Immunohistochemically Detected Thymidylate Synthase in Colorectal Cancer: An Independent Prognostic Factor of Survival[1]

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ABSTRACT

Intratumoral thymidylate synthase (TS) expression and M_r 53,000 phosphoprotein (p53) overexpression were studied immunohistochemically in sections from stored paraffin-embedded primary colorectal cancers in 70 patients who had undergone surgery during the years 1987–1990. These cancers were classified according to Dukes' stage A–D, using monoclonal antibodies TS 106 and DO-7. In patients with Dukes' stage A–C tumors, univariate analyses showed that there was a significant correlation ($P = 0.048$) between disease-free survival and TS expression and between TS expression and time to death with colorectal cancer ($P = 0.038$). In patients with Dukes' stage A–D tumors, overall survival was correlated to TS expression ($P = 0.015$), Dukes' stage ($P < 0.001$), and level of tumor differentiation ($P = 0.044$) but not to p53 overexpression. Patients with low intratumoral TS expression survived significantly longer than patients with high expression. Cox multivariate analysis showed that Dukes' stage ($P < 0.001$) and TS expression ($P = 0.043$) could independently serve as prognostic factors for time to death with colorectal cancer in patients with Dukes' stage A–D tumors.

INTRODUCTION

In primary colorectal cancer, the degree of extension of neoplasia through the bowel wall is the most reliable prognostic factor (1, 2). Despite this, there is an urgent need to define additional factors that may help with both prognosis and effective monitoring of response to therapy. In the present investigation, we have analyzed the prognostic value of two markers of potential interest in primary colorectal cancer, *i.e.*, the expression of p53 protein and TS.[3] *p53*, a tumor suppressor gene, is a cell-cycle check point regulator (3) that is frequently altered in cancer cells (4–6). There are several reports suggesting that p53 overexpression may be associated with reduced survival in primary colorectal cancer; however, conflicting results have been published (7). The intratumoral concentration of TS, an enzyme that catalyzes the reductive methylation of dUMP to dTMP (8), has been suggested as a prognostic factor of survival in colorectal cancer (9–12) and of the response of tumor cells to 5-FU therapy (9, 10, 13, 14).

Both the M_r 53,000 phosphoprotein of the altered tumor suppressor gene *p53* (15) and TS (16) can now be semiquantitatively assessed immunohistochemically in paraffin-embedded tumor sections. The primary aim of this study was to determine the prognostic significance of intratumoral TS expression in colorectal cancer. The secondary aim was to evaluate the possible prognostic values of p53 expression and differentiation of the tumor and their possible relationships with TS expression.

PATIENTS AND METHODS

Patients. The patient population consisted of 70 patients who underwent surgery for primary colorectal cancer at the Uppsala Akademiska Hospital during the years 1987–1990. The median age was 69 years with a range between 43 and 87 years. Twenty-two had carcinoma of the rectum, and 48 had carcinoma of the colon (16 in right colon, 9 in transverse colon or left flexure, 3 in descending colon, and 20 in sigmoid colon). Details concerning gender, Dukes' stage, site of tumor, and tumor differentiation are presented in Tables 1 and 2. The median postoperative follow-up time was 94.5 months with a range between 56 and 120 months. Five of the rectal cancer patients received preoperative radiation, one received palliative radiation, and two received palliative 5-FU-leucovorin chemotherapy. Seven of the colon cancer patients received palliative 5-FU-leucovorin chemotherapy, and three patients received palliative radiation therapy. Palliative therapy was given to cure pain caused by recurrence and distant metastasis and was given late in life. All patients were included in a previously presented

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[3] The abbreviations used are: TS, thymidylate synthase; 5-FU, 5-fluorouracil.

Table 1 Univariate analysis on categorical background factors with respect to disease-free survival and time to death with colorectal cancer in 61 patients with colorectal cancer Dukes' stages A–C

	No. of patients	No. of deaths or recurrence	χ^2	*P*	No. of deaths in colorectal cancer	χ^2	*P*
Gender			0.7	0.418		1.9	0.166
Female	37	18/37 (49%)			9/37 (24%)		
Male	24	14/24 (58%)			8/24 (33%)		
Dukes' stage			4.3	0.116		5.7	0.056
A	12	4/12 (33%)			1/12 (8%)		
B	30	15/30 (50%)			8/30 (27%)		
C	19	13/19 (68%)			8/19 (42%)		
Site of tumor			0.0	0.972		0.7	0.787
Colon	44	24/44 (55%)			12/44 (27%)		
Rectum	17	8/17 (47%)			5/17 (29%)		
Tumour differentiation			1.3	0.523		4.4	0.108
High	14	6/14 (43%)			2/14 (14%)		
Moderate	38	21/38 (55%)			11/38 (29%)		
Poor	9	5/9 (56%)			4/9 (44%)		
TS expression			3.9	0.048		4.3	0.038
Low	17	8/17 (47%)			2/17 (12%)		
High	44	24/44 (55%)			15/44 (34%)		
p53 expression			0.1	0.721		1.3	0.263
Negative	24	12/24 (50%)			4/24 (17%)		
Positive	35	18/35 (51%)			13/35 (37%)		

Table 2 Univariate analysis on categorical background factors with respect to time to death with colorectal cancer in 70 patients with colorectal cancer Dukes' stages A–D

	No. of patients	No. of deaths in colorectal cancer	χ^2	*P*
Gender			0.6	0.435
Female	43	15/43 (35%)		
Male	27	11/27 (41%)		
Dukes' stage			33.3	<0.001
A	12	1/12 (8%)		
B	30	8/30 (27%)		
C	19	8/19 (42%)		
D	9	9/9 (100%)		
Site of tumor			0.7	0.403
Colon	49	17/49 (35%)		
Rectum	21	9/21 (43%)		
Tumour differentiation			10.0	0.007
High	15	3/15 (20%)		
Moderate	41	14/41 (34%)		
Poor	14	9/14 (64%)		
TS expression			7.9	0.005
Low	17	2/17 (12%)		
High	53	24/53 (45%)		
p53 expression			0.39	0.532
Negative	28	8/28 (29%)		
Positive	40	17/40 (43%)		

study in which immunohistochemically detected p53 was evaluated (17).

Histopathology. Paraffin-embedded, formalin-fixed specimens of the resected tumors were analyzed immunohistochemically. A mean number of 2.5 sections/person (1–4), taken from different parts of a primary tumor, were analyzed for TS expression. One section from the same tumor was used for p53 expression evaluation.

Immunohistochemistry. The monoclonal antibody TS 106 (16) was used to detect TS, and the monoclonal antibody DO-7 (Ref. 15; Dakopatts, Glostrup, Denmark) was used to detect p53. The standard avidin-biotin-peroxidase complex (Vectastain Elite; Ref. 18) technique was used. Tumor sections (4 μm thick) were deparaffinized in xylene and then hydrated in decreasing concentrations of ethanol. Sections to be stained for p53 expression were heated in a microwave oven for 5 min twice for antigen retrieval. To quench the endogenous peroxidase activity, the sections were incubated in a solution of 3% hydrogen peroxide for 15 min. For reduction of nonspecific background staining, sections to be stained for TS expression were exposed to 20% horse serum for 30 min. The sections were then incubated with TS 106 at room temperature for 90 min or with DO-7 at 4°C overnight. Sections were then rinsed in PBS, incubated with biotinylated horse antimouse secondary antibodies, rinsed in PBS, and then incubated with avidin-biotin-peroxidase complexes. After rinsing, immunostaining was developed by immersion in 0.05% 3.3′-diaminobenzidine tetrahydrochloride solution for TS detection and ethyl-carbazole for p53 detection and then counterstained with a modified Harris-hematoxylin.

Scoring of Immunohistochemical Staining. Each time a set of tumor samples was stained, we included positive and negative reference slices from tumors that were classified previously as low or high intensity staining for TS expression and negative or positive for p53 expression. The intensity of TS staining of the tumor cells was arbitrarily graded from 0 to 3. As described previously, 0–1 was defined as low intensity and 2–3 as high intensity staining (Ref. 9; Fig. 1). The highest staining intensity (low/high) found in a tumor was used for classification of the tumor. The agreement of TS intensity reached by two independent observers was >90%. Where there was disagreement, intensity was determined by consensus. Tumors were scored as positive for p53 overexpression if >15% of the tumor cells showed immunoreactivity. p53 scoring was made by one observer (17).

Heterogeneity in TS expression was defined as different TS

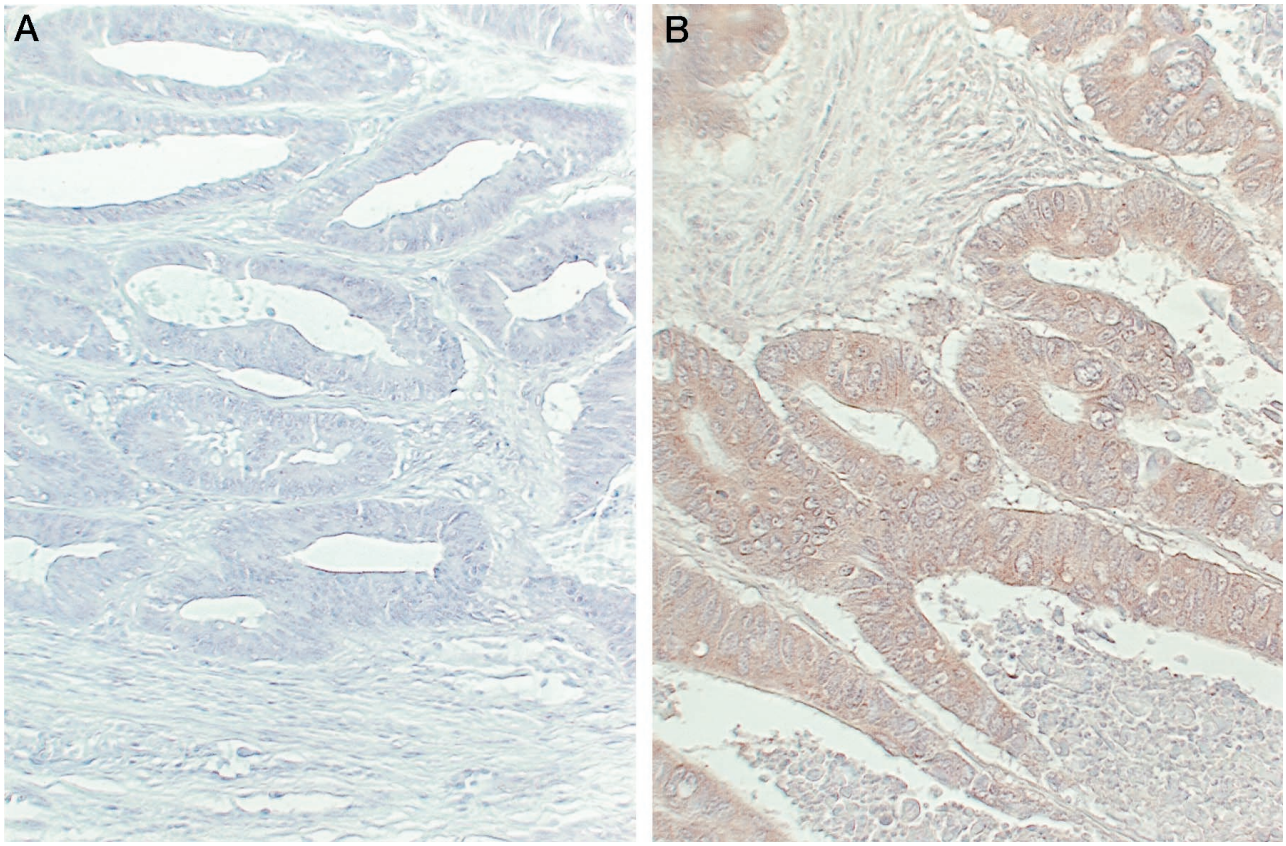

Fig. 1 Immunohistochemical detection of TS on paraffin sections of colorectal carcinoma. *A*, low TS staining; *B*, high TS staining.

expression (high/low) in two sections taken from different parts of a primary tumor.

Statistical Analysis. The Gehan Wilcoxon univariate test (19) was used to examine the possible relationships between disease-free survival, overall survival, gender, Dukes' stage, site of tumor, tumor differentiation, TS expression, and p53 expression. When disease-free survival was used as an end point, an event included recurrence of disease, death from cancer, and death from noncancer causes. Multivariate analysis was performed using Cox regression (20). Survival curves were constructed using the Kaplan-Meier method (21).

RESULTS

During the follow-up period, 26 (37%) of the 70 patients died of colorectal cancer. Fourteen patients (20%) who were judged as tumor free died of diseases unrelated to colorectal cancer. Only 2 of those 14 patients were autopsied.

TS expression was high in 53 tumors (76%) and low in 17 tumors (24%). There was homogeneous TS expression in 54 of 68 (79%) tumors, and heterogeneous expression in 14 of 68 (21%).

TS and Survival in Dukes' Stages A–C. When calculating only Dukes' stages A–C tumors, TS showed a significant correlation ($P = 0.048$) to disease-free survival (Table 1). There was a significant correlation between TS expression and time to death with colorectal cancer ($P = 0.038$) but not to overall survival. Eighty-eight % of patients (Dukes' A–C) whose tumors expressed low TS were alive 5 years after primary surgery, compared with 66% with high TS expression ($P = 0.043$). Eighty-eight % of patients (Dukes' A–C) with low TS expression were disease free at 5 years compared with 55% of those with high levels ($P = 0.010$).

Dukes' stage did not correlate significantly with disease-free and overall survival but showed a borderline correlation ($P = 0.056$; Table 1) to time to death with colorectal cancer.

TS and Survival in Dukes' Stages A–D. When calculating patients with Dukes' stages A–D tumors, univariate analyses showed that overall survival was significantly correlated to TS expression ($P = 0.015$), Dukes' stage ($P < 0.001$), and tumor differentiation ($P = 0.044$). Time to death with colorectal cancer correlated to TS expression ($P = 0.005$), Dukes' stage ($P < 0.001$), and tumor differentiation ($P = 0.007$; Table 2).

Disease-free survival (Dukes' stages A–C), disease-specific survival (time to death with colorectal cancer as detected during clinical follow-up or at autopsy; Dukes' stages A–C), and overall survival (Dukes' stages A–D) of patients with low and high TS intensities are illustrated graphically in Fig. 2. Patients whose tumors had high TS expression showed poorer clinical outcome.

A Cox multivariate analysis showed that among the variables tested, only two of them could independently serve as prognostic factors for death with cancer in Dukes' stages

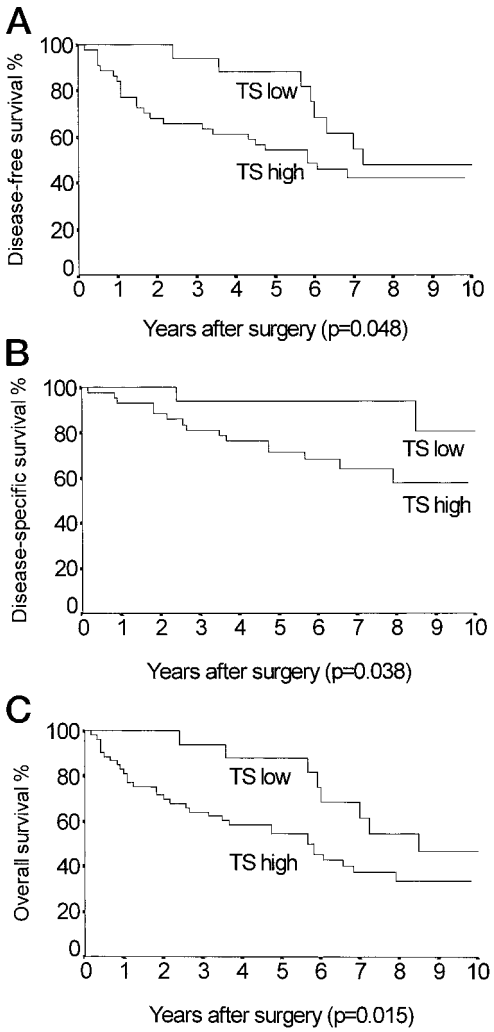

Fig. 2 Kaplan-Meier life table analysis of disease-free survival in 61 patients with colorectal cancer Dukes' stages A–C (*A*), disease-specific survival (time to death with colorectal cancer; *B*), and overall survival in 70 patients with colorectal cancer Dukes' stages A–D according to their TS expression (low or high) in primary tumor (*C*). Patients with low intratumoral TS expression had a significantly better outcome than those with high TS expression.

A–D. These were Dukes' stage ($P < 0.001$) and TS expression ($P = 0.043$; Table 3). Only Dukes' stage was an independent factor for overall survival ($P < 0.001$; $P = 0.08$ for TS expression).

DISCUSSION

There is an increasing need for defining new factors that may be used to forecast prognosis in colorectal cancer and its response to therapy. Measurements of TS in colorectal cancer have been shown to be of interest because of the possible role of this enzyme in the clinical response to 5-FU-based therapy (9, 10, 13, 14).

We have found previously that the present immunohistochemical method to detect TS is suitable for archival paraffin-embedded colorectal cancer tissue. There is a correlation between immunohistochemically detected TS expression in primary colorectal cancer and intratumoral TS enzyme activity (22), TS protein concentration (16), and TS mRNA (13).

Table 3 Cox multivariate regression analysis with respect to death with colorectal cancer.

Relative hazards (RH) and 95% confidence intervals (CI) in 70 patients with primary colorectal cancer Dukes' stages A–D are presented.

	RH	CI	χ^2	*P*
Dukes' stage, overall			31.2	<0.001
Dukes' stage A *versus* B	3.4	0.4–27.5	1.4	0.244
Dukes' stage A *versus* C	8.3	1.02–68.1	3.9	0.048
Dukes' stage A *versus* D	108.6	11.3–1041.1	16.5	<0.001
TS expression, low *versus* high	4.8	1.05–22.4	4.1	0.043

The results of the present investigation demonstrate that there is a correlation between TS expression in primary colorectal cancer Dukes' stages A–D and time to death with colorectal cancer. Patients whose intratumoral TS expression was high had a poorer clinical course. TS expression was found to add prognostic information independent to that provided by Dukes' stage. No other factor has yet emerged as consistently adding prognostic information (2). Johnston *et al.* (9) and Lenz *et al.* (11) reported a correlation between immunohistochemically detected TS expression and disease-free survival and overall survival in patients with rectal and colon cancer. We have found a correlation between TS and disease-free survival and between TS and time to death with colorectal cancer. We could not show a correlation between TS and overall survival in Dukes' stages A–C, as reported by Johnston *et al.* (9); however, this may be attributable to the limited numbers of patients in our study.

In this study, TS expression did not correlate with stage of disease, p53 expression, tumor differentiation, or site of tumor. In a previous study, however, a correlation was observed between TS expression and Dukes' stage in 48 primary colorectal tumors (22). A larger number of tumors may be required to determine this relationship. Johnston *et al.* (9) have reported a correlation between TS expression and Dukes' stage in 294 patients with rectal cancer.

The results from the present study indicate that there is no correlation between p53 over expression and survival rate in primary colorectal cancer, which is in agreement with the results of some investigators but in disagreement with others (7). It has been suggested that *p53* gene sequence analyses, which are able to detect mutations that do not necessarily influence p53 protein levels, may have a higher prognostic value in colorectal cancer.

Our lack of correlation between the expression of TS and p53 is in disagreement with recent reported studies (10, 11) in which an association between TS and p53 was found in a selected group of 46 patients with stage II colon cancer and in a group of 36 patients with disseminated colorectal cancer.

TS is the target for the new drug termed raltitrexed (Tomudex; Refs. 23 and 24) as well as for 5-FU. The latter is still the most important drug in the treatment of colorectal cancer. Advances in adjuvant therapy of colorectal cancer require the precise determination of the group of patients that may benefit from it.

There are several reports indicating that immunohistochemically detected TS in colorectal cancer may be a useful predictor of survival (11, 12). However, Findlay *et al.* (25) have reported a lack of correlation between immunohistochemically detected TS in primary tumor and disease-free survival, overall survival, and response to chemotherapy. This may be attributable to the fact that the patient population of Findlay *et al.* 25) was selected for local relapse or distant metastasis, and moreover because TS expression in distant metastatic lesions may be different from that of the primary tumor. Our sample of patients was unselected.

Because TS is the target for 5-FU, studies are now in progress to determine whether there is any relationship between TS contents and clinical outcome in 900 randomized colorectal cancer patients who are included in a Scandinavian prospective randomized trial aimed at determining the clinical value of adjuvant 5-FU-based chemotherapy.

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