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From Cell Biology to Tissue Engineering

Immunohistochemical analysis of thymidylate synthase expression in gastric carcinoma: correlation with clinicopathological parameters and survival

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Summary. The correlation of thymidylate synthase (TS) expression in gastric cancers with tumor histology and prognostic or predictive information remains unclear. Most studies have involved Asian populations, with few conducted in European cohorts. Moreover, all published studies analyze TS expression using semi-quantitative methods. This retrospective study evaluated the association of TS expression in tumor cells with gastric carcinoma histological type, with selected clinico-pathological parameters, and with the prognosis of patients who underwent surgical treatment. TS expression was detected using immunochemistry and objectively assessed by computerized image analysis of tumor cells in 100 gastric cancers. We found that high TS expression was significantly more common in intestinal than in diffuse type of gastric cancer according to Lauren classification (P=0.0003); in type I carcinomas compared to type IV according to Goseki classification (P=0.002); and in gastric cancers in men than women (P=0.04). Low TS expression was found more often in carcinomas in the middle and lower third of the stomach than in cancers in the upper third of the stomach (P=0.009 and P=0.001, respectively). In the subgroup of 25 patients without lymph node metastases (stage I+II), high TS expression was associated with better DFS (83% for high TS expression versus 38,5% for low TS expression, P=0.03). The results: (1) indicate significant correlation between the Lauren and Goseki histopathological classifications of gastric cancer and TS expression in tumor cells, (2) suggest that high TS expression may be a positive prognostic marker with regard to DFS in patients with gastric cancer without involvement of regional lymph nodes who underwent radical surgical treatment and were not treated with preoperative chemotherapy. Prognostic results need confirmation in larger cohorts.

Key words: Thymidylate synthase, Gastric carcinoma, Prognostic factors, Lauren, Goseki

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Introduction

Gastric cancer is characterized by a variety of histological types and clinical courses. In gastric cancer, the most reliable prognostic factor is clinical stage based on TNM classification. However, the TNM system does not include morphological, histological and molecular factors that may have prognostic significance. Thymidylate synthase (TS) is one of the principle enzymes involved in DNA synthesis and repair. Specifically, TS is responsible for the *de novo* synthesis of pyrimidine nucleotides and serves as a molecular target of 5-fluorouracil, a cytostatic drug of the fluoropyrimidine group that is most often used in the treatment of gastric cancer (Montfort and Weichsel, 1997).

Studies suggest that TS expression in digestive tract cancers may correlate with prognosis and may be a

predictive factor for the outcome of 5-fluorouracil-based chemotherapy (Johnston et al., 1995; Tsouroufl is et al., 2008; Sulżyc-Bielicka et al., 2014). However, the results of previous studies that have analyzed the prognostic and predictive significance of TS expression in gastric cancers have been conflicting. Some studies found a correlation between high TS expression and poorer patient prognosis (Kuniyasu et al., 1998; Suda et al., 1999a; Tsujitani et al., 2000; Grau et al., 2004), while others found no association between TS expression and prognosis (Choi et al., 2001; Skarlos et al., 2007; Lee et al., 2008; Kim et al., 2009). Most of the studies involved Asian populations, with only a few conducted in European cohorts (Grau et al., 2004; Skarlos et al., 2007). Furthermore, virtually nothing is known regarding the association of TS expression with histological type of gastric carcinoma. Moreover, all of the published studies analyzed TS expression subjectively using semi-quantitative methods, which may have influenced the results.

The aim of this study was to evaluate the association of TS expression in tumor cells, which was quantified objectively using computerized image analysis, with the histological type of gastric carcinoma according to Lauren and Goseki classifications, with selected clinicopathological parameters and with the prognosis of patients who underwent surgical treatment for gastric cancer.

Materials and methods

Ethics

The Research Ethics Committee of the Pomeranian Medical University approved this study according to national regulations (decision nr KB-0080/105/09).

Patients

This retrospective study was based on tumor tissue from 100 unselected consecutive patients with a histological diagnosis of gastric carcinoma who underwent gastric resection between 1994 and 2006 in the Departments of General and Hand Surgery and Hepatobiliary Surgery of the teaching hospitals of the Pomeranian Medical University in Szczecin, Poland. A total of 59 patients underwent radical surgical treatment: 32 underwent total and 27 underwent subtotal gastrectomy with lymphadenectomy, with no metastases apparent in the microscopic evaluation. A total of 41 patients underwent palliative surgical treatment: 26 total and 15 subtotal gastrectomy with tumor mass removal but without resection of all metastases (i.e. R1 resection) because intraoperative macroscopic and/or microscopic evaluation showed tumor dissemination. There were 31 women and 69 men with a mean age of 59 years (range, 16–78 years; median, 61 years). Before surgery, the patients were not treated with chemotherapy or radiotherapy. Post-surgery, 39 patients received adjuvant chemotherapy: 26 patients were treated with the EAP regimen (epirubicin 20 mg/m^2, cisplatin 40 mg/m^2, etoposide 120 mg/m^2); 3 patients were treated with the PELF regimen (cisplatin 30 mg/m^2, calcium folinate 100 mg/m^2, 5-FU 500 mg/m^2, epirubicin 50 mg/m^2); and 10 patients received 5-fluorouracil and cisplatin (5-fluorouracil 1000 mg/m^2, cisplatin 100 mg/m^2).

In this cohort of 100 patients, 99 completed follow-up. One patient who underwent palliative surgical treatment was lost to follow-up. Time from the surgery until the time of death due to cancer or to last known follow-up was regarded as overall survival (OS), and the time until the first appearance of metastasis or local recurrence was regarded as disease-free survival (DFS). The median OS and DFS were 71 and 42 months respectively in the group of patients who underwent radical surgery (n=59), and 76.5 and 46 months respectively in the subgroup of these patients with negative lymph nodes (n=25).

DFS was not analyzed in 40 patients who underwent palliative surgical treatment because this subgroup had *a priori* poor prognosis. During 3 years of follow-up, 60 of the 99 (60.6%) patients died of gastric cancer, while 39 (39.4%) were alive and symptom-free. Of those who died, 23 underwent radical surgical treatment and 37 underwent palliative treatment. Of those who survived, 36 underwent radical surgery, while 3 underwent palliative treatment.

Pathology and immunohistochemistry

Tumor tissue from 100 gastric carcinomas was fixed in buffered 10% formalin and embedded in paraffin. Sections (4-μm thick) were stained with hematoxylin and eosin for histopathological diagnosis. Pathology review was conducted by two pathologists (PD, WD) who were associated with the study. The slides were deparaffinized and rehydrated, and the endogenous peroxidase activity was blocked. Next they were immersed in pH 9.0 buffer, and heat-induced antigen retrieval was performed in a pressure cooker (Pascal, Dako, Glostrup, Denmark). Monoclonal TS-106 antibody was used (Chemicon, Temecula, CA, USA; dilution 1:50; incubation time 30 min), and the slides were immunostained using a Dako EnVision™ kit according to the manufacturer's instructions (EnVision™ + peroxidase anti-mouse polymer labeled with horseradish peroxidase; Dako Co., Carpinteria, CA, USA). We used sensitive EnVision visualization system because detection methods using signal amplification with HRP-labeled polymers have been shown to be more sensitive than systems without such a layer of amplification. The reaction was developed with a diaminobenzidine substrate-chromogen solution and counterstained with hematoxylin. Strong positive staining in stromal macrophages, lymphocytes and plasma cells served as a built-in positive control. Appropriate positive and negative controls were run as well.

Image analysis

Microscope slides were scanned using an Aperio ScanScope CS (Aperio, Vista, CA, USA) using a 20×/0.75 Plan Apo lens. The scanned images were analyzed by a pathologist (PD) with ImageScope™ (Aperio) software using an algorithm for automatic analysis of the immunochemistry marker's expression (Aperio IHC algorithm analysis v. 9.0). This software enables the evaluation of TS expression in both nucleus and cytoplasm. Unselected peripheral tumor areas were marked for analysis (Fig. 1A) and a minimum of 1000 carcinoma cells, were analyzed. Those areas were marked manually; thus, only carcinoma cells were assessed, and benign stromal cells were not analyzed (Fig. 1A). Furthermore, tumor cells were automatically discriminated from benign cells (e.g. lymphocytes, fibroblasts) based upon size and shape (roundness, compactness, elongation) algorithm parameters.

The intensity of the nuclear and cytoplasmic staining and the percentage of TS-positive cells were determined automatically. The image analysis algorithm parameter "intensity" was selected to generate markup images. Intensity uses the total intensity i.e. the sum of all stained cellular components (nucleus and cytoplasm). Brown (DAB) and blue (hematoxylin) nuclei were identified and were spectrally separated with ImageScope™ software to evaluate immunohistochemical markers. Blue nuclei were counted as negative (0) and brown nuclei (DAB) as positive (Fig. 1B). The intensity of the brown nuclear staining was automatically scored as low (1), moderate (2) and high (3) (Fig. 1C, yellow, orange, dark red colors respectively).

The results were reported using an H-score that takes into account both staining intensity and the percentage of positive cells according to the following formula: H-score = (% of cells stained at intensity category 1x1) + (% of cells stained at intensity category 2x2) + (% of cells stained at intensity category 3x3) (Detre et al., 1995, Wong et al., 2001). We applied H-score adapted to automated assessment by McClelland et al. (1990) with modification in that not only the nucleus but the entire cell as an integral unit was counted because TS may be expressed in the nucleus and in the cytoplasm. The H-scores ranged from 0 to 176.4, and Fig. 2 shows a histogram of the distribution of H-scores. The median and mean H-scores were 2.79 and 26.1, respectively. Low TS expression was defined as H-score ≤26.1, and high TS expression was defined as an H-score above the

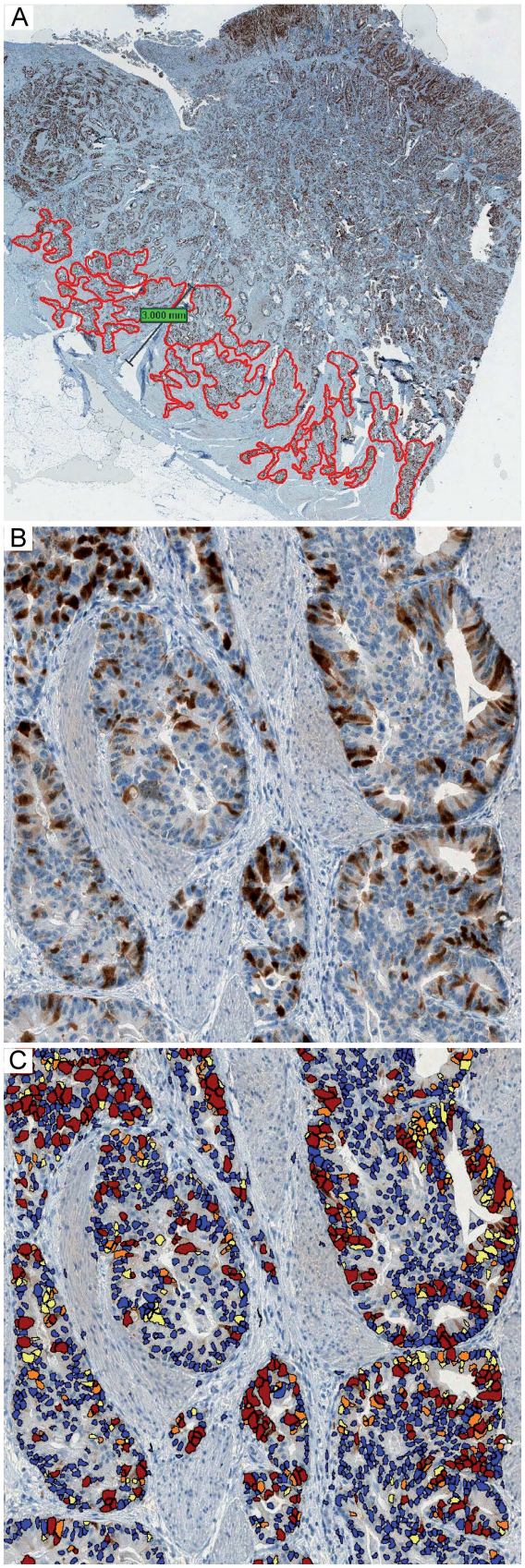

Fig.1 Image analysis of TS expression in a representative section of gastric carcinoma. Peripheral tumor areas marked for analysis **(A)**. Brown (DAB, TS positive) and blue (hematoxylin, TS negative) tumor cells **(B)** were identified and were spectrally separated with image analysis software. The intensity of the brown nuclear staining was automatically scored as low (yellow), moderate (orange) and high (dark red) **(C)**. A, x 5; B, C, x 20

mean value (i.e. >26.1).

Statistical analysis

Statistical analysis was performed using STATISTICA 9 software. The relationships between TS expression and clinicopathological parameters were determined using the chi-squared test or Fisher's exact test. Patient survival rates were determined using Kaplan-Meier estimates. The Wilcoxon rank sum test was used to compare patient survival curves, and the Cox log-rank test was used for multivariate analysis. A P-value less than 0.05 was considered significant, except for multiple comparison testing in which the Bonferroni-Holm correction was assessed. The survival curves were determined for 3-year DFS and for 3-year OS in the group of patients who underwent radical surgery and for 1-year OS rate in the group who received palliative surgical treatment.

Results

TS expression and clinicopathological parameters

Low TS expression (H-score ≤26.1; TS-) (Fig. 3) was found in 69 carcinomas (69%), and high TS expression (H-score >26.1; TS+) (Fig. 4) in 31 cases (31%). Statistically significant correlations of TS expression with clinicopathological parameters are shown in Table 1.

High TS expression was significantly more common in intestinal than in diffuse type of gastric cancer according to Lauren classification (P=0.0003); in type I carcinomas compared to type IV according to Goseki classification (P=0.002); and in gastric cancers in men than women (P=0.04). Low TS expression was found more often in carcinomas in the middle third (corpus) and lower third (antrum) of the stomach compared to cancers in the upper third (cardia and fundus) of the stomach (P=0.009 and P=0.001, respectively). We did not find a statistically significant relationship between TS expression and the remaining studied parameters.

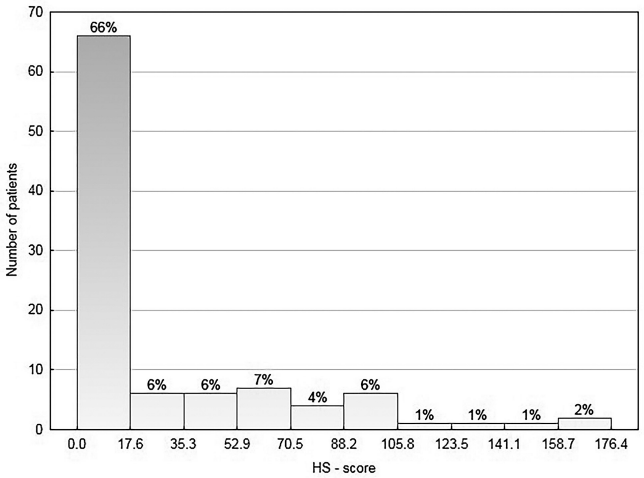

Fig. 2. Distribution of TS histoscores in the study group (n=100).

Table 1. Correlation of thymidylate synthase (TS) expression with clinicopathological parameters.

Parameter	N	Low TS N (%)	High TS N (%)	P
Sex:				
Female	31	26 (37.7)	5 (16.1)	0.04
Male	69	43 (62.3)	26 (83.9)	
Histological type (Lauren class):				
Intestinal type [1]	42	20 (29)	22 (71)	0.0003
Diffuse type [2]	45	39 (56.5)	6 (19.3)	[1] vs. [2] 0.02[a]
Mixed type [3]	13	10 (14.5)	3 (9.7)	
Histological type (Goseki class):				
Type I [1]	16	6 (11.5)	10 (43.5)	0.002
Type II [2]	8	5 (9.6)	3 (13)	[1] vs. [4] 0.0002[ab]
Type III [3]	30	21 (40.4)	9 (39.1)	
Type IV [4]	21	20 (38.5)	1 (4.4)	
Localization:				
Upper third of stomach [1]	14	5 (7.3)	9 (29)	0.005
Middle third of stomach [2]	33	25 (36.2)	8 (25.8)	[1] vs. [2] 0.009[a]
Lower third of stomach [3]	32	27 (39.1)	5 (16.2)	[1] vs. [3] 0.001[a]
≥2 anatomical areas [4]	19	10 (14.5)	9 (29)	
Gastric stump	2	2 (2.9)	0 (0)	

[a]: Significant difference after the Bonferroni-Holm correction; [b]: Fisher's exact test.

TS expression and the survival of patients with gastric cancer

In the group of patients who underwent radical surgery (n=59) no association was found between TS expression and DFS or OS (P=0.47 and P=0.74 respectively). However, in the subgroup of 25 patients without lymph node metastases (stage I+II), high TS expression was associated with better DFS (83,3% for high TS (n=10) versus 38,5% for low TS expression (n=5), P=0.03, Fig. 5). Of these 25 patients, 12 had tumors with high TS expression (10 patients survived 3 years and two patients died, the median OS and DFS: 77 and 67 months respectively) and tumors of 13 patients exhibited low TS expression (5 patients survived 3 years and 8 patients died, the median OS and DFS: 69 and 23 months respectively).

Tumors of all 13 patients who received 5-FU-based adjuvant chemotherapy exhibited low TS expression. Of these, 6 patients underwent radical surgical treatment (5/6 patients had 3-year OS and 3/6 – 3-year DFS) and 7 patients underwent palliative surgical treatment (2/7 patients had 1-year OS).

In the subgroup of 34 patients with regional lymph node metastases (stage III), TS expression did not significantly stratify patients with regard to DFS (P=0.18) or OS (P=0.17).

In multivariate analysis which included all patients who underwent radical surgery (stage I+II+III), none of the parameters, i.e. TS expression in tumor cells, the depth of cancer invasion, the presence of tumor metastasis to regional lymph nodes and lymph vessel invasion by cancer cells, had any correlation with survival, probably because the number of patients was too small. Hence, TS was a prognostic marker in univariate analysis but it was not an independent prognostic marker in multivariate analysis. No association was found between TS expression and 1-year survival in the group of patients who underwent palliative surgical treatment (n=40; P=0.85).

Discussion

The results of studies correlating TS expression with the prognosis of patients with gastric cancer are conflicting, with some showing such an association

Table 2. Correlation of thymidylate synthase (TS) expression with clinicopathological parameters and patient prognosis in studies that included over 100 cases of gastric carcinoma.

Study	Number of patients	Clinical Stage	TS expression	Results
Kuniyasu et al.	134	I–IV	IHC	High TS expression was associated with disease progression and poorer prognosis.
Tsujitani et al.	216	I–IV	IHC	High TS expression was associated with disease progression and poorer prognosis.
Lee et al.	463	IB–IV (M0)	IHC	No prognostic significance of TS expression.
Choi et al.	103	IB–IV (M0)	IHC	No prognostic significance of TS expression. High TS expression was associated with male sex, Lauren's mixed histological type and low tumor differentiation.
Kim et al.	153	III–IV (M0)	IHC	No prognostic significance of TS expression.
Yeh et al.	124	II–III	IHC	High TS expression was associated poorer prognosis, male sex and low tumor differentiation.

IHC: immunochemistry.

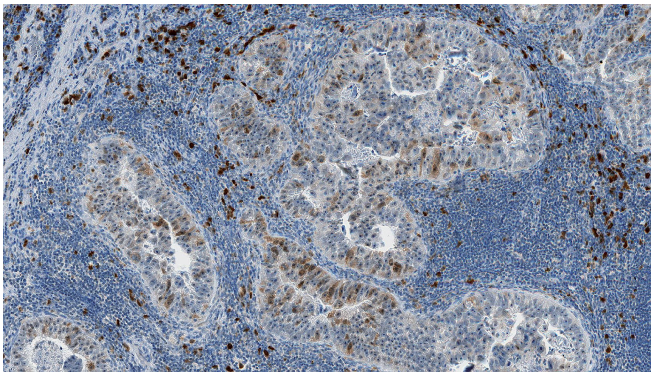

Fig. 3. TS immunostaining in a representative section of gastric carcinoma. Low TS expression (brown) in nuclei and cytoplasm of tumor cells. x 20

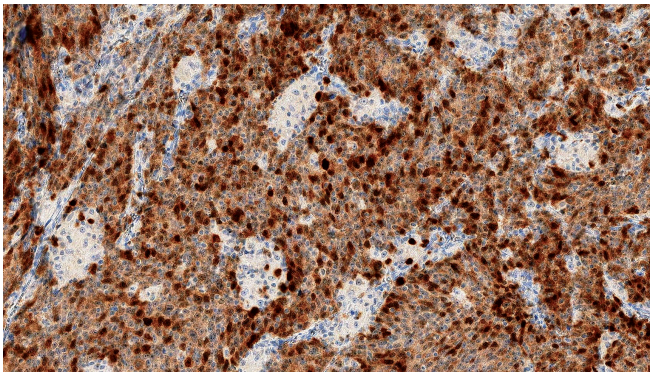

Fig. 4. TS immunostaining in a representative section of gastric carcinoma. High TS expression (brown) in nuclei and cytoplasm of tumor cells. x 20

(Kuniyasu et al., 1998; Suda et al., 1999a; Tsujitani et al., 2000; Yeh et al., 2010), and others showing an absence of association (Choi et al., 2001; Skarlos et al., 2007; Lee et al., 2008; Kim et al., 2009). In this study, most of the evaluated gastric carcinomas (69%) had low TS expression, in agreement with a majority of reports (Boku et al., 1998; Kuniyasu et al., 1998; Suda et al., 1999a,b; Tsujitani et al., 2000; Fukuda et al., 2006; Kamoshida et al., 2007; Kwon et al., 2007; Skarlos et al., 2007; Lee et al., 2008) in which the proportion of cases with low TS expression ranged from 54% to 79%. The majority of cancers showed high TS expression only in a few studies: 63% (Choi et al., 2001), 57% (Yeh et al., 2010) and 53.3% (Yeh et al., 1998).

We found a higher incidence of high TS expression in intestinal type than in diffuse type of cancer according to Lauren classification as well as in type I cancer (i.e. in tumors containing a small amount of mucus and well-differentiated tubular glands) than in type IV carcinoma according to Goseki classification. Choi et al. (2001) found an association between high TS expression and mixed histological type of cancer according to Lauren classification. In contrast, other studies showed no significant correlation between TS expression and the histological type of gastric cancer based on Lauren classification (Yeh et al., 1998; Tsujitani et al., 2000; Skarlos et al., 2007). The relationship between TS expression and histological type of gastric cancer according to Goseki has not yet been studied. Choi et al. (2001) and Yeh et al. (2010) showed a correlation between high TS expression and a low degree of tumor differentiation according to Kubo classification but this relationship was not confirmed by other studies (Kuniyasu et al., 1998; Suda et al., 1999b; Tsujitani et al., 2000; Skarlos et al., 2007).

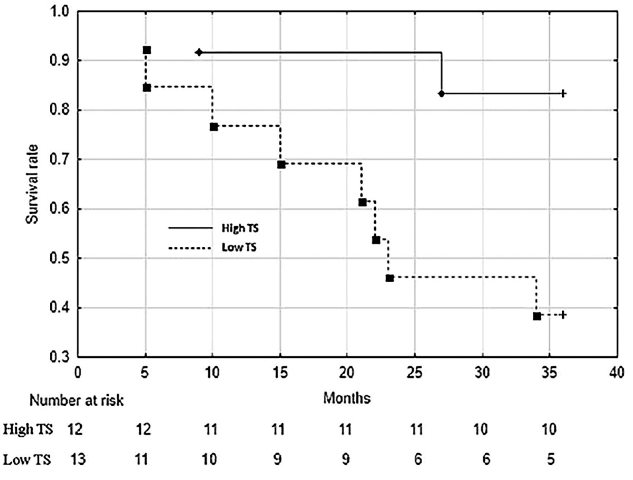

Fig. 5. DFS of patients with gastric cancer without metastases to regional lymph nodes who underwent radical surgery, categorized according to TS expression (P=0.03).

No previous studies have described a relationship between TS expression and gastric cancer localization. Our study showed a correlation between low TS expression and localization to the middle and lower thirds (corpus and antrum) of the stomach.

We found that high TS expression was significantly more common in men. The association of TS expression and the sex of the patient has been described by several studies (Choi et al., 2001; Yeh et al., 2010), although others found no association (Kuniyasu et al.,1998; Tsujitani et al., 2000; Kwon et al., 2007).

Three of six previous studies that included at least 100 gastric cancer patients (Table 2) showed that high TS expression had an impact on poorer prognosis (Kuniyasu et al., 1998; Tsujitani et al., 2000; Yeh et al., 2010). Other studies (Boku et al., 1998; Suda et al., 1999b) reported an association of high TS expression with shorter survival in advanced stages of gastric cancer, i.e. in stages IIIB and IVB. In the other three studies listed in table 2, including one based on 463 patients, no association with prognosis was found (Choi et al., 2001; Lee et al. 2001; Kim et al., 2009). In all of these published studies, TS expression was analyzed subjectively using a semi-quantitative method. In contrast, we applied an objective method based on computerized image analysis of TS expression on scanned digital slides. Using this method we found no association between TS expression and the survival rate in the whole group of patients that underwent radical surgery and did not receive preoperative chemotherapy. However, in the subgroup of patients without regional lymph node involvement, high TS expression was related to relatively good prognosis, whereas in the subgroup of patients with regional lymph node metastases, TS expression had no impact on survival. Previous studies gave no information about the prognostic significance of high TS expression in patients with gastric cancer who were treated with radical surgery in the two subgroups i.e. patients with and without regional lymph node metastases. Our finding, demonstrating improved DFS for patients with gastric cancer with high TS expression who did not receive preoperative chemotherapy is in contrast to other studies reporting improved survival for patients with low tumoral TS expression. However, our results are in line with other reports. Similarly to our results high TS protein expression was prognostic of long survival of patients with surgically resected NSCLC who did not receive preoperative chemotherapy. High TS protein expression was shown to be significantly associated with better survival of patients with surgically resected stage I NSCLC than low TS expression (Zheng et al., 2008). One possible explanation may be efficient nucleotide metabolism of tumors with increased level of TS expression and increased ability to repair DNA damage (TS is crucial for the formation of deoxynucleosides required for DNA synthesis and repair) as suggested by Zheng et al. (2008). Also, low TS expression was detected in aggressive thymic tumors (Monica et al.,

2013). Hence, our results generate a hypothesis that high TS expression in patients with gastric cancer stage I and II who were treated with surgical resection but did not receive preoperative chemotherapy is associated with better survival. However, since our cohort of patients is rather small a larger study including patients who meet the above mentioned criteria (stage I and II, surgical resection, no preoperative chemotherapy) seems to be necessary.

Several factors may be responsible for the differences among studies reporting correlations of TS expression with clinicopathological parameters and patient prognosis: 1) different methods of evaluating TS expression (semi-quantitative methods versus the computerized image analysis); 2) the use of different anti-TS antibodies; 3) tumor heterogeneity (Chung-Kang et al., 2006); 4) evaluation of nuclear or cytoplasmic or both nuclear/cytoplasmic TS expression. Most published studies evaluated only cytoplasmic expression (Johnston et al., 1995; Kuniyasu et al., 1998; Yeh et al., 1998; Grau et al., 2004; Fukuda et al., 2007; Kamoshida et al., 2007; Skarlos et al., 2007), nuclear/cytoplasmic expression was assessed by Huang et al. (2009) while one study did not mention site of TS expression at all (Suda et al., 1999b); 5) population differences related to TS coding gene polymorphisms, which might affect the expression of the TS protein; 6) different cut-off points for TS expression level. In the present study we used the mean value of H-score as the cut-off. Currently there are no generally accepted cut-off levels for immunohistochemically assessed TS protein expression in tumor cells. Therefore, cut-off levels are usually chosen empirically. A number of different threshold levels have been reported. A median (Chen et al., 2011) or arbitrary thresholds of 10%, 15%, 20% (Popat et al., 2004; Langer et al., 2010; Mirza et al., 2013), ≥30% (Miyamoto et al., 2000; Kamoshida et al., 2004) or 50% (Kamoshida et al., 2007) of positive tumor cells were applied. Histoscores were calculated (Wong et al., 2001; Lindebjerg et al., 2006). We applied histoscore because it has been suggested that a histoscore better reflects biologic associations than a mere percentage count (Wong et al., 2001).

It is very difficult to assess whether adjuvant 5-FU-based-chemotherapy that was applied in a certain percentage of patients might have influenced the correlation of TS expression and prognosis. Association of TS expression in gastric carcinoma cells and the response to 5-FU-based-chemotherapy remains controversial. Terashima showed that higher TS activity is associated with poorer prognosis but has no association with sensitivity to 5-FU-based-chemotherapy (Terashima et al., 2002, 2003). Kuniyasu et al. (1998) reported that patients with low TS expression who were treated with 5-FU-based chemotherapy had a better prognosis. In our study, 6 patients who underwent radical surgical treatment received 5-FU-based adjuvant chemotherapy. Of these, 5/6 patients had 3-year OS and 3/6 – 3-year DFS. On the other hand, high TS mRNA expression has been suggested to correlate with worse prognosis and with poor response to 5-FU-based-chemotherapy (Lenz et al., 1996; Ishikawa et al., 1999; Hua et al., 2007). Notably, however, this correlation was not confirmed in other studies (Metzger et al., 1998; Toriumi et al., 2004). Toriumi et al. did not find that TS mRNA expression or genotype correlated with prognosis or with sensitivity to 5-FU-based-chemotherapy (Toriumi et al., 2004).

The main weakness of our study is a rather small cohort of patients, therefore the results only generate the hypothesis with respect to prognosis. However, there are important strengths of this study. First, survival analysis is based on a homogenous group of gastric cancer patients (node negative stage I and II, radical surgery, no preoperative chemotherapy) whereas previous studies were based on mixed groups including node negative and node positive patients and patients with advanced gastric cancer. Second, this is the first study of prognostic significance of TS in gastric cancer based on objective assessment of TS expression (computerized image analysis). In previous studies subjective manual counting was applied which is probably one of major reasons responsible for discrepant results. Objective image analysis sets a certain standard of assessment which can enable a comparison of results of future studies in respect of TS expression and prognosis.

In summary, the results of our study indicate significant correlation between the Lauren and Goseki histopathological classifications of gastric cancer and TS expression in tumor cells. Further, the results suggest that high TS expression may be a positive prognostic marker with regard to DFS in patients with gastric cancer without involvement of regional lymph nodes (clinical stage I+II) who underwent radical surgical treatment and did not receive preoperative chemotherapy. However, these prognostic results require confirmation in larger cohorts of patients.

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