Prognostic implications of thymidylate synthase gene polymorphisms in patients with advanced small bowel adenocarcinoma treated with first-line fluoropyrimidine-based chemotherapy

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Abstract. Thymidylate synthase (*TS*) gene polymorphisms such as tandem repeat (TR) polymorphisms and single-nucleotide polymorphisms (SNPs) affect transcriptional efficiency of the *TS* gene and may be prognostic markers for fluoropyrimidine-based therapy in various gastrointestinal cancers. However, data for *TS* polymorphisms on clinical outcomes in advanced small bowel adenocarcinoma (SBA) are limited. We retrospectively enrolled 58 locally advanced/metastatic SBA patients treated with first-line fluoropyrimidine-based chemotherapy and analyzed the relationship between *TS* genotypes and clinical outcomes in 30 patients who were available for tumor tissue. Based on TR polymorphisms and a G>C SNP in the promoter region of the *TS* gene, 74% of patients had high TS expression genotypes (2R/3RG, 3RG/3RC, 3RG/3RG); the remainder had low TS expression genotypes (2R/2R, 2R/3RC, 3RC/3RC). After a median follow-up of 48.8 months, median progression-free survival (PFS) and overall survival (OS) in all patients were 6.0 and 11.3 months, respectively. However, patients with low TS expression genotypes had better median PFS (12.8 vs. 4.3 months, P=0.027) and OS (28.8 vs. 8.9 months, P=0.025) than those with high TS expression genotypes. In multivariate analysis, poor Eastern Cooperative Oncology Group performance status [hazard ratio (HR), 2.85; 95% CI, 1.02-7.93] and high TS expression genotypes (HR, 3.49; 95% CI, 1.13-10.78) were independent prognostic factors for worse OS. Therefore, *TS* genotypes, based on a G>C SNP in the TR sequence of the *TS* gene, may be a useful biomarker for predicting outcomes for fluoropyrimidine-based chemotherapy in patients with locally advanced/metastatic SBA.

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Abbreviations: CEA, carcinoembryonic antigen; CRC, colorectal cancer; CT, computed tomography; ECOG, Eastern Cooperative Oncology Group; IHC, immunohistochemistry; ORR, overall response rate; OS, overall survival; PCR, polymerase chain reaction; PFS, progression-free survival; RECIST, Response Evaluation Criteria in Solid Tumors; SBA, small bowel adenocarcinoma; SNP, single-nucleotide polymorphism; TR, tandem repeat; TS, thymidylate synthase; UTR, untranslated region; 5-FU, 5-fluorouracil

Key words: genotype, polymorphism, prognosis, small bowel adenocarcinoma, thymidylate synthase

Introduction

Small bowel adenocarcinoma (SBA) is a rare malignancy, accounting for <5% of all gastrointestinal cancers, and is associated with a poor prognosis (1). Standard chemotherapeutic regimens for SBA have not been established due to the lack of prospective randomized studies. Fluoropyrimidines,

including 5-fluorouracil (5-FU) and capecitabine, have been the most commonly studied cytotoxic chemotherapeutic agents for advanced SBA. Fluoropyrimidines in combination with oxaliplatin, irinotecan, or doxorubicin/mitomycin have shown clinical benefits in small phase II studies (2-5). In addition, several retrospective studies have revealed that pretreatment performance status (6-8), primary tumor site (7), and serum carcinoembryonic antigen (CEA) level (6,7) might have a prognostic role in patients receiving fluoropyrimidine-based chemotherapy. However, molecular markers reflective of tumor biology and predictive of chemotherapeutic sensitivity/resistance in patients with advanced SBA are lacking. Such molecular markers predicting treatment response will help physicians to select treatment strategies for this rare disease.

The antitumor effect of fluoropyrimidines has been associated with competitive inhibition of thymidylate synthase (TS) (9,10). Preclinical studies have shown that increased TS expression is associated with resistance to 5-FU in cancer cell lines (11,12). Subsequent clinical studies have demonstrated that TS expression may play an important role in determining tumor sensitivity to fluoropyrimidines (13-15). Therefore, the TS tissue level may modulate the efficacy of fluoropyrimidine therapy against tumor cells. Polymorphisms within the 5'-untranslated region (UTR) of the *TS* gene have been shown to be important in controlling TS expression. Polymorphisms in the 5'-UTR regulate *TS* transcription and have been suggested to be potential predictors of response to 5-FU chemotherapy (16-18). Two main polymorphisms in the 5'-UTR that modulate *TS* transcription have been described: the tandem repeat (TR) polymorphism and G>C single-nucleotide polymorphism (SNP). The TR polymorphism comprises double (2R) or triple (3R) repeats of a 28-base pair (bp) sequence in the 5'-UTR (16,17). This polymorphism is associated with *TS* gene transcription efficiency, which is lower with the double repeat than with the triple repeat (16). The G>C SNP in the second repeat of the 3R alleles is associated with decreased transcriptional activity of the 3R alleles (18). Consequently, the transcriptional efficiency of 3R alleles with the G>C substitution (3RC) is similar to that of 2R alleles. Recently, several studies have demonstrated that *TS* SNP status might be associated with clinical outcomes in colorectal cancer (CRC) patients receiving 5-FU-based chemotherapy or preoperative chemoradiotherapy (19-22). However, the prognostic and predictive relevance of *TS* gene polymorphisms in SBA have not yet been investigated. Thus, in the present study, we aimed to evaluate the prognostic implications of polymorphisms in the 5'-UTR of the *TS* gene on treatment outcomes in patients with locally advanced/metastatic SBA receiving fluoropyrimidine-based chemotherapy.

Materials and methods

Study design and patient population. We performed a multicenter, retrospective cohort study of patients with pathologically confirmed locally advanced (unresectable or incompletely resected) or metastatic SBA who received ≥1 cycle of first-line fluoropyrimidine-based chemotherapy at 10 medical centers in Korea between Jan 2003 and Dec 2012. Patients with localized or completely resected disease and those who received front-line treatment with non-fluoropyrimidine-containing chemotherapy were excluded. Patients with cancer of the ampullar of Vater or periampullary cancer were also excluded. Prior history of chemotherapy for metastatic disease was not allowed, but previous use of fluoropyrimidines was permitted if fluoropyrimidines were used as adjuvant therapy and the interval between completion of adjuvant therapy and recurrence of disease was more than 6 months. Data were collected using study-specific case record forms from participating institutions. Collected data included patient demographics, tumor characteristics, first-line chemotherapy regimens and dose intensities, response to first-line therapy, progression-free survival (PFS) and overall survival (OS). The study protocol was reviewed and approved by the institutional review board at each participating institution, and the study was conducted in accordance with the recommendations of the Declaration of Helsinki for biomedical research involving human subjects.

Treatment and outcome measurement. First-line chemotherapy consisted of the following 4 regimens: the cisplatin and fluoropyrimidines (FP) regimen, cisplatin (75 mg/m^2) day 1 plus infusional 5-FU (1,000 mg/m^2) for 4 consecutive days or oral capecitabine (1,000 mg/m^2 twice a day) for 14 consecutive days every 3 weeks; the FOLFOX regimen, 2-h infusion of oxaliplatin (85 or 100 mg/m^2) on day 1 plus folinic acid (200 mg/m^2) and bolus 5-FU (400 mg/m^2) followed by a 46-h infusion of 5-FU (2,400 mg/m^2) every 2 weeks; the FOLFIRI regimen, 90-min infusion of irinotecan (180 mg/m^2) on day 1 plus folinic acid (200 mg/m^2) and bolus 5-FU (400 mg/m^2) followed by a 46-h infusion of 5-FU (2,400 mg/m^2) every 2 weeks; fluoropyrimidine alone, protracted venous infusion of 5-FU using a portable pump at a dose of 300 mg/m^2/day or oral capecitabine 1,250 mg/m^2 twice daily on day 1 through 14 every 3 weeks. The treatment regimen was selected at the discretion of the treating physician. First-line chemotherapy was discontinued in patients who progressed during treatment or experienced unacceptable toxicities. Management of adverse events and subsequent dose reduction of chemotherapeutic agents was performed at the discretion of the physician based on hematologic or non-hematologic adverse events. Tumor responses were assessed in patients with measurable lesions using contrast-enhanced computed tomography (CT) every three or four cycles or earlier when signs of progression were evident. Objective tumor response was evaluated using the Response Evaluation Criteria in Solid Tumors (RECIST) criteria (23). PFS and OS were calculated from the day of diagnosis until progression, death or last follow-up as appropriate.

Immunohistochemistry (IHC) for TS. TS expression was analyzed in 3-μm-thick sections from pretreatment formalin-fixed paraffin-embedded (FFPE) tissues. Sections were deparaffinized and subjected to heat-induced antigen retrieval by microwaving in Tris-EDTA buffer (EnVision FLEX High pH; Dako, Glostrup, Denmark) for 15 min. Tissue sections were incubated with 3% hydrogen peroxide for 10 min to inhibit endogenous peroxidase activity. After washing in Tris-buffered saline, tissue sections were incubated with monoclonal anti-TS (TS-106; Dako) for 90 min at room temperature. TS immunohistochemical staining was detected using the Dako REAL EnVision Detection System. After

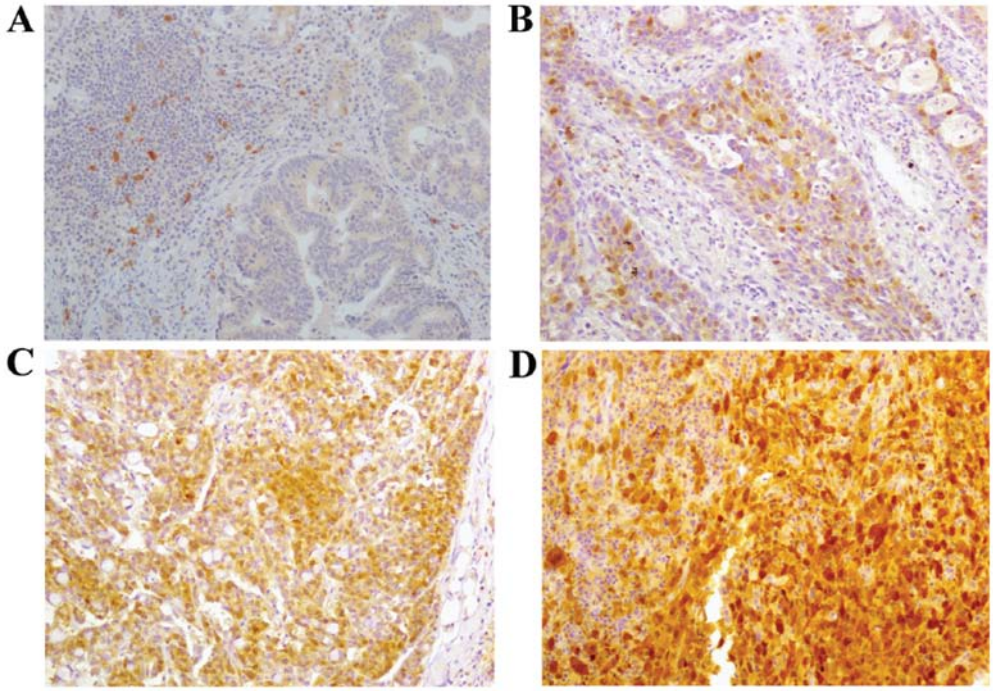

Figure 1. Representative examples of immunohistochemical staining for thymidylate synthase (TS) (magnification, x400). Immunohistochemistry results were graded by the cytoplasmic staining intensity as follows: (A) no staining, (B) 1+, (C) 2+ and (D) 3+. The grade of 2+ or above was regarded as positive expression for TS.

washing, the sections were incubated with the Envision kit for 30 min at room temperature and 3,3'-diaminobenzidine was applied as the chromogen. After washing, the tissue sections were counterstained with Mayer's hematoxylin. To ensure consistent staining, a positive control (an adenocarcinoma sample with well-characterized staining) and negative control (no primary antibody) were included in each staining run. Adjacent lymphocytes within each section were identified as internal references. Immunohistochemical staining was evaluated by a single pathologist blinded to the clinical parameters. The intensity of TS cytoplasmic staining was graded on a 4-point scale (0, 1+, 2+ and 3+), and a grade of ≥2+ was considered as positive (Fig. 1).

TS TR polymorphism and G>C SNP analysis

DNA extraction. Genomic DNA was prepared from FFPE tissue samples using the QIAamp DNA FFPE Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Briefly, the specimen was cut to 20-μm tissue sections using a microcutter, and three subsequent sections were transferred to microcentrifuge tubes. Then, PBS was added to each 20-μm tissue section and heated for 10 min at 75˚C. After centrifuging at 13,000 rpm, the supernatant was carefully discarded and fresh PBS was added. Tissue samples were then incubated with lysis buffer and proteinase K for 30 min at 56˚C. Subsequently, the mixture was applied to the spin column and centrifuged into a collection tube according to the manufacturer's protocol. The purified DNA was used for subsequent analyses.

TS genotyping. *TS* TR polymorphism and G>C SNP were identified by capillary electrophoresis and direct sequencing of genomic DNA, respectively. For the *TS* TR assay, DNA was amplified by polymerase chain reaction (PCR) using the HotStarTaq Master Mix kit (Qiagen) with the primer pair 5'-FAM-GTGGCTCCTGCGTTTCCCCC-3' (forward) and 5'-GAGCCGGCCACAGGCATG-3' (reverse). Reactions were set up in a final volume of 25 μl with 50 ng of DNA and primers and performed in the 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The PCR conditions were as follows: initial denaturation at 94˚C for 15 min, followed by 35 cycles of denaturation at 95˚C for 30 sec, primer annealing at 60˚C for 30 sec, and extension at 72˚C for 1 min and final extension at 72˚C for 5 min. The PCR product (1 μl) was added to a mixture of diformamide and GeneScan -500 LIZ Size Standard (Applied Biosystems), denatured at 95˚C for 2 min, and analyzed using the ABI 3130xl Genetic Analyzer (Applied Biosystems). A peak at 210 base (2R/2R), 238 base (3R/3R), or both of these peaks (2R/3R) were obtained depending on the *TS* TR status.

Next, to reveal the *TS* SNP status in the samples containing 3R in the *TS* TR assay, direct sequencing was performed. PCR for genomic DNA amplification was performed as described for the *TS* TR assay using the following primer pair: 5'-GT GGCTCCTGCGTTTCCCCC-3' (forward) and 5'-GCTCCG AGCCGGCCACAGGCATGGCGCGG-3' (reverse). Purified PCR products were obtained using an Exonuclease I and Shrimp Alkaline Phosphatase mixture (Fermentas, Vilnius, Lithuania) and subsequently sequenced using the ABI PRISM BigDye Terminator version 3.1 kit (Applied Biosystems). Forward and reverse sequences were analyzed separately under the same conditions with the same primers used in the PCR reaction. Cycle sequencing was performed for 25 cycles of 96˚C for 30 sec, 50˚C for 15 sec, and 60˚C for 4 min. Sequencing analysis was performed using the ABI 3130XL Genetic Analyzer (Applied Biosystems).

Table I. Patient and tumor characteristics according to *TS* SNP status.

		N=30[a]		
	Total (n=58)	Low TS expression genotypes (n=8)	High TS expression genotypes (n=22)	P-value
Gender				
Male	39 (67)	5 (62.5)	12 (54.5)	1.0
Female	19 (33)	3 (37.5)	10 (45.5)	
Age, years				
Median	61	62	63.5	0.906
Range	32-83	55-72	32-76	
F/U duration (months)				
Median	48.8	38.2	48.5	0.888
Range	3.3-121.9	16.8-113.3	3.3-121.9	
ECOG performance status				
0-1	42 (72)	7 (87.5)	16 (72.7)	0.638
2-3	16 (28)	1 (12.5)	6 (27.3)	
Disease status				
Metastatic	43 (74)	7 (87.5)	16 (72.7)	0.638
Locally advanced	15 (26)	1 (12.5)	6 (27.3)	
Tumor location				
Duodenum	50 (86)	8 (100)	18 (81.8)	0.550
Jejunum/ileum	8 (14)	0 (0)	4 (18.2)	
Pathologic differentiation				
Well	9 (16)	3 (37.5)	4 (18.2)	0.352
Moderate	23 (40)	2 (25.0)	7 (31.8)	
Poor	16 (28)	1 (12.5)	9 (40.9)	
Unknown	10 (17)	1 (12.5)	2 (9.1)	
Pretreatment CEA level (ng/ml)				
Median	2.2	1.8	2.5	0.489
Range	0.5-100.0	1.2-14.8	0.5-100.0	
Prior fluoropyrimidine use				
No	46 (79)	7 (87.5)	17 (77.3)	1.0
Yes	12 (21)	1 (12.5)	5 (22.7)	
First-line chemotherapy				
Cisplatin + fluoropyrimidine	31 (53)	6 (75.0)	14 (63.6)	0.837
Oxaliplatin + fluoropyrimidine	11 (19)	1 (12.5)	5 (22.7)	
Irinotecan + fluoropyrimidine	6 (10)	0 (0)	1 (4.5)	
Fluoropyrimidine alone	10 (17)	1 (12.5)	2 (9.1)	
No. of chemotherapy cycles				
Median	4	9	3.5	0.003
Range	1-50	3-50	1-10	

Values are expressed as the number of patients (%) unless stated otherwise. [a]Patients who were available for *TS* genotyping were analyzed. Based on *TS* TR and SNP status, patients were classified into low TS expression genotypes (2R/2R, 2R/3RC, and 3RC/3RC) and high TS expression genotypes (2R/3RG, 3RC/3RG, and 3RG/3RG). TS, thymidylate synthase; SNP, single-nucleotide polymorphism; F/U, follow-up; ECOG, Eastern Cooperative Oncology Group; CEA, carcinoembryonic antigen.

Based on the *TS* TR and SNP status, *TS* genotypes were classified as high TS expression genotypes (2R/3RG, 3RC/3RG and 3RG/3RG) or low TS expression genotypes (2R/2R, 2R/3RC and 3RC/3RC).

Statistical analysis. PFS and OS were calculated using the Kaplan-Meier method and compared using the log-rank test. Clinical variables, such as age, gender, disease status, Eastern Cooperative Oncology Group (ECOG) performance status,

Table II. TS protein expression according to *TS* TR and SNP status.

	TS TR status			
TS protein expression[a]	2R/2R, 2R/3R	3R/3R	Total	P-value
Negative	9 (69)	7 (41)	16 (9)	0.127
Positive	4 (31)	10 (59)	14 (10)	

	TS SNP status								
	Low-expression genotypes				High-expression genotypes				
TS protein expression[a]	2R/2R	2R/3RC	3RC/3RC	Total	2R/3RG	3RC/3RG	3RG/3RG	Total	P-value
Negative	2 (67)	2 (100)	3 (100)	7 (88)	5 (63)	2 (33)	2 (25)	9 (41)	0.039
Positive	1 (33)	0 (0)	0 (0)	1 (13)	3 (38)	4 (67)	6 (75)	13 (59)	

Values are expressed as the number of patients (%). [a]TS protein expression was evaluated by the intensity of TS immunohistochemical staining. TS, thymidylate synthase; SNP, single-nucleotide polymorphism; TR, tandem repeat.

location of the primary tumor, histologic grade, pretreatment CEA and systemic chemotherapy were included for analysis. Descriptive statistics were summarized as frequencies and percentages for categorical variables and as median and range for continuous variables. Comparison of clinical variables according to *TS* genotypes and comparisons between TS immunohistochemical staining intensity and *TS* polymorphisms (TR, SNP) and tumor response were made using χ^2 test or Fisher's exact test. Multivariate analysis was carried out using the Cox proportional hazards models. Variables with P<0.05 in the univariate analysis were included in the multivariate model using a forward conditional method, and hazard ratio (HR) and 95% confidence interval (CI) were calculated. A two-tailed P-value of <0.05 was considered statistically significant. All data analyses were carried out using SPSS software (SPSS, Inc., Chicago, IL, USA).

Results

Patient cohort. We identified 64 patients at 10 Korean institutions who were diagnosed with locally advanced/metastatic SBA and treated with first-line fluoropyrimidine-based regimens between 2003 and 2012. Of the 64 patients, four patients were not included because they were initially treated with fluoropyrimidines as a radiosensitizer during concurrent chemoradiotherapy. Two patients were excluded, because they did not complete their first cycle of capecitabine monotherapy owing to non-hematologic toxicity. Thus, 58 patients were included in this analysis.

Patient characteristics. The demographic and clinical characteristics of the patients are listed in Table I. Of the 58 patients, 39 (67%) were male and 19 (33%) were female, with a median age of 61 years (range, 32-83 years). The most common primary tumor location was the duodenum (50/58, 86%). Metastatic disease was present in 43 patients (74%), and the ECOG performance status was 0 or 1 in 42 patients (72%). The median CEA level was 2.2 ng/ml (range, 0.5-100.0 ng/ml). Twelve patients (21%) had a history of prior fluoropyrimidine adjuvant therapy. Thirty-one patients (53%) received the FP regimen, 11 patients (19%) received the FOLFOX regimen, 10 patients (17%) received fluoropyrimidine alone, and 6 patients (10%) received the FOLFIRI regimen as first-line chemotherapy.

Association between immunohistochemical expression of TS and TS genotype. TS IHC and genotyping were successfully performed in 30 cases (52%); the remaining 28 cases lacked sufficient tissue samples for analysis. TS immunohistochemical staining exhibited a predominantly diffuse pattern throughout the nucleus and cytoplasm of the tumor cells. TS immunohistochemical staining intensity was follows: 0 in 8 cases (27%), 1+ in 8 cases (27%), 2+ in 11 cases (37%), and 3+ in 3 (8%). Among these cases, 14 (47%) were considered positive for TS expression (≥2+). The *TS* TR status was 2R/2R in 3 patients (10%), 2R/3R in 10 patients (33%), and 3R/3R in 17 patients (57%). The SNP status was 2R/3RC in 2 patients (7%), 2R/3RG in 8 patients (27%), 3RC/3RC in 3 patients (10%), 3RC/3RG in 6 patients (20%), and 3RG/3RG in 8 patients (27%). Based on the *TS* SNP status in the TR sequence, 22 patients (73%) had high TS expression genotypes (2R/3RG, 3RC/3RG, 3RG/3RG) and the remaining 8 patients had low TS expression genotypes (2R/2R, 2R/3RC, 3RC/3RC; Table II).

The prevalence of positive TS immunohistochemical expression was not significantly different between patients with the 3R/3R genotype and those with the 2R/2R and 2R/3R genotypes [59% (10/17) vs. 31% (4/13); P=0.127; Table II]. In contrast, the prevalence of positive TS immunohistochemical expression was significantly higher in patients with high TS expression genotypes than in those with low TS expression genotypes (59 vs. 13%; P=0.039; Table II).

Correlations of TS protein expression and genotype with treatment response, PFS and OS. Tumor response was evaluable in 29 (97%) of the 30 patients in whom TS immunohistochemical expression and genotype data were available. One patient did

Table III. Treatment response according to TS protein expression and *TS* TR and SNP status.

		TS protein expression			*TS* TR status			*TS* SNP status		
Treatment response	Total	Negative	Positive	P-value	2R/2R, 2R/3R	3R/3R	P-value	Low-expression genotypes	High-expression genotypes	P-value
CR/PR	10 (35)	7 (47)	3 (21)	0.245	5 (42)	5 (29)	0.694	5 (71)	5 (23)	0.030
SD/PD	19 (66)	8 (53)	11 (79)		7 (58)	12 (71)		2 (79)	17 (77)	

Values are expressed as the number of patients (%). TS, thymidylate synthase; TR, tandem repeat; SNP, single-nucleotide polymorphism; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

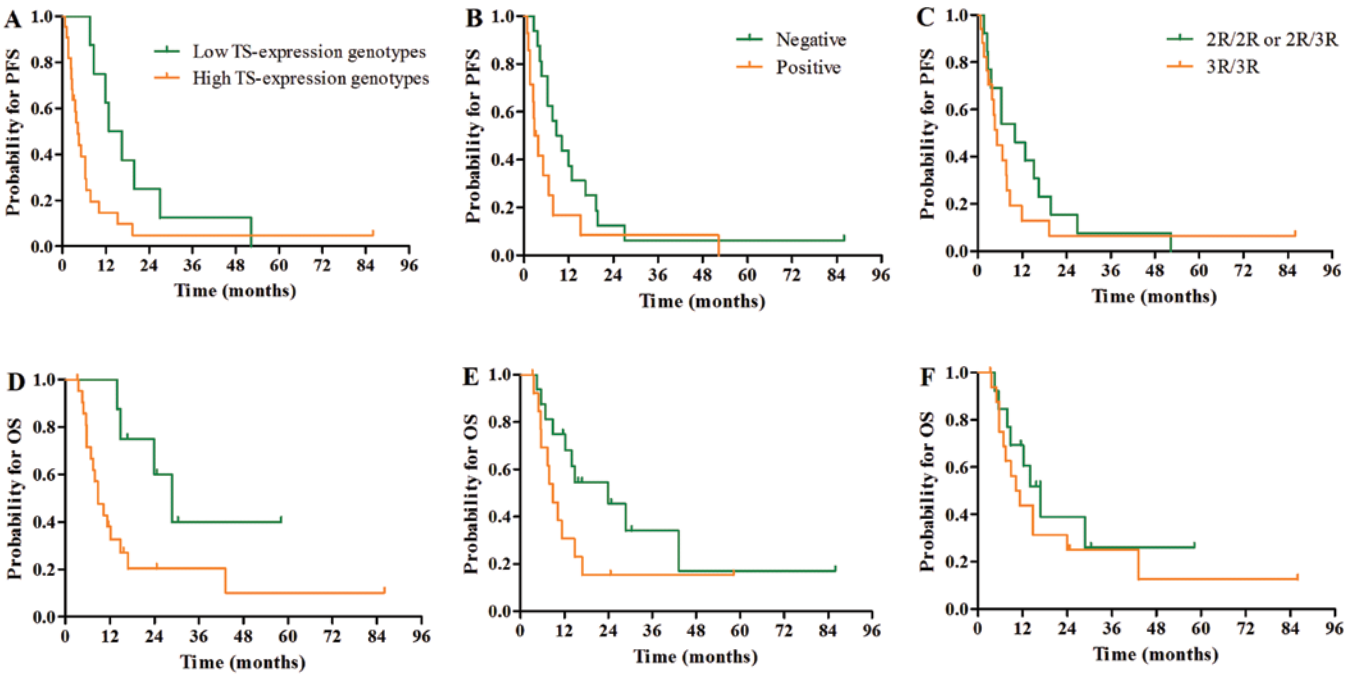

Figure 2. Comparison of progression-free survival (PFS, upper) and overall survival (OS, lower) according to the thymidylate synthase (*TS*) genotypes based on G>C single-nucleotide polymorphism (SNP) within the tandem repeat (TR) sequence, TS immunohistochemistry, and *TS* TR polymorphism. (A) PFS and (D) OS were significantly longer in patients with low TS expression genotypes than in those with high TS expression genotypes. A non-significant trend towards a better (B) PFS and (E) OS was observed in patients with negative TS protein expression. *TS* TR polymorphism status did not significantly affect (C) PFS or (F) OS.

not have any evaluable lesions at baseline. Among the evaluable patients, 10 patients (1 complete response and 9 partial response) responded to treatments, resulting in an overall response rate (ORR) of 35%. The ORR was not significantly different according to TS protein expression and TR polymorphism status (Table III). However, the ORR was significantly higher in patients with low TS expression genotypes than in those with high TS expression genotypes (71 vs. 23%; P=0.030; Table III).

With a median follow-up duration of 48.8 months (range, 3.3-121.9 months), median PFS and OS were 6.0 months (95% CI, 4.5-7.5) and 11.3 months (95% CI, 8.1-14.5), respectively. Median PFS and OS were 12.8 months (95% CI, 6.4-19.2) and 28.8 months (95% CI, 19.2-38.4) in patients with low TS expression genotypes and 4.3 months (95% CI, 2.9-5.7) and 8.9 months (95% CI, 5.5-12.3) in patients with high TS expression genotypes, respectively. Patients with low TS expression genotypes had significantly better PFS and OS than those with high TS expression genotypes (PFS, P=0.027; OS, P=0.025; Fig. 2A and D).

Although there was a trend towards a better PFS (median, 8.7 vs. 2.8 months; P=0.060) and OS (median, 24.0 vs. 8.9 months; P=0.090) in favor of the negative TS protein expression group, PFS and OS were not significantly different according to TS protein expression (Fig. 2B and E) and TS TR polymorphism status (Fig. 2C and F).

The results of the univariate and multivariate analyses for PFS and OS are shown in Tables IV and V. In univariate analysis, ECOG performance status (P<0.001) and *TS* SNP status (P=0.025) were significantly associated with OS (Table IV). In the multivariate analysis, poor ECOG performance status (HR, 2.85; 95% CI, 1.02-7.93) and high TS expression genotypes (HR, 3.49; 95% CI, 1.13-10.78) were independent prognostic factors for worse OS (Table V).

Table IV. Univariate analysis for PFS and OS.

Variables	No. of patients	Median PFS (ms, 95% CI)	P-value	Median OS (ms, 95% CI)	P-value
Age at diagnosis (years)					
≤60	27	6.4 (4.1-8.7)	0.910	14.8 (11.1-18.5)	0.995
>60	31	5.2 (3.0-7.4)		10.2 (7.7-12.7)	
Gender					
Male	39	6.0 (4.2-7.8)	0.977	12.2 (8.4-16.0)	0.630
Female	19	6.4 (2.6-10.2)		14.0 (6.1-21.9)	
ECOG performance status					
0-1	42	7.3 (5.8-8.8)	<0.001	14.8 (11.6-18.0)	<0.001
2-3	16	2.7 (2.1-3.3)		5.0 (3.4-6.6)	
Disease status					
Locally advanced	15	8.7 (0.1-17.3)	0.064	19.2 (5.5-32.9)	0.265
Metastatic	43	5.5 (3.4-7.6)		10.6 (9.0-12.2)	
Tumor location					
Duodenum	50	6.3 (4.8-7.8)	0.277	11.3 (7.2-15.4)	0.863
Jejunum/ileum	8	3.8 (3.3-4.3)		12.2 (8.9-15.5)	
Pathologic differentiation					
Well	9	11.9 (4.7-19.1)	0.104	24.0 (0.2-47.8)	0.284
Moderate	23	3.9 (2.0-5.8)		11.1 (6.7-15.5)	
Poor	16	4.3 (1.7-6.9)		10.6 (8.4-12.7)	
Pretreatment CEA level (ng/ml)					
≤2.2	23	3.8 (3.4-4.2)	0.364	11.1 (5.8-16.4)	0.860
>2.2	22	6.3 (4.8-7.8)		8.9 (4.1-13.7)	
Chemotherapy					
Cisplatin + fluoropyrimidine	31	6.7 (5.3-8.1)	0.128	14.0 (9.3-18.7)	0.083
Oxaliplatin + fluoropyrimidine	11	3.9 (2.2-5.6)		11.3 (2.6-20.0)	
Irinotecan + fluoropyrimidine	6	3.7 (2.0-5.4)		4.7 (0-9.6)	
Fluoropyrimidine alone	10	5.5 (0-25.1)		30.5 (0-75.6)	
Prior fluoropyrimidine use					
No	46	5.8 (3.9-7.7)	0.642	10.6 (6.5-14.7)	0.952
Yes	12	6.3 (1.5-11.1)		13.6 (9.5-17.7)	
TS protein expression					
Negative	16	8.7 (4.0-13.4)	0.060	24.0 (9.6-38.4)	0.090
Positive	14	2.8 (0.9-4.7)		8.9 (5.7-12.1)	
TS TR status					
2R/2R, 2R/3R	13	10.1 (2.0-18.2)	0.336	16.9 (10.3-23.5)	0.397
3R/3R	17	5.2 (3.5-6.9)		10.2 (5.5-14.9)	
TS SNP status					
Low expression genotypes	8	12.8 (6.4-19.2)	0.027	28.8 (19.2-38.4)	0.025
High expression genotypes	22	4.3 (2.9-5.7)		8.9 (5.5-12.3)	

PFS, progression-free survival; OS, overall survival; CI, confidence interval; ms, months; ECOG, Eastern Cooperative Oncology Group; CEA, carcinoembryonic antigen; TS, thymidylate synthase; TR, tandem repeat; SNP, single-nucleotide polymorphism.

Discussion

Thymidylate synthase (TS) is a target of fluoropyrimidines, and TS expression has been demonstrated to be a determinant of fluoropyrimidine sensitivity *in vitro* (11,12). Subsequent clinical studies have also suggested that the TS level is associated with fluoropyrimidine sensitivity (13-15). Fluoropyrimidines have been essential backbone drugs for the treatment of gastrointestinal malignancies, including SBA (24). Since SBA is a rare malignancy, it may be meaningful to identify biologic

Table V. Multivariate analysis for PFS and OS.

Variables	HR	95% CI	P-value
PFS			
ECOG performance status			
0-1	1		
2-3	2.70	1.08-6.75	0.034
TS SNP status			
Low expression genotypes	1		
High expression genotypes	2.47	1.05-5.79	0.038
OS			
ECOG performance status			
0-1	1		
2-3	2.85	1.02-7.93	0.046
TS SNP status			
Low expression genotypes	1		
High expression genotypes	3.49	1.13-10.78	0.030

PFS, progression-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; ECOG, Eastern Cooperative Oncology Group; TS, thymidylate synthase; SNP, single-nucleotide polymorphism.

prognostic factors for current therapy. However, to date, data regarding the prognostic role of TS in patients with SBA receiving fluoropyrimidine-based chemotherapy are lacking. Therefore, we investigated the impact of immunohistochemical expression of TS protein and TS 5'-UTR polymorphisms on treatment outcomes of fluoropyrimidine-based chemotherapy in patients with advanced SBA. To the best of our knowledge, this is the first study to investigate the role of drug sensitivity in regards to TS protein expression and *TS* polymorphisms (SNP and TR) in patients with advanced SBA.

In the present study, we demonstrated that the *TS* genotype, identified by the G>C SNP status in the *TS* TR sequence, may correlate with TS protein expression and influence ORR, PFS, and OS in patients with locally advanced/metastatic SBA treated with first-line fluoropyrimidine-based chemotherapy. Notably, the present study also showed that the *TS* TR polymorphism alone is not a sufficient marker to predict response to fluoropyrimidine-based chemotherapy. These findings are consistent with recent studies demonstrating that TS activity was regulated by the G>C SNP as well as the TR polymorphism in the 5'-UTR of the *TS* gene (19-21). The number of TR in the *TS* gene is associated with TS activity. The translational efficiency of *TS* mRNA is generally three to four times higher with the 3R sequence than with the 2R sequence (16,17,25). Recent clinical studies in patients with metastatic CRC have shown that patients with the 3R/3R genotype were significantly associated with a poorer response to 5-FU than those containing the 2R genotype (26,27). However, some studies have found no association between *TS* TR polymorphism and response to 5-FU-based chemotherapy (28,29). A G>C SNP in the second repeat of the 3R alleles may partially explain these discrepancies (18). Functional analysis has demonstrated that translational efficiency of the 3R sequence is three to four times greater without the G>C SNP than with the G>C SNP (18,19). As a result, the 3RC alleles may have a similar translational efficiency to that of the 2R alleles.

Given that fluoropyrimidine-based cytotoxic chemotherapy is currently regarded as the mainstay of treatment in patients with advanced SBA (24), the present study suggests that the *TS* genotype may serve as a biomarker of response to fluoropyrimidine-based chemotherapy. In this analysis, median OS among patients with low TS expression genotypes was more than 28 months. Thus, the therapeutic approach in these patients should take into account not only an efficacy of chemotherapeutic agents but also toxicity and quality-of-life implications. Therefore, trials involving such patients should incorporate both patient-reported quality-of-life end points and survival parameters to aid therapeutic decision making. On the other hand, the outcomes of patients with high TS expression genotypes were dismal; thus, the development of novel targeted treatment strategies is urgently needed for this group of patients. Although targeted therapies incorporating bevacizumab or cetuximab are commonly used for the treatment of CRC, the role of targeted therapies in advanced SBA has not yet been established. However, studies investigating the molecular pathology of SBA suggest that targeted therapies hold promise in the treatment of this disease. Notably, vascular endothelial growth factor is highly expressed in SBA, and the frequency of *KRAS* mutations is similar in SBA and CRC (30-32). Furthermore, several case series have reported the promising efficacy of cetuximab in patients with *KRAS* wild-type SBA (33,34). Therefore, the *TS* genotype will allow for the identification of patients with a poor prognosis who preferentially require novel targeted treatment other than fluoropyrimidine-based cytotoxic chemotherapy. Prospective studies are urgently needed to validate our results.

Even though data on the *TS* genotypes in patients with SBA are lacking, previous studies in patients with CRC have suggested the ethnic difference in the distribution of *TS* genotypes (35). In the present study, 3R/3R was the most prevalent allele with a frequency of 57%, whereas 2R/2R and 2R/3R occurred at frequencies of 10 and 33%, respectively. Furthermore, the frequency of high TS expression genotypes was much higher than that of low TS expression genotypes (73 vs. 27%). This result is consistent with previous studies of Korean CRC patients (22,36). However, the proportion of patients with high and low TS expression genotypes was reported to be similar in Caucasian ethnic group (18,20,35). Because of the small sample size, we could not conclusively predict the difference in the distribution of *TS* genotypes according to ethnicity. Therefore, further studies are required to understand whether this difference may affect the different prognosis following fluoropyrimidine-based chemotherapy between Asian and Caucasian ethnic groups.

The results of the present study suggest that the *TS* genotype based on both *TS* SNP and TR status is correlated with the immunohistochemical expression of TS protein. However, the results should be interpreted with caution, since previous studies investigating immunohistochemical expression of TS as a surrogate marker for TS expression have been inconclusive. Until recently, the predictive value of TS expression for response to 5-FU-based chemotherapy was mainly investigated in metastatic CRC (37,38). These studies produced conflicting rather than conclusive results. Recent meta-analyses concluded

that advanced CRC with high TS expression levels seem to be less sensitive to fluoropyrimidine-based chemotherapy, although evidence of heterogeneity and possible publication bias was observed (39,40). Differences in methodology and TS status assessment criteria might have contributed to the heterogeneity between studies (39). The most commonly used method to determine TS expression is IHC. IHC is a convenient technique with multiple advantages, thus making it a desirable approach for biomarker study. However, significant limitations exist due to the variability in its technical aspects, such as the antibodies used and staining technique. Additionally, differences in the IHC scoring methods used to dichotomize TS expression might contribute to the conflicting results (39,40). Therefore, TS immunohistochemical expression as a surrogate biomarker of TS expression might need further clarification and validation in patients with advanced SBA.

The present study has several limitations. First, the present analysis is based on retrospective data obtained from a small number of patients; therefore, unexpected selection bias may be present. Second, our study focused solely on the prognostic role of the G>C SNP and TR polymorphism of the 5'-UTR of the *TS* gene in SBA patients treated with fluoropyrimidines-based chemotherapy. However, the effects of other genetic polymorphism of the *TS* gene (i.e., 6-bp deletion in the 3'-UTR) were not investigated in this analysis. Moreover, an evaluation of the impact of other fluoropyrimidine metabolic enzymes, such as thymidylate phosphorylase, dihydropyrimidine dehydrogenase, and methylenetetrahydrofolate reductase, on treatment outcomes was beyond the scope of this study. Additional studies are necessary to examine the impact of these enzymes on clinical outcomes in patients with advanced SBA. Finally, prospective randomized studies are needed to validate our results. However, the rarity of advanced SBA makes randomized trials virtually difficult. Hence, cooperation between study groups is crucial to conduct such studies. Nevertheless, our study is the first to demonstrate the prognostic relevance of the *TS* genotype in patients with advanced SBA treated with first-line fluoropyrimidine-based chemotherapy.

In conclusion, the *TS* genotype based on G>C SNP and TR polymorphism appears to be an independent predictor of PFS and OS in patients with locally advanced/metastatic SBA treated with first-line fluoropyrimidine-based chemotherapy. Our findings represent a promising step toward the optimization of treatment strategies in advanced SBA. Further prospective studies with international collaboration are required to verify the prognostic role of the *TS* genotype in advanced SBA.

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