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Oncology Research, Vol. 11, pp. 437–445, 1999
Printed in the USA.

0965-0407/99 $20.00 + .00

Marked Variation of Thymidylate Synthase and Folylpolyglutamate Synthetase Gene Expression in Human Colorectal Tumors

Enrico Mini,* Cristina Biondi,* Maria Morganti,* Cristina Napoli,* Paolo Mazzoni,† Fabio Cianchi,‡ Francesco Tonelli,† Camillo Cortesini,‡ Sergio Capaccioli,§ Ferdinando Ficari,† Alessandro Quattrone,§ Susanna Rossi,* and Teresita Mazzei*[1]

*Unità di Chemioterapia, Dipartimento di Farmacologia Preclinica e Clinica, †Sezione di Chirurgia, Dipartimento di Fisiopatologia Clinica, ‡Istituto di Clinica Chirurgica Generale e Discipline Chirurgiche, §Istituto di Patologia Generale, Università degli Studi di Firenze, Italy

(Submitted September 20, 1999; sent for revision October 14; received December 23; accepted December 28, 1999)

Patients with advanced colorectal cancer are currently being treated with 5-fluorouracil (5-FU)-based chemotherapy. A growing number of patients with resectable disease receive adjuvant therapy with 5-FU/levamisole (LEV) or 5-FU/folinic acid (LV). However, many patients still fail on these treatments, due to occurrence of natural or acquired tumor resistance. Among clinically relevant mechanisms of resistance to fluoropyrimidines, increased expression of thymidylate synthase (TS) has been emphasized. Another potentially relevant mechanism involves a decrease in folylpolyglutamate synthetase (FPGS) expression. To establish the value of these genes as prognostic factors and predictors of the outcome of 5-FU-based chemotherapy in colorectal cancer, we measured their expression in colorectal tumors from patients undergoing surgery and postoperative chemotherapy and compared it with that in normal colonic mucosa. This was done by a semi-quantitative, nonradioisotopic polymerase chain reaction (PCR) method using β-actin as an internal standard and expressed as a TS/β-actin or a FPGS/β-actin mRNA ratio. In tumor samples from 21 colorectal cancer patients, TS gene expression varied 118-fold. The median TS/β-actin ratio was, in fact, 41.36×10^{-3} (range 2.49×10^{-3} to 294.54×10^{-3}). Little variation in TS gene expression was observed in corresponding normal colonic mucosa; the TS/β-actin gene ratio was lower (median 26.16×10^{-3}; range 8.49×10^{-3} to 69.49×10^{-3}). Among tumor explants from 20 patients, FPGS expression varied over 161-fold. A similar marked variation was also observed in normal colonic mucosal samples (over 185-fold). Overall and disease-free survival data suggest an inverse association between the level of tumor TS and FPGS expression and clinical prognosis. The availability of this sensitive and accurate assay for gene expression should now make it possible to extend these laboratory/clinical correlations to larger populations.

Key words: Thymidylate synthase; Folylpolyglutamate synthetase; 5-Fluorouracil; Resistance; Colorectal carcinoma

Patients with advanced colorectal cancer are being effectively treated with 5-fluorouracil (5-FU[2])-containing regimens, achieving an objective response in about 20–30% of cases (1,2). It has also been recently demonstrated that 5-FU and folinic acid (LV) or levamisole (LEV) markedly decrease the incidence of disease recurrence and mortality in patients with radically resected colon cancer (Dukes stage B and C or Dukes stage C, respectively), compared to untreated controls (3). However, better insight into the factors that determine the efficacy of 5-FU-based palliative and adjuvant chemotherapy is clearly warranted, because many patients will still fail on these treatments.

Although 5-FU has a complex mechanism of action, evidence is accumulating that the actual antitumor effect of 5-FU in patients is predominantly mediated by the extent and duration of the inhibition of thymidylate synthase (TS) by the 5-FU metabolite fluorodeoxyuridine monophosphate (FdUMP) (4–7). TS is a key enzyme in the de novo synthesis of deoxythymidine monophosphate (dTMP), an essential precursor of DNA synthesis, for which deoxyuridine monophosphate (dUMP) is the nucleotide substrate and 5,10-$CH_2H_4PteGlu_n$ the co-substrate. 5,10-$CH_2H_4PteGlu_n$ acts as the methyl-group donor, both in the monoglutamate form (8) and with increased efficiency in the polyglutamate form (9). Polyglutamates of natural folates, including 5,10-$CH_2H_4PteGlu_n$, formed by the enzyme FPGS, are better retained intracellularly and are better cosubstrates for enzymes (10).

In experimental tumor model systems, the principal mechanisms of resistance to 5-FU have been attributed to alterations in TS, such as: altered enzyme structure due to gene point mutation with decreased affinity for the inhibitor, FdUMP (11,12); increased enzyme levels with increased mRNA levels, sometimes attributed to gene amplification (13). Other mechanisms of resistance to 5-FU include: diminished activation of 5-FU due to reduction of enzymatic activity (14–17) or increased catabolism (e.g., by alkaline phosphatase or dihydropyrimidine dehydrogenase) (18,19); increased levels of dUTPase preventing accumulation of FdUTP and dUTP in cellular DNA (20).

[1]Address correspondence to Teresita Mazzei, Unità di Chemioterapia, Dipartimento di Farmacologia Preclinica e Clinica, Università degli Studi di Firenze, Viale Pieraccini 6, 50139 Firenze, Italy. Tel. +39(0)55-416680; Fax +39(0)55-4271280.
[2]Abbreviations used: 5-FU, 5-fluorouracil; LV, folinic acid; LEV, levamisole; TS, thymidylate synthase; FdUMP, fluorodeoxyuridine monophosphate; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; 5,10-$CH_2H_4PteGlu$, 5,10-methylenetetrahydrofolate; 5,10-$CH_2H_4PteGlu_n$, 5,10-$CH_2H_4PteGlu$ polyglutamates; FPGS, folylpolyglutamate synthetase; IFN-α2a, interferon-α2a; FUDR, floxuridine.

Lower folypolyglutamate synthetase (FPGS) activity and FPGS gene expression also confer resistance to 5-FU (21), presumably because the resulting lower intracellular levels of 5,10-$CH_2H_4PteGlu_n$ destabilize the inhibitory TS–FdUMP–5,10-$CH_2H_4PteGlu_n$ ternary complex (22).

In human colorectal cancer a large variation in TS level (6,7) and in the kinetics of FdUMP enzyme inhibition by FdUMP (5,23), and more recently, a large variation in the expression of TS mRNA (24–26) have been observed. These variations may be related to the often poor response to treatment in the advanced disease setting (23–26). Recently, it has also been demonstrated that the expression of TS is an important, independent prognosticator of survival in patients with rectal cancer undergoing adjuvant 5-FU-based chemotherapy (27).

Although there are valid indications that TS protein activity is related to response to 5-FU, data for this hypothesis are still limited. Analysis of TS from a large patient population would enable us to answer this question.

A broad range of FPGS activity (28–30) or of FPGS gene expression (31) in patients affected by various tumor types, including colon cancer, has been reported. In one of these investigations a relationship between resistance to 5-FU–LV combination chemotherapy and low FPGS activity in advanced colorectal cancer patients was observed (30).

We have employed a sensitive and accurate method of semiquantitative PCR for measuring TS and FPGS gene expression after the mRNA has been reverse-transcribed to cDNA (RT-PCR), derived from that of Horikoshi et al. (24). This procedure is capable of reliably and accurately determining the relative study gene mRNA levels in surgery biopsy-size tissue samples (≤50 mg) using β-actin as the internal standard.

We report here the results of TS and FPGS expression obtained in patients with colorectal cancer undergoing surgery by applying this analytical technique to tumor and normal colonic mucosal surgery specimens.

MATERIALS AND METHODS

Chemicals

Plasticware was purchased from Nunc (Roskilde, Denmark). RNAzol B was obtained from Cinna Biotecx (Houston, TX). RNA guard RNase inhibitor, ultrapure dNTP set 2′-deoxynucleoside 5′-triphosphate, random hexamers, and bovine serum albumin were from Pharmacia (Uppsala, Sweden). RQ1 RNase-free DNase, M-MLV reverse transcriptase, and Taq DNA polymerase were purchased from Promega (Madison, WI). PCR primers were from Genosys (The Woodlands, TX). All other reagents were molecular biology grade.

Tissue Collection

Samples of normal and tumoral colorectal mucosa or hepatic metastasis and normal liver parenchyma were taken from patients undergoing surgery. All primary tumor biopsies were taken prior to 5-FU-based treatment, while the liver metastasis sample tested was taken at relapse following adjuvant systemic treatment with a LV–5-FU combination. The malignant appearance of the tissue was confirmed by inspection by a surgical pathologist. The portions of the specimens to be used for gene expression measurements were washed with saline solution to remove hemorrhagic or necrotic parts and frozen in liquid nitrogen within 10 min from removal from the operative field, until molecular analyses were performed.

Quantitative PCR for Determination of Relative TS and FPGS Expression

The procedure for RT-PCR-based quantitation was performed essentially as previously described by Horikoshi et al. (24) with β-actin as internal standard. The PCR theory predicts that the increase in the amount of amplified DNA generated at any set number of cycles that are within the region of exponential amplification is linearly proportional to the amount of starting DNA (32). In practice, because the PCR is an enzyme reaction, product formation as a function of starting substrate concentration (or as a function of PCR cycle number) is not linear indefinitely, due to saturation of binding sites, consumption of substrate, build-up of inhibitory products, and other factors. Thus, the linear ranges of amplification of each cDNA must be established separately.

Gene expression was high enough to be detected by ethidium bromide staining of the agarose gel after PCR amplification; therefore, no additional steps (i.e., transcription of cDNA to RNA) (24) were performed to increase the sensitivity of the method.

In brief, the method involved the following steps:

(a) Isolation of RNA. Total cellular RNA was extracted from tissue samples as small as 50 mg according to the guanidine thyocyanate method (33) using RNAzol B as the extracting medium. RNA was then treated with RQ1 RNase-free DNase (34) to eliminate DNA contamination, dissolved in water, and spectrophotometrically quantified. RNA quality was assayed on 0.8% agarose gel.

(b) Reverse transcription of RNA. Reverse transcription of 20 μg of total RNA was performed in a final volume of 100 μl in the presence of random hexamers (0.045 μg/μl), RNAguard (0.7 U/μl), dNTPs (1 mM), M-MLV reverse transcriptase (20 U/μl), bovine serum albumin (0.2 μg/μl), reverse transcription buffer (50 mM tris HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT) at 37°C for 1 h and stopped by heating at 90°C for 5 min. cDNA was purified from the reaction mixture according to a nonorganic DNA purification protocol (35).

(c) Choice of PCR primers. PCR primers for TS and FPGS were chosen according to the published sequence of the coding region (36,37) and those for β-actin according to that of the genomic sequence (38).

TSA: AGATCCAACACATCCTCCGCT
(upstream, bases 107–127 of the TS cDNA gene) (24).
TSB: CCAGAACACACGTTTGGTTGTCAG

(downstream, bases 220–243 of the TS cDNA) (24).

FPGS1: AGCCCGGACCTCTGGAGTG (upstream, bases 1391–1409 of the FPGS cDNA) (31).

FPGS2: CTGCCAGTGACTAGCACATGG (downstream, bases 1633–1653 of the FPGS cDNA) (31).

βAC1: GCGGGAAATCGTGCGTGACATT (upstream, bases 2104–2127 of the β-actin genomic sequence) (24).

βAC2: GATGGAGTTGAAGGTAGTTTCGTG (downstream, bases 2409–2432 of the β-actin genomic sequence) (24).

TS, FPGS, and β-actin primers were designed to amplify a segment of 136, 262, and 328 mer, respectively.

(d) PCR amplification. Increasing volumes of cDNA were amplified in a reaction mixture containing primers (0.4 μM), dNTP (200 μM), $MgCl_2$ (2 mM), reaction buffer (50 mM KCl, 10 mM Tris HCl, pH 9.0, 1% Triton X-100), Taq polymerase (0.05 U/μl). Final volume was 50 μl. Amplification was performed according to a hot start protocol to avoid aspecific amplification and primer dimer formation. The following PCR conditions were found to be optimal for amplification: 1 cycle of denaturation for 7 min at 94°C, annealing for 1 min at 58°C (TS) or 55°C (β-actin) and extension for 1 min at 72°C; 32 cycles (TS) or 28 cycles (β-actin) of denaturation for 40 s at 94°C, annealing for 1 min at 58°C (TS) or 55°C (β-actin) and extension for 1 min at 72°C. For FPGS gene amplification optimal conditions were: 1 cycle of denaturation for 7 min at 96°C, annealing for 1 min at 65°C and extension for 1 min at 72°C; 34 cycles of denaturation for 1 min at 96°C, annealing for 1 min at 65°C and extension for 1 min (7 min last cycle) at 72°C.

(e) Quantitation of the amplification products. The PCR product was analyzed by agarose gel electrophoresis and stained with ethidium bromide. The gel photographs were densitometrically analyzed by the Bio-Rad system GS-670 Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA).

The linear amplification ranges of the genes of interest (TS or FPGS) and β-actin were found by plotting the intensity of fluorescence of the bands against the volumes of the cDNA solution added to the PCR reaction. Our experimental conditions gave reproducible linear amplification of different starting DNA concentrations and generated a PCR product in high yield with a minimum of nonspecific amplification bands. To calculate the relative gene expression the slope of the linear portion of TS and FPGS cDNA amplification was divided by the slope of the linear range of β-actin amplification for each sample to give an empirical ratio between PCR products generated in the regions of concentration-proportional amplification.

Dividing this ratio by that from another sample gives the relative gene expression in the two samples, provided that the expression of the denominator gene remains relatively invariant. Repeated determinations of the ratios performed on different days with these primers did not vary by more than 15%.

Statistical Analysis

Overall and disease-free survival in low and high TS or FPGS expressors were estimated by the Kaplan-Meier method and compared using the logrank test. A multivariate analysis of the correlation between TS and FPGS expression and the two above-mentioned clinical parameters was also performed using the Cox regression model. The Wilcoxon signed-rank test was used for comparing TS and FPGS levels in tumor and corresponding normal tissues (paired samples) and to evaluate the correlation of TS and FPGS expression with clinical and pathologic characteristics (tumor site, Dukes stage, and grading). To determine the possible correlation between TS and FPGS expression within the same tumor sample, the Spearman test was used. Values of $P \leq 0.05$ were considered statistically significant.

RESULTS

Patient Characteristics

We evaluated the expression of TS and FPGS genes relative to the internal standard β-actin in 21 patients with colorectal cancer, of which 20 had undergone surgical resection of the primary tumor and one exploratory laparotomy for hepatic metastases, which were not resectable.

The principal clinical and pathological characteristics are reported in Table 1. The case series was comprised of 12 females and 9 males; the site of primary tumor was the rectum in nine cases, transverse colon in three cases, sigma in five cases, cecum in one case, and right colon in four cases. In one patient who underwent surgical resection of the primary tumor (No. 9) a double tumor (at the level of the rectum and of the right colon) was found. A specimen of metachronous hepatic metastasis obtained from patient No. 10, who had a primary tumor of the rectum, was also analyzed. Primary tumor stage was Dukes A in one patient, B in eight patients, C in eight patients, and D in three patients. Fifteen patients out of 21 received fluoropyrimidine-based chemotherapy postoperatively: in 12 cases as adjuvant and in 3 as palliation for hepatic disease. No medical therapy was administered to six patients because of early tumoral stage (one case) or clinical patterns (e.g., advanced age, presence of severe cardiac disease, poor performance status). In 10 cases systemic chemotherapy consisted of combined LV and 5-FU. In four cases another modulating agent (IFN-α2a: two cases; LEV: two cases) was also added to this combination. In one patient intrahepatic artery chemotherapy with FUDR was performed.

TS and FPGS Gene Expression

In patients undergoing surgical resection of the primary tumor, specimens of normal colorectal mucosa were collected along with tumor specimens. Analysis of TS mRNA levels in relation to the internal standard (β-

Table 1. Clinical and Pathological Characteristics of 21 Patients With Colorectal Carcinoma Undergoing Analysis of TS and FPGS Gene Expression

No. of patients	21
Age (years)	
Median	61
Range	23–82
Sex (M/F)	9/12
Site of primary tumors	
Cecum	1
Right colon	4*
Transverse colon	3
Sigma	5
Rectum	9*
Dukes stage†	
A	1
B	8
C	8
D	3
Histotype	
Adenocarcinoma	19
Colloid	2
Grading	
G1	1
G2	15
G3	2
ND‡	3
Postoperative chemotherapy	
None	6
Adjuvant (5-FU based)	12
Palliative (5-FU or FdUrd based)	3

*Patient with double neoplasm (colon and rectum).
†Twenty patients with primary tumor tested.
‡Not determined.

actin gene expression) revealed a relatively homogeneous and, on average, lower (TS/β-actin: 26.16×10^{-3} median; 8.49×10^{-3}–69.49×10^{-3} range) expression in normal colonic mucosa ($n = 13$) and liver parenchyma ($n = 1$) than in tumor tissue (TS/β-actin: 41.36×10^{-3} median; 2.49×10^{-3}–294.54×10^{-3} range of 21 samples). The difference between TS expression in tumor and in corresponding normal tissues in the 14 paired samples (i.e., primary tumor in 13 patients and liver metastasis in 1 patient vs. normal colonic mucosa and liver) was statistically significant ($P = 0.03$). This difference was also significant in the 13 patients in which primary tumor and colonic mucosa were available ($P = 0.04$) (Table 2).

The median ratio between the relative TS expression in the tumor compared to normal tissue was 1.98 (range 0.21–13.92). In the patient with hepatic metastases (No. 10) there was no substantial difference between expression of TS in the tumor tissue and that in normal liver tissue. In five primary tumor samples (No. 1, 4, 8, 9, and 16) a TS/β-actin ratio greater than 100×10^{-3} was observed. In 11 cases (No. 2, 3, 6, 7, 11, 12, 13, 14, 15, 19, and 21) the TS/β-actin ratio varied between 10×10^{-3} and 100×10^{-3}, while in 4 cases (No. 5, 17, 18, and 20) it was less than 10×10^{-3}. In the hepatic metastasis sample (No. 10) TS expression was intermediate (ratio between 10 and 100×10^{-3}).

In 20 out of 21 patients tumor samples were also analyzed for FPGS gene expression; this analysis was accomplished in 13 cases on corresponding normal mucosa samples (Table 2). The FPGS mRNA levels were similar in tumor tissue (FPGS/β-actin = 8.14×10^{-3} median) and normal mucosa (FPGS/β-actin = 3.79×10^{-3} median). Ratios varied from 0 to 247.05×10^{-3} and from 1.09×10^{-3} to 201.56×10^{-3} in tumor and in normal colonic mucosa, respectively. The median ratio between relative FPGS expression in the tumor and in mucosa was 0.85 (range 0–152.5). By comparing FPGS expression in the 13 paired samples (primary tumor and colonic mucosa) no significant differences were obtained ($P = 0.916$). There were 1 high (ratio $> 100 \times 10^{-3}$; No. 4), 7 medium (ratio between 10×10^{-3} and 100×10^{-3}; No. 3, 7, 8, 12, 17, 20, and 21), and 12 low expressors (ratio $< 10 \times 10^{-3}$; No. 1, 2, 5, 6, 9, 11, 13, 14, 15, 16, 18, and 19).

No significant differences were observed in the level of TS and FPGS gene expression in biopsy samples from primary colorectal tumors according to site, Dukes stage, and grading. Also, no correlation was observed between TS and FPGS expression in the same tumor sample (Spearman's $r = 0.0932$).

Clinical Results and Correlates

The overall median survival of the 17 patients undergoing radical surgery for a Dukes stage A, B, or C tumor is at present 19 months (range 12+–42+). Fourteen patients are still alive, of whom 11 are disease free. The zero time point for disease-free and overall survival was the date of the biopsy.

Two patients died because of nonneoplastic disease. One patient (No. 6) had disease recurrence 13 months after resection of the primary tumor and died shortly thereafter. He was a Dukes C stage and a medium TS and low FPGS expressor. In patient No. 7 (Dukes C stage, medium TS and FPGS expressor), 6 months after surgery, hepatic disease recurrence occurred following adjuvant chemotherapy with a survival of 21+ months; patient No. 16 (Dukes C stage, high TS and low FPGS expressor) and patient No. 19 (Dukes C stage, medium TS and low FPGS expressor) had paraortocaval lymphonode recurrence 31 and 27 months after surgery with a survival of 37+ and 30+ months, respectively.

Disease progression was observed in two of the three patients with liver metastases at surgery (No. 3 and 14) who received chemotherapy. The third patient (No. 9), who did not undergo chemotherapy due to poor performance status, died 1.5 months after surgery. The first two patients were medium TS and low FPGS expressors, while the third was a high expressor of the TS gene and a low expressor of the FPGS gene.

In the patient who underwent intrahepatic artery chemotherapy with FUDR, disease progression in the liver was observed after only three cycles. In this patient a

Table 2. TS and FPGS Gene Expression in Human Colorectal Tumor and Normal Tissues

Patient No.	Stage	5-FU-Based Chemotherapy	TS/β-Actin ($\times 10^{-3}$)		T/M Ratio*	FPGS/β-Actin ($\times 10^{-3}$)		T/M Ratio*
			Tumor	Mucosa		Tumor	Mucosa	
1	B	Yes, adjuvant	160.51	25.56	6.28	2.58	5.11	0.5
2	B	Yes, adjuvant	27.58	19.51	1.41	0	1.09	0
3	D	Yes, palliative	33.98	46.34	0.73	32.21	201.56	0.16
4	B	No	294.54	42.14	6.99	247.05	1.62	152.5
5	B	No	6.28	30.38	0.21	3.33	17.55	0.19
6	C	Yes, adjuvant	79.43	16.45	4.83	3.25	13.32	0.24
7	C	Yes, adjuvant	36.97	69.49	0.53	15.86	42.52	0.37
8	B	Yes, adjuvant†	234.44	26.76	8.76	27.64	15.85	1.74
9	D	No	226.48	16.27	13.92	7.05	3.79	1.86
10	Liver metastases	Yes, palliative‡	56.84	43.69	1.30	ND§	ND	ND
11	B	No	58.84	ND	ND	3.97	ND	ND
12	A	No	29.89	18.36	1.63	25.28	1.67	15.14
13	B	Yes, adjuvant	68.53	37.49	1.83	3.65	1.97	1.85
14	D	Yes, palliative	87.45	23.22	3.77	9.24	1.81	5.1
15	B	Yes, adjuvant	18.13	8.49	2.13	1.53	1.8	0.85
16	C	No	134.44	ND	ND	4.98	ND	ND
17	C	Yes, adjuvant	3.89	ND	ND	32.05	ND	ND
18	C	Yes, adjuvant	6.92	ND	ND	3.85	ND	ND
19	C	Yes, adjuvant	21.57	ND	ND	9.96	ND	ND
20	C	Yes, adjuvant	2.49	ND	ND	15.01	ND	ND
21	C	Yes, adjuvant	41.36	ND	ND	17.80	ND	ND
Median			41.36	26.16	1.98	8.14	3.79	0.85
Range			2.49–294.54	8.49–69.49	0.21–13.92	0–247.05	1.09–201.56	0–152

Statistical analysis (Wilcoxon signed-rank test): comparison of the distribution of the TS gene expression values in tumor vs. normal tissues (14 paired data): $P = 0.03$, and in primary tumor vs. colonic mucosa (13 paired data): $P = 0.04$; comparison of the distribution of the FPGS gene expression values in primary tumor vs. colonic mucosa (13 paired samples): $P = 0.916$.
*Tumor/mucosa.
†One cycle only; interruption due to toxicity.
‡FUDR-based intrahepatic chemotherapy.
§Not determined.

medium degree of TS expression was noted. FPGS gene expression was not tested.

The preliminary analysis of disease-free survival (patients with resectable disease only, $n = 17$) and overall survival (patients with either resectable or metastatic disease, $n = 21$) (Fig. 1A, B) showed that patients with lower TS gene expression had a better prognosis than those with higher TS gene expression. These differences were not statistically significant, however ($P = 0.14$ and 0.053, respectively). Also, restricting overall survival analysis to patients with potentially resectable disease, the observed difference between high and low TS expressors became lower (data not shown, $P = 0.11$); this could be due to a too short follow-up for this class of patients.

Similar trends, although slighter, in disease-free and overall survival (Fig. 2A, B) were observed between low and high FPGS expressors but with no statistical significance.

By performing multivariate analysis according to Cox regression model, no significant correlation between TS and FPGS expression and the above-mentioned clinical end points was observed.

DISCUSSION

Substantial experimental evidence has accumulated in recent years indicating that the most clinically relevant mechanism of resistance to 5-FU is represented by insufficient TS inhibition (4–7,23), which may be due to qualitatively and/or quantitatively altered effects on TS (39). Traditionally, TS inhibition has been determined by measuring the number of FdUMP binding sites or the conversion of dUMP to dTMP (5). Standard biochemical assays for this enzyme, although quite informative, are time consuming and difficult due to the generally low level of enzyme expression in samples obtained from human solid tumors (5,23). In addition, studies of clinical specimens from patients have been limited by the availability of sufficient tumor tissue for performing this type of assay (≥50 mg).

Recently new methods have become available such as ELISA assays (40,41), immunohistochemistry (42),

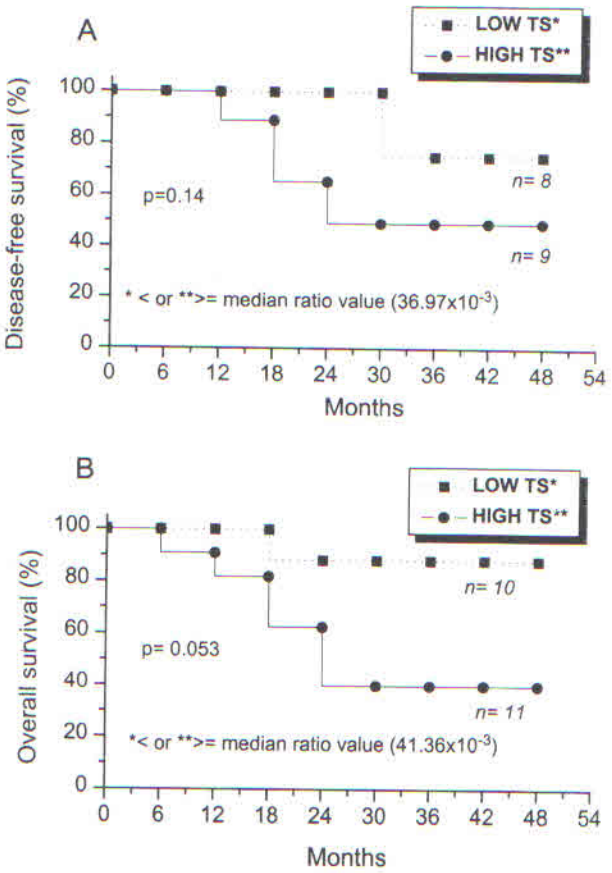

Figure 1. Association of the pattern of TS gene expression, analyzed by a semiquantitative RT-PCR method, with disease-free survival (A) and overall survival (B) in the study group. Survival was estimated by use of the Kaplan-Meier method.

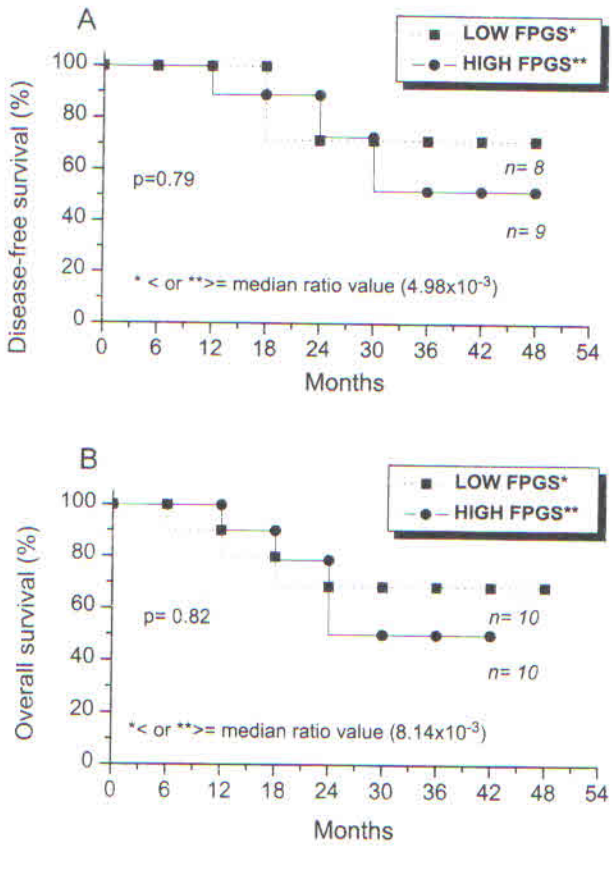

Figure 2. Association of the pattern of FPGS gene expression, analyzed by a semiquantitative RT-PCR method, with disease-free survival (A) and overall survival (B) in the study group. Survival was estimated by use of the Kaplan-Meier method.

Western blotting (25,43), or quantitative PCR for TS (24). In particular, the measurement of TS mRNA levels by a quantitative RT-PCR assay requires a very small quantity of tumor (<50 mg), permitting determination of TS expression in human tumor biopsy specimens (24).

Recent studies have demonstrated that TS immunohistochemical staining, Western blotting, or quantitation of TS mRNA expression can be used to predict the outcome of chemotherapy in colorectal cancer (25,26,44), gastric and esophageal tumors (25,43,45), and head and neck tumors (46), and they appear to have a prognostic value in patients with colorectal (27,47), gastric (45), and breast cancer (48).

On the contrary, Findlay et al. (49) and Cheradame at al. (50) found no association between TS protein or activity levels and response to 5-FU-based chemotherapy in colorectal and head and neck tumor patients, respectively.

Our data concerning the screening by RT-PCR of TS expression in tumor explants from patients with colorectal neoplasms reveal a higher variability in tumor tissue compared to normal mucosa, with more marked mRNA levels on average, in agreement with results obtained previously by Peters et al. (5) studying TS enzyme activity by classical biochemical methods in untreated patients. A large variation in the total levels of TS was also observed in 5-FU-pretreated tumors (23). In the latter investigation the total level and inhibition of TS was correlated with the outcome of 5-FU-based therapy (23).

TS gene expression values (TS/β-actin ratios) obtained in this investigation were on average higher than those observed by other authors in colorectal tumor tissues using RT-PCR analysis (24–26). These differences might be explained by changes in various steps of the RT-PCR technique employed compared to Horikoshi et al. (24) [e.g., omission of transcription of DNA to RNA after PCR amplification in our experience, differences in PCR primers in the studies of Leichman et al. (26) and Johnston et al. (25)].

The rapid recurrence or disease progression following surgery and/or chemotherapy in patients with medium degree TS expression observed in our series suggests that other clinical resistance mechanisms to 5-FU may have relevance, as demonstrated recently for high dihydropyrimidine dehydrogenase and/or thymidine phosphorylase expressors (51,52). It also identifies the need for a more precise definition of the sensitivity/resistance threshold in relationship to TS gene expression, requiring tests in a larger series of patients.

Among other mechanisms of resistance to 5-FU in tumor model systems, the relative deficiency of the intracellular folate cosubstrate 5,10-CH_2FH_2PteGlu has been clearly demonstrated (53). Increased efficiency in the formation and stabilization of the inhibitory TS-FdUMP–5,10-CH_2H_4PteGlu$_n$ ternary complex is provided by the expression of polyglutamate forms of the folate coenzyme (9) synthesized by the enzyme FPGS. Deficient FPGS activity appears to represent a common mechanism of resistance to antifolates, which are substrates of this enzyme (54,55). It has also been demonstrated that decreased FPGS activity and FPGS gene expression confer resistance to fluoropyrimidines (21), resulting in limited formation and stability of the ternary complex (22). However, in other human and murine tumor systems the lack of correlations between FPGS gene expression, protein expression, and enzyme activity has been described (56). It is conceivable that FPGS expression might be a clinically relevant determinant of response to chemotherapy with 5-FU-containing regimens and with potentially more effective regimens, including more specific folate-based TS inhibitors (57).

We observed a marked variation in FPGS gene expression, in both colorectal tumor explants (161-fold) and in correspondent normal colonic mucosa (185-fold), with no significant differences in mean FPGS relative expression levels between the two tissue types. These are the first reported data concerning FPGS mRNA levels in colorectal cancer.

A substantial range of variation in FPGS enzyme activity (about 70-fold) was previously shown by Janssen et al. (28) in nine head and neck tumor specimens. Lenz et al. (31) have demonstrated a similar broad range (about 500-fold) of FPGS mRNA levels in patients with acute lymphocytic leukemia and acute myelogenous leukemia.

More recently, Chazal et al. (30) have shown that low FPGS activity was correlated to lack of clinical response to standard 5-FU-LV therapy in advanced colorectal cancer patients. The apparent disparity of our data with those reported by Chazal et al. (30) may be due to the different clinical stage of our cases (i.e., early disease as compared to metastatic disease). An explanation for our observations could be the role of FPGS in contributing to sustained tumor cell proliferation via an enhancement of folate-dependent metabolic pathways; this phenomenon would be favored in high FPGS expressors, who may be characterized by a more rapid growth rate of the disease. However, no correlation with cell cycle parameters is available in our series.

The number of observations in our colorectal cancer case series, as well as in those of other types of tumors, is still too limited to demonstrate that such variation is a general phenomenon, but results suggest that basal levels of FPGS expression could contribute to clinical response to fluoropyrimidines or classical folate-based TS inhibitors. Several new antifolate inhibitors of TS have been synthesized and demonstrate promising antineoplastic activity in preclinical tumor models (57). They are currently in clinical trials (58). One of them, raltitrexed, exhibiting FPGS substrate properties, has been proved active in patients with advanced colorectal cancer in phase II and III trials (59).

Our preliminary data on patient survival suggest that both TS and FPGS gene expression may represent a prognostic parameter for patients undergoing surgery for primary colorectal cancer. Although not significant, in our experience the higher levels of these two proliferation-associated enzymes were associated with poorer outcome.

Recent advances in biochemical and molecular biology methodologies have allowed a comprehensive laboratory approach to the study of clinical fluoropyrimidine resistance.

The precise predictive role of TS, FPGS, and other molecular determinants of fluoropyrimidine sensitivity resistance in colorectal cancer patients can be tested in the future within the context of appropriate, large-scale clinical studies, applying the technology described here, along with others cited, which require limited amounts of tissue. The results of these future studies will very likely enable patients to be selected for fluoropyrimidine therapy in the adjuvant, or advanced stage disease setting, or for alternative treatments, as preliminarily suggested by Farrugia et al. (60).

ACKNOWLEDGMENTS: This work was supported by grants from the Associazione Italiana per la Ricerca sul Cancro (AIRC), from the Consiglio Nazionale delle Ricerche (Progetto Finalizzato Applicazioni Cliniche della Ricerca Oncologica), from the Ministero dell'Università e della Ricerca Scientifica e Tecnologica (ex 40%), and Azienda Ospedaliera Careggi-Regione Toscana, "Progetto Qualità." At the time of this work C. Biondi and M. Morganti were recipients of fellowships from the AIRC. The authors wish to thank Professor Vieri Boddi of the Istituto di Patologia Generale, University of Florence, for contributing to the statistical analysis of the data, and Mary Forrest for revising the English.

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