

Prognostic Role of Thymidylate Synthase, Thymidine Phosphorylase/Platelet-derived Endothelial Cell Growth Factor, and Proliferation Markers in Colorectal Cancer¹

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ABSTRACT

5-Fluorouracil (5FU)-based therapy is given to patients with advanced colorectal cancer and as adjuvant treatment. Thymidylate synthase (TS) is the target for 5FU, and may have a prognostic role for the outcome of 5FU-based therapy together with proliferation markers such as p53 and Ki67. Thymidine phosphorylase (TP, also known as platelet-derived endothelial cell growth factor) may be of importance both in the 5FU drug activation pathway and in tumor angiogenesis, similar to vascular endothelial growth factor (VEGF). TS and TP levels were determined biochemically in fresh-frozen tumor specimens of 32 untreated patients with colorectal cancer, whereas in paraffin-embedded tissue samples, immunohistochemistry was performed for TS, TP, and additional prognostic markers such as p53, Ki67, and VEGF as well as microvessel density. All factors were correlated with patient characteristics such as age, gender, Dukes' stage, angio-invasion, and differentiation grade. TS and TP as measured by various assays were correlated with overall and disease-free survival in this patient group. TP enzyme activity and protein expression correlated with each other. A significant correlation was found between TP enzyme activity and 5-fluoro-2'-deoxyuridine-5'-monophosphate binding activity. VEGF expression correlated significantly with TP immunostaining and Ki67 index. Survival analysis revealed a significant relation of TS levels to the overall survival in this small patient group and a significant correlation be-

tween TP activity and disease-free survival. TS and TP both were of prognostic significance in these patients with colorectal cancer. The interesting relationship of TS and TP with angiogenesis and proliferation needs further investigation.

INTRODUCTION

For treatment of metastatic colorectal cancer, 5FU³ in combination with LV is still the most commonly used regimen, with 20–30% response rates (1). Adjuvant therapy of patients with Dukes' C colon cancer with 5FU and levamisole was superior to surgery (2) but inferior to the combination of 5FU-LV (3). At present, adjuvant therapy trials are also performed using different modes of 5FU administration (3), the specific TS inhibitor Tomudex (Raltitrexed), and immunotherapy (4, 5).

The cytotoxic action of 5FU is mostly dependent on TS inhibition, mediated by the 5FU metabolite FdUMP, which blocks *de novo* synthesis of dTMP, which is essential for DNA synthesis. FdUMP forms a ternary complex with TS and the essential cofactor CH₂-THF [reviewed by Peters and Jansen (6)].

Because response to 5FU-based chemotherapy is low, prognostic criteria are being evaluated based on biological or molecular parameters with the aim to select a patient for a specific treatment schedule. TS levels have been related to response to 5FU therapy in different tumor types. In patients with metastatic colonic cancer, a high TS level measured biochemically correlated with a poor response to 5FU-based chemotherapy (7). TS mRNA expression as measured by reverse transcription-PCR was also prognostic for the outcome of 5FU therapy in metastatic colorectal cancer (8) and in patients treated with hepatic artery infusion therapy (9). With IHS using the monoclonal antibody TS-106, a significant relationship was found between TS levels and prognosis in rectal cancer (10).

Preclinical studies have shown a correlation between fluoropyrimidine sensitivity and TP levels. TP catalyzes the reversible phosphorolysis of thymidine and its analogues to their respective bases and 2-deoxyribose-1-phosphate (11). After transfection with TP cDNA, human epidermal KB carcinoma (12), MCF-7 breast carcinoma (13), and PC-9 lung adenocarcinoma (14) cells were more sensitive to the 5FU prodrug 5'-deoxy-5-fluorouridine (Doxifluridine) compared with untransfected cell lines. A bystander effect was also observed in cocultured untransfected PC-9 cells. Induction of TP by IFN- α

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³ The abbreviations used are: 5FU, 5-fluorouracil; LV, leucovorin; TS, thymidylate synthase; FdUMP, 5-fluoro-2'-deoxyuridine-5'-monophosphate; CH₂-THF, 5,10-methylenetetrahydrofolate; IHS, immunohistochemical staining; TP, thymidine phosphorylase; MVD, microvessel density; VEGF, vascular endothelial growth factor; DFS, disease-free survival; OS, overall survival.

increased the sensitivity to 5FU in HT29 human colon carcinoma cells in the presence of deoxyribose donors (15) because deoxyribose-1-phosphate is the rate-limiting cosubstrate in this activation pathway (16). These *in vitro* studies suggest a role for TP in the activation of certain fluoropyrimidines.

TP was also identified as an angiogenic factor and identical to platelet-derived endothelial cell growth factor (17–19). TP activity is indispensable for its angiogenic action *in vivo* (19) and stimulates chemotaxis of endothelial cells *in vitro* (20, 21). Although neovascularization is a necessity for tumor growth and metastasis (22), studies on the prognostic role of MVD counts in colorectal cancer are inconclusive. MVD of 178 colon tumor samples (23) was higher in adenoma *versus* carcinoma but was not prognostic. MVD was, however, prognostic for hematogenous spread in 133 patients with colorectal cancer (24). In colon cancer, MVD correlated with TP immunostaining, but TP was inversely correlated with VEGF (25). High TP immunostaining also correlated with more extensive angiogenesis and poor clinical outcome of patients with colorectal cancer (26). These data indicate that TP levels and TP-associated neovascularization may play a role in the prognosis of cancer. In addition, patients with a low expression of both TS and TP mRNA in the colon tumor were the best responders to 5FU-LV therapy (27).

In the present study, we evaluated TS and TP levels in primary tumor and normal mucosa samples from patients with colorectal cancer and compared enzyme activity with protein TS/TP expression and with that of p53, Ki67, VEGF, and MVD measured by IHS. We evaluated the relation of TP and TS to outcome of chemotherapy and markers of tumors progression, such as p53 and proliferation (Ki67), and angiogenic parameters such as VEGF and MVD.

PATIENTS AND METHODS

Patients

During the period November 1994 to August 1996, primary tumor biopsy specimens were collected from patients who underwent surgery because of colorectal cancer: 26 patients in the Onze Lieve Vrouwe Gasthuis hospital and 6 patients in the University Hospital Vrije Universiteit. None of the patients received preoperative chemotherapy or radiotherapy. All samples were histologically proven colon or rectal cancer. After removal, the tumor was immediately placed on ice, and a representative sample of tumor tissue was snap frozen as soon as possible and stored at -80°C until enzyme activity analysis. All patients gave informed consent. Patient characteristics are given in Table 1. The mean follow-up time was 32 months, with a range of 1–41 months and a median of 33 months. The intention of this study was to collect the material of patients with Dukes' stage B and C who were candidate for adjuvant chemotherapy; therefore, the preoperatively expected Dukes' stage was the only criterion to select patients from whom the tumor material was collected. Different adjuvant treatment protocols were running: in the Onze Lieve Vrouwe Gasthuis hospital, patients for whom adjuvant therapy was indicated were entered in the Netherlands Adjuvant Colorectal Cancer Project (28) in which surgery alone was compared with surgery followed by adjuvant 5FU and levamisole. In the University Hospital Vrije Universiteit, patients were entered in the adjuvant active specific immunization

Table 1 Patients and tumor characteristics

Characteristic	Patients	
	n	%
Total patients	32	100
Sex		
Male	13	41
Female	19	59
Age at operation (years)		
Median	74	
Range	51–91	
Follow-up (months)		
Median	33	
Range	1–41	
Tumor type		
Rectal cancer	6	19
Colon cancer	26	81
Localization in colon		
Right side	10	38
Left side	16	62
Tumor size (cm)		
Mean	5	
Range	2–12	
<5	17	53
≥ 5	15	47
Dukes' stage		
A	1	3
B1 + B2	13	41
C1 + C2	15	47
D	3	9
Differentiation		
Moderate	23	72
Poor	9	28
Vaso-invasion		
Yes	9	28
No	23	72
Chemotherapy: 5FU-LV	7	22
Radiotherapy	2	6

trial (29). Not all patients with Dukes' stage C colon cancer were given adjuvant treatment because this is not standard for stage C colon cancer (28). When the Dukes' stage was confirmed, some patients appeared to have Dukes' stage A or D. All patients were in a good performance status (0–1), according to WHO criteria.

Materials

[6- ^3H]-FdUMP (MT-692; specific activity, 19 Ci/mmol) was from Moravex Biochemicals Inc. (Brea, CA). [5- ^3H]-dUMP (TRK-287; specific activity, 19 Ci/mmol) was from Amersham International (Buckinghamshire, United Kingdom). DL-Tetrahydrofolic acid (Sigma Chemical Co, St. Louis, MO) was converted to $\text{CH}_2\text{-THF}$ by the addition of formaldehyde (30). The liquid scintillation fluid Ultima Gold was obtained from Packard (Tilburg, the Netherlands). We used the Bio-Rad protein assay for protein determination (31). Antibodies were obtained from different sources as described below. Unless otherwise specified, all other chemicals were of analytical grade and commercially available.

Tissue Preparation

Frozen tissues were pulverized using a microdismembrator as described previously (32). The frozen powder was weighed

and suspended in ice-cold assay buffer at a concentration of 1 g of tissue in 3 ml of assay buffer: a Tris-HCl buffer [200 mM Tris-HCl, 20 mM β -mercaptoethanol, 100 mM NaF, 15 mM CMP (pH 7.4)] for the TS enzyme assays and a Tris-EDTA buffer [50 mM Tris, 1 mM EDTA (pH 7.4)] for the TP enzyme assay. The suspensions were centrifuged for 10 min at $3200 \times g$ at 4°C and subsequently for 10 min at $20,000 \times g$ at 4°C. The supernatants were used for the enzyme assays.

TS Assays

FdUMP-binding Assay. The binding assay, with [6-³H]-FdUMP as a ligand, for determining the number of free FdUMP-binding sites of TS was carried out as described previously (32). Briefly, the reaction mixture contained 20 μ l of enzyme suspension from supernatants (150–400 μ g protein/assay), 10 μ l of 6.4 mM CH₂-THF, 3 μ l of 570 nM [6-³H]-FdUMP (final specific activity, 19 Ci/mmol) and 17 μ l of the 200 mM Tris-HCl assay buffer (pH 7.4). The reaction was started by addition of the enzyme, was incubated at 37°C for 1 h, and was stopped by addition of 100 μ l of 10% activated charcoal (to remove free FdUMP). After centrifugation, the radioactivity was estimated by liquid scintillation counting of 50 μ l of supernatant.

TS Catalytic Assay. This assay determines the catalytic activity of TS by means of tritiated water released during the TS-catalyzed conversion of [5-³H]-dUMP to dTMP (16). The TS catalytic activity was measured at a saturating substrate concentration (specific activity, 90 Ci/mol; final concentration, 10 μ M dUMP) and at the approximate K_m of TS (specific activity, 820 Ci/mol; final concentration, 1 μ M dUMP). Briefly, the assay consisted of 25 μ l of enzyme suspension (200–500 μ g protein/assay), 5 μ l of 6.4 mM CH₂-THF, and 10 μ l of Tris-HCl assay buffer or 10 μ l of 0.05 μ M FdUMP. The reaction was started by adding the enzyme and incubating for 60 min at 37°C and stopped by adding 50 μ l of ice-cold 35% trichloroacetic acid and 250 μ l of 10% neutral activated charcoal. After centrifugation, 150 μ l of the supernatant were collected and counted by liquid scintillation.

TP Activity

This assay was based on the enzyme determination as reported previously (33). Briefly, enzyme activity was measured using thymidine as a substrate and by calculation of the conversion of thymidine to thymine. The reaction mixture, consisting of 10 μ l of 2 mM thymidine, 10 μ l of 800 mM K₂HPO₄, 130 μ l of 50 mM Tris-1 mM EDTA buffer (pH 7.4), and 50 μ l of enzyme suspension in Tris-EDTA (400–1200 μ g protein/assay), was incubated for 15 and 30 min at 37°C; the reaction was terminated by the addition of 50 μ l of 40% trichloroacetic acid. After cooling on ice for 20 min, the samples were centrifuged for 5 min at $21,000 \times g$ at 4°C. The supernatant was neutralized with 400 μ l of a mixture of triethylamine and 1,1,2-trichlorotrifluoroethane (1:4, v/v), mixed, and centrifuged for 5 min at $21,000 \times g$ at 4°C. Separation was achieved using high-pressure liquid chromatographic analysis with a reversed-phase μ Bondapak C₁₈ column (3.9 \times 300 mm; particle size, 10 μ m; Waters, Milford, MA), detection at 254 nm and 280 nm, and isocratic elution with Pic B7, (Waters) containing heptane sulfonic acid in 15% methanol at 1.0 ml/min. Peaks were quanti-

fied, using a data acquisition system, by comparison with calibration samples of thymine. Enzyme activity was calculated by the conversion of thymidine to thymine.

IHS

IHS was performed for TS, TP, Ki67, p53, CD31, and VEGF on the paraffin-embedded tissue material from the patients described above. Deparaffinization of the 4- μ m thick sections was followed by rehydration. Thereafter, the sections were subjected to antigen retrieval by microwave thermocycling two times for 5 min each in 0.01 M citrate buffer (pH 6.0) for the TS, TP, Ki67, p53, and VEGF staining. The following primary antibodies were used: a polyclonal TS antibody (dilution, 1:100; a gift Dr. G. W. Aherne, Sutton, United Kingdom; Ref. 34); a recombinant TP antibody, P-GF.44C (dilution, 1:5; a gift from Dr. A. L. Harris, Oxford, United Kingdom; Ref. 35); clone Do-7 for p53 staining (dilution, 1:100; Ref. 36), a polyclonal Ki67 antibody (dilution, 1:100; Ref. 37), and clone JC/70A (dilution, 1:40) for CD31 staining (38), all three from Dakopatts (Glostrup, Denmark); and recombinant human VEGF (dilution, 1:50; R&D Systems, Minneapolis, MN) for VEGF staining (39). For detection of the primary antibody complexes, the avidin-biotin immunoperoxidase method (dilution, 1:100; Dakopatts) for TS, TP, Ki67, and p53, whereas for VEGF and CD31 the streptavidin-biotin complex (Vectastain, dilution, 1:200; Vector Laboratories, Inc., Burlingame, CA;) was used. All biotinylated secondary antibodies were purchased from Dakopatts. Staining was developed by using the 3-amino-9-ethylcarbazole substrate kit (Dakopatts) for TS, TP, Ki67, and p53, or 0.05% 3,3'-diaminobenzidine tetrahydrochloride dihydrate (Sigma) with 0.02% hydrogen peroxide for VEGF and CD31. For the CD31 staining, an additional amplification step was added after the streptavidin-biotin complex was incubated. This step consisted of an incubation with biotinylated tyramide (Department of Pathology, University Hospital Vrije Universiteit, Amsterdam, the Netherlands) at a dilution of 1:1000 for 10 min, followed by a second streptavidin-biotin complex step. PBS was used for the washing procedures. All slides were counterstained with hematoxylin and mounted with either Glycergel (for TS, TP, p53, and Ki67; Dakopatts) or Depex mounting medium (for CD31 and VEGF; Gurr, BDH Laboratories Supplies, Poole, United Kingdom). Quality/positive control samples were used as follows: for TS, a colonic cancer tissue sample with a known high intensity; for TP, a normal liver tissue section because Kupffer cells have a high expression of TP (35); for p53, a colonic tumor sample with high-intensity staining; for Ki67, a tonsil section and the lymph nodes in the specimens as external or internal positive controls, respectively; for VEGF, a section of a primary breast carcinoma with known high VEGF expression as a positive control, and a section of an epididymis as a negative control. Positive blood vessels were used as an internal positive control. Negative controls were performed for each sample by omitting the primary antibody and using a substrate of the same IgG subclass as the primary antibody (for TS, TP, p53, Ki67, and VEGF) or by using a PBS-1% BSA solution instead of the primary antibody (for VEGF and CD31). Samples were scored for each antibody separately by two independent investigators, blinded to clinical outcome.

Table 2 TS and TP levels and results from immunostaining in patients with colorectal tumors
Enzyme assays and IHS were performed under optimal assay conditions as described in "Patients and Methods."

A. Enzyme activity	Range	Mean (SD)	Median
TS catalytic activity (pmol/h/mg protein)			
1 μM dUMP	4.6–118.5	56 (45)	46
10 μM dUMP	18.9–1001.8	391 (276)	192
FdUMP binding (fmol/mg protein)	4.8–69	26 (12)	23
TP activity (nmol/h/mg protein)			
Tumor tissue	42.9–642	201 (126)	192
Normal mucosa	30.9–246.2	87 (40)	85
B. IHS		<i>n</i>	%
TS	1+	18	56
	2+	14	44
TP	Positive	14	44
	Negative	18	56
p53	Positive	18	56
	Negative	14	44
Ki67 labeling index	$\leq 40\%$ positive cells	15	47
	$> 40\%$ positive cells	17	53
VEGF	Positive	22	69
	Negative	10	31
CD31	MVD hotspot		
	$\leq 40/\text{mm}^2$	11	34
	$> 40/\text{mm}^2$	21	66
	MVD global		
	$\leq 16/\text{mm}^2$	17	53
	$> 16/\text{mm}^2$	15	47

Evaluation of Immunostaining and Microvessel Count

TS Immunostaining. The intensity of the staining was scored as high (2+) or low (1+) as determined in the tumor cells within the area with highest intensity in the specimen.

TP Immunostaining. The samples were scored for positive tumor cells and infiltrating stromal cells such as fibroblasts, macrophages, and plasma cells.

P53 Immunostaining. The samples were scored as positive when $>10\%$ of the tumor cells were positive.

Ki67 Immunostaining. Percentages of positive cells were scored in 500 cells, and a cutoff value of 40% positive tumor cells in the area with most positive cells was chosen (the median).

VEGF Immunostaining. The slides were scored as positive when $>10\%$ of the tumor cells were positive; otherwise, they were scored as negative.

Microvessel Counts. Microvessel counts as stained with the CD31 antigen were scored as described by Honkoop *et al.* (40). Microvessels were first counted in 15 fields of vision systematically spread over the whole tumor area at $\times 400$ magnification using a $\times 40$ objective; the outcome was referred to as global microvessel count. In the tumor area with the highest microvessel count (hotspot), four consecutive fields were counted at the same magnification; the outcome was referred to as hotspot microvessel count (41). All microvessel counts were converted to vessels/ mm^2 .

Statistical Methods

For a correlation analysis between the various parameters, the χ^2 test or Spearman's rank correlation test were used for the dichotomized values; when continuous values were available,

linear regression analysis was used. Analysis of survival was performed according to Kaplan-Meier. Differences between survival curves were analyzed with log rank analysis. As end points, development of metastasis or cancer-related death were taken for the disease-free and overall survival, respectively. Follow-up data were calculated from the date of operation to April 1, 1998, the date of last follow-up. For comparison between two parameters, $P < 0.05$ was considered significant. When more parameters are tested, a correction for multiple testing may be included to adapt the level of significance ($\alpha = 0.05$); this consists of the number of tests ($n = 8$) used for statistical analysis on the same samples. Thus for multiple testing, $\alpha = 0.05/8 = <0.01$ may be used as the level of significance. All statistical procedures were carried out with SPSS 7.0 (SPSS Inc., Chicago IL).

RESULTS

TS and TP Levels. The results for TS and TP are summarized in Table 2. For both TS assays, a large variation was observed; the FdUMP-binding levels varied 14-fold, whereas for the TS catalytic activity, the variation was 2–53-fold for the assays at 1 and 10 μM dUMP, respectively. The ratio of activity between 1 and 10 μM was ~ 7 .

The variation for TP levels was larger in the tumor (15-fold) compared with the adjacent normal tissue (8-fold). TP activity was 2.3-fold higher in the tumor tissue compared with the normal tissue ($P < 0.001$, Mann-Whitney *U* test; Fig. 1). TS activity as measured by the FdUMP-binding or the TS catalytic assay and TP activity were not correlated with differentiation grade, Dukes' stage, or angio-invasion.

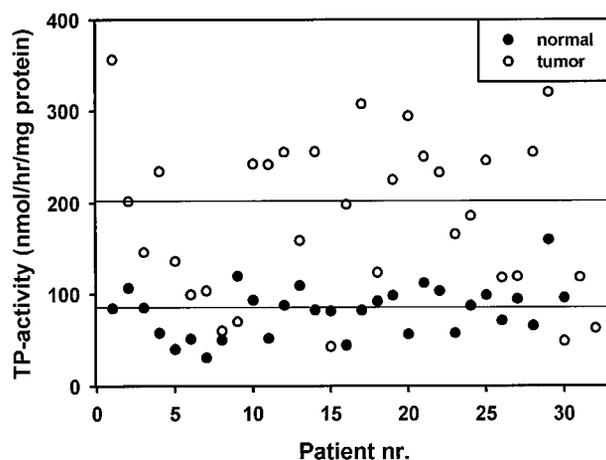


Fig. 1 TP levels in colon tumor and adjacent normal tissue. Each number on the X axis represents a patient and is plotted against the values for TP in normal tissue (●) or in tumor tissue (○) from the same patient. The lines represent the mean levels in normal tissue (lower line; 87 ± 40 nmol/h/mg protein) and tumor (upper line; 201 ± 126 nmol/h/mg protein). The levels were significantly different ($P < 0.01$) when tested with the Mann-Whitney U test for paired samples.

IHS. A summary of the various immunostainings is given in Table 2, and examples of the results of the immunostaining are shown in Fig. 2.

TS Immunoreactivity. Positive TS staining was seen in the cytoplasm of the tumor cells, with a granular staining pattern (41). Most tumor samples showed a diffuse staining, although heterogeneity was also observed. Normal colonic tissue showed a fairly weak positive staining but to a much lower extent than the tumor cells. In this group, the majority of the patients showed a low intensity (1+) for the TS staining in the tumor. The relationship between TS staining and TS levels as measured by the FdUMP-binding assay was positive but did not reach significance ($r = 0.53$; $P = 0.07$). No association was found between TS staining and tumor histology, Dukes' stage, or angio-invasion.

TP Immunoreactivity. Only in a relatively small proportion of patient samples (44%) did the tumor epithelial cells stain positive for TP with a cytoplasmic or perinuclear staining pattern. Evidence was obtained that infiltrating cells (mostly fibroblasts and plasma cells) showed TP immunoreactivity. Several patterns could be distinguished: no tumor cells positive, stromal cells positive; tumor and stromal cells positive; tumor cells positive, stromal cells much more positive. High-intensity staining of the infiltrating cells (mostly fibroblast and plasma cells) was observed in 41% of the samples, moderate intensity staining was observed in 53%, and in 6% of the samples, no staining of the infiltrating cells was seen. In most samples with positive infiltrating cells, the cells at the infiltrating edge showed the highest intensity. In patients with the highest intensity of TP-positive infiltrating cells, a separate section of normal colonic mucosa was stained, but the TP intensity of these infiltrating cells was far less than that of the stromal cells around the tumor. The large heterogeneity of TP staining was the most pronounced finding.

Ki67 and Proliferative Activity. Tumor cells showed a nuclear staining pattern when positive for Ki67. No positive staining was seen in the cytoplasm of the tumor cells. The number of positive cells ranged from 0 to 90%. With a cutoff point of 40%, we could distinguish slightly (0–40% positive cells) from highly (>40% positive cells) proliferative samples, and this value was used to analyze the samples in a dichotomized way. The number of the patients with a low index of positive cells was comparable to that with a high proliferation rate. No correlation was found between Ki67 positivity and clinical variables.

p53 Accumulation. Nuclear p53 accumulation in >10% of the tumor cells was measured in 18 of the 32 (56%) of the tumor samples. In the other patient samples, p53 was not detected by immunostaining, indicating the absence of mutated p53. No cytoplasmic immunoreactivity was encountered. p53 positivity was unrelated to histology of the tumor, Dukes' stage, or angio-invasion.

VEGF Immunostaining. Cytoplasmic staining was observed in the tumor cells. In addition, blood vessels were positive for VEGF. Some tumor samples showed a gradient of increased intensity toward the infiltrating edge of the tumor cells. Cytoplasmic staining was not homogeneous for all samples. In some samples, higher intensity was observed at the brush zone at the luminal border.

CD31 Immunoreactivity and Microvessel Count. MVD was evaluable in all samples. Background staining was absent in most tumor sections; cross-reactivity with plasma cells was very infrequent and could be distinguished easily on morphological grounds. Microvessel counts in the tumor sections ranged from 18 to 120/mm² in the hotspot counts, with an average of 40 ± 18 (mean \pm SD) and a median of 37. For the global counts, the values ranged from 12 to 25 vessels/mm², with 16 ± 4 on average (mean \pm SD) and a median count of 17 vessels/mm². For neither the global counts nor the hotspot microvessel counts was an association between clinical parameters such as differentiation, Dukes' stage, or angio-invasion and MVD found.

Correlations among TS and TP Enzyme Activity and TS, TP, Ki67, p53, and VEGF Immunoreactivity and Microvessel Counts. When dichotomized values were analyzed for correlation, the values were divided in less than or equal to the mean and higher than the mean, using the mean values given in Table 3. Significant correlations are depicted in Table 3.

A positive significant correlation was seen between the TP level in tumor tissue and the FdUMP-binding assay (Fig. 3 and Table 3). In addition, IHS of TS and TP was correlated. However, no significant correlation was seen when tumor TP levels were related with the TS catalytic activity at 10 or 1 μ M. TP levels in the tumor were not significantly correlated with that in the normal tissue. TP activity positively correlated with the protein expression as measured by IHS. No significant correlations were found between either the TS or TP enzyme levels in relation to the immunoreactivity for Ki67 index, p53, and VEGF or to microvessel count. However, TP and Ki67 immunoreactivity correlated significantly with that of VEGF.

Analysis of Survival. Differentiation grade of the tumor, Dukes' classification, or angio-invasion were not correlated with DFS or with OS in these patients. FdUMP-binding levels in

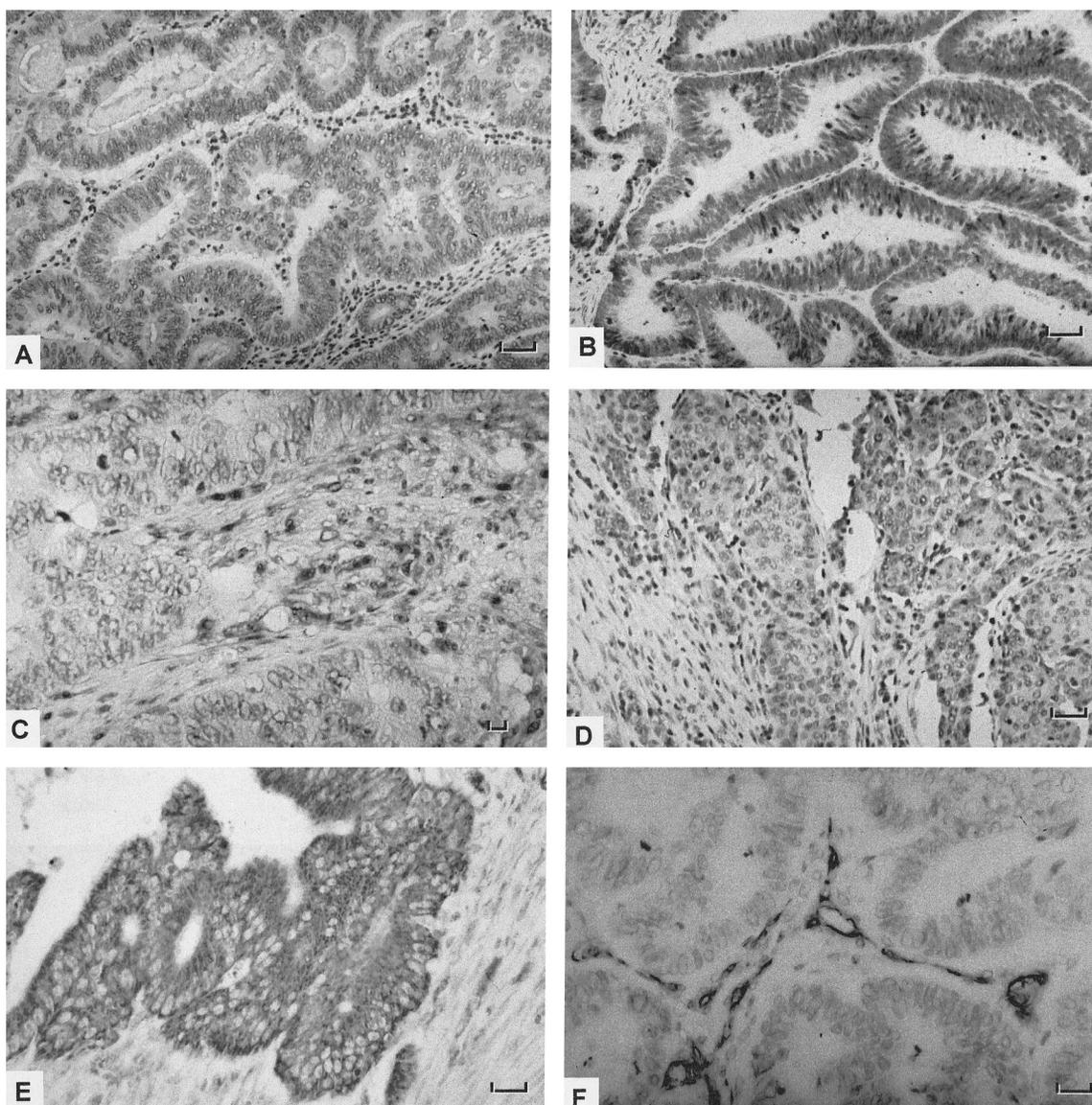


Fig. 2 IHS of colorectal cancer samples. A and B, TS polyclonal antibody with highly positive (A) and negative (B) TS intensity at $\times 165$ magnification. C and D, TP monoclonal antibody with TP-negative tumor cells and TP-positive infiltrating cells at $\times 330$ magnification (C), and TP-positive tumor at $\times 165$ magnification (D). E, sample with VEGF-positive tumor cells at $\times 165$ magnification; F, sample with CD31-positive microvessels at $\times 165$ magnification. Color pictures are available on request.

all patients were significantly correlated to OS (log rank, 3.98; $P = 0.046$) when dichotomized at the mean FdUMP level, but no correlation was observed with DFS (log rank, 2.14; $P = 0.14$). When the analysis was limited to Dukes' B and C patients, this correlation was still present (Fig. 4A). Thus, patients with a FdUMP-binding level above the mean had a worse prognosis compared with patients with a low FdUMP binding. For the TS catalytic level, no such correlation was encountered with either OS or DFS. When TP enzyme levels of the all-patients (Dukes' A–D) group were related to patient survival, a significant correlation was found between DFS and TP levels (log rank, 4.54; $P = 0.03$) but not for OS. In addition, the results of the TP immunostaining were correlated to DFS (log rank,

4.09; $P = 0.04$). Moreover, when limited to Dukes' B and C patients, a significant correlation was found (Fig. 4B). The proliferative activity as measured with Ki67 immunostaining was nearly significantly correlated with the OS (log rank, 3.5; $P = 0.06$) and DFS (log rank, 3.99; $P = 0.046$); for Dukes' B and C patients, the relationship with DFS was significant (log rank, 4.98; $P = 0.03$).

A higher percentage of Dukes' stage C and D was seen among the poor survivors. In this group, several parameters were also increased compared with the good survivors, such as high tumor TP levels (64%), high FdUMP-binding levels (73%), positive p53 (64%), and high MVD as measured by global counts (73%) or hotspot counts (55%). Only seven patients in

Table 3 Correlation of TS, TP, and other parameters in various assays

	TS IHS	TP IHS	TP activity	MVD hotspot	Ki67
FdUMP binding	$r^a = 0.53$ $P = 0.07$		$r = 0.53$ $P = 0.002$		
TP activity	$r = 0.53$ $P = 0.002$	$r = 0.38$ $P = 0.03$			
TP IHS	$r = 0.37$ $P = 0.04$				
Ki67	$r = 0.39$ $P = 0.03$			$r = 0.37$ $P = 0.04$	
VEGF	$r = 0.46$ $P = 0.008$	$r = 0.46$ $P = 0.01$		$r = 0.54$ $P = 0.04$	$r = 0.54$ $P = 0.001$

^a r , the correlation coefficient as calculated by Spearman's rank correlation analysis.

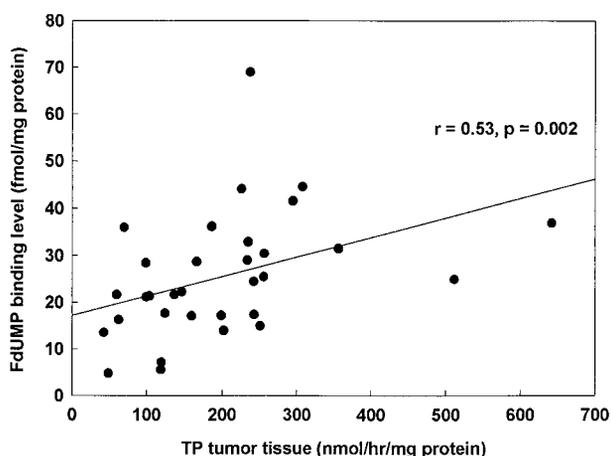


Fig. 3 Correlation between TP levels in tumor tissue and the FdUMP-binding level in patients with colorectal cancer.

this group received chemotherapy: one patient with Dukes' stage B2, three with Dukes' C2, and three with Dukes' D. These patients had a poor survival, with OS of 13 months and DFS of 11 months; only one patient was still alive. In this group of seven patients, all tumor parameters were distributed similarly compared with the total study group of 32 patients. However, for the TP protein expression, the number of samples with TP-positive expression was higher compared with the total study group, with six of seven samples being highly positive for TP.

DISCUSSION

This study evaluated various types of parameters in patients with colorectal cancer in relation to the clinical outcome: TS and TP enzyme levels, proliferation, and tumor vascularization. A relationship was observed between parameters from different classes such as between TP activity and FdUMP binding; between IHS of TS, TP, and VEGF; and between Ki67 and VEGF. TS and TP levels correlated with several survival parameters. A major result of this study seems the importance of both proliferation and angiogenic parameters for the prognosis of colorectal cancer.

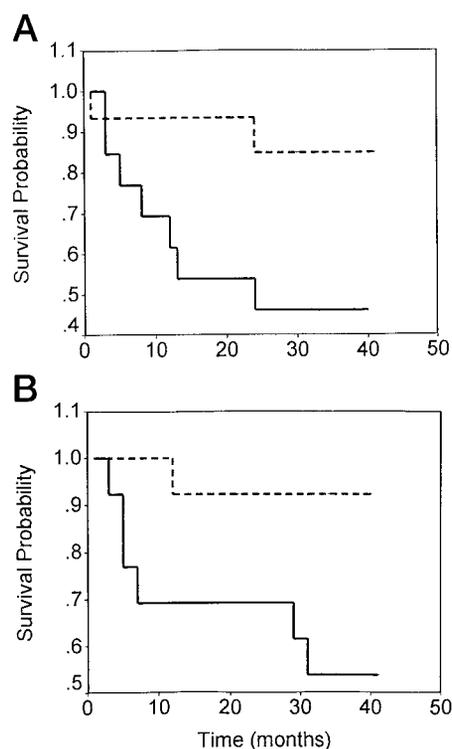


Fig. 4 A, OS curves of Dukes' B and C patients with high (>26 fmol/mg protein) FdUMP-binding levels (—) in tumor tissue and low FdUMP-binding levels (---; log rank, 4.75; $P = 0.03$). B, DFS curves of Dukes' B and C patients with high (>201 nmol/mg protein/hr) TP levels in the tumor tissue (—) and low TP levels (---; log rank, 4.08; $P = 0.044$).

A high TS level seems to be one of the important prognostic parameters; poor response to 5FU-based chemotherapy in patients with colorectal cancer was associated with a high TS level (7). High TS levels, as determined by immunostaining in 294 patients with rectal cancer (10) or biochemically and by immunostaining (42) in 58 patients with colorectal cancer, were correlated with poor survival. In the latter study, only a small proportion of the patients received 5FU-LV treatment, precluding a reliable relationship between TS levels and response to 5FU-LV. However, this study (42) and our study indicate a relationship between low FdUMP-binding levels and both longer survival and the natural behavior of the disease. TS levels may not only be predictive for 5FU response but also may be of prognostic value for survival in nontreated patients, possibly because low TS levels correspond to less aggressive tumor types or to a low growth potential. The latter may also be an explanation for the relationship between TP activity and TS protein expression and FdUMP binding. Infiltrating cells or paracrine effects may also play a role.

A high TP level seems to be an important parameter indicative of a shorter DFS. In addition, patients with renal cell carcinoma with high TP levels, evaluated enzymatically or by immunoblotting/immunostaining (43), had a 4-fold higher risk for death than patients with low or no TP expression. Patients with node-negative non-small cell lung cancer and positive TP

immunostaining had a poor prognosis (44). TP expression was elevated in node-positive primary breast carcinoma as measured with immunohistochemistry ($n = 240$) and by RNase protection assay ($n = 64$; Ref. 45). In addition, in 163 patients with colorectal cancer, high TP levels were associated with poor clinical outcome (26). It seems evident that TP levels are related with OS or DFS, although the correlation is different among the various tumor types. Different regulatory pathways of TP activation may play a role, possibly leading to development of liver metastases, explaining the shorter DFS in the present study.

Taken together, the data seem to indicate a dual role for TP in predicting the outcome of 5FU-based therapy. TP may play a role in 5FU chemotherapy because patients with invasive breast cancer ($n = 328$) with TP-positive tumors have a significant survival benefit compared with TP-negative tumors when treated with cyclofosfamide-methotrexate-5FU (46). Danenberg *et al.* (27) observed that high TP mRNA levels (reverse transcription-PCR) were predictive for a poor response to 5FU-LV therapy of colorectal cancer. TP may have a role in drug activation either by playing a crucial role in pyrimidine metabolism or as an angiogenic factor; possibly the different pathways are dependent on the tumor type. In our patient group, six of the seven patients receiving 5FU-LV had high TP levels and a poor survival. This suggests that high TP levels are a poor prognostic factor in patients with colorectal cancer treated with 5FU-LV.

A point of major interest is the role of infiltrating cells in TP expression. In the present study, 44% of the tumor cells were TP positive as measured by IHS, similar to IHS results for 163 colorectal cancer patients (26). Fox *et al.* (46) observed 51% TP-positive breast cancers with nuclear and/or cytoplasmic staining, which was occasionally focal but often up-regulated at the infiltrating tumor edge. In non-small cell lung cancer patients, 25% of the tumors were positive with invariably stained alveolar macrophages and weak immunoreactivity of the stromal fibroblasts (44). TP expression was also high in infiltrating cells (83%, macrophages and lymphocytes) of 96 colorectal cancer patients (25), whereas only 5% of the tumor epithelium was positive for TP. Similarly in patients with gastric cancer, only 10% of the tumors were positive for TP, but 54% of the infiltrating cells (predominantly macrophages) were TP positive. TP expression was high in infiltrating cells of intestinal-type gastric cancer (66%) but not in diffuse-type gastric cancer (40%; Ref. 47). In our study, 41% of the samples showed TP-positive infiltrating cells, but no positive stromal cells were found in separate normal tissue samples. TP expression in polyploid or nonpolyploid carcinoma was significantly higher than in premalignant adenomas (48), whereas polyploid adenomas had a higher TP expression than nonpolyploid growth adenomas. Thus, the degree of atypia and TP expression in adenomas were related (48). In particular, because infiltrating cells in the tumor were highly positive, this suggests a specific role of the infiltrating cells in the tumor area in producing angiogenic factors such as TP, although TP in tumor cells might also affect expression of angiogenic factors in tumor cells.

Mutant p53 has been reported to regulate VEGF-activating pathways (49), whereas after loss of wild-type p53, a decrease in angiogenesis inhibitors such as thrombospondin-1 was observed (50). We did not observe a relationship with p53, which may be related to the discrepancy between p53 immunostaining and

mutation analysis. Immunostaining for p53 is positive for up-regulated p53, but it may not reflect a mutated status of the protein.

This study showed a significant correlation between the proliferation marker Ki67 and the angiogenic factor VEGF. The mechanism is unclear. Vermeulen *et al.* (51) observed the highest percentage of endothelial proliferating cells (Ki67 positive) together with MVD hotspots (CD31 positive) at the luminal margins of the tumors, with a significant correlation between tumor proliferation and tumor vascularity. TP may play an essential role in this process by specifically activating cells from inside the tumor and from the extracellular compartment. Cytokines such as tumor necrosis factor- α , interleukin-1 α , or interleukin-1 γ may play an essential role in this process because they can increase TP activity (52).

It can be concluded that several prognostic factors for chemotherapy, such as TS and TP levels and proliferation, are related to angiogenic factors. Each factor showed a correlation with survival of patients with colorectal cancer. Tumor and stromal cells seem to have some interactions with respect to proliferation and angiogenesis. The prognosis of colorectal cancer may be improved by the use of combinations of drug-target levels and angiogenesis factors.

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Prognostic Role of Thymidylate Synthase, Thymidine Phosphorylase/ Platelet-derived Endothelial Cell Growth Factor, and Proliferation Markers in Colorectal Cancer

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