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Prognostic value of thymidylate synthase expression in colorectal cancer

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Aim. The aim of the study was to define the prognostic value of thymidylate synthase (TS) expression in colorectal cancer (CRC), and its role as a predictor of the chemotherapeutic benefit in patients treated with fluorouracil (5-FU).
Material and methods. TS expression was immunohistochemically assessed on primary tumor archival specimens from 120 patients with CRC.
Results. For the entire study population the univariate analysis revealed that TNM stage, surgical margin, vessel invasion, and TS expression were significant prognostic factors. In a subgroup treated with 5-FU (n=98) overall survival correlated with tumor location, TNM stage, surgical margin, and TS expression. Multivariate analysis demonstrated that tumor stage, TNM stage, and TS expression were independent prognostic factors. In the subgroup treated with 5-FU only tumor location, tumor stage, and TNM stage correlated with survival.
Conclusions. TS expression in primary tumors was a significant, independent prognostic factor in colorectal cancer. The evaluation of its predictive role requires further investigations.

Znaczenie syntetazy tymidylowej w rokowaniu u chorych z rakiem jelita grubego

Cel. Celem pracy było określenie wartości prognostycznej i predykcyjnej ekspresji syntetazy tymidylowej (TS) w raku jelita grubego.
Materiał i metody. Ekspresję TS oznaczano immunohistochemicznie w bloczkach parafinowych z guzów pierwotnych u 120 chorych z rakiem jelita grubego.
Wyniki. Analiza jednocechowa wykazała, że w grupie 120 chorych na całkowite przeżycie wpływały istotnie: zaawansowanie procesu nowotworowego, radykalność mikroskopowa zabiegu, obecność zatorów z komórek nowotworowych w naczyniach oraz charakter ekspresji TS. W grupie 98 chorych leczonych chemicznie fluorouracylem na przeżycie wpływały istotnie: lokalizacja guza, zaawansowanie procesu, radykalność mikroskopowa zabiegu oraz charakter ekspresji TS. Analiza wielocechowa wykazała, że głębokość nacieku raka, stopień zaawansowania wg TNM oraz charakter ekspresji TS są istotnymi niezależnymi czynnikami rokowniczymi. W grupie chorych leczonych fluorouracylem jedynie lokalizacja nowotworu, głębokość nacieku i zaawansowanie procesu nowotworowego są niezależnymi istotnymi czynnikami rokowniczymi.
Wnioski. Charakter ekspresji TS jest istotnym, niezależnym czynnikiem rokowniczym w raku jelita grubego. Określenie roli predykcyjnej TS wymaga dalszych badań.

Key words: colorectal cancer, thymidylate synthase
Słowa kluczowe: rak jelita grubego, syntetaza tymidylowa

Introduction

Colorectal cancer is one of the most common cancer sites diagnosed throughout the world, accounting for approx. 10% of all incident cases [1]. About 50-60% of patients are cured by surgery and adjuvant chemotherapy. The remaining group will, sooner or later, need palliative chemotherapy [2, 3]. The most common chemotherapeutic agent in the treatment of colorectal cancer is still fluorouracil. The efficiency of this treatment is similar to the treatment with new antifolate agents, like ralitrexed (Tomudex) or capecitabine (Xeloda), depends on the activity of thymidylate synthase (TS) [2, 4]. This enzyme is responsible for the provision of thymidylate required for DNA synthesis and repair [2, 5].

Multiple studies have shown that patients with high levels of intratumoral TS expression in their cancers have a significantly worse clinical outcome and worse response to fluoropyrimidine-containing regimens as compared to patients with cancers of a relatively low intracellular level. However, the results of these studies are not consistent. Currently, only the stage of disease is widely accepted as a prognostic factor [6, 7].

In the present investigation, we have retrospectively examined the prognostic value of thymidylate synthase

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expression in primary colorectal cancer, and the role of this expression as a predictor of the chemotherapeutic benefit in patients treated with fluorouracil.

Material and methods

Tumor specimens examined for TS expression were obtained from 120 patients with colorectal cancer, who were diagnosed between 1992 to 2001. Their characteristics according to sex, age, localization of tumor, histology and grading, TNM and Dukes classification, microscopic radical margin of surgery and vessel invasion are listed in Table I. Mean duration of follow-up was 28.6 months (range: 0.3 – 114 months; median: 28 months), from August 1992 to March 2003.

Among 120 patients after radical surgery of the primary tumor 98 (81.7%) were treated with 5-FU-based chemotherapy regimens (45 were treated with chemo-radiotherapy in 1999-2001; 36 with adjuvant chemotherapy and 17 as palliation in disseminated disease). 61 patients received leucovorin-modulated 5-fluorouracil, 25 patients 5-FU plus leucovorin and levamisol, and 12 patients were treated with 5-FU and levamisol.

Table I. Characteristics of the group of 120 patients with colorectal cancer treated between the years 1992 and 2001

Characteristic	No. of patients	% of patients
Sex		
Male	74	62%
Female	46	38%
Age (median age 59,4 years)		
≤ 60 years	64	53.3%
> 60 years	56	46.7%
Localization		
colon	42	35%
rectum	78	65%
Histology		
Adenocarcinoma	117	97.5%
other	3	2.5%
Tumor differentiation		
G1	63	52.5%
G2	56	46.7%
G3	1	0.8%
Tumor size pT		
pT2	12	10%
pT3	72	60%
pT4	36	30%
Lymph node metastases pN		
pN0	64	53.3%
pN1	39	32.5%
pN2	17	14.2%
Distant metastases		
M0	101	84.2%
M1	19	15.8%
Dukes' stage / TNM stage		
A / I°	4	3.3%
B / II°	52	43.3%
C / III°	45	37.5%
D / IV°	19	15.8%
Radical resection margin		
yes	73	60.8%
no	47	39.2%
Vessel invasion		
no	96	80%
yes	24	20%

12 patients with Dukes A and B cancer were without any adjuvant treatment.

TS immunohistochemical analysis

Paraffin-embedded, formalin fixed specimens of the resected tumors were analyzed immunohistochemically [2-4, 6]. Two sections (4 μm thick), taken from different parts of the primary tumor were analyzed for TS expression.

Tissue specimens were first deparaffinized in 100% xylene and rehydrated through graded alcohol solutions. Endogenous peroxidase activity was inhibited by incubating the slides in 3% hydrogen peroxidase for 10 minutes, followed by a 5-minute rinse in dH_2O. The slides were heated in a microwave oven in EDTA, pH 8.0, and stored in TBS (Tris-buffered saline) and proteinase K. The tissues were incubated for 60 minutes with TS-106 (mouse monoclonal antibody, Chemicon International, Temecula, CA 92590) at room temperature at a 1:50 dilution. The slides were washed with TBS and then incubated in DAKO EnVision+™, Peroxidase Mouse complex (DAKO Corporation, USA) for 30 minutes. The slides were again washed a few times with TBS and then incubated with AEC Substrate-chromogen system (DAKO AEC) for 10 minutes. Following another wash cycle, the tissues were counter-stained with hematoxilin (DAKO). The slides were rinsed and closed in glicerogel.

Tissue evaluation

Each slide was examined for intensity and staining pattern by two investigators, who were blinded to all clinical information. Discrepant scores (about 5% of cases) were resolved by consensus.

Intensity scores ranged from 0 to 3 (0=no staining, 1=trace staining, 2=definite staining of light to moderate intensity, and 3=bright intensity). The staining pattern was either F (focal) or D (diffuse). Samples with 50% or fewer malignant cells stained at the assigned intensity level were considered F (0=less than 25% cells stained, 1=25-50% cells stained). Samples with more than 50% of stained cells were scored as D (2=50-75% cells and 3=more than 75% cells stained). Examples of the TS staining pattern are presented in Figures 1-4.

Statistical analysis

The outcome variable was overall survival (OS) with death from any cause as an end point. Follow-up time was measured from the date of surgery.

The analysis was performed for the entire study population (n=120) and in the subgroup treated with fluorouracil-based chemotherapy at any time (n=98). Owing to the relatively small

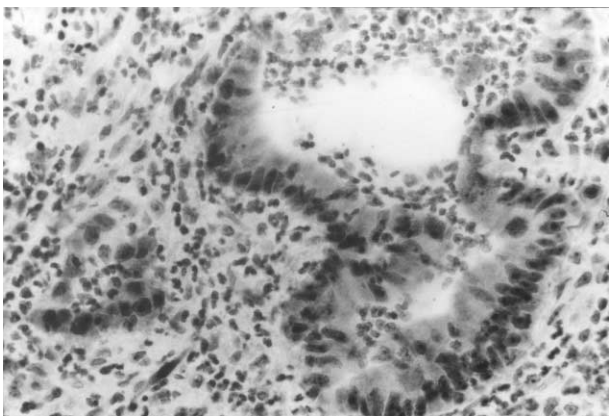


Figure 1. The focal TS staining pattern (TS=0)

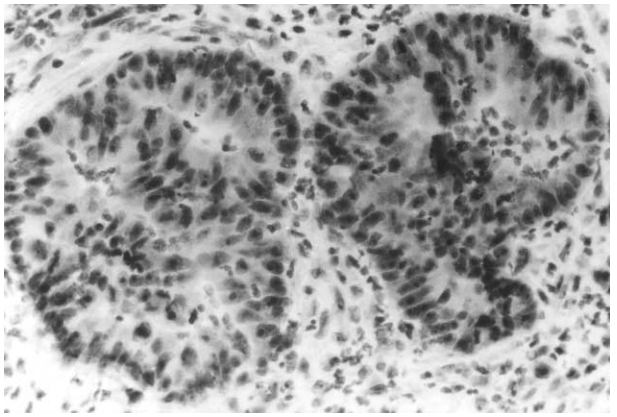


Figure 2. The focal TS staining pattern (TS=1)

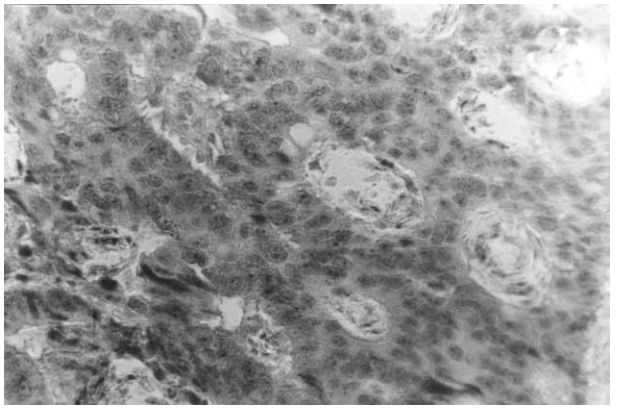


Figure 3. The diffuse TS staining pattern (TS=2)

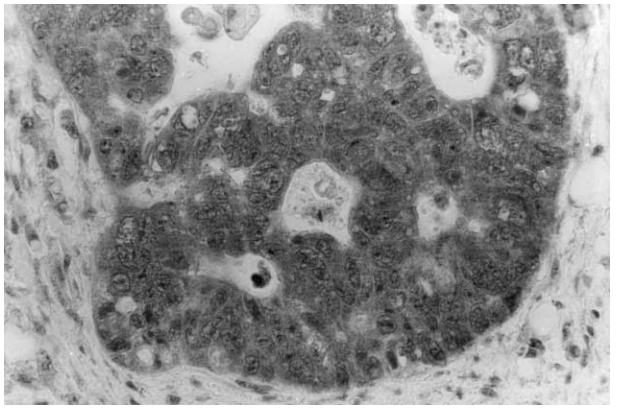


Figure 4. The diffuse TS staining pattern (TS=3)

numbers of patients treated with chemo-radiotherapy, adjuvant and palliative chemotherapy we did not perform the analysis in these particular subgroups. Survival distributions were estimated using the Kaplan-Meier method.

In the univariate analysis the possible relationships between overall survival and age, sex, localization, histological grading, TNM and Dukes' stage, the microscopic radical margin of surgery, vessel invasion, TS staining intensity and pattern were examined by log-rank test. Multivariate analysis was performed using Cox's regression model. All *P* values below 0.05 were considered statistically significant.

Results

In the univariate analysis for the entire study population (n=120) overall survival was significantly linked to Dukes' and TNM stage, the microscopic radical margin of surgery, the vessel invasion and the staining pattern of TS ($p<0.05$ – Table II). Multivariate analysis showed that only the stage and the staining pattern of TS remained as the significant prognostic factors (Table III).

In the subgroup treated with 5-FU-based therapy at any time (n=98) the univariate analysis revealed the prognostic importance of localization, TNM and Dukes' stage, the microscopic radical margin of surgery and the staining pattern of TS (Table II). By multivariate analysis localization, TNM and Dukes' stage remained as important prognostic factors of survival ($p<0.05$). The staining pattern of TS had no significant prognostic value in this group ($p=0.0853$, Table III).

Discussion

In 1994 Johnston et al. [8] were the first to suggest that the level of TS expression in primary colorectal cancer may be of prognostic importance. During the next 10 years at least 28 studies concerning this problem were published [2-29]. 16 of them confirmed the observations of Johnston et al. [2, 4-6, 8, 9, 11, 12, 14-16, 18, 21, 23, 26, 28].

The results of our study confirm that the level of TS expression in primary colorectal cancer correlates with survival. Patients with a low level of TS have a better overall survival compared with patients with high TS levels. This observation was restricted only to the TS diffuse staining pattern (Figure 5 and 6). Similarly as Gonen et al. [18] and Allegra et al. [2] we were unable to identify the prognostic role of TS staining intensity. Multivariate analysis has demonstrated that the ability of the TS diffuse staining pattern to predict survival was independent of the tumor size and the stage of disease.

The results of our investigation seem to be resemble most those presented by Allegra et al. [2]. They assessed the TS staining pattern immunohistochemically and divided patients into two groups with more or less than 50 % of cancer cells with TS expression (diffuse vs. focal TS staining pattern). Similarly, TS expression was detected in the primary tumors with the use of TS 106 monoclonal antibody.

Gonen and others have estimated TS expression in frozen samples of liver metastases with the use of a polyclonal antibody [18]. Due to those technical differences the results confirmed the prognostic significance of thymidylate synthase in colorectal cancer.

The Edler et al. study revealed the prognostic value of TS in the group of 862 patients with Dukes' B and C colorectal cancer [6]. The material and methods were similar as in our study. Similarly, most of patients had high TS level that correlated with overall survival. As in our investigation Edler was not able to prove the prognostic role of TS expression in the subgroup of patients

Table II. Univariate overall survival analysis for examined parameters

Parameter	All patients (n=120)			Chemotherapy group (n=98)		
	n	OS (%)	*P*	n	OS (%)	*P*
Sex						
Male	74	63	.5160	58	64	.9041
Female	46	62		40	59	
Age						
≤ 60 years	64	64	.2027	56	61	.6604
> 60 years	56	60		42	62	
Localization						
colon	42	52	.1484	39	47.5	.0166*
rectum	78	69		59	72	
Tumor differentiation						
G1	63	73	.2357	49	73.5	.2277
G2 + G3	57	52		49	53	
Tumor size pT						
pT2	12	75		7	71.5	
pT3	72	71		61	70	
pT4	36	40		30	42	
pT2+pT3 v. pT4		71 v 40	.0005*		70 v 42	.0029*
Lymph node metastases pN						
pN0	64	77	.0054*	46	75	.0338*
pN1 + pN2	56	45		52	49	
Distant metastases						
M0	101	74	.0000*	81	75	.0000*
M1	19	0		17	0	
Dukes' stage / TNM stage						
A+B / I° + II°	56	85	.00007*	38	86.5	.0003*
C+D / III° + IV°	64	2		60	45	
Radical surgical margin						
yes	73	75	.0031*	61	74	.0066*
no	47	40		37	32.5	
Vessel invasion						
no	96	70	.0018*	78	67	.0584
yes	24	36		20	43	
TS intensity						
Low (0, 1)	43	74	.1896	35	75	.0908
High (2, 3)	77	55		63	52	
TS pattern						
Focal (0, 1)	50	82	.0024*	40	85	.0013*
Diffuse (2, 3)	70	48		58	43.7	

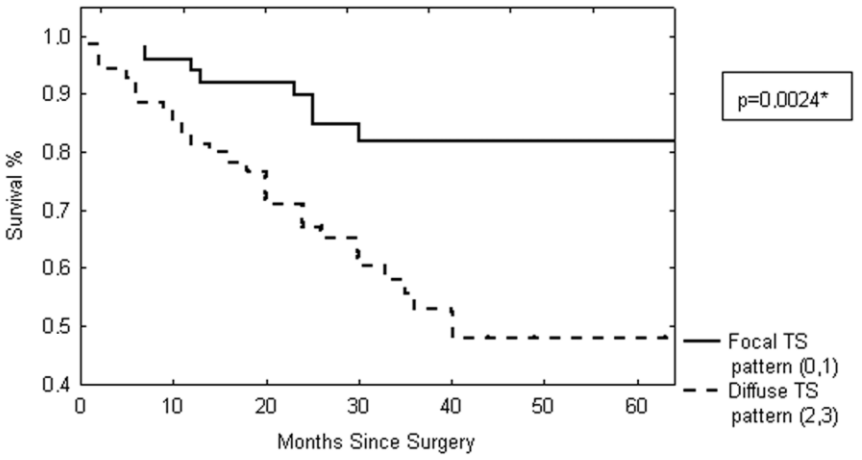
* significant difference ($P<.05$)

Table III. Cox's regression model multivariate analyses with respect to overall survival in the entire study population (n=120) and in the group treated with 5FU-based chemotherapy (n=98)

Parameter	All patients n=120			Chemotherapy group n=98		
	HR**	95% CI	*P*	HR**	95% CI	*P*
Localization						
colon				1.0		
rectum				1.91	1.12 – 2.70	.0452*
Tumor size pT						
pT2 + pT3	1.0			1.0		
pT4	1.82	1.43 – 2.22	.0028*	1.80	1.41 – 2.20	.0034*
TNM stage						
I° + II°	1.0			1.0		
III° + IV°	1.97	1.59 – 2.35	.0004*	6.52	5.30 – 7.74	.0026*
TS staining pattern						
F (focal; 0, 1)	1.0			1.0		
D (diffuse; 2, 3)	2.36	1.56 – 3.16	.0355*	2.44	1.42 – 3.46	.0853

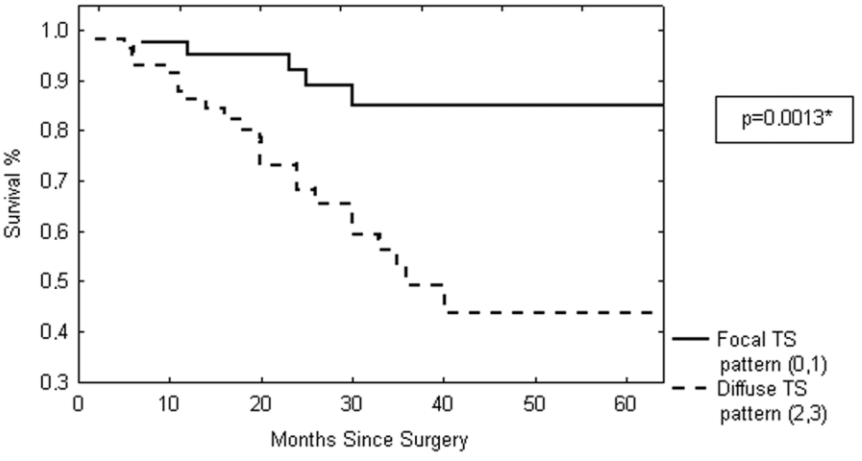
* significant difference ($P<.05$)

** HR, hazard ratio ($HR = e^{\beta}$, where e=2.71828); CI, confidence interval



* significant difference ($p<0.05$)

Figure 5. The 5-year overall survival curve for thymidylate synthase (TS) staining pattern (focal versus diffuse) for the study population (n=120)



* significant difference ($p<0.05$)

Figure 6. The 5-year overall survival curve for thymidylate synthase (TS) staining pattern (focal versus diffuse) for 98 patients treated with chemotherapy

treated with adjuvant 5-FU chemotherapy. The earlier observations of Edler and others in the group of colorectal cancer patients with Dukes' stage A-D revealed that 5-year overall survival of patients with high TS expression reached about 50% (in our study 48%) versus 85% in patients with low TS level (82% in our study) [15].

Several previous reports have suggested that patients whose primary tumors had low TS expression may be more sensitive to 5-FU-based chemotherapy [3, 4, 6, 9, 10, 12, 13, 18-20, 22, 24].

There are also studies indicating that patients with high TS levels may benefit from adjuvant 5-FU chemotherapy [6]. In this retrospective investigation we found that patients with diffuse TS staining pattern had the lesser but not significant benefit from the use of

5-FU chemotherapy compared with those patients whose tumors contained the focal TS staining pattern. However, the number of our patients in the chemotherapy group was relatively small.

Previous studies have demonstrated that in cases of disseminated disease the high TS mRNA level and the high TS protein expression assessed on the metastatic tumor indicated resistance to treatment based on 5-FU [11, 18, 20, 21]. Johnston et al. compared the TS expression in the primary and metastatic tumors in colorectal cancer patients and found a lack of correlation between TS levels [3]. Aschele et al. assessed TS expression immunohistochemically on the primary tumors and liver metastases obtained from 18 patients with colorectal cancer [10]. In 11 of 18 patients the TS expression was different in primary and metastatic tumor, and in 10 patients the TS level was lower in the metastasis than in the primary site. Those results were observed only in patients with metastatic disease treated with fluorouracil. In our investigation among 98 patients treated with 5-FU only 17 had metastatic colorectal cancer (all with liver metastases). TS expression was assessed on the primary tumors. Similarly as in the study of Aschele et al., where the high TS levels were observed in primary tumors

in 70 percent of patients, in our material the diffuse TS staining pattern was found in 13 of 17 patients treated with palliative chemotherapy (76.5%). The TS expression in the metastasis would be different than in the primary tumor, which could determine the response to 5-FU-based chemotherapy.

Gorlick et al. have demonstrated significantly higher TS expression in pulmonary metastases than in liver metastases in advanced colorectal cancer [19]. Cascinu et al., on the contrary, have observed lower TS expression in liver metastases than in other abdominal metastases in patients with advanced colon cancer [12]. In addition, the investigation performed by Findlay and others has revealed that the TS level in the primary tumor could not serve as the predictor of response to 5FU-based chemotherapy in case of the disease dissemination [17].

Summarizing, the results of the above studies suggest that TS expression assessed on the primary tumor may not correlate with that measured in the metastasis of the same patient. Similar observation can relate to patients with locally advanced colorectal cancer, where the TS expression in the primary tumor may differ from the TS level on the site of micrometastases. So estimation of the TS expression in the primary tumor would not predict the response of micrometastases to the adjuvant chemotherapy.

Thus the issue of the optimal selection of patients for adjuvant treatment is still vague.

The results of our study suggest that the diffuse TS staining pattern may help in the identification of high risk colorectal cancer patients who should receive more aggressive treatment. Such patients seem to be good candidates for chemotherapy regimens with irinotecan or oxaliplatin.

In conclusion, our study has confirmed the prognostic value of the TS staining pattern in colorectal cancer. Predictive role of TS expression assessed on the primary tumors still needs future investigations.

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