Prognostic and Predictive Value of Thymidylate Synthase Expression in Primary Colorectal Cancer

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Abstract. *Aim: To investigate thymidylate synthase (TS) expression in primary colorectal cancer (CRC) as a prognostic and predictive marker of benefit for adjuvant chemotherapy. Patients and Methods: TS expression was immuno0-histochemically (IHC) assessed on tumors from 1,389 patients with stage II and III CRC randomly assigned to either surgery alone or surgery plus 5-fluorouracil (5-FU)-based adjuvant chemotherapy. Results: In the subgroup treated with surgery alone (n=708), TS expression was prognostic using the classification of TS 0-1 versus 2-3 (p=0.045) as well as TS classified as 0-2 versus 3 (p=0.002). A high TS expression was associated with a shorter overall survival. Among patients with TS grade 3 (n=460), the subgroup treated with adjuvant chemotherapy had a significant longer OS (p=0.005). Conclusion: In this study TS, immunohistochemically assessed, is a prognostic factor in CRC patients treated with surgery alone. Patients with the highest level of TS expression (grade 3) had an improved clinical outcome following adjuvant 5-FU-based chemotherapy.*

Colorectal cancer (CRC) is a major health problem in the Western world (1). CRC diagnosed at an early stage is often treatable with surgery alone. The current challenge is to identify the category of patients who might benefit from 5-fluorouracil (5-FU)-based adjuvant chemotherapy. In stage II CRC approximately 75% of the patients are cured by surgery alone. Adjuvant chemotherapy would only cure an additional 1%-6% in this group of patients (2). However, patients with stage III CRC are known to have a survival benefit of up to 30% after adjuvant chemotherapy (3). The current staging and risk stratification methods in CRC are insufficient in predicting tumour aggressiveness as well as response to chemotherapy. Recent CRC research has therefore focused on finding useable predictive and prognostic factors at the molecular and genetic level. One promising prognostic marker is the expression of thymidylate synthase (TS), a rate-limiting enzyme involved in DNA synthesis and repair. TS is also the intracellular target enzyme for 5-FU where the main antitumoral effect is a competitive inhibition of TS.

Several studies have investigated the relationship between TS expression and survival in CRC. Most studies have reported that TS expression is a prognostic indicator, where patients with a high expression of TS in their tumors have a worse prognosis which is described in a meta-analysis by Popat *et al*. (4). The role of TS as a predictive marker in CRC is dependent on the type of disease to be treated. In patients with locally advanced CRC adjuvantly treated with 5-FU a high TS expression in the primary tumor seem to be predictive for a better chemotherapy response (5-11). However, the results are conflicting and the potential use of TS as a predictor of benefit from adjuvant 5-FU still remains unclear. In metastastic CRC the results are more convincing, since in most studies a low TS expression in the metastases has been associated with a better response to 5-FU (12-14).

This is a retrospective study examining 1389 CRC patients, included in Nordic trials randomly assigned to surgery alone or surgery plus adjuvant chemotherapy. The results from the analysis of 862 of the patients in this current study have been reported previously (15). The purpose of this study was to evaluate the value of TS expression in the primary tumor as a prognostic marker in patients with stage II and III CRC in a large group of patients with a longer follow-up time. We also investigated whether TS expression is a predictive factor in adjuvant therapy according to the dose of 5-FU.

Patients and Methods

We examined the primary tumors of 1389 patients with CRC stage II and III who were ≤75 years and underwent curative surgery between 1991 and 1997. The surgical specimens were derived from adjuvant Nordic trials including 2224 patients with CRC, of which 1389 patients from Sweden and Denmark were available for this

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Table I. *Patients' and tumor characteristics, Gehan-Wilcoxon univariate analyses, and Cox regression multivariate ananlyses with respect to DFS and OS.*

Characteristic	No. of patients (%)	Recurrences and Deaths no. (%)	*p*-Value (univariate analysis)	*p*-Value (multivariate analysis)	Deaths no. (%)	*p*-Value (univariate analysis)	*p*-Value (multivariate analysis)
Age (years)			0.005	0.001		0.001	0.0001
<65	635 (46%)	257 (40%)			248 (39%)		
>65	753 (54%)	377 (50%)			368 (49%)		
Gender			0.02	0.004		0.03	0.005
Male	782 (56%)	388 (50%)			378 (48%)		
Female	607 (44%)	246 (41%)			238 (39%)		
Tumor site			NS	NS		NS	NS
Colon	984 (71%)	444 (45%)			432 (44%)		
Rectum	405 (29%)	190 (47%)			184 (45%)		
Stage			<0.0001	<0.0001		<0.0001	<0.0001
II	678 (49%)	218 (32%)			208 (31%)		
III	711 (51%)	416 (59%)			408 (57%)		
Numbers of analyzed LN			0.01	0.02		0.003	0.007
0-11	896 (88%)	418 (47%)			407 (45%)		
≥12	123 (12%)	41 (33%)			37 (30%)		
Missing data	370						
Grade of differentiation			<0.0001	0.0006		<0.0001	<0.0001
Poor	274 (21%)	149 (54%)			147 (54%)		
Moderate	958 (73%)	419 (44%)			406 (42%)		
Well	87 (6%)	28 (32%)			27 (31%)		
Missing data	70						
Treatment			NS	NS		NS	NS
Surgery alone	708 (51%)	329 (46%)			322 (45%)		
Adjuvant CT	681 (49%)	305 (45%)			294 (43%)		
TS grade			NS			NS	
Low (0-1)	399 (29%)	177 (44%)			171 (43%)		
High (2-3)	990 (71%)	457 (46%)			445 (45%)		
TS grade			NS			NS	
Low (0-2)	929 (67%)	413 (44%)			397 (43%)		
High (3)	460 (33%)	221 (48%)			219 (48%)		

DFS, Disease-free survival; OS, overall survival; NS, not significant ($p<0.05$), TS, thymidylate synthase, LN, lymph nodes, CT, chemotherapy.

study (16). The patients included in this study cohort are representative of those participating in the Nordic trials. Parameters of outcome were obtained from seven different regional centres of epidemiological oncology in Sweden and Denmark. The median age was 65 years (range 29 to 75 years). The patients' demographics and tumor characteristics are listed in Table I. The patients were randomly assigned to either surgery alone or surgery plus adjuvant 5-FU-based chemotherapy according to the Moertel schedule with levamisole, the Mayo regimen with or without levamisole or the Nordic schedule with or without levamisole (16). The chemotherapy was initiated within 11 weeks after surgery and the follow-up time was 120 months.

The chemotherapy arm (n=681) was divided into patients who received ≥90% of the planned 5-FU dose (n=335) *versus* those who received <90% (n=288) of the planned 5-FU dose. We were unable to obtain the dose of 5-FU for 58 patients, thereby excluding them from the dose calculation.

Laboratory methods. The specimens derived from formalin-fixed, paraffin-embedded tumors in which two sections, each 4 μm-thick, were taken from different parts of the tumor. Immunohistochemical (IHC) analysis of the TS expression in the additional 527 (862+527=1389) specimens was performed with the same TS 106 monoclonal antibody as used in the previous study using the commercially available avidin-biotin-peroxidase complex technique (Vectastain Elite ABC kit). The tumor section slides were deparaffinized in xylene and rehydrated in ethanol. Thereafter, the slides were incubated in 3% hydrogen peroxide to inhibit the endogenous peroxidase activity. Antigen retrieval was carried out in citrate buffer (pH 6.0) for 20 minutes in a microwave oven followed by 20 minutes of cooling at room temperature. In order to reduce nonspecific background staining, the slides were blocked with horse serum followed by an overnight incubation at 4˚C with the TS 106 monoclonal antibody (17). The samples were then rinsed and incubated with biotinylated horse antimouse secondary antibodies and thereafter rinsed and incubated with avidin-biotin-peroxidase complexes. Visualization of immunostaining was achieved by immersion in 0.05% 3,3'-diaminobenzidine tetrahydrochloride followed by counterstaining with hematoxylin.

Evaluation of immunohistochemistry. TS staining intensity was defined by a visual grading scale 0-3 (grade 0=no staining, grade 1=weak, grade 2=moderate, grade 3=intense staining) according to the method developed by Johnston *et al*. (5). The intensity of TS expression has been classified into the following categories: TS grade 0-1 *vs*. TS grade 2-3 as well as TS grade 0-2 *vs*. TS grade 3 (18, 19). In the previous study as well as in the present study two reference slides were included each time a set of tumor samples was stained. The grade of expression of TS was based on the highest intensity found in the tumor, even if the high-staining area was small. Four of the authors performed the scoring blinded to clinical data (DE, MH, MK, KÖ). The level of agreement between the observers was ≥90%. Scoring discrepancies were resolved by consensus after reexamination.

Statistics. The Gehan-Wilcoxon univariate test was used to examine the relationships between survival and patients' demographics and tumor characteristics. Multivariate analyses were performed using Cox regression. The Kaplan-Meier method was used to construct the survival curves. Distribution differences between groups were compared with the χ^2 test and mean differences with Student's *t*-test.

Overall survival (OS) was defined as time from surgery to death and disease-free survival (DFS) was defined as time from surgery to the first event of local recurrence, presence of distant metastases or death of any reason.

Results

Patients. This study included 1389 patients with equally represented stage II and stage III CRC (Table I). The results from 862 of these patients have been presented in a previous report (15). During a median follow-up time of 75 months (range 1-120) 616 patients died, of whom 497 died (81%) of CRC. Nine hundred and eighty-four (71%) of the primary tumors were located in the colon and 405 (29%) in the rectum. Two hundred and fourteen (53%) of the rectal cancer patients were treated with preoperative radiation (5Gy ×5).

In multivariate analyses, OS was significantly linked to gender in which females had a better OS, age, where the younger patients had a longer OS, differentiation, with the high grade having an OS advantage, stage, with a longer OS in stage II compared to stage III, and finally removal of ≥12 lymph nodes which correlated with a better OS (Table I).

TS expression in the entire group. TS expression grade 0-1 was found in 399 (29%) and grade 2-3 was found in 990 (71%) of the primary tumors. Using the classification of TS expression 0-2 *vs*. 3, we found 929 (67%) primary tumors with TS grade 0-2 compared to 460 (33%) tumors with grade 3. The expression of TS grade was independent of tumor localization, as well as stage of disease (II or III). In rectal cancer, the TS expression did not differ between the patients who had received preoperative radiotherapy and those who had not.

TS expression was not a prognostic factor for OS or DFS in the entire study group regardless of the two different methods of TS classification.

Table II. *Clinical outcome in patients with high TS expression (grade 3) treated with adjuvant chemotherapy compared with surgery alone, p-values according to stage of disease and tumor site.*

Tumor site	All patients	Stage II	Stage III
Colon	n=337	n=161	n=176
Univariate analysis	p=0.05	NS	p=0.018
Multivariate analysis	p=0.007	NS	p=0.005
Rectum	n=123	n=54	n=69
Univariate analysis	p=0.03	p=0.004	NS
Multivariate analysis	NS	p=0.01	NS
Colon+rectum	n=460	n=215	n=245
Univariate analysis	p=0.005	NS	n=0.017
Multivariate analysis	p=0.0008	NS	p=0.008

NS, not significant (p<0.05).

TS expression in the group treated with surgery alone. In the group of patients treated with surgery alone (n=708) there was a significant longer OS and DFS for patients whose tumors expressed TS grade 0-1 compared to patients whose tumors expressed TS grade 2-3 (OS p=0.045, Figure 1A, multivariate analysis p=0.043). This was further enhanced using the TS classification grade 0-2 *vs*. grade 3 (OS p=0.002, Figure 1B, multivariate analysis 0.002).

TS Expression in the group treated with adjuvant chemotherapy. The expression of TS had no prognostic value in the group of 681 CRC patients treated with adjuvant chemotherapy independent of tumor localization and stage of disease.

There was no difference in OS among the patients who received chemotherapy ≥90% of the planned 5-FU dose, (n=335) *vs*. <90% (n=288) of the planned 5-FU dose.

Low TS expression (grade 0-1 or grade 0-2). In patients with TS grade 0-1 as well as TS grade 0-2 there was no difference in OS and DFS between the surgery group alone and the group of patients treated with surgery plus adjuvant chemotherapy.

High TS expression (grade 2-3 or grade 3). In the group of 990 patients with TS grade 2-3, a tendency to a longer OS was observed for the group treated with surgery plus adjuvant chemotherapy *versus* surgery alone (p=0.07, Figure 2A). This finding was even more enhanced in the group of 460 patients with a TS grade 3 expression, where a significant longer OS was noted in the adjuvant chemotherapy group compared to the surgery alone group (p=0.005, Figure 2B), a result which also remained in the multivariate analysis (p=0.0008, Table II).

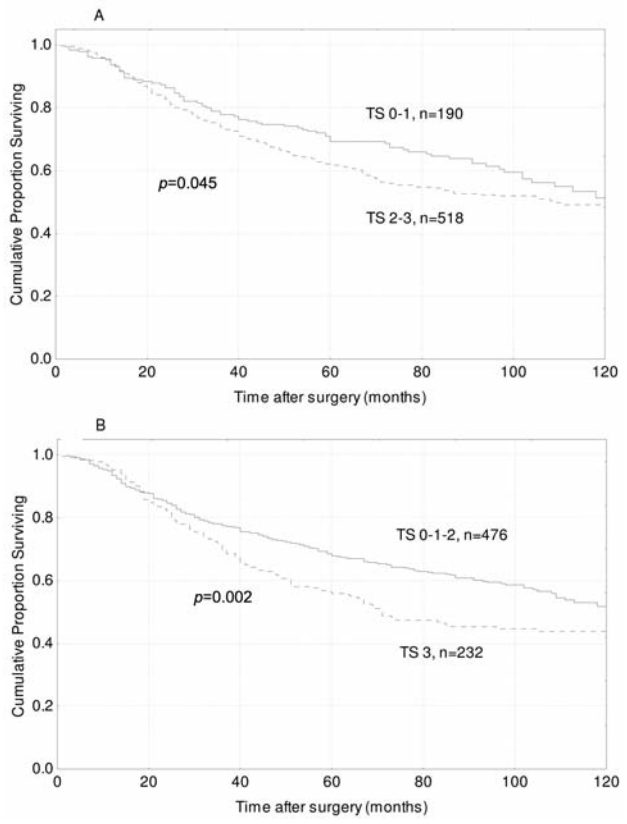

Figure 1. *(A). Overall survival (OS) in 708 patients with colorectal cancer stage II and III treated with surgery alone according to expression of TS 0-1 versus 2-3. (B) Overall survival (OS) in 708 patients treated with surgery alone according to expression of TS 0-1-2 versus 3.*

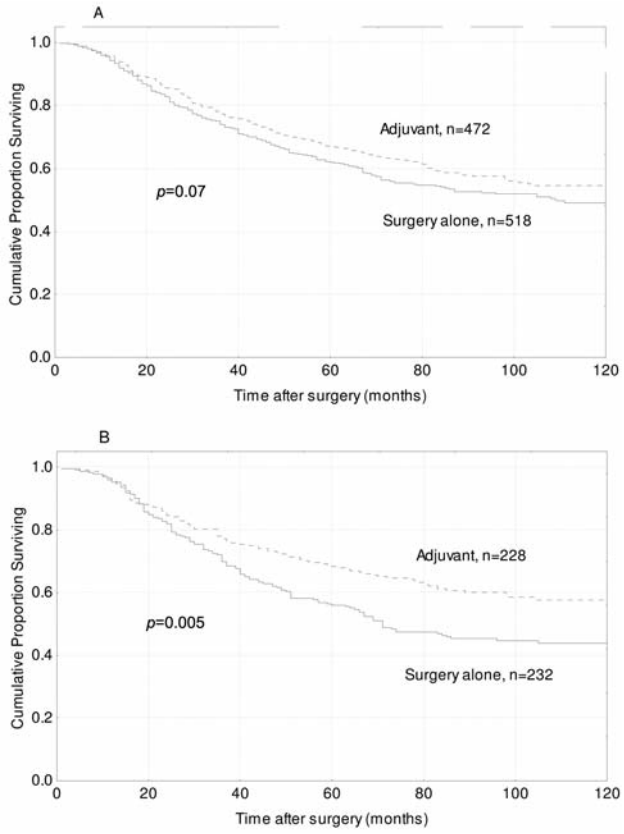

Figure 2. *(A) Overall survival (OS) in the group of 990 patients with high TS expression (TS 2-3) tumors according to treatment with surgery alone versus surgery plus adjuvant chemotherapy. (B) Overall survival in the group of 460 patients with TS grade 3 tumours and treatment with surgery alone versus surgery and adjuvant chemotherapy.*

Discussion

In this study, we report a total of 1389 CRC patients who were included in Nordic trials from 1991 to 1997 randomized between surgery alone or 5-FU-based chemotherapy after surgery with a median follow-up time of 75 months (16). As already mentioned, the results from a study including 862 of these patients have been reported previously (15). Our aim was to test the hypothesis of TS expression as a prognostic marker as well as a predictor of OS or DFS in the adjuvant 5-FU-based chemotherapy setting in a large study cohort. In the patients who received adjuvant 5-FU chemotherapy we have also added valuable information about the expression of TS in relation to OS according to the given 5-FU dose.

TS expression was not a prognostic factor for OS or DFS in the entire study group. Patients in the surgery group alone with a TS expression grade 0-1 and grade 0-2 had a significant longer OS and DFS compared to TS grade 2-3 respective TS grade 3. When using the classification of TS expression 0-2 *vs*. 3, TS had a stronger prognostic value compared to the classification of TS expression in the category 0-1 *vs*. 2-3. In patients with tumors expressing TS grade 3, the adjuvant chemotherapy group showed a significant longer OS compared to the surgery alone group. No such survival advantage was found in the group of patients with TS expression grade 0-1 or grade 0-2. No differences in OS or DFS in relation to TS expression were found when comparing patients treated with chemotherapy who received ≥90% of the planned 5-FU dose with those receiving <90% of the planned 5-FU dose.

Different methods are used to quantify TS protein and TS gene expression such as IHC and polymerase chain reaction (PCR) (17, 20).The development of antibodies to detect the TS protein has made it possible to easily define TS expression in paraffin-embedded formalin-fixed tissue sections. Other advantages with IHC is the ability to evaluate the histological features of the tumors and tumor heterogeneity. However, evaluation with IHC will always be semi-quantitative and therefore difficult to standardize.

In recent years, tissue microarray (TMA) has been more frequently used in protein marker studies. The TMA technique is good at testing if genes found to be differentially expressed in CRCs by cDNA expression profiling are also distinct at the protein level (21). The major disadvantage of TMA is the deficient evaluation of the presence and extent of heterogeneity in protein expression within a carcinoma as the analysed area of the tumor tissue is limited.

In previous studies using IHC to analyse TS, various criteria have been used to grade TS expression and to determine cut-off points. The majority of these studies have used a grading system with four intensity grades in which grade TS 0-1 has been classified as low and grade TS 2-3 classified as high (4). The classification of TS expression grade 0-2 *vs*. grade 3 has been described earlier by Allegra *et al*. (19). Some reports have also taken into account the percentage and nature of TS staining (9, 10, 18). The use of different antibodies and differences in protein degradation may also explain the variance in the results between different research groups. Furthermore, TS is known to be heterogenically expressed in primary CRC (22) which might have an implication in the measurement of TS. In this report, we have analysed two different areas from each primary tumor with the intention to reduce the effect of TS heterogeneity. Taken together, there is not yet any accepted golden standard for IHC assay of TS expression.

The present study, which to our knowledge is the largest randomized CRC study assessing TS with IHC, showed that TS expression had a significant prognostic value in the subgroup of patients treated with surgery alone. On the other hand the expression of TS was not prognostic in the entire study group or in the group treated with surgery and adjuvant chemotherapy. This is in contrast to the results from our previous study including 862 of these 1389 patients, where the entire group of patients whose tumors expressed a low TS (grade 0-1) had a longer OS (p=0.04) (15). Our finding in the enlarged study group that TS expression was of no prognostic value in the group of patients treated with adjuvant chemotherapy group is in accordance with the results from several other adjuvant studies (18, 23, 24).

In a meta-analysis from 2004 including both the adjuvant and advanced disease settings (n=3497), Popat *et al*. (4) concluded that patients with tumors expressing high levels of TS have poorer OS compared with those with tumors expressing low levels. In the adjuvant setting, where data from a total of 2610 patients were available for pooling, high TS expression only seemed to predict poorer OS for CRC patients treated by surgery alone, which is in agreement with the results from our study. On the other hand, in a meta-analysis one will always have different sources of bias. For example, this meta-analysis demonstrated a marked methodological heterogeneity in studies that have addressed the relation between TS expression and prognosis, *e.g*. IHC, PCR and enzyme assays.

The two largest IHC studies analyzing TS in this meta-analysis are two studies published by Allegra *et al*. (18, 19). In the first study with 465 colon cancer patients in which 84% had stage III disease, 68% were treated with surgery plus adjuvant chemotherapy and 32% with surgery alone. They also failed to show a significant association between TS expression and outcome (18).

In the second IHC study with 706 randomized patients, TS was found to have a prognostic value with high TS (grade 3) patients having a shorter OS (19). In this study, the benefit of 5-FU-based adjuvant chemotherapy was independent of TS expression. After the meta-analysis was published, the largest TS IHC study included 967 Chinese patients, 779 with TS analyzed. This study evaluated the prognostic value of TS IHC assessed using a polyclonal antibody, without finding any evidence that TS was associated with survival (23). However, most of the patients in the study were treated with systemic and/or adjuvant chemotherapy, whereas the effect of TS expression in patients treated by surgery alone was not specifically discussed.

In a recent study with 945 non-randomized CRC patients treated with or without 5-FU-based chemotherapy using TMA, a low TS (grade 0-2, 61%) was associated with worse prognosis in stage II CRC (p=0,019) in the surgery alone arm (25). The use of TMA could have underestimated the true frequency of high TS (grade 3-4, 39%) expressing tumors, particularly in patients with focal staining (25).

Among the 2224 patients in the Nordic trials, where the 1389 patients in our study represent a group of Swedish and Danish patients, a non-significant absolute difference of 7% favouring adjuvant 5-FU-based chemotherapy was found in colon cancer stage III (16). According to the previously known consensus there is a significant survival advantage in colon cancer stage III treated with adjuvant chemotherapy (3). The absence of a significant survival benefit from adjuvant chemotherapy in the entire Nordic study cohort has to be taken into account while analyzing our data.

There is no clear evidence as yet that TS expression in the primary tumor can predict response to FU-based regimens in the adjuvant setting (4-9, 18, 19, 26). In the present study (n=1389) the trend of benefit of 5-FU based chemotherapy is more obvious among patients whose tumors express a TS level grade 2-3 than in our previous report (n=862) (15). The benefit of 5-FU-based chemotherapy was significant in the group of 460 patients with the highest TS expression grade 3. The finding that patients with high TS expression benefit more from adjuvant 5-FU-based chemotherapy has been reported previously (5-11). A theory that supports this finding states that cells with low TS have a low proliferation rate that in turn prevents induction of DNA damage and 5-FU toxicity (27, 28). Other studies have found no difference in benefit of adjuvant chemotherapy between tumors expressing low or high TS (18, 19, 23, 24).

In our previous report (n=862), we found that patients whose tumors expressed TS level 0-1 had a worse outcome when treated with adjuvant chemotherapy compared with surgery alone (15). In this enlarged study (n=1,389), we were unable to confirm any deleterious effect of adjuvant chemotherapy in patients with low intratumoral TS expression.

To date, the benefit from adjuvant chemotherapy is reported to relate to the histopathological criteria of the tumor and patients at highest risk of relapse are those who seem to gain most from adjuvant therapy (2). There is still no consensus as to an adequate prognostic and predictive marker in CRC and if one were found it, would be especially of use in stage II patients (29-31). The challenge ahead is to find a set of biomarkers that can help to individualize the adjuvant treatment with fluoropyrimidines alone or in combination with other drugs for patients with locally advanced CRC. In summary, we demonstrated that TS, IHC assessed, is an independent prognostic factor in the group of patients with CRC treated with surgery alone. We also showed a benefit of adjuvant 5-FU-based chemotherapy in the group of CRC patients with high TS expression.

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