND, not detectable, i.e., <10 fmol/min/mg protein for TS and <400 fmol/min/mg protein for FPGS activity.

Clinical response on liver metastases was 1 CR, 12 PRs, 14 Stabs, and 17 Progs, leading to a response rate of 29.5%. Table 1 depicts the analysis of tumoral TS and FPGS activities according to the clinical response on liver metastases. Median values of TS activity measured in liver metastases was 78 fmol/min/mg protein in responsive patients and 199 fmol/min/mg protein in nonresponsive patients. Although the highest TS activities were observed in nonresponsive tumors, the difference in the distribution of liver TS activity was not significant between responsive and nonresponsive patients (\( P = 0.28 \)).

With regard to liver FPGS activity, the median value in responsive patients was 1550 fmol/min/mg protein versus 1100 fmol/min/mg protein in nonresponsive patients. Interestingly, FPGS activity expressed in liver metastases was significantly higher in responsive patients as compared with nonresponsive patients (\( P = 0.046 \)). In addition, a FPGS threshold allowing nonresponsive patients to be discriminated from responsive patients was demonstrated at 1100 fmol/min/mg protein (\( \chi^2; 7.45, P = 0.0063 \)). Among the 17 patients exhibiting a liver FPGS activity lower than the above threshold, 16 (i.e., 94%) were FU-FA-resistant patients. The relative risk of resistance was 1.7. Note-worthy, neither TS activity nor FPGS activity measured in the primary was linked to treatment efficacy in liver metastases (Table 1).

The plot of liver FPGS versus liver TS activity in responsive and nonresponsive patients shows that patients with either a low FPGS activity and/or a high TS activity were mainly nonresponders (Fig. 1). In fact, 24 of the 25 patients exhibiting a FPGS ≤ 1100 and/or a TS > 320 fmol/min/mg protein were FU-FA-resistant patients (\( \chi^2; 18.14, P = 0.00002 \), predictive value of resistance 96%). On the other hand, the predictive value of responsiveness in the remaining patients having both FPGS >1100 and TS ≤320 fmol/min/mg protein was 63.2%. The relative risk of resistance was 2.61.

DISCUSSION

Today, colorectal carcinoma is the second cancer in western countries (19). Ten to 25% of them are detected in the metastatic phase, involving the liver in the majority. Surgical exeresis of liver metastases can be performed in 10–15% of the cases (20); chemotherapy thus plays a major role in this pathology. During the past few years, chemotherapy of advanced colorectal cancer was limited to FU-based protocols (21). Recently, new anticancer drugs have demonstrated interesting activity in this pathology, and, among them, some like the camp-tothecin analogue CPT 11 act through different cellular targets than those involved in FU activity (22). On this basis, a rational...
approach should be to identify, before starting therapy, the tumors which are likely to be resistant to FU-based chemotherapy to direct clinicians toward other anticancer drugs.

Both experimental and clinical investigations on tumoral predictors of FU resistance have led to identifying TS as such a predictive parameter. Clark et al. (8) have shown that TS gene amplification might be a relevant mechanism of acquired FU resistance in the clinical setting. Clinical investigations on limited numbers of patients by Horikoshi et al. (9), Johnston et al. (10), Peters et al. (11), Lenz et al. (12), and more recent data (13, 14) have confirmed that overexpression of tumoral TS is linked to FU resistance. The present study does not contradict the potential usefulness of TS as a predictor for response to FU-based chemotherapy; in a group of 44 patients, the highest TS tumoral activities were observed in nonresponding patients. However, the distribution of TS tumoral activities was not significantly different between responders and nonresponders. A part of the discrepancy between present data and other clinical reports on TS may be explained by differences inherent to the analytical methods used since we measured TS enzyme activity when others considered TS expression (reverse transcriptase-PCR; Refs. 9 and 12) or TS protein levels (immunohistochemistry; Ref. 10). In fact, TS should be considered as a primary target but not as the sole determinant factor leading to cell death after FU exposure. Secondary events include cellular damage recognition pathways and death program inducers like p53 and Bcl2 which should also be taken into account. For instance, Lenz et al. (23) have recently proposed a dual approach considering both p53 point mutations and TS for predicting clinical response to FU in gastrointestinal cancer. In 22 patients, we were able to compare TS activity expressed in liver metastases to that expressed in primaries. We did not observe any correlation between metastatic and primary TS activity, although TS activity was significantly overexpressed in primaries as compared with metastases. This result is in line with recent data from Davis et al. (24) who reported higher TS levels in primary colorectal tumors as compared with liver metastases. We have no explanation for this paradoxical observation. In fact, TS expression is well known to be related to cell proliferation, and there is no evidence that cell proliferation in primaries is enhanced as compared with metastases.

Two independent enzyme systems are responsible for formation and degradation of polyglutamate forms of folates and antifolates: the FPGS catalyzes the γ-glutamylolation of folates and the γ-glutamyl hydrolase (or folypolyglutamyl hydrolase) catalyzes the hydrolisis of the polyglutamates. Both enzymes are expressed in tumor cells (15, 16, 25); however, very little data on γ-glutamyl hydrolase expression in tumors have been published. Up to now, the role of FPGS in cancer chemotherapy has been mainly focused on methotrexate therapy (26). In fact, FPGS is directly involved in methotrexate activation mechanisms which lead to cytotoxicity. With regard to FU activity, CH₂FH₄ is a necessary cofactor for TS inhibition by FdUMP, and the stability of the ternary complex has been shown to be enhanced with long polyglutamated forms of CH₂FH₄ (5). A decrease in FPGS activity has been reported in a cell line that became resistant to FU (16). In addition, a decrease of the polyglutamate-reduced folate pool was reported in a cell line resistant to FU modulation by FA (15). We recently demonstrated on a large panel of 14 human cancers expressing a spontaneous sensitivity to FU that the higher the FPGS activity, the greater the FU sensitivity; moreover, FPGS activity was also a significant predictor for FU-FA sensitivity (27). Data concerning FPGS activity in human solid tumors are quite scarce and concern few patients with head and neck tumors (28) and colon cancers (29). In these cases, tumoral FPGS activity exhibited a marked variability. The present study confirms on a larger set of patients the wide variability of FPGS activity expressed in primary colorectal cancer and in liver metastases with values ranging from the limit of detection (≤400 fmol/min/mg protein) up to 3730 fmol/min/mg protein). This 9-fold range of variability could motivate the determination of tumoral FPGS in the assessment of clinical trials currently undertaken with new TS inhibitors, such as tomudex (ZD1694), which require intracellular activation by FPGS. Importantly, the present study has revealed for the first time a relationship between FPGS expression and FU-based chemotherapy resistance in the clinical setting. FPGS activity measured in liver metastases before treatment was significantly higher in responsive patients than in nonresponsive patients. Also, the response rate in patients exhibiting a high FPGS activity (>1100 fmol/min/mg protein) was significantly higher than that observed in patients with a low FPGS activity (44.4% and 5.9%, respectively). FPGS activity expressed in primaries was significantly correlated to the activity expressed in metastases, but was not linked to the response on metastatic disease. Even though in our limited set of 44 patients we were not able to demonstrate the predictive value of TS activity taken as a single parameter, the dual analysis of TS and FPGS activities in liver metastases increased the prediction of FU-FA resistance: χ² values and P levels changed from 7.45 and 0.0063 with the single threshold (FPGS) to 18.14 and 0.0002 with the double threshold (TS and FPGS). The response rate for the whole group of patients was 29.6% (i.e., the risk of resistance was 70.4%). Interestingly, in the group of patients exhibiting a low FPGS (≤1100) and/or a high TS (>320), the risk of FU-FA resistance was 96%, whereas in the remaining patients (FPGS >1100 and TS ≤320) the probability of response was 63.2%. The predictive value of FPGS and TS activities measured in liver metastases will merit further confirmation; however, present results are very promising for stimulating such investigations in the future. The fact that FU was modulated by high-dose FA probably strengthens the role of FPGS because elevated tumor FPGS activity would favor a higher tumor retention of reduced folates under polyglutamated form and thus would lead to an optimal modulation of FU. Further studies should be encouraged for testing the value of FPGS activity in FU responsiveness without FA modulation or with other modulating agents.

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