GENETIC TESTING AND MOLECULAR BIOMARKERS
Volume 17, Number 11, 2013

Pp. 799–806
DOI: 10.1089/gtmb.2013.0171

Thymidylate Synthase Gene Polymorphism and Survival of Colorectal Cancer Patients Receiving Adjuvant 5-Fluorouracil

Violetta Sulzyc-Bielicka,[1] Dariusz Bielicki,[2] Agnieszka Binczak-Kuleta,[3] Mariusz Kaczmarczyk,[3] Wiesława Pioch,[3] Anna Machoy-Mokrzynska,[4] Andrzej Ciechanowicz,[3] Magdalena Gołębiewska,[1] and Marek Drozdzik[4]

Limited studies indicate a possible association of 5′-UTR thymidylate synthase enhancer region polymorphism and treatment outcome in patients medicated with 5-fluorouracil (5-FU). The study was designed to verify the relationship in patients with colorectal cancer (CRC), a Polish population that received 5-FU-based adjuvant chemotherapy. The study analyzed 145 Astler-Coller B2 and C CRC patients. Genotyping for a variable number of tandem repeats and G to C single-nucleotide polymorphism in the 5′-UTR of the thymidylate synthase (*TS*) gene was carried out. *TS* genotypes were classified into high expression (high TS) and low expression types (low TS). High TS was found in 22.8% of patients. The right-side tumors were more frequently associated with high TS than the left-side tumors ($p = 0.024$). High TS was only found in 9.3% of rectal tumors, but in 29.7% of colon cancers ($p = 0.0042$). Disease-free survival after 20 months (DFS 20) was longer in subjects with low TS than in high TS ($p = 0.043$). Patients who underwent chemotherapy had longer DFS 20 in the low TS than in the high TS subgroup ($p = 0.051$). The low TS was found to be an independent good prognostic factor for DFS 20 in the whole group as well as in the subgroup treated with chemotherapy ($p = 0.024$ and $p = 0.034$, respectively). Patients with low TS did not show any differences in DFS 20 whether they were treated with adjuvant chemotherapy or not. Proximal CRC tumors are characterized by higher *TS* expression genotypes than distal tumors, and are at significantly greater risk of early recurrence during the first 20 months after surgery.

Introduction

Colorectal cancer (CRC) belongs to one of the most common human malignancies and the stage of the disease decides the mode of treatment. Surgery is essential for the localized form, and chemotherapy is indicated in stage III (node positive) patients, although some stage II (node negative) patients may also benefit from its application (Quasar Collaborative Group *et al.*, 2007; Wolpin and Mayer, 2008; Midgley *et al.*, 2009). In both adjuvant and palliative settings, 5-fluorouracil (5-FU) plays a central role in chemotherapy regimens. However, only about one-third of the patients treated with adjuvant chemotherapy benefit from it. Thus, there is an urgent need to identify markers of tumor sensitivity to proposed chemotherapy.

Thymidylate synthase (TS) is the enzyme that catalyzes the reductive methylation of deoxyuridine monophosphate to form deoxythymidine monophosphate and dihydrofolate (Carreras and Santi, 1995). This reaction provides a *de novo* source of thymidylate, an essential precursor for DNA biosynthesis (Carreras and Santi, 1995). TS functions as an RNA binding protein that, at the translational level, regulates the expression of its own mRNA translation and other cellular mRNAs, including that of p53 (Ju *et al.*, 1999; Xi *et al.*, 2006). Recently, it has been found that TS may act as an oncogene as well (Rahman *et al.*, 2004). TS is the target enzyme for 5-FU (Danenberg, 1977). Several studies have demonstrated that TS tissue levels may modulate prognosis, irrespectively of 5-FU and the response to 5-FU medication, with high expression levels generally associated with a poor response, especially in advanced disease (Leichman *et al.*, 1997). Yet, the predictive role of the TS level for 5-FU sensitivity in adjuvant treatment is controversial. In a recent meta-analysis by Popat *et al.* (2004), 13 studies consisting of 887 patients with metastatic CRC and 7 studies involving 2610 patients with localized CRC were analyzed. The authors showed that tumors expressing high levels of TS seemed to have poor overall survival (OS) compared with tumors expressing low TS levels (Popat *et al.*,

Departments of [1]Oncology, [2]Gastroenterology, [3]Clinical and Molecular Biochemistry, and [4]Clinical and Experimental Pharmacology, Pomeranian Medical University, Szczecin, Poland.

Table 1. Patient Characteristics

		TS *expression type*		
	n	*Low (3C/3C, 2R/3C, 2R/2R)* n *(%)*	*High (3G/3G, 3G/3C, 2R/3G)* n *(%)*	p
Sex				
Male	80	59 (73.8)	21 (26.2)	0.18
Female	65	54 (83.1)	11 (16.9)	
Astler-Coller				
B2	81	65 (80.2)	16 (19.8)	0.45
C	64	48 (75.0)	16 (25.0)	
Grade				
1/2	102	81 (79.4)	21 (20.6)	0.42
3	10	9 (90.0)	1 (10.0)	
Tumor location				
Right-side tumor	33	21 (63.6)	12 (36.4)	0.024
Left-side tumor	112	92 (82.1)	20 (17.9)	
Tumor location				
Rectum	54	49 (90.7)	5 (9.3)	0.0042
Colon	91	64 (70.3)	27 (29.7)	
Chemotherapy				
Yes	117	92 (78.6)	25 (21.4)	0.68
No	28	21 (75.0)	7 (25.0)	
Age				
<63	70	56 (80.0)	14 (20.0)	0.56
≥63	75	57 (76.0)	18 (24.0)	

TS, thymidylate synthase.

2004). *TS* gene expression is modulated by functional, significant germ-line polymorphisms in the 5′-UTR thymidylate synthase enhancer region (TSER), and 3′-UTR (*TS* 1494del6b) of the gene (Horie *et al.*, 1995; Mandola *et al.*, 2003; Lurje *et al.*, 2009). A variable number of tandem repeats (VNTR) in the promoter region of *TS* (TSER), mainly 2 (2R) and 3 repeats (3R), and cytosine versus guanine single-nucleotide polymorphism (SNP) can influence the efficiency of translation (Mandola *et al.*, 2003; Lurje *et al.*, 2009). The presence of a G to C SNP within the second repeat of the 3R allele (3G/3C) alters mRNA stability, and therefore enzyme activity (Mandola *et al.*, 2003; Lurje *et al.*, 2009). Thus, *TS* polymorphisms in tumor cells might influence the clinical outcome under 5-FU treatment through the altered TS activity. *TS* genotypes of patients with CRC were classified into high expression types (high TS): 2R/3G, 3C/3G, 3G/3G and low expression types (low TS): 2R/2R, 2R/3C, 3C/3C (Kawakami and Watanabe, 2003).

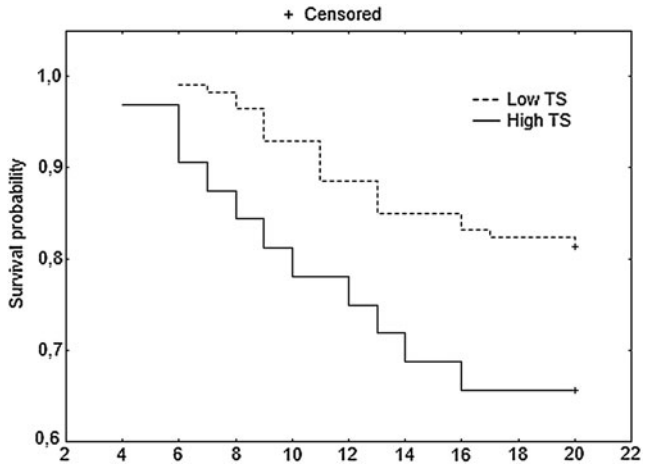

FIG. 1. Disease-free survival (DFS) at 20 months for patients with Astler-Coller B2 and C stages of colorectal cancer (CRC).

The present study was designed to investigate whether 5-TSER polymorphisms (TSER 2R/3R and TSER 3G/3C) are associated with disease-free survival (DFS) and OS in patients with CRC who received 5-FU-based adjuvant chemotherapy.

Materials and Methods

Participants and study design

The study included 145 patients, aged 63 years (range 28–81 years) with Astler-Coller B2 and C CRC, who underwent radical resection (R0) at the Department of Surgery of the Pomeranian Medical University, Szczecin, Poland. The clinicopathological characteristics of patients are summarized in Table 1; 117 (59 stage B2 and 58 stage C Astler-Coller). Patients received 5-FU-based adjuvant chemotherapy according to the Mayo regimen (FA 20 mg/m^2, 5-FU 425 mg/m^2/day, on days 1–5 every 28 days, six cycles). For stage B, chemotherapy was applied only for high risk patients (obstruction, T4 tumor, perforation). Twenty-two patients with stage B2, and six with stage C Astler-Coller CRC were not treated with adjuvant

Table 2. Univariate Analysis of Survival for all Patients

	DFS 20		*DFS 36*		*DFS 60*	
	p[a]	*HR*	p[a]	*HR*	p[a]	*HR*
Sex						
Male	0.39	1.37	0.69	1.12	0.94	0.98
Female		1		1		1
Astler-Coller						
B2	0.0027	0.32	0.0065	0.45	0.0004	0.41
C		1		1		1
Grade						
1/2	0.50	0.66	0.92	1.07	0.19	0.58
3		1		1		1
Tumor location						
Right-side tumor	0.96	0.98	0.34	0.69	0.12	0.59
Left-side tumor		1		1		1
Tumor location						
Rectum	0.66	1.17	0.22	1.43	0.0088	1.92
Colon		1		1		1
Chemotherapy						
Yes	0.92	1.04	0.65	1.19	0.53	1.23
No		1		1		1
Age						
<63	0.36	0.72	0.74	0.91	0.66	1.11
≥63		1		1		1
5′-TSER TS						
High TS 3G/3G, 3G/3C, 2R/3G	0.043	2.13	0.17	1.57	0.22	1.42
Low TS 3C/3C, 2R/3C, 2R/2R		1		1		1

[a]Test long-rank.

DFS 20, DFS 36, DFS 60, disease-free survival at 20, 36, and 60 months; HR, hazard ratio.

Table 3. Univariate Analysis of Survival for Patients Treated with Adjuvant Chemotherapy

	DFS 20		*DFS 36*		*DFS 60*	
	p[a]	*HR*	p[a]	*HR*	p[a]	*HR*
Sex						
Male	0.70	1.17	0.89	0.96	0.61	0.87
Female		1		1		1
Astler-Coller						
B2	0.0035	0.26	0.0068	0.40	0.00069	0.38
C		1		1		1
Grade						
½	0.49	0.65	0.85	1.12	0.23	0.61
3		1		1		1
Tumor location						
Right-side tumor	0.58	0.76	0.18	0.55	0.033	0.42
Left-side tumor		1		1		1
Tumor location						
Rectum	0.55	1.26	0.25	1.45	0.01	2.02
Colon		1		1		1
Age						
<63	0.20	0.60	0.59	0.84	0.83	1.06
≥63		1		1		1
5′-TSER TS						
High TS 3G/3G, 3G/3C, 2R/3G	0.051	2.23	0.14	1.69	0.23	1.47
Low TS 3C/3C, 2R/3C, 2R/2R		1		1		1

[a]Test long-rank.

chemotherapy (patients with stage C did not receive adjuvant chemotherapy due to contraindications). Chemotherapy and survival details were obtained from the hospital medical case histories. Patients with rectal cancer also received preoperative radiotherapy (5×5 Gy).

The time from surgery until the time of death due to cancer or to the last known follow-up was regarded as OS, and the time until the first appearance of metastasis or local recurrence was regarded as DFS.

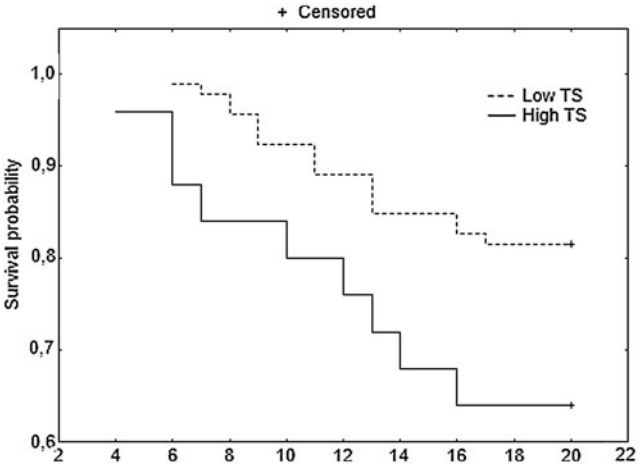

FIG. 2. DFS at 20 months for patients with stage B2 and C CRC who underwent adjuvant chemotherapy.

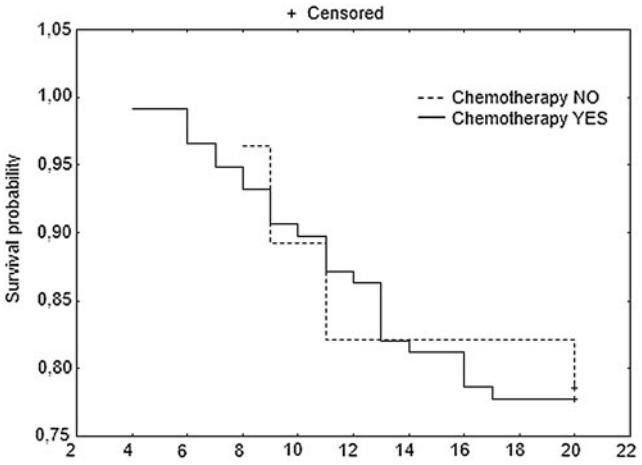

FIG. 3. DFS at 20 months for patients with stage B2 and C CRC with low thymidylate synthase (*TS*) polymorphisms.

The project was approved by the Ethics Committee of Pomeranian Medical University, and all patients signed informed consent.

Genotyping

Genomic DNA was extracted from 0.2 mL of K_3EDTA-anticoagulated blood with a QIAamp DNA Mini Kit (Qiagen)

Table 4. Multivariate Analysis of Survival for All Patients

	DFS 20		*DFS 36*		*DFS 60*	
	p[a]	*HR*	p[a]	*HR*	p[a]	*HR*
Sex						
Male	0.19	1.83	0.63	1.19	0.93	0.98
Female		1				
Astler-Coller						
B2	0.016	0.20	0.0055	0.36	0.0033	0.40
C		1				
Grade						
½	0.72	0.78	0.58	1.43	0.56	0.77
3		1				
Tumor location						
Right-side tumor	0.50	0.63	0.34	0.57	0.52	0.73
Left-side tumor		1				
Tumor location						
Rectum	0.61	1.29	0.22	1.59	0.058	1.88
Colon		1				
Chemotherapy						
Yes	0.15	0.43	0.75	0.85	0.82	0.91
No		1				
Age						
<63	0.30	0.63	0.95	1.02	0.86	1.06
≥63		1				
5′-TSER TS						
High TS 3G/3G, 3G/3C, 2R/3G	0.024	2.91	0.12	1.86	0.14	1.70
Low TS 3C/3C, 2R/3C, 2R/2R		1				

[a]Test long-rank.

and from serum using the Sherlock AX kit (A&A Biotechnology). Genotyping for the VNTR/SNP polymorphism in the 5′-UTR of the *TS* gene was carried out based on the Kawakami and Watanabe method (Kawakami and Watanabe, 2003). Polymerase chain reaction (PCR) of the polymorphic region in the promoter of *TS* was performed using previously published primers: forward 5′-AGGCGCGCG GAAGGGGTCCT-3′ and reverse 5′-TCCGAGCCGGCCA CAGGCAT-3′. The reaction was carried out in a total volume of 20 μL containing 40 ng of template DNA, 4 pM of each primer, 1× PCR buffer (Qiagen), 1× Q-Solution (Qiagen), 1.5 mM $MgCl_2$ (Qiagen), 200 μM each dNTP (MBI Fermentas), and 2.5 U of HotStarTaq polymerase (Qiagen). The amplification was performed with initial denaturation at 94°C for 15 min, and then 34 cycles of denaturation at 94°C for 20 s, annealing at 57°C for 40 s, and extension at 72°C for 40 s. The final 72°C incubation was extended by 8 min. The double repeat (2R) of the promoter region resulted in a 116-bp PCR product and the triple repeat (3R) in a 141-bp product. The amplified fragments were separated by electrophoresis in a 3% agarose gel stained with ethidium bromide. The G>C substitution within the triple repeat was determined by restriction fragment length polymorphism (RFLP). For the RFLP assays, a 16-μL aliquot of PCR product (which showed genotypes with the 3R allele) was incubated at 37°C for 18 h with 10 U of *Hae*III enzyme. The restriction fragments sizes were 66, 37, 28, and 10 bp for the 3G allele and 94, 37, and 10 bp for the 3C allele. The fragments were separated by electrophoresis in a 4% agarose gel stained with ethidium bromide. The results were recorded by photographing the gels in UV light.

Table 5. Multivariate Analysis of Survival for Patients Treated with Adjuvant Chemotherapy

	DFS 20		*DFS 36*		*DFS 60*	
	p[a]	*HR*	p[a]	*HR*	p[a]	*HR*
Sex						
Male	0.50	1.38	0.97	1.01	0.75	0.90
Female		1				
Astler-Coller						
B2	0.0027	0.14	0.0037	0.31	0.0037	0.37
C		1				
Grade						
½	0.69	0.75	0.54	1.48	0.60	0.79
3		1				
Tumor location						
Right-side tumor	0.58	0.63	0.30	0.50	0.31	0.55
Left-side tumor		1				
Tumor location						
Rectum	0.64	1.30	0.32	1.52	0.078	1.91
Colon		1				
Age						
<63	0.23	0.55	0.89	1.05	1.04	0.90
≥63		1				
5′-TSER TS						
High TS 3G/3G, 3G/3C, 2R/3G	0.034	2.87	0.18	1.76	0.34	1.44
Low TS 3C/3C, 2R/3C, 2R/2R		1				

[a]Test long-rank.

Statistical analysis

The analysis was performed using STATISTICA software (StatSoft, Inc., 2008, version 8.0). Nominal variables were tested using chi-squared analysis. Risk factors for OS and DFS were determined by univariate and multivariate Cox proportional hazards regression analysis. The results of Cox regression are expressed as a hazard ratio (HR). The differences between univariate variables were illustrated by Kaplan–Meier plots. A *p*-value of less than 0.05 was considered significant.

Results

The clinicopathological characteristics of 145 patients and the distribution of 5′-TSER polymorphisms are presented in Table 1. The high expression *TS* polymorphisms (3G/3G, 3G/3C, 2R/3G) were found in 22.1% of patients and the low *TS* polymorphisms (3C/3C, 2R/3C, 2R/2R) in 77.9% of patients. Tumors originating from the proximal colon (right side) were more frequently associated with high TS (36.4%) than the left-side tumors (17.9%) ($p=0.024$). High *TS* was only found in 9.3% of rectal tumors, but in 29.7% of colon cancers ($p=0.0042$). No significant differences in the frequencies of 5′-TSER genotypes were associated with sex, Astler-Coller stage, age, grade, or introduced chemotherapy.

In the group of patients with B2 tumors, DFS detected 20, 36, and 60 months after the potentially curative resection was longer than in patients with Astler-Coller group C tumors.

Table 6. Univariate Analysis of Survival of all Patients

	OS 36		*OS 60*	
	p[a]	*HR*	p[a]	*HR*
Sex				
Male	0.14	2.66	0.17	1.86
Female		1		1
Astler-Coller				
B2	0.35	0.59	0.24	0.61
C		1		1
Grade				
½	0.10	0.26	0.19	0.43
3		1		1
Tumor location				
Right-side tumor	0.54	1.45	0.89	0.93
Left-side tumor		1		1
Tumor location				
Rectum	0.46	1.51	0.018	2.74
Colon		1		1
Chemotherapy				
Yes	0.73	0.79	0.79	0.87
No		1		1
Age				
<63	0.43	0.64	0.71	0.85
≥63		1		1
5′-TSER TS				
High TS 3G/3G, 3G/3C, 2R/3G	0.93	1.06	0.88	1.08
Low TS 3C/3C, 2R/3C, 2R/2R		1		1

[a]Test long-rank.
OS 36 and OS 60, overall survival at 36 and 60 months.

The 20-month DFS was significantly longer in patients with high *TS* polymorphisms ($p = 0.043$, HR 2.13) than those with low *TS* expression (Fig. 1 and Table 2). Patients with colon tumors had better DFS at 60 months (DFS 60) than subjects with rectal tumors ($p = 0.0088$) (Table 2).

Patients with stage B2 and C CRC who underwent adjuvant chemotherapy had better DFS 20 in the subgroup with low versus high *TS* (Table 3) ($p = 0.051$). In the 36 and 60 months after the surgical treatment, a trend of longer DFS for the low *TS* subgroup was still observed, but was not statistically significant. DFS 60 in patients who underwent adjuvant chemotherapy was influenced by tumor location, with better prognosis for colon cancers than rectal tumors ($p = 0.01$), and longer DFS 60 for proximal tumors than distal tumors ($p = 0.033$).

Furthermore, patients with stage B2 and C CRC who underwent adjuvant chemotherapy had better DFS 20 in the subgroup with low versus high *TS* (Fig. 2). When B2 and C groups were analyzed separately, no significant differences in DFS 20 were found between patients with low versus high *TS*, but a trend toward longer DFS 20 in patients with low *TS* was observed (data not shown). When patients with B2 and C tumors with low *TS* were grouped together, no differences in DFS 20 were found between the subgroups that received and did not receive adjuvant therapy (Fig. 3). A similar analysis for the B2 and C groups with high *TS* could not be performed due to the small number of patients.

Astler-Coller stage B2 was an independent, significant factor for better DFS 20, 36, and 60 ($p = 0.016$, $p = 0.0055$, and $p = 0.0033$, respectively) in all B2 + C patients ($n = 145$). The low *TS* polymorphism type was an independent, significant factor for better DFS 20 in all B2 + C patients ($n = 145$, $p = 0.024$) (Table 4) and in the patient group treated with adjuvant chemotherapy ($p = 0.034$) (Table 5).

Statistical analysis of OS at 20 months was not possible due to a small number of patients. Any analyzed parameter had an impact on OS at 36 months. The OS at 60 months (OS 60) was influenced only by tumor location with better prognosis for colon cancers than rectal tumors ($p = 0.018$) (Table 6). Female sex and colon location of tumors were significant and independent good prognostic factors for OS 60 ($p = 0.044$ and $p = 0.006$, respectively) (Table 7). In the subgroup treated with chemotherapy, solely the colon location was an independent favorable prognostic factor for OS 60 ($p = 0.037$) (data not shown).

Discussion

In the present study, patients were stratified into two groups according to *TS* gene polymorphisms associated with high or low TS expression, as indicated by Kawakami and Watanabe (2003). In our study, the following distribution of the 5′-TSER polymorphisms was documented: 22.8% of polymorphisms were associated with high TS expression (3G/3G, 3G/3C, 2R/3G; high TS) and 77.2% were associated with low TS expression (3C/3C, 2R/3C, 2R/2R; low TS). The frequency of these alleles differs with ethnicity. Homozygous triple repeat subjects were twice as common in Chinese subjects (67%) than in Caucasian subjects (38%) (Marsh *et al.*, 1999). In the white population, 2R/3R heterozygosity occurs in ~50% of the population and each of the two homozygous genotypes is found in ~25% of the population (Marsh *et al.*, 2001). The frequency of the 3C allele among all 3R alleles showed a variation of 56%, 47%, 28%, and 37% for whites, Hispanics, African–Americans, and Chinese, respectively (Lurje *et al.*, 2009).

Tumors originating from the proximal colon in respect to the splenic flexure (proximal CRC) were significantly more frequently associated with high *TS* than tumors originating from the distal colon in respect to the splenic flexure. In addition, rectal tumors were associated with high *TS* in only 9.3% of cases, whereas colon tumors were associated with high *TS* in 29.7% of patients. Similar results were reported by Fernández-Contreras *et al.* (2009). In a previous study (Sulzyc-Bielicka *et al.*, 2009), we found that proximal CRC was characterized by a higher TS protein expression, as detected by immunohistochemistry, than distal tumors. Elsaleh *et al.* (2000) found a striking survival benefit after adjuvant 5-FU-based therapy in patients with Dukes C CRC who had right-side tumors, especially in women.

The general finding of this study is that DFS 20 was significantly better for tumors with low *TS* polymorphisms than for high *TS* polymorphisms. In addition, all patients with B2 and C Astler-Coller CRC who underwent adjuvant chemotherapy had significantly better DFS 20 in the low *TS* subgroup compared to the high *TS* subgroup. The low *TS* polymorphism type was an independent and significant good prognostic factor for DFS 20 in the whole analyzed group and in the subgroup treated with chemotherapy. Therefore, the high *TS* polymorphism type may be a risk factor for early

Table 7. Multivariate Analysis of Survival of All Patients

	OS 60	
	p[a]	*HR*
Sex		
Male	0.044	3.27
Female		1
Astler-Coller		
B2	0.11	0.40
C		1
Grade		
½	0.30	0.49
3		1
Tumor location		
Right-side tumor	0.46	2.12
Left-side tumor		1
Tumor location		
Rectum	0.006	9.52
Colon		1
Chemotherapy		
Yes	0.17	0.35
No		1
Age		
<63	0.29	0.57
≥63		1
5′-TSER TS		
High TS 3G/3G, 3G/3C, 2R/3G	0.41	1.76
Low TS 3C/3C, 2R/3C, 2R/2R		1

[a]Test long-rank.

recurrence of CRC during the first 20 months after operation. The observations of the present study revealed that the colon location and female sex were independent, and good prognostic factors for OS 60. These findings are in agreement with other studies that show lower survival rates in patients with distal tumor locations than those with proximal tumors (Laurie *et al.*, 1989).

There were no differences in DFS 20 for patients with low *TS* treated with adjuvant chemotherapy compared to those not medicated. This observation suggests that patients with low TS may not benefit from 5-FU-based chemotherapy. In the study conducted by Allegra *et al.* (2003), no interaction between TS expression measured by immunohistochemistry in primary CRC tumors and chemotherapy outcome was shown. In contrast, Edler *et al.* (2002) on the basis of immunohistochemical assessment of TS in CRC, suggested a worse outcome in subjects with low TS treated with 5-FU than those not treated with chemotherapy.

Our results based on *TS* polymorphisms are consistent with those from immunohistochemical assessment of TS protein expression, and show that the high *TS* polymorphism leads to early cancer recurrence, as measured by DFS 20. In immunohistochemical studies, patients with high TS protein expression either do not develop recurrences or they tend to develop them rather quickly (Edler *et al.*, 2002; Kornmann *et al.*, 2002). Patients with low TS expression tend to have later recurrences, but they are more frequent (Kornmann *et al.*, 2002). Low TS expression may be associated with a low spontaneous recurrence rate and longer survival, also indicating less benefit from adjuvant 5-FU treatment (Edler *et al.*, 2002).

Differences in outcome between the investigated groups seem to disappear with time. This may be a consequence of our second line of treatment of a recurrent disease, that is, surgery or other types of chemotherapy both aiming at improving the chances of OS. A relatively small number of patients or too short a time of observation could also influence our observations.

High TS levels (mRNA, protein, enzyme activity) in advanced disease seem to predict nonresponsiveness to 5-FU and a worse prognosis (Leichman *et al.*, 1997; Paradiso *et al.*, 2000; Etienne *et al.*, 2002; Ichikawa *et al.*, 2003; Popat *et al.*, 2004). For adjuvant treatment, it is controversial whether TS levels measured in primary CRC predict clinical benefit from 5-FU-based treatment. In several studies, a high TS level was associated with poor postoperative outcome, independent of the Duke's stage (Kralovánszky *et al.*, 2002; Formentini *et al.*, 2004), and leading to poor DFS and OS in an adjuvant setting (Cascinu *et al.*, 2001; Kralovánszky *et al.*, 2002; Allegra *et al.*, 2003; Popat *et al.*, 2004; Broll *et al.*, 2005). In contrast, other reports indicated that high TS is predictive of 5-FU treatment and patients with a high TS level may benefit from adjuvant 5-FU therapy (Edler *et al.*, 2002; Kornmann *et al.*, 2003). Allegra *et al.* (2003) in a study on 706 patients with Dukes B and C CRC, found no predictive value of TS protein expression determined by immunohistochemistry in primary tumors, but showed a good prognostic impact of low TS protein expression on survival. The impact of TS gene polymorphisms on prognosis and 5-FU-based chemotherapy in CRC patients was reviewed by Lurje *et al.* (2009). The largest study concerning adjuvant 5-FU was conducted by Iacopetta *et al.* (2001) and showed better survival in the low *TS* polymorphism subset receiving adjuvant 5-FU compared to those not receiving adjuvant chemotherapy. Tsuji *et al.* (2003) found no survival differences among patients who received adjuvant 5-FU after curative CRC surgery, when stratified by *TS* gene polymorphism. Similar observations were also reported by Prall *et al.* (2007). Kawakami and Watanabe (2003) documented a negative predictive effect of the C/G SNP for adjuvant 5-FU chemotherapy, but Prall *et al.* (2007) reported no predictive value for such therapy.

Hitre *et al.* (2005) in a prospective study of patients with CRC treated with 5-FU adjuvant therapy found that subjects with germline high *TS* polymorphisms had longer DFS and OS. The authors concluded that the combination of 5'-TSER and 3'-TSER *TS* polymorphisms, measured from PBMC of CRC patients receiving adjuvant 5-FU-based chemotherapy, is an independent prognostic marker (Hitre *et al.*, 2005). In addition, the authors suggest that the germline *TS* genotypes leading to high TS expression (Danenberg, 2004; Mandola *et al.*, 2004) can predict significantly better DFS and OS.

It is very important to point out that the published data refer to various populations with different ethnic background that may impact on the study outcome. As an example, the Hungarian study by Hitre *et al.* (2005) included a majority of patients (82%) with high *TS* polymorphisms, whereas in our study only 22.1% had high *TS* polymorphisms. The more ethnic differences in the metabolism of the folate cofactor of 5-FU may also affect the final study results, as well as findings suggesting a relationship between the folate level and *TS* polymorphisms (Chen *et al.*, 2003).

It should be stated that some other genetic factors may influence the 5-FU response, like microsatellite stability (MSI) and *TP53* mutations. Some studies suggest a relationship between p53 status, MSI, and TS expression. Kristensen *et al.* (2010) revealed that TS expression is significantly higher in MSI tumors compared with microsatellite stable tumors, and Popat *et al.* (2006) found that CRC cells with high TS levels are more likely to overexpress p53. However, clinical studies are not sufficient to reliably predict a response to 5-FU on the basis of MSI, TP53, and TS status.

On the basis of our results, it can be concluded that CRC tumors located proximal to the splenic flexure were more frequently associated with high *TS* polymorphisms than distal tumors. Patients with high *TS* polymorphisms had a significantly greater risk of early recurrence during the first 20 months after surgery. The high *TS* polymorphism patients should be subjected to a strict follow-up protocol for early detection of cancer recurrence.

Acknowledgment

This work was supported by the Pomeranian Medical University Research Program grant no. WLBiML-401-01/3/12.

Author Disclosure Statement

No competing financial interests exist.

References

Allegra CJ, Paik S, Colangelo LH, *et al.* (2003) Prognostic value of thymidylate synthase, Ki-67, and p53 in patients with Dukes' B and C colon cancer: a National Cancer Institute-National Surgical Adjuvant Breast and Bowel Project collaborative study. J Clin Oncol 21:241–250.

Broll R, Busch P, Duchrow M, *et al.* (2005) Influence of thymidylate synthase and p53 protein expression on clinical outcome in patients with colorectal cancer. Int J Colorectal Dis 20:94–102.

Carreras CW, Santi DV (1995) The catalytic mechanism and structure of thymidylate synthase. Annu Rev Biochem 64:721–762.

Cascinu S, Graziano F, Valentini M, *et al.* (2001) Vascular endothelial growth factor expression, S-phase fraction and thymidylate synthase quantitation in node-positive colon cancer: relationships with tumor recurrence and resistance to adjuvant chemotherapy. Ann Oncol 12:239–244.

Chen J, Hunter DJ, Stampfer MJ, *et al.* (2003) Polymorphism in the thymidylate synthase promoter enhancer region modifies the risk and survival of colorectal cancer. Cancer Epidemiol Biomarkers Prev 12:958–962.

Danenberg PV (1977) Thymidylate synthetase—a target enzyme in cancer chemotherapy. Biochim Biophys Acta 473: 73–92.

Danenberg PV (2004) Pharmacogenomics of thymidylate synthase in cancer treatment. Front Biosci 9:2484–2494.

Edler D, Glimelius B, Hallström M, *et al.* (2002) Thymidylate synthase expression in colorectal cancer: a prognostic and predictive marker of benefit from adjuvant fluorouracil-based chemotherapy. J Clin Oncol 20:1721–1728.

Elsaleh H, Joseph D, Grieu F, *et al.* (2000) Association of tumour site and sex with survival benefit from adjuvant chemotherapy in colorectal cancer. Lancet 355:1745–1750.

Etienne MC, Chazal M, Laurent-Puig P, *et al.* (2002) Prognostic value of tumoral thymidylate synthase and p53 in metastatic colorectal cancer patients receiving fluorouracil-based chemotherapy: phenotypic and genotypic analyses. J Clin Oncol 20:2832–2843.

Fernández-Contreras ME, Sánchez-Hernández JJ, González E, *et al.* (2009) Combination of polymorphisms within 5′ and 3′ untranslated regions of thymidylate synthase gene modulates survival in 5 fluorouracil-treated colorectal cancer patients. Int J Oncol 34:219–229.

Formentini A, Henne-Bruns D, Kornmann M (2004) Thymidylate synthase expression and prognosis of patients with gastrointestinal cancers receiving adjuvant chemotherapy: a review. Langenbecks Arch Surg 389:405–413.

Hitre E, Budai B, Adleff V, *et al.* (2005) Influence of thymidylate synthase gene polymorphisms on the survival of colorectal cancer patients receiving adjuvant 5-fluorouracil. Pharmacogenet Genomics 15:723–730.

Horie N, Aiba H, Oguro K, *et al.* (1995) Functional analysis and DNA polymorphism of the tandemly repeated sequences in the 5′-terminal regulatory region of the human gene for thymidylate synthase. Cell Struct Funct 20:191–197.

Iacopetta B, Grieu F, Joseph D, Elsaleh H (2001) A polymorphism in the enhancer region of the thymidylate synthase promoter influences the survival of colorectal cancer patients treated with 5-fluorouracil. Br J Cancer 85:827–830.

Ichikawa W, Uetake H, Shirota Y, *et al.* (2003) Combination of dihydropyrimidine dehydrogenase and thymidylate synthase gene expressions in primary tumors as predictive parameters for the efficacy of fluoropyrimidine-based chemotherapy for metastatic colorectal cancer. Clin Cancer Res 9: 786–791.

Ju J, Pedersen-Lane J, Maley F, Chu E (1999) Regulation of p53 expression by thymidylate synthase. Proc Natl Acad Sci USA 96:3769–3774.

Kawakami K, Watanabe G (2003) Identification and functional analysis of single nucleotide polymorphism in the tandem repeat sequence of thymidylate synthase gene. Cancer Res 63:6004–6007.

Kornmann M, Link KH, Galuba I, *et al.* (2002) Association of time to recurrence with thymidylate synthase and dihydropyrimidine dehydrogenase mRNA expression in stage II and III colorectal cancer. J Gastrointest Surg 6: 331–337.

Kornmann M, Schwabe W, Sander S, *et al.* (2003) Thymidylate synthase and dihydropyrimidine dehydrogenase mRNA expression levels: predictors for survival in colorectal cancer patients receiving adjuvant 5-fluorouracil. Clin Cancer Res 9:4116–4124.

Kralovánszky J, Köves I, Orosz Z, *et al.* (2002) Prognostic significance of the thymidylate biosynthetic enzymes in human colorectal tumors. Oncology 62:167–174.

Kristensen MH, Weidinger M, Bzorek M, *et al.* (2010) Correlation between thymidylate synthase gene variants, RNA and protein levels in primary colorectal adenocarcinomas. J Int Med Res 38:484–497.

Laurie JA, Moertel CG, Fleming TR, *et al.* (1989) Surgical adjuvant therapy of large-bowel carcinoma: an evaluation of levamisole and the combination of levamisole and fluorouracil. The North Central Cancer Treatment Group and the Mayo Clinic. J Clin Oncol 7:1447–1456.

Leichman CG, Lenz HJ, Leichman L, *et al.* (1997) Quantitation of intratumoral thymidylate synthase expression predicts for disseminated colorectal cancer response and resistance to protracted-infusion fluorouracil and weekly leucovorin. J Clin Oncol 15:3223–3229.

Lurje G, Manegold PC, Ning Y, *et al.* (2009) Thymidylate synthase gene variations: predictive and prognostic markers. Mol Cancer Ther 8:1000–1007.

Mandola MV, Stoehlmacher J, Muller-Weeks S, *et al.* (2003) A novel single nucleotide polymorphism within the 5′ tandem repeat polymorphism of the thymidylate synthase gene abolishes USF-1 binding and alters transcriptional activity. Cancer Res 63:2898–2904.

Mandola MV, Stoehlmacher J, Zhang W, *et al.* (2004) A 6 bp polymorphism in the thymidylate synthase gene causes message instability and is associated with decreased intratumoral TS mRNA levels. Pharmacogenetics 14:319–327.

Marsh S, Collie-Duguid ES, Li T, *et al.* (1999) Ethnic variation in the thymidylate synthase enhancer region polymorphism among Caucasian and Asian populations. Genomics 58:310–312.

Marsh S, McKay JA, Cassidy J, McLeod HL (2001) Polymorphism in the thymidylate synthase promoter enhancer region in colorectal cancer. Int J Oncol 19:383–386.

Midgley RS, Yanagisawa Y, Kerr DJ (2009) Evolution of nonsurgical therapy for colorectal cancer. Nat Clin Pract Gastroenterol Hepatol 6:108–120.

Paradiso A, Simone G, Petroni S, *et al.* (2000) Thymidilate synthase and p53 primary tumour expression as predictive factors for advanced colorectal cancer patients. Br J Cancer 82:560–567.

Popat S, Matakidou A, Houlston RS (2004) Thymidylate synthase expression and prognosis in colorectal cancer: a systematic review and meta-analysis. J Clin Oncol 22:529–536.

Popat S, Wort R, Houlston RS (2006) Inter-relationship between microsatellite instability, thymidylate synthase expression, and p53 status in colorectal cancer: implications for chemoresistance. BMC Cancer 6:150.

Prall F, Ostwald C, Schiffmann L, Barten M (2007) Do thymidylate synthase gene promoter polymorphism and the C/G single nucleotide polymorphism predict effectiveness of adjuvant 5-fluorouracil-based chemotherapy in stage III colonic adenocarcinoma? Oncol Rep 18:203–209.

Quasar Collaborative Group, Gray R, Barnwell J, *et al.* (2007) Adjuvant chemotherapy versus observation in patients with colorectal cancer: a randomised study. Lancet 370:2020–2029.

Rahman L, Voeller D, Rahman M, *et al.* (2004) Thymidylate synthase as an oncogene: a novel role for an essential DNA synthesis enzyme. Cancer Cell 5:341–351.

Sulzyc-Bielicka V, Domagala P, Majdanik E, *et al.* (2009) Nuclear thymidylate synthase expression in sporadic colorectal cancer depends on the site of the tumor. Virchows Arch 454:695–702.

Tsuji T, Hidaka S, Sawai T, *et al.* (2003) Polymorphism in the thymidylate synthase promoter enhancer region is not an efficacious marker for tumor sensitivity to 5-fluorouracil-based oral adjuvant chemotherapy in colorectal cancer. Clin Cancer Res 9:3700–3704.

Wolpin BM, Mayer RJ (2008) Systemic treatment of colorectal cancer. Gastroenterology 134:1296–1310.

Xi Y, Nakajima G, Schmitz JC, *et al.* (2006) Multi-level gene expression profiles affected by thymidylate synthase and 5-fluorouracil in colon cancer. BMC Genomics 7:68.

Address correspondence to:
Marek Drozdzik, MD, PhD
Department of Clinical and Experimental Pharmacology
Pomeranian Medical University
Powstancow Wlkp 72
Szczecin 70-111
Poland

E-mail: drozdzik@pum.edu.pl